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The role of Nitric Oxide in Pre-eclampsia

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Abstract

Hypertension complicates approximately 10% of all pregnancies and is a leading cause of maternal and foetal mortality and morbidity world-wide. Pre-eclampsia is a major subgroup of these hypertensive disorders. It is defined as a rise in blood pressure to 140/90mm Hg or greater accompanied by proteinuria and usually presents after 20 weeks of pregnancy. Much of the early research into this disorder has concentrated on the determination of vasoactive compounds such as the renin-angiotensin system and prostacyclin.

In the 1980's it was discovered that an inorganic free radical molecule, nitric oxide (NO), was released from the endothelium cell lining of the vasculature and was involved in regulating vasodilation of the vasculature walls via smooth muscle . It was also shown to have cytotoxic effects on bacteria, to inhibit platelet aggregation and to act as a neurotransmitter. The aim of this research was to assess the role of nitric oxide in pre-eclampsia. This was accomplished by the analysis of its oxidation products nitrite and nitrate in plasma from women with pre-eclampsia compared with those from normotensive pregnancies.

A simple and robust assay for nitrite and nitrate was developed using ion chromatography. Initial experiments using isocratic elution with conductivity detection on a Dionex QIC system with an AS4A-SC column showed promise but were unsatisfactory due to the interference from chloride ions. Successive improvements to the technique involved changing the elution system to a gradient, initially to one with carbonate and subsequently to chloride, changing the detector system to direct UV detection at 214nm and changing the column to a high capacity, strong exchanger type. The resulting method shows good resolution, does not suffer from chloride overload and was simple to use. Control results for 200 serum samples showed that the male mean nitrite and nitrate levels were 3.34 ± 5.17 $\mu mol\ L^{-1}$ and 42.1 ± 33.1 $\mu mol\ L^{-1}$ respectively while female levels were 4.74 ± 11.7 $\mu mol\ L^{-1}$ and 37.5 ± 27.9 $\mu mol\ L^{-1}$ respectively.

Addition information on the free-radical status of the pregnant study groups was assessed by determination of lipid peroxides and the peroxynitrite product, 3-nitrotyrosine. An improved GC-MS method was developed to quantify total fatty acids and lipid peroxides. A new reversed phase HPLC technique for the analysis of free 3-nitrotyrosine in human plasma/serum was also developed although sample numbers were not as great as expected.

Statistical analysis using F-tests, t-tests and the Mann-Whitney analysis did not show any difference in nitric oxide metabolites, lipids, lipid peroxides or peroxynitrite between gestation matched normotensive pregnant women and those with pre-eclampsia or pregnancy-induced hypertension.

Published research has shown a vital role for nitric oxide in the maintenance of blood flow in normal pregnancy. This research does not support evidence for diminished or enhanced nitric oxide production in pre-eclampsia compared with normal pregnancy.

Contents

Glossary

AED – Atomic emission detector
BLD – Below limits of detection
BSTFA – bis(trimethylsilyl)trifluororaceamide
cNOS – Constitutive nitric oxide synthase
DCM – Dichloromethane
ECD – Electron capture detector
EI – Electron ionisation
eNOS – Endothelial nitric oxide synthase
GC – Gas chromatography
HPIC – High performance ion chromatography
HPLC – High performance liquid chromatography
iNOS – Inducible nitric oxide synthase
LPO – Lipid peroxides
MS – Mass spectrometry
MSD – Mass spectrometry detector
NICI – Negative ion chemical ionisation
nNOS – Neural nitric oxide synthase
NO – Nitric oxide
NOx – Sum of nitrite and nitrate
PBS – Phosphate buffered saline
PE – Pre-eclampsia
PFB – Pentafluorobenzene
PIH – Pregnancy induced hypertension
SIM – Selected ion monitoring
SPE – Solid phase extraction
TBARS – Thiobarbituric acid reactive substances
TG – Triglycerides
TMCS – Trimethylchlorosilance
TMS - Trimethylsilane
UV – Ultra-violet

Chapter 1

Nitric oxide biology and chemistry

Overview of nitric oxide

During 1987/88 it was discovered that an inorganic free radical molecule, nitric oxide (NO), is involved in endothelium-dependent vasodilation [1,2]. Early biological work had discovered that a substance released from the endothelium cell lining of the vasculature is involved in regulating vasodilation of the vasculature walls via smooth muscle [3]. This substance was found to have a very short half-life and was called endothelium derived relaxing factor (EDRF) [4]. In the late 1980's EDRF was found to be nitric oxide [4]. Since that time extensive research has attributed many vital physiological functions to this molecule including inflammatory reactions [5] , a role in learning and memory [6], toxic shock [1,7,8], neutrophil chemotaxis and adhesion, regulation of lymphocyte proliferation [9,10], platelet aggregation [1] and also as an important part of the non-specific immunity system [11,3].

Chemistry of nitric oxide

Nitric oxide is a molecule that contains an odd number of valence shell electrons and is therefore a radical. It is less reactive than many other radicals (e.g. hydroxyl (OH•)), so is defined as a 'stable radical' [12]. Once produced from the source it diffuses from a higher to lower concentration in a random manner and due to its low solubility in water (1.7×10^{-3} M at 25°C and P_{NO} of 1 atm) [13] can travel up to 50μm [14]. It is now believed that NO does not elicit all of its biological functions directly but reacts with itself or other compounds to form more reactive intermediates, including NO_2, NO_3, N_2O_3 and N_2O_4 [15]. This is because even though NO is thermodynamically unstable its decomposition is kinetically hindered and so it can be stored indefinitely as a gas at 1 atm and room temperature. Under elevated pressure NO can disproportionate to yield N_2O and NO_2. NO_2 can undergo polymerising reactions and react with molecular oxygen and water. The degradation sequence is shown in fig 1.1

$$2NO + O_2 \longrightarrow 2NO_2$$
$$2NO_2 + 2NO \longrightarrow 2N_2O_3$$
$$2N_2O_3 + 2H_2O \longrightarrow 4HNO_2$$

Figure 1.1 Oxidation reaction of nitric oxide (taken from Bohle [16]).

Formation of nitrite occurs from the radical reaction of nitrogen dioxide with nitric oxide [17] to produce an intermediate that quickly reacts with water [12]. Under these conditions virtually all of the starting material is converted to nitrite [18]. When NO reacts with water directly, nitrite and nitrate are both produced [12]. When blood is analysed the major anion found is nitrate, formed by oxidation of nitrite or by the interaction of NO and oxyhaemoglobin producing nitrate without nitrite formation [19]. As well as its degradation, its reaction with other species has been investigated. NO itself is relatively unreactive, however an easy oxidation by removal of the single unpaired π^* (antibonding) electron results in the formation of a nitrosyl cation NO^+, which is highly reactive [18]. It is possible that this intermediate is involved in nitrosothiol formation [17]. Reduction of NO by addition of a twelfth electron produces a nitroxyl anion radical ($NO^{-\bullet}$) [18], which is isoelectronic with molecular oxygen. However, the extra electron is soon lost [18] and is questionable whether this process could occur *in vivo* [14]. Whatever the intermediates, the targets are well known. Among these oxygen, superoxide, oxyhaemoglobin, thiols and transition metals have been extensively studied [18, 20]. The reaction between superoxide and nitric oxide to form peroxynitrite is the fastest of all, being only diffusion limited [18] ($k=6.7x10^9$ M^{-1} sec^{-1} [21]). Peroxynitrite can rearrange on protonation to form nitrate or OH• and NO_2 , promoted by trace amounts of Cu^{2+} [18]. It has a range of targets but much research has concentrated on its action in the nitration of tyrosine resides. However it is questionable whether NO is the sole source as the air pollutant products NO_2, N_2O_3 and N_2O_4 can also produce nitrotyrosine [21]. Oxyhaemoglobin reacts rapidly reacts with NO to form methaemoglobin and nitrate ($k = 3x10^7$ M^{-1} sec^{-1} [21]) . In this chemical reaction the bound O_2 acts in the same way as superoxide, producing a peroxynitrite intermediate which isomerizes to nitrate [20]. Nitrosation of thiols has been postulated as an important reservoir of basal endothelial produced NO [17], this is further discussed in Chapter 3. The reactivity of NO with transition

complexes (especially Fe^{2+}) is central to many of its biological functions [12]. Several key enzymes contain a Fe centre that reacts with NO to form metal-nitrosyls. In some cases, such as haem iron and guanylate cyclase, these are reversible but in those with an Fe-S cluster such as mitochondrial aconitase the action is non-reversible [17]. This action on essential enzymes is used in the control of cell death and in other similar enzymes can regulate mRNA production [17].

Biochemistry of nitric oxide

Nitric oxide is formed from two classes of enzymes, constitutive and inducible which are calcium-dependent (cNOS) and calcium-independent (iNOS) respectively [15]. Of the cNOS group there are two isoforms which are given the first initial from the cells they were first discovered in, nNOS from neurons and eNOS from endothelial cells. It should be noted that each enzyme is not exclusive to an organ type e.g. eNOS and nNOS are present in the brain as well as the endothelium, but in much smaller quantities [22]. They were also given a numerical number based on the order in which they were cloned [15]. All three enzymes have a genetic code sharing 51-57% homology [23]. All have the same binding sites for the cofactors including calcium/calmodulin; eNOS has an extra site for myristoylation as this is membrane bound while the other two are found in the cytosol [23]. iNOS also requires calcium-calmodulin but its affinity for these factors is so strong that they are almost permanently bound to the enzyme [15]. In the early work on these enzymes it was thought that iNOS produced more NO than the other two [24]. However more recent research has shown that they all produce approximately 1μM/min/mg protein of NO at 37°C. The reason for the initial discrepancy was attributed to high level of expression of this enzyme with its ability to produce NO without restrictions on intracellular free calcium [15]. The nitric oxide synthases (NOSs) all share similar homology with the cytochrome P_{450} enzyme system . However they require extra cofactors from flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN) and tetrahydrobiopterin (BH_4) to shunt the electrons from the nicotinamide adenosine dinucleotide phosphate (NADPH) substrate to the haem active site [15]. In the first step of the biochemical reaction one of the guanidino-nitrogens from L-arginine is oxidised with loss of two electrons and gain of oxygen to form a short-lived N-

hydroxy-L-arginine compound that is further oxidised with loss of three electrons to produce L-citrulline and nitric oxide [15].

	Neural NOS (nNOS) (type I)	**Inducible NOS (iNOS) (type II)**	**Endothelial NOS (eNOS) (type III)**
Main Location	Brain, cerebellum, CNS and peripheral nervous tissue [6,22]	Macrophages, neutrophils, lymphocytes, hepatocytes and can be expressed by most cells [1,15]	Heart, endothelial cells [15,22]
Activated by	Oestrogen/ Testosterone lithium,tacrine or glutamate [23,25]	IFN-γ, TNF-α, IL-1 and 2, MIGF-β, bradykinin and LPS at receptor level [5,7,23]	Glucocorticoids, oestrogen, acetylcholine, bradykinin and shear stress [23,26]
Inhibited by	--	IL-4 and 10, TGF-β, MDF, IGF, thrombin at transcriptional level [4,27]	--
Role	Synaptic plasticity, learning, neurotransmission [28] and cerebral blood flow control [22]	Neutrophil adhesion, chemotaxis and regulator of proliferation [29] Inhibitor of platelet aggregation and proliferation [29] Free radical damage to pathogenic organisms [22]	Regulator of blood flow [29] Protective role from superoxide, lipid peroxides [30]
Mode of action	Binds to soluble guanylate cyclase enzymes for second messenger activation [3]	Inhibits enzymes by binding to Fe/Fe-S enzymes of mitochondria and nucleus [15]	Binds to soluble guanylate cyclase enzymes for second messenger activation [3]

Table 1.1 Biochemical properties of nitric oxide synthases

IFN-γ - Interferon γ, TNF-α - Tumour necrosis factor α, MIGF-β - Migratory inhibitory growth factor β, LPS - Lipopolysaccaride, IL-x - Interleukins, TGF-β - Transforming growth factor β, MDF - macrophage deactivating factor, IGF - insulin like growth factor.

Neural Nitric oxide synthases

In the brain, periphery and the central nervous system (CNS) NO is produced by non-adrenergic non-cholinergic (NANC) nerves. It is believed that the role of this free radical in the periphery is as a neurotransmitter where it augments the release of glutamate from the pre-synaptic neurones. This glutamate then diffuses across the synaptic cleft to activate receptors on the dendrites of the post synaptic neurones. When glutamate binds to the N-methyl-D-aspartate (NMDA) receptor it causes an increase in intracellular calcium which in turn activates the nNOS to produce nitric oxide. The nitric oxide diffuses back into the pre-synaptic nerve to further augment the release of glutamate thereby increasing the response of that signal [31,4]. This process of 'long-term potentiation' has been linked to memory function as the use of NO inhibitors has revealed impairment in learning and feeding in animal studies [1].

In the central nervous system it is thought that nitric oxide binds to soluble guanylate cyclase (sGC) in neighbouring cells to produce second messengers involved in memory and learning [3]. NO binds to the haem iron of sGC, weakening its co-ordinate bonding within the porphyrin ring and bringing about a conformational change which leads to its activation [15,26]. This activation produces 400x more cGMP than in the unbound basal conditions [22]. The production of NO is thought to be self-regulated by negative feedback from nNOS's own production and from adjacent cells [6] but this theory is still controversial [15]. The binding of NO to sGC haem is reversible and disassociates more rapidly than was initially considered [6], especially in the presence of haemoglobin or other higher-affinity compounds [15].

In stroke patients glutamate levels are increased, thereby causing a large increase in the cellular concentration of nitric oxide which leads to free radical damage [3]. It has been proposed [3] that nitric oxide is used to augment the neural signals and also to regulate nerves so that any over-stimulated cells are self-destroyed by this regulatory action.

Inducible Nitric oxide synthases

Inducible NOS differs from the other two constitutive forms (nNOS and eNOS) in that it is not calcium dependent. However this enzyme is not normally present in non-immune cells and is only transcribed after several hours of stimulus activation [5]. Even though the endothelium and other cells can produce iNOS, most of the research in this field has focused on the immune system, relating to the macrophage and the neutrophil.

It is considered that NO is a vital part of the non-specific immune system where its action is concentrated in the liver and lungs as an 'immunological filter' [1]. Upon activation by endotoxins, cytokines etc., macrophages produce high levels of NO, superoxide and other radicals and cytokines. Nitric oxide binds rapidly to any iron moiety thereby inactivating the vital enzymes of the invading pathogen or tumour cells. The main targets include aconitase, mitochondrial enzymes and DNA which are vital to cellular respiration and reproduction [15]. Precipitation of iron by excess NO is a strong initiator of enzyme inhibition [27] as well as an initiator of lipid peroxidation (chapter 2). Several parasitic species are highly susceptible such as Leishmania or Listeria; a comprehensive list is reported by O'Donnell [10]. The reaction of NO and O_2 is third order indicating that the half-life of NO is proportional to its concentration. This illustrates its dual activity, first as a cytotoxic agent with high production by immune cells and second allowing this molecule to travel significant distances in its role of regulating blood pressure [21]. As well as having a cytotoxic effect directly, nitric oxide reacts rapidly with superoxide to form peroxynitrite which decomposes to form the more aggressive hydroxyl (OH•) and $NO_2^{\bullet}$ radicals [32].

Endothelial Nitric oxide synthases

Endothelial NOS is activated in a similar method to that of nNOS, in that any stimulus which brings about an increase in intracellular calcium will activate the enzyme [26]. Nitric oxide is a lipid soluble gas that rapidly crosses the endothelial cell membranes where it binds to the sGC in the adjacent smooth muscle cells. Second messengers cascading from cGMP activation bring about vasodilation of the vasculature [15]. This mechanism is localised as any NO entering the lumen of the artery vessel will be quickly oxidised by

oxyhaemoglobin to nitrate and methaemoglobin [26] in oxygenated blood and to nitrosylhaemoglobin in venous blood (chapter 3). Methaemoglobin requires a $NADP^+$-dependent enzyme to convert this reduced molecule back to haemoglobin. In normal states the blood levels of methaemoglobin are undetectable. In extreme disease states with high NO production this restoring system cannot be maintained and methaemoglobin levels can be readily detected [4]. Arteries, arterioles, veins, venules and the umbilical vein have been shown to secrete NO [6] with the major contribution from the arterial vessels [29]. It is thought that NO may bind to thiols such as glutathione, cysteine and albumin forming more stable nitrosothiols to act as reservoirs for maintaining basal blood flow homeostasis [33] increasing stability up to 30 minutes [27]. As well as a direct role in vasodilation, NO also acts on the endothelium lining inhibiting the adhesion of platelets to its surface thereby avoiding damage to the lining and formation of atherosclerotic plaques [6].

The eNOS (membrane bound) enzyme is activated by acetylcholine, bradykinin, oestrogen and shear stress [23,26] via controlled influx of calcium ions. Oestrogen and shear stress also regulate eNOS expression in these cells [6]. It is suggested that this enzyme is membrane bound so it can detect changes in the outer membrane stress and pulsatile stretch [27]. Maintenance of the cardiovascular system is controlled by all three enzymes [22] with the primary regulation from eNOS, the support for this being from studies showing that mice deficient in the eNOS gene are hypertensive [22]. Innervated vessels exposed to cytokines can express iNOS as well as nNOS so both can play a role in regulating blood pressure in normal and diseased states [6].

In the heart eNOS plays a negative inotrophic role by increasing myocyte contraction. In disease states such as heart failure iNOS has been detected in this organ [22]. There is a strong association of hypertension with impaired coronary release of NO by acetylcholine in patients with mild coronary atherosclerosis, and with reduced formation of NO in patients suffering from essential hypertension [24], emphasising the vital role of these enzymes in the normal homeostasis of blood pressure [25, 34]. Indeed, NO generating compounds have long been used for the management of ischaemic syndromes related to coronary heart disease [35] via the formation of active, unstable, intermediate nitrosothiols [36,37].

Endothelial cells can also produce iNOS if activated by a suitable stimulus such as endotoxin. In endotoxic shock, endotoxin activates endothelial cells to produce iNOS [8]. As

well as acting as a pathogen inhibitor this high production of NO in the endothelium brings about sustained system vasodilation where the normal blood pressure cannot be maintained. Inhibitors of NO synthesis such as analogues to L-arginine have been successful in treating this disorder [38].

It has also been shown that nitric oxide can regulate its own production by a negative feedback mechanism directly to the NOS [39].

NO is also a potent platelet disaggregator and inhibitor of aggregation. There is a fine balance between the ratio of NO and superoxide [30]. If NO is limiting it encourages a pro-atherogenic phase but if in excess it promotes an anti-thrombotic environment, therefore NO has a role in the formation of atherogenic plaques.

Peroxynitrite

Peroxynitrite is formed from the reaction of nitric oxide with superoxide [40,41]. Nitric oxide synthase [42] in neural as well as endothelial [43], neutrophil [44] and macrophage [45,46] cells is capable of producing both NO• and O_2^-. The reaction proceeds at a near diffusion limited rate of 6.7×10^9 M^{-1} s^{-1} [47] to form the peroxynitrite anion. Removal of superoxide by superoxide dismutase effectively doubles the half life of nitric oxide [48] by stopping the subsequent formation of peroxynitrite.

Peroxynitrite has a pKa of 7.49 at 37°C and a half-life of 1.9 s^{-1} in solution at pH 7.4 [40]. At physiological pH it will be protonated to form peroxynitrous acid which decays by homolytic fission to form the strong oxidants HO• and NO_2• [(40)] or rearranges to form nitrate and a proton. The pathway followed is governed by the pH; the less harmful rearrangement process being favoured at high pH [40]. At low pH the nitrogen dioxide radical and the hydroxy radical formed are potent oxidising agents which target DNA [46], lipids [40], sulfhydryls and metaloproteins. Reaction with iron forms a nitronium ion intermediate that readily nitrates tyrosine and tyrosyl groups of proteins leading to the formation of 3-nitrotyrosine [47]. An alternative nitrating radical, the nitrocarbonate anion, is formed from the reaction of peroxynitrite with carbon dioxide [49]. Nitric oxide can react with hypochlorous acid (from the reaction of hydrogen peroxide and HCl) to form chloride and nitrogen dioxide free radicals, possibly with a role in inhibiting bacterial survival [50].

Peroxynitrite is not considered an inflammatory molecule in all systems since its effect is dependent on the environment and concentration [51]. Oxidation of thiols [52] and other NO-donors at intermediate concentrations (30-100 μmol L^{-1}) has a vasodilatory effect. However at higher concentrations it can cause sufficient endothelial damage to impair vascular relaxation [51].

Measurement of nitrite and nitrate as an indicator of NOS activity

As previously discussed, NO combines with molecular oxygen to form NO_2 at a rate that is dependent on the oxygen concentration and the square of the NO concentration [53]. Therefore, the rate of this reaction is very slow at the low physiological levels produced. The rate determining step is in the formation of NO_2 which rapidly hydrolyses to nitrite and nitrate [53]. From this it would be expected that if a low nitrite/nitrate diet was sustained then the circulating plasma and urine nitrite/nitrate would be an index of NOS nitric oxide production. Indeed several investigators have shown this [54, 55]. Maintaining a controlled nitrate diet reduced overall urinary nitrite [56] and plasma nitrite/nitrate [1.46] variation between samples. In humans approximately 16% of the daily nitrate output is derived from dietary L-arginine [53].

Florin [57] reported that ingested nitrate was largely excreted into human urine giving recoveries of 83, 100 and 82%. This overall loss has also been seen when nitrate was injected IV into human [58] (>85%) and rat [53] (93%) patients or when NO was directly inhaled by rats and mice [59] (55,53, 78%).

These small losses have been attributed to redistribution of NOx into other fluids such as saliva [53], stomach [60], skin [60], lungs [53] and other metabolic pathways (such as urea cycle) [53].

In blood, up to 25 to 33% of circulating nitrate is absorbed by the salivary glands [53,60] where bacteria can reduce it back to nitrite [60]. The bacterial reduction is dependent on oxygen concentration, pH and the bacterial flora [53]. When nitrite reaches the stomach it is converted back to NO at the acid pH [60], and in the presence of reducing agents such as ascorbic acid [60]. It is believed that this process, combined with the low pH, is more effective in the destruction of pathogens than acidic pH alone [60]. Rats are unique in their ability to secrete nitrate into the small intestine, which must be considered when comparing metabolism results from rats with those from humans [53]. NO released by this reaction has been proposed

as a method of increasing superficial mucosal blood flow [53]. The remaining nitrate entering the gastrointestinal tract is reabsorbed into the blood stream where it circulates before being excreted into urine, saliva, skin, lungs or further metabolised [60]. Excretion onto the skin has shown that bacteria convert nitrate from sweat back to nitrite while the acidic nature of the skin further reduces it to NO [60]. As in the stomach it has been proposed as a cytotoxic agent to harmful pathogens. NO exhaled from the lungs is possibly derived from macrophages present in the airways and those cells involved in modulation of bronchial resistance [53]. Nitrite can be converted to hydroxylamine and eventually to ammonia in the intestine where levels of ammonia correlate with those of nitrate [61].

Net nitrate production ranges from 150 to 1000 μmol/day per person [53] in normal uncontrolled replete subjects, while in fasted subjects it can range from 100 to 200 μmol/day [60]. In urine, the total nitrate concentration can range from 250-2000 μM whereas nitrite and NO are not usually detected except when there is bacterial contamination [53]. The normal dietary intake ranges from 75 to 150 mmol/day while the output exceeds the input 2 to 60 fold; the excess being attributed to NOS activity [53]. Interestingly, the amount of NO produced by eNOS in the average human has been reported to be 1728 μmol/day per person [53], indicating that the L-arginine:NO pathway contributes significantly to net nitrate production.

Another factor is the contribution of the total NO from iNOS since any pathogenic infection would activate expression of iNOS and would produce more NO than nNOS or eNOS [53].

Normal reported levels of serum nitrite and nitrate

Author	Mean Nitrite Concentration (μmol/L)	Mean Nitrate Concentration (μmol/L)	Total NOx (μmol/L)	n
Michigami [62]	4.4 +/- 2.8	61 +/- 37	65 +/- 40	34
Jong [37]	------	36 +/- 19*	------	20
Lyall [63]	------	------	29 +/- 1.1*	36
Curtis [64]	9.5 +/- 0.58*	85 +/- 8.0	95 +/- 8.6*	59
Moshage [19]	4.2 (1.3 - 13)	19.7 (40 - 45.3)	24 (41 - 58)	26
Kelm [58]	0.1 - 0.3	30 - 60	30 - 60	??

Table 1.2 published data on nitrite and nitrate levels in normal human serum.

*-result quoted for normal pregnant females, all other data obtained from non-pregnant females. NOx = nitrite + nitrate.

The amount of variation is clearly considerable, probably due in part to variation in dietary intake, smoking [24] and other environmental factors [65] which will vary from day to day and from person to person. To obtain meaningful results it is necessary to monitor a patient's anion levels prior to and throughout any clinical study.

As the data show in table 1.2, definitive normal ranges for nitrite and nitrate are very difficult to establish. It is evident that any study of the function of nitric oxide would require a high net production of NO metabolised into nitrite and nitrate to fall with statistical significance outside the normal ingested nitrate levels as diet control is difficult in human subjects. An alternative procedure could monitor an individual's NOx (nitrite + nitrate) levels over time to see the effect of diet on that person.

Chapter 2

Pre-eclampsia

Definitions

Hypertension - By convention hypertension is defined as a rise in blood pressure to 140mmHg systolic and 90mmHg diastolic or greater. Hypertension may arise *de novo* in pregnancy or be pre-existing i.e. chronic hypertension. Chronic hypertension may be due to essential (idiopathic) hypertension or due to underlying renal or adrenal disease [66].

Pregnancy induced hypertension (PIH) sometimes also referred to as pregnancy associated hypertension is defined as a rise in blood pressure to 140/90mm Hg or greater presenting after 20 weeks of pregnancy. There should be evidence of normotension pre-pregnancy and in the first 20 weeks of pregnancy [66].

Pre-eclampsia (PE) is defined as a rise in blood pressure to 140/90mm Hg or greater accompanied by proteinuria. Pre-eclampsia usually presents after 20 weeks of pregnancy and characteristically occurs in primigravid women. There should be evidence of normotension pre-pregnancy and in the first 20 weeks of pregnancy [66].

Pre-eclampsia is a syndrome i.e. a collection of signs and symptoms. If the central nervous system is affected then generalised seizures may occur - a complication known as **Eclampsia**. "Superimposed" pre-eclampsia may develop in women with chronic hypertension [66].

Clinical importance of hypertension in pregnancy.

Hypertension leads to complications in approximately 10% of pregnancies [66b]. It is a leading cause of maternal and foetal mortality and morbidity world-wide. The maternal mortality rate associated with hypertension has hardly changed since the first triennial maternal mortality report in 1952 [66c]. Pre-eclampsia is responsible for most of the adverse outcomes. In contrast, pregnancy induced hypertension is usually a relatively benign complication presenting late in pregnancy. Pre-eclampsia is a multisystem disorder. Placenta function is often impaired leading to restriction of foetal growth. There is a risk of intrauterine foetal death especially if the placental dysfunction is not recognised. In the mother severe hypertension, if uncontrolled, may precipitate cerebral haemorrhage. The maternal kidneys, liver, nervous system and clotting mechanisms may all be disturbed leading to life threatening complications.

The cause of pre-eclampsia is unknown. At present, the only cure is delivery of the foetus and placenta. If the disorder presents early in pregnancy the delivery of a premature baby may be necessary. Pre-term delivery may lead to neonatal death or long term disability from the complications of prematurity.

Difficulties in assessing studies of hypertension in pregnancy

Although the definitions appear clear cut, the diagnosis of hypertensive disorders in pregnancy is not always straightforward. For example, eclampsia may occur post-delivery in a women with no apparent antenatal signs and symptoms of pre-eclampsia. Pre-eclampsia may present with severe restriction of foetal growth and it is only after one or two weeks that maternal hypertension and proteinuria becomes evident. In some cases increased serum uric acid and low platelet counts may aid the diagnosis [66]. It may not be known if a woman has pre-existing hypertension if she does not attend for a blood pressure measurement before 20 weeks gestation.

Thus in some cases the diagnosis of pre-eclampsia can only be made several weeks after delivery of the baby. Studies on pre-eclampsia should be restricted to women in their first pregnancy with new onset hypertension and proteinuria remote from term.

The pathophysiology of pre-eclampsia.

Many years of study have been devoted to determining the cause of pre-eclampsia. Although much of the associated pathophysiology is now understood, the cause remains unknown [78].

Certain factors associated with PE:

a. Genetic predisposition

A genetic predisposition to PE has been established but the mechanism of inheritance has yet to be elucidated. The daughters of women who have suffered PE have an increased risk of developing the condition. A change in partner increases the risk of PE. This would suggest that paternal genes also have a role to play in this disorder [532]. Prior blood transfusion and increased preconceptual exposure to sperm or seminal plasma are also associated with a decreased risk of PE.

b. Immunological factors

It has been suggested that PE results from an inadequate natural immune adaptation to pregnancy. Immunological mechanisms could be responsible for abnormal trophoblastic implantation. However the immunological features may be secondary rather than primary to the disease.

c. Defective placental implantation

The normal human placenta has separate uteroplacental circulation and fetoplacental circulation. Development of the uteroplacental circulation begins in the first trimester and is completed by 20-24 weeks gestation. By contrast the foetal villous tree continues to branch and differentiate throughout gestation.
The placenta in PE is characterised by failure of normal trophoblast invasion of the maternal spiral arteries (defective uteroplacental circulation) and maldevelopment of the terminal foetal villi (defective fetoplacental circulation).

The fetoplacental circulation lacks autonomic innervation. Vascular tone may be influenced by a variety of vasoactive agents e.g. prostaglandins, nitric oxide. In PE there is a disturbance of normal balance between vasoactive and vasoconstrictor agents

d. Endothelial dysfunction

There is evidence of PE being associated with endothelial cell dysfunction. Alteration in endothelial cell function has implications for immune and inflammatory responses, intravascular coagulation and the contractile response of smooth muscle.

A theory linking the various pathological findings in PE has been suggested as follows.
A genetic predisposition leads to defective placentation which is immunologically-mediated. Poor placental perfusion results in the release of blood borne factor(s) which in turn cause widespread endothelial cell dysfunction. As a consequence, vascular tone increases (balance of vasoconstrictors to vasodilators altered in favour of vasoconstriction), and activation of the coagulation system occurs with loss of endothelial wall integrity leading to escape of fluid into the extravascular space. The severity of the disease is governed by the individual's ability to compensate for the consequences of endothelial cell dysfunction. The placenta plays a pivotal role in the disorder as all studies have shown that after removal of the placenta, blood pressure eventually returns to normal. It must be

stressed that hypertension is not the cause of the disease but is an effect of altered levels and actions of vasodilators [78,72,73,196,135].

Much of the research relating to the cause and prevention of PE has focused on vascular changes in normal pregnancy compared with PE and the influence of vasoactive agents derived from endothelial cells. This work will be reviewed with a particular focus on nitric oxide.

Vascular changes in pregnancy

In normal pregnancy the blood volume increases from about 6 weeks until 30-34 weeks when it is maintained at 40-50% above that of non-pregnant females [78b]. This increase in blood volume is accompanied by a fall in blood pressure in the midtrimester and then a rise to the early pregnancy level in the third trimester. During this time stroke volume and cardiac output increase with an overall decrease in the peripheral resistance caused by local and/or systemic vasodilation after 5 weeks of gestation [79,66]. In severe PE, studies on cardiac output have produced varied results but the consensus is that it is reduced as a result of increased systemic vascular resistance and reduced maternal plasma volume [72]. Oestrogen is a likely stimulus for the increase in the renin-angiotension II and aldosterone (RAA) system causing salt and water retention [79]. Angiotensin II (AII) is a potent vasoconstrictor and it's concentration, like renin, is reduced in PIH and PE patients compared to normotensives after 18 weeks of gestation [66,72]. In this condition concentration and pressor effects decrease, eventually returning to a level similar to that before pregnancy. Renin, AII and aldosterone are all suppressed once the disease is established [78b]. Studies on these substances concluded that the increased sensitivity to AII was due the local responsiveness of the vessel wall rather than changes in blood volume or renin/AII levels [66]. Pre-eclamptic serum has shown increases in the circulating levels of the vasoconstricting endothelins [72] but this is thought not to be important in the pathogenesis of the disease, but produced secondarily to endothelial damage [522,78]. Atrial natriuretic peptide promotes vasodilation and has been shown to be increased in PE [72].

Prostaglandins and thromboxane

The prostaglandins PGE_2 and prostacyclin both modulate plasma AII and renin production in normal pregnancy and are involved in regulation of vascular reactivity [66,67]. PGE_2 production is largely reduced in renal injury. It is also short-lived being rapidly deactivated by the lungs. Prostacyclin (PGI_2) is a more potent vasodilator and inhibitor of platelet aggregation than PGE_2 [72, 82]. Thromboxane (TxA_2) is a pro-aggregatory/vasoconstricting substance released from platelets, kidney, endometrium, trophoblast [524] and placenta [84]. Like prostacyclin (which is produced from the intact endothelium, trophoblast [524] and smooth muscle [84]) it is derived from arachidonic acid/cyclooxygenase metabolism. In normal pregnancy the balance of PGI_2:TxA_2 and lipid peroxide:vitamin E ratios in favour of PGI_2 and vitamin E which increases vasodilation with advancing pregnancy [85,86,523]. With PE and PIH the production of PGI_2 by the maternal vasculature and cord vessels is significantly reduced, particularly if the disease is severe [7,87,529], while TxA_2 biosynthesis can be normal or increased [72,67]. There is conflicting evidence about whether PGI_2 production is decreased or its degradation is increased [67,78]. Decreased levels [78] of urinary metabolites of PGI_2 have been reported. It is known that this shift in ratio leads to a more vasoconstrictive/pro-aggregation state and to increased vascular sensitivity in the maternal circulation. PE serum can stimulate human umbilical vein endothelial cells to produce more prostacyclin than serum from normotensive patients [89]. Intact umbilical arteries taken from pre-eclamptic patients have been shown to be unresponsive when stimulated to produce PGI_2 compared that those of normal samples. This data suggest endothelial damage and dysfunction since PE serum contains factors to promote PGI_2 synthesis but the responding cells are in reduced numbers [72,67]. Additionally, concentrations of von Willebrand's factor, plasminogen activator inhibitors and lipid peroxides are all increased in PE maternal serum [67]. Evidence of cytotoxic disorder has come from studies revealing that PE sera contain activated neutrophils [68] and from observations that endothelial changes are brought about by plasma-borne factors [69].

It has been postulated that endothelial damage leads to a reduction in circulating levels of PGI_2 [70]. It was therefore considered that low doses of aspirin could be used to inhibit the production of thromboxane while not affecting that of prostacyclin and therefore alleviate vascular resistance and pressor response to angiontensin II. Initial studies showed that low-

dose aspirin had a 'favourable' effect [71] on the vasculature, but larger studies failed to confirm this [66,72,73,74] and suggested an increased risk of abrupted placentae [75]. Comparison of the vasodilatory effect of PGI_2 and nitric oxide on endothelial intact human umbilical artery and vein revealed that a nitric oxide generator was five times more potent than prostacyclin [76, 77] and that PGI_2 synthesis was not involved in maintaining the physiological vasodilation of pregnancy in the rat [78].

PGI_2 is an important dilator of the uterine and renal vascular beds [79] while nitric oxide effects the umbilical placental circulation and systemic peripheral resistance [80,79] directly [81] by attenuating the action of vasoconstrictors [82]. In the foetal vasculature system, inhibition of the eicosanoids has little effect while inhibitors of nitric oxide cause significant increases in perfusion pressure of the placental suggesting a vital role for NO in the foetal vasculature [83]. Hence attention has now shifted to the investigation of nitric oxide function in normal and pre-eclamptic pregnancies.

Lipids

In normal pregnancy the total amount of maternal fatty acids increases significantly through pregnancy [525] as a result of hormone sensitive lipases [526] in the very low-density lipoprotein (VLDL) and low-density lipoprotein (LDL) fractions [527,528]. This produces hyperlipidemia in the late period of pregnancy [88]. Lipid peroxidation increases in pregnancy and more so in pre-eclampsia, attributed to increases from VLDL in normotensive serum and to an increase from HDL in pre-eclamptic serum [527]. This correlates with the increased levels of circulating triglycerides [527]. In normal pregnancy the maternal levels of arachidonic and docosahexaenoic acids both decline from 10-40 weeks onwards to term. The levels in the foetal circulation mirror the maternal lipid profile, containing lower total amounts of fatty acids (including linoleic acid) but greater levels of arachidonic and docosahexaenoic acids. This has been attributed to preferred transport and sequestration of these fatty acids by the developing foetus [97]. In pre-eclampsia there is a dramatic increase in the amount of free fatty acids and triglycerides (but not cholesterol) at 16-18 weeks before clinical symptoms become apparent [90,91,92,526,527,530], while there is a small increase in the VLDL triglycerides in PIH patients [93]. This alteration has been attributed to an impairment in the β-oxidation of fatty acids leading to greater circulating levels of triglycerides and VLDL [526]. Combined

with a reduction in maternal lipolysis regulated by TNF-α, IL-1, IL-6 (increased in PE sera), this leads to an overall increase in VLDL [526]. However there is an overall reduction in the concentrations of linoleic and linolenic (C18:3) acids with an increase in cervonic acid (C22:6). Serum triglycerides have been shown to correlate with the lipid peroxidation marker malondialdehyde in PE [91] which has led to suggestion of a connection between triglyceride levels and lipid peroxidation.

Interestingly the free unsaturated fatty acids, palmitic, oleic and linoleic acids have been shown to alter the expression of endothelial cell adhesion molecules. This may increase susceptibility to immune reactions [94]. Incubation of cells with these unsaturated acids and triglycerides [526] produces a dose-dependent reduction in PGI_2 and nitric oxide production in response to stimuli [92,95]. Prostacyclin has been shown to correlate with the essential fatty acids mead/arachidonic acid in plasma and tissue [96]. Significantly higher levels of mead acid in umbilical plasma suggest an overall improved fatty acid status of the neonate [97], even though neonatal essential fatty acid status is not negatively affected by pre-eclampsia [525]. Abnormally high levels of mead acid (C22:3) is regarded as a indicator of essential fatty acid deficiency [98,70,78b]. There is evidence of 6-desaturase deficiency in PE women [78b] where supplemention with linoleic acid in the third trimester has shown to be beneficial by partial restoration of prostanoid synthesis [99, 523]. Linoleic acid (9,11) increases through gestation and is significantly increased in PE compared to normotensive pregnant women, this difference increasing with the severity of the disease [100, 101]. Linoleic acid (9,11) is formed by free-radical transfer from normal linoleic acid (9,12) to proteins [100]. This is a possible predictor of the disease [101]. Arachidonic acid has been shown to induce relaxation of several vascular preparations including human placental vessels *in vitro* [102].

Lipid peroxides

In normal pregnancy states, levels of lipid peroxides are relatively low and are controlled by antioxidant inhibition of free radical propagation. If antioxidant levels decrease or lipid peroxidation increases this mechanism fails and excessive membrane damage/enzyme inhibition can occur. Investigations into the similar actions of lipid peroxides (LPOs) in pregnancy as a possible factor in PE has led to studies on the placental and maternal/foetal circulation levels and action. Walsh [103] reported that normal human placenta infused with t-butyl hydroperoxide stimulated endogenous cyclooxygenase-dependent production of lipid peroxides which were secreted primarily into the maternal circulation. PE placental tissue and trophoblasts produce abnormally high levels of lipid peroxides while their diene conjugates products return to normal after 24 hours of delivery [104]. These findings suggest that the placenta is the main source of lipid peroxidation in pregnancy. This is not surprising as this organ is highly susceptible to superoxide dependent peroxidation producing intermediate and end-products of lipid peroxidation [105]. In normal pregnancy lipid peroxides in the maternal circulation were reported as being relatively stable [86] throughout gestation. In a large study [106] higher variation in LPOs occurred from 5-21 weeks, with levels 1.6 times higher than non-pregnant subjects [106]. These levels may be a result of increased circulating lipids which decrease significantly after delivery of the placenta [107,108], further suggesting the placenta as the source of lipid peroxides in pregnancy. Levels of LPOs in cord blood are approximately 70% lower than in the maternal circulation [106,103] and the placenta has a natural ability to suppress lipid peroxidation in late gestation [106] . This is possibly because the cord circulation has a much lower oxidant activity [109] . It should be noted that the placenta is a major source of polyunsaturated lipids, making it highly susceptible to oxidation by free radicals.

Lipid peroxide levels are increased in mild and severe PE maternal sera [85,110, 527, 107, 111,112,91,113], umbilical cord blood [107] and placental tissue [114, 115] (as judged by malondialdehyde (MDA), thiobarbituric acid reactive substances (TBARS) and conjugated diene analysis) when compared to normal pregnant sera and tissue. MDA levels correlated positively with diastolic blood pressure and negatively correlated with vitamin E [110] and

foetal blood pH [116]. Whether these increased levels of MDA are proportional to the increase in circulating polyunsaturated fatty acids or to the increase in free radical activity does not seem to have been addressed. However, MDA levels have been shown to correlate with serum triglyceride and fatty acids [91], and triglycerides contain the major fraction of lipid peroxides in pregnancy [107]. It must be noted that most of these assays used TBARS and therefore are prone to overestimation as explained in chapter 3. These estimations may therefore reflect the relative amount of lipid peroxidation rather than absolute concentrations.

Vitamin E is a powerful antioxidant that donates hydrogen to form a relatively stable radical which stops free radical propagation. This is not the only radical scavenger but is one of the major markers of antioxidant status in humans. Vitamin E has been reported to increase in normal maternal blood as gestation progresses [86, 117]. It is decreased in PE [110], severe PE [118,85, 113], PIH serum [110] and PE/PIH placental tissue [122], especially in late gestation [123]. However not all studies have shown this [87]. These discrepancies may result from the ability to mobilise vitamin E in blood while membranes and other compartments show a reduction [113], possibly due to the poor membrane stability seen in PE [119, 120]. Ascorbic acid, another free radical scavenger, is also decreased in women with PE [118]. The enzymatic scavenger of lipid peroxides, glutathione peroxidase [121], has been reported to be significantly reduced, unchanged [87] or increased [113] . This enzyme is dependent on selenium levels which may account for this discrepancy [120]. A reduction in superoxide dismutase activity reported in PE was not statistically significant [119]. In the placenta both glutathione peroxidase and (red cell) superoxide dismutase activities are decreased [120]. These studies suggest that the overall loss of antioxidant activity could be due to over-production of free radical species and lipid peroxides. The stability of lipid peroxides is very important as most free radicals are very short-lived, although they can last upto 3 hours if bound to LDL [120].

Lipid peroxides damage endothelial cells by affecting proteins and lipids on the membrane surface and by oxidation of vitamin E, leading to increased membrane fluidity, permeability and low stability [120]. Oxidised LDL/VLDL's have been shown to stimulate expression of VECAM-1 on endothelial cells, a monocyte adhesion molecule which is increased in PE patients [526]. Neutrophil adhesion to endothelial cells is also activated by oxidised LDL, as is

activation of platelets [526]. This might be a cause of angiopathy through injury to the blood vessels, since cultured human umbilical vein endothelial cells exposed to 7.5nmol/ml of linoleic acid hydroperoxides revealed detachment of the cells, with deleterious effects seen even at 1nmol/ml [124]. PE is classically associated with necrotising arteriopathy which shares many features with coronary atherosclerosis. This suggests a strong connection between increased lipid peroxides levels and the necrosis seen in PE [526]. Experimental elevation of lipid peroxides in animals produces endothelial cell dysfunction and mirrors the effects observed in human PE [104].

Other effects of oxidised LDL's include vasoconstriction of vascular smooth muscle both directly [125, 120, 126] and by enhancement of agonist-induced vasoconstriction such as noradrenaline and serotonin [127, 104]. Saade [128] argued that oxidised lipids inhibit rather than antagonise contraction of the human umbilical artery through an effect on the calcium-dependent contractile mechanism. However this may be confined to the foetal vasculature which would be expected to maintain low vasculature resistance [129]. Observations by Davidge [130] offer some support to Saade's theory [128] with vitamin E deprivation leading to an increase in lipid peroxidation, enhanced endothelial dependent relaxation and suppression of arachidonic acid-induced constriction. This evidence, combined with the fact that PE sera activate over-production of PGI_2 in cultured endothelial cells [89, 131, 132] and cause less relaxation in myometrial arteries [69], suggests a two tier system where the low levels of lipid peroxides naturally seen in normal pregnancy stimulate PGI_2 production and cause smooth muscle relaxation. Over-production leads to damage of endothelial cells, reducing PGI_2 production and leading to the syndrome of PE.

Early studies suggested that serum from maternal and infant [133] PE cases contained substance(s) that were cytotoxic to cultured endothelial cells [134, 135], although this has been disputed [135, 136]. All these observations of endothelial damage [137], vasoconstriction [125] and neutrophil activation [526] have led to the conclusion that these cytoactive substances are included in the "factor X" of PE [120].

Nitric oxide

Animal studies for hypertension in pregnancy and the role of nitric oxide

Since it is unethical to perform invasive *in vivo* experiments in human pregnant subjects many of the effects and actions of nitric oxide in normal and hypertensive conditions have been determined through animal studies. These have shown strong evidence that chronic inhibition of NO synthesis produces PE like symptoms in pregnant rats [138, 65, 139, 140, 141], including dose-dependent increase in blood pressure [142, 65], proteinuria [65], thrombocytopenia [141], decrease in glomerular filtration rate [143], reduction in the normal volume expansion [65,143, 144], and increase in foetal/maternal morbidity and mortality [145, 139]. All these effects of NOS inhibitors were reversed when the animals were treated with L-arginine [146].

The levels of NO and metabolites in normal pregnancy have also been extensively studied in the rat model. Plasma and 24hr urinary excretion levels increase significantly over gestation and cannot be accounted for by dietary intake [147, 148]. This increase in urinary nitrate was shown to correlate with urinary cGMP levels, providing further evidence of an increase in tissue production of NO during pregnancy [65, 147, 149] and an involvement in vascular smooth muscle adaptation to pregnancy [522, 150]. In the rat model, NO and cGMP levels decreased during late gestation, returning to non-pregnant levels 6 days post-partum [147]. The changes in cGMP were attributed to changes in blood flow [151]. The increased immuno-suppressive response has been linked to the increased production of NO during the initial vasodilator changes in normal gestation [147]. *In vitro* studies have shown other roles of NO in the attenuation of pregnancy-associated resistance to the pressor action of vasoconstrictors [65, 152, 153], noradrenaline [155] , general vascular reactivity to angiotensin II [154, 153] and in human placenta [156] and the effects of acetylcholine [129, 65, 157]. The reason for the reduced basal release of NO at term in pregnant rats is unknown but is connected to the altered vascular smooth muscle function [65]. NO [158] and L-arginine infusion [159] in rats, rabbits, guinea pigs and humans has been shown to inhibit uterine contractility until term but not during delivery, suggesting that NO autocrine activity maintains low uterine activity and suppresses parturition until term [135, 139, 140, 160]. There are changes in the NOS isoforms in the cervix and uterus where iNOS expression increases up to labour then declines after term in normotensive rats, possibly under progesterone control [161, 150].

Analysis of guinea pig uterine artery, kidney, heart and other tissues have shown an increase in cNOS of both endothelial and neural origin in early and late pregnancy [65, 129]. This calcium-dependent isoform is believed to be regulated by oestrogen [157, 522] which parallels NO by modulating its transcription. NOS activity is also regulated by increased shear stress [80]. iNOS is highly expressed in rat peripheral placental layer, and this isoform is responsible for the gestational changes in NO production, exhibiting major down-regulation before term [135]. Further evidence of a higher activity of NOS in pregnant rats is revealed by L-arginine deficiency that progresses during pregnancy [162, 163], while chronic reduction in this substrate leads to low foetal birth weight and high perinatal mortality [65].

Human studies

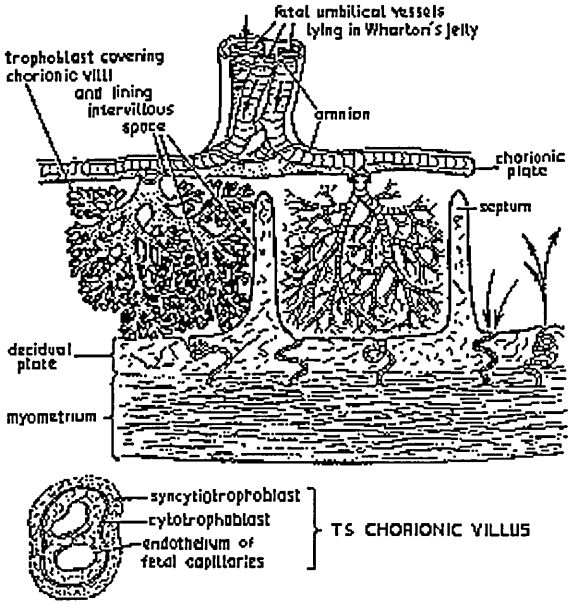


Figure 2.1 Simplified anatomy of the human placenta from reference [164].

The basic anatomy of the human placenta is shown in figure 2.1.

In human studies, eNOS has been isolated from placental extracts [522], villous vasculature [522,82], syncytiotrophoblasts [522] and uterus [150] while activity in the myometrium is low [150]. Immunocytochemical and mRNA techniques have detected eNOS in the umbilical artery

and vein [165, 166, 76], syncytiotrophoblasts [166, 167] and trophoblast plasma membrane [168], while it has a variable distribution in the chorionic arteries and veins [169]. It was not detected in cytotrophoblasts [167] or small foetal vessels [166]. This may be due to the lack of smooth muscle to act upon in these vessels [170]. It is believed that the uterus and syncytiotrophoblasts produce NO as a paracrine substance acting on the blood vessels of the myometrium [150], chorionic plate arteries [171,80] and the EG terminal villi (small feto-placental vessels) [166, 65]. Another possible role of high NOS activity in the syncytiotrophoblast is to reduce platelet pro-aggregatory activity which may be involved in the reduction of an immune response to the placenta [83, 170]. Most of the activity of NOS is seen in the placental villous vasculature, with a low activity in the placental bed [83].

There is conflicting evidence on the isoforms present in placental tissue. Garvey [165] and Kristoffersen [168] have detected mRNA from eNOS but not nNOS or iNOS. Gude [172] found that most but not all of the activity was absent when a cNOS inhibitor was used, suggesting the possibility of a small amount of iNOS activity. In the studies discussed above placentae were taken from normal term pregnancies.

If the animal model studies are representative of the human situation then the alteration in NO over gestation could be due to altered NOS composition. Human umbilical cords from 8-17 and 38-41 weeks have shown little evidence of NOS activity although cGMP has been shown to increase and be maintained after 18-21 weeks by NO stimulation [173]. nNOS was not present but there was a detectable amount of eNOS in arteries and slightly more in the veins [174]. Discrepancies may be due to differing methodologies or to differences between *in vivo* and *in vitro* studies. In placental homogenates from weeks 8-10 the cNOS and iNOS isoforms are present in the ratios of 90-95% and 5-10% respectively [175].

NOS activity [177,176] and cGMP [176] levels are highest in early gestation but decrease after 12-14 weeks. NOS activity decreases with placental bed impedance providing further evidence of NO's role in vascular relaxation and modulation of angiogenesis, possibly regulated by shear stress [82, 176]. There is no change in NOS activity in the human amniotic and placental tissue prior, during or after labour [135, 178], although activity in the myometrium was not examined in this study. Izumi [179] showed that the amount of NO and the sensitivity of smooth muscle both decrease with advancing gestation while the L-arginine-NO-cGMP pathway regulates uterine contractility throughout gestation [180].

NO regulation in the foetal circulation

On the foetal side of this circulation there is a low impedance system which is controlled by vasoactive substances. NOS inhibitors are associated with an increase feto-placental vascular resistance [180] while NO-generators are associated with reduced umbilical artery blood flow in humans and sheep [180]. Although the umbilical cord vessels produce NO they are resistant to the effect of agonists such as acetylcholine, bradykinin, histamine, ATP, ADP, substance P and A-23187. These vessels relax in response to nitroso-vasodilators suggesting a paracrine function downstream to maintain low vascular resistance [129]. Studies on full term placentae have shown that sodium nitrite and sodium nitroprusside [181] reduce feto-placental vascular resistance by vasodilation of the vascular smooth muscle interaction [182, 83]. This has led to speculation that foetal NO production is controlled not by agonists but by shear stress or transmural pressure on vessel walls [80].

These observations suggest that NO has an important function in the maintenance of low basal vascular tone and the attenuation of the actions of several vasoconstrictors [82, 183] throughout early and mid [77] pregnancy in both the maternal and foetal circulation [170]. The marked increase in total placental NOS activity in early pregnancy from eNOS and iNOS decreases throughout gestation leading to lower activity, from eNOS alone, closer to term [184].

Nitric oxide and pre-eclampsia

Evidence supporting a role for nitric oxide in the basal homeostasis of the foetal-placental circulation in normal pregnancy has been presented. Its contribution to the pathogenesis of pre-eclampsia is now reviewed.

McCarthy [185] found that the effect of acetylcholine on endothelium dependent relaxation was reduced in PE. Evidence linking NO to this reduction was provided by Pinto [186] and Knock [187]. They employed a bioassay to show that human umbilical artery (HUA) produced 80-90% less NO when stimulated by bradykinin compared with normotensive HUA; this deficiency was not overcome by infusion of L-arginine, excess bradykinin or shear stress [79]. The lack of response to stimulus was also observed in the umbilical artery but not vein (Akar et al [188]). Rutherford [189] has shown that binding of radio-labelled ([^{3}H]-L-NOARG) substrate is decreased in pregnancies complicated by PE and IUGR (intra-uterine growth retardation). Orpana [190] showed that total nitric oxide production in umbilical vein endothelial cells was lower in PE compared to normal pregnancy. Pinto [186] concluded that there was a reduced or altered endothelial cell number, while the cytotoxic nature of PE sera adds support to the concept of "factor X" leading to the clinical symptoms of PE through endothelial dysfunction and consequent decreases in PGI_2, NO and other vasoactive substances.

Typing of NOS isoforms from PE, IUGR and normotensive placentae has produced conflicting results for the role of NOS in PE and normal pregnancies. Morris [191] reported that NOS activities from placental villous homogenates were significantly reduced in PE and IUGR placentae compared normotensives, while there was no difference in NOS activities from umbilical arteries or veins [173,191, 190]. This was confirmed by Ghabour [192], Di Lorio [193] and Sooranna [177] who found PE-related differences in NOS expression only in the placental villous vessels and syncytiotrophoblastic layer. Brennecke [194] also observed a reduced NOS activity in PE human villous tissue, with a decrease in V_{max} but not K_m attributed to reduced NOS activity. There was no change in the ratio of cNOS/iNOS as iNOS has a higher K_m than cNOS; this reduced activity may have been promoted by foetal hypoxia [192].

These, and other studies [195] have shown that the syncytiotrophoblast layer contains eNOS which is diffuse and located at the top of this layer in normal pregnancy whereas in the PE syncytiotrophoblast layer, eNOS is more intense and is in the basal region. Myatt [196] also found iNOS in the villous tissue using immunostaining but it was observed in both PE and normal placenta. However, the eNOS isoform investigated by the same group [195] showed expression in villous vessels that was much greater in the PE group than normal subjects. Their data suggest that increased iNOS expression is not due to an immunological factor but possibly another stimulator of eNOS expression such as shear stress. This may also be evident in the maternal system since arteries from normal pregnant women show greater relaxation to shear stress compared to PE arteries [197].

Conrad [198] produced conflicting results showing that the activity of NOS in the villous, basal plate, the total NOS K_m/V_{max} and the cNOS/iNOS ratio is not different in PE, PIH or normal placentae. However, they suggested that other tissues may show a difference in NOS activity. Wang [199] confirmed these results by showing that there was no difference in the amount of nitrite produced from incubated whole placental villi between PE and normal groups.

A potential problem with all the placental studies is the gestational time that the tissue was taken. All those discussed used term placentae. Since the majority of studies looking at the effect of normal gestation NOS expression/activity have indicated a change in these factors over pregnancy this may account for the differences in the results.

Studies of enzyme activity through human gestation are not feasible. Thus, several studies have used less-invasive assays such as the measurement of stable end products of nitric oxide; nitrite, nitrate as markers of NO activity in serum/plasma and urine. The current information is displayed in table 2.1

Author	Sample	n	NO_x µmol L^{-1} Normal	n	NO_x µmol L^{-1} PE	Signifi-cance
Di Iorio [193] *	Maternal	27	~ 11 (6-29)	26	~ 11 (6-25)	ns
	Mixed Cord blood		~ 12 (6-37)		~ 9 (6-31)	ns
	Urine (24hrs)		~ 2 (2-11)		~ 3 (2-6)	ns
	Amniotic	27	5.6 (2-25) µmol/mg creatinine	31	10.3±4-43 µmol /mg creatinine	P<0.001
Davidge [200] *	Maternal	20	~ 30 (10-42)	14	~ 31 (14-59)	ns
	Random urine		~ 0.7 (0.2-1.3) µmol/mg creatinine		~ 0.3 (0.2-0.9) µmol/mg creatinine	P<0.05
Smarason [201] **	Maternal	20	31.2±9.1	20	47.4±13.6	P<0.05
Silver [202] **	Maternal	35	0.98±0.088	34	1.01±0.041	ns
Curtis [64] *,***	Maternal non-pregnant (NP)	14	105.04±15	6		
	1st trimester		96.47±16			
	2nd trimester		88.28±12			
	3rd trimester		98.91±15		107.44±16	ns
Seligman [203] *	Maternal	26	27.38±2.23	26	20.04±1.25	P<0.02
Garmenda [204] *	Maternal	20	~25±5	33	~18±5 Mild PE	P<0.005
					~23±6 Severe PE	P<0.05
Nobunaga [205] *	Maternal NP	37	18.3±1.0			
	Maternal		30.3±1.0	38	45.6±2.3	P<0.0001
					19.1±3.0 PIH	P<0.005
Lyall [63] *	Maternal	36	29.8±1.07	32	29.5±1.06	ns
	Cord vein		34.59±1.12		23.90±1.05	P<0.01

Author	Sample	n	NO_x µmol L^{-1} Normal	n	NO_x µmol L^{-1} PE	Signifi-cance
Cameron [206] **	Random urine	12	28 (5-63) mmol/mmol creatinine	13	38 (0.3-112) mmol/mmol creatinine	ns
Brown [207] *	Urine (24hr) NP	56	982 (763-1534) µM/day			
			980 (673-1274) µM/day	30	912 (684-1307) µM/day Mild PE	ns
					989 (689-1204)µM/day Severe PE	ns
Begum [208] *	Random urine	24	1501±823 nmol/min	12	872.59±523.35 nmol/min	??
Begum [209] *	Urine 1st trimester	30	610±409 nmol/min	19		
	2nd trimester		963±385 nmol/min			
	3rd trimester		1001±417 nmol/min		912±357 nmol/min	P<0.04
Conrad [210] *	24hr urine NP	17	551 µmol/24hrs	6		
	1st trimester		371 µmol/24hrs			P<0.05
	2nd trimester		526 µmol/24hrs			ns
	3rd trimester		610 µmol/24hrs		451 µmol/24hrs	P<0.05
	Plasma NP		35 µmol			ns
	1st trimester		30 µmol			ns
	2nd trimester		33 µmol			ns
	3rd trimester		32 µmol		36 µmol	ns

Table 2.1 Summary of currently published research on serum, cord and urinary NO_x in normal pregnancy and pre-eclampsia.

*-Griess assay (after reduction of nitrate), **-chemiluminescence, ***-ion chromatography, ns-not significant, NP-non-pregnant, concentrations quoted with standard deviation or range depending on data from reference.

Note that all the comparisons made in table 2.1 between the gestation matched normotensive and PE subjects were performed during the third trimester.

Di Iorio [193], Davidge [200], Silver [202], Curtis [64], Lyall [63] and Conrad [210] did not find any difference in serum NOx levels between pre-eclamptic and normotensive patients. However Smarason [201] and Norbunga [205] reported an increase while conversely Seligman [203] and Garmenda [204] observed a decrease in serum NOx levels between the two groups. With respect to urine studies Di Iorio [193], Cameron [206] and Brown [207] all noticed no difference in urinary excretion of nitrate between the diseased and normal group although Davidge [200], Begum [208], Begum [209] and Conrad [210] observed a statistically significant decrease in NOx values.

From the results of Curtis [64] and Davidge [200] it would appear that the normal changes in NOS activity over gestation are not reflected in the circulating levels of the NO metabolites. However, Garmendia [204] reported a negative correlation between gestation and nitrite/nitrate levels in normal but not hypertensive pregnancies. Inspection of reported nitrate/nitrate levels in maternal and foetal cord blood reveals major differences ranging from a increase, decrease or no change in PE patients. The 24hr urine samples are less prone to variation and are seen as a much more stable detector of metabolism, although care must be taken to avoid bacterial contamination. Davidge [211] suggested that urine studies only should be used because NO production is best indicated by a 'steady-state' excretion, rather than that in blood in subjects with abnormal renal function. Begum [208] reported a progressive increase in the excretion of nitrite/nitrate that was significantly reduced in random pre-eclamptic urine [208, 200]. The evidence of an reduction of 24 hour NO excretion was not observed by Brown [207], Di Iorio [193], Conrad [210] or in a random urine test [206]. Brown [72, 207] stated that normal subjects eating a 'uncontrolled' diet can have nitrate levels that vary by 32-56% in four days. Thus a change of greater than 50% was seen as significant, but this did not occur even when corrected for with creatinine. Interestingly Cameron [206], Smarason [201], Seligman [203], Garmendia [204] and Begum [209] all saw a significant correlation between blood/urinary NOx and systolic pressure change although Davidge [200] and Silver [202] did not observe any correlation.

It is difficult to derive a definitive conclusion(s) from the summary in table 2.1. There is no consensus on whether nitric oxide production is altered or not in PE maternal blood and what (if any) a normal range could be. Three different techniques have been used to determine nitrite and nitrate levels, the problems associated with each of them are discussed in Chapter 3. In summary, the Griess assay encounters problems with the chemical/enzymatic reduction of nitrate to nitrite while also suffering from interfering substances, especially in urine [279]. The chemiluminescence assay usually requires a separation step to remove interference from thiols, nitrosothiols and nitrating agents and other ions (Chapter 3). None of the studies cited in table 2.1 using this technique performed any separation. In addition to difficulties with assay techniques many of these studies have statistical limitations because of small sample numbers ranging from 6-32 in the disease group(s). Disease severity was not always considered. Only Garmenda [204] and Seligman [203] separated the mild from the severe pre-eclamptics. Other factors such as influence from dietary intake, cigarette smoking, alcohol consumption, atmospheric pollution and exercise could also contribute to variations in NO levels [65] and NOS activity [177]. These inter-subject variations cannot be eliminated as the use of low nitrate-diet for pregnant women is not ethically acceptable. The next best approach is to employ a longitudinal study on selected subjects so that inter-/intra-personal variation can be seen as well as any differences in nitric oxide synthesis through pregnancy between normal and pre-eclamptic groups.

Another indirect marker of NO activity is cGMP. NO acts by activation of guanylate cyclase to produce cGMP. Studies in PE patients have shown conflicting results in the circulating levels of this metabolite; Schneider [212] reported on an increased in maternal serum while Lopez-Jaramillo [213] detected a decrease in plasma and urinary cGMP levels. Kovacs [214] reported reduced cGMP levels in umbilical venous plasma while Barton [215] and Begum [208] saw no change in urinary excretion between the normal and PE groups, but not during PE pregnancies [209].

Nitrotyrosine is produced from the reaction of the potent oxidant peroxynitrite with tyrosine and can be used as a marker for peroxynitrite (chapters 1&4). In pregnant rats fed

with a combination of L-NAME and LPS (iv), nitrotyrosine immunoreactivity was detected in the uterus but not in the placenta and was below detectable levels in control rats (not fed L-NAME or LPS (iv)) [216]. In the human placentae superoxide dismutase scavenges O_2^-, preventing the formation of peroxynitrite. There was no difference in the distribution of activity of this enzyme in normal and PE placenta implicating its importance in reducing damaging oxidants [217]. Faint immunostaining for nitrotyrosine was seen in normal placental tissues while in IUGR or PE this immunostaining was moderate to intense in the villous vascular endothelium, the surrounding vascular smooth muscle and the villous stroma [218] compared with controls.

These data suggest that basal and stimulated NO levels in the fetoplacental circulation are impaired during PE [129]. The beneficial effects of nitric oxide donors as therapeutic agents in PE have been investigated by several groups. The results show that there is reduced maternal arterial pressure [219,176], platelet activity [220] and uterine artery resistance [220, 221, 222] without compromise of foetal Doppler flow [220]. However, circulatory distress and paradoxical bradycardia can develop in PE patients who have not been pre-treated with plasma volume expansion agents [180] and side effects such as headaches are common [221]. Another possible role of NO-generating drugs is in the onset of parturition by initiation of myometrial contraction [223].

Another factor that affects NO activity is the amount of circulating endogenous inhibitors. Thus N^G-monomethyl-L-arginine (L-NMMA) and N^GN^G-dimethylarginine (asymmetric dimethylarginine) (ADMA) have been detected in human plasma and urine [224, 225]. In PE there is a significant increase in the amount of ADMA (which is found at ten times the concentration of L-NMMA [226] compared with normotensive patients) but in PIH patients there were no differences [226, 227]. In normal pregnancy there are significant increases in the plasma concentrations of these inhibitors through gestation [226] but these concentrations are much lower than in non-pregnant controls. King [228] has shown that the possibility of higher ADMA levels detrimentally affecting the placenta is unlikely as they have shown that removal of the substance did not affect the reduced placental NOS activity.

Plasma from PE women stimulates prostacyclin and nitric oxide production and activity [229] in cultured endothelial cells. The activator for prostacyclin is non-lipid while the activator for NO synthesis is found in the lipid fraction possibly a lipoprotein or hydroperoxy lipid [230]. In normal plasma, shear stress increased this activation to levels near those of non-shear stressed PE plasma, indicating the possible role of increased luminal shear stress in the aetiology of pre-eclampsia.

Overall, there are good theoretical reasons why NO, a potent vasodilator should play a pivotal role in the pathogenesis of PE. *In vivo* studies have however, clearly produced conflicting results. Hence, the aim of the work described in this thesis was to develop a novel and more reliable method of assay than was previously available and study "pure" groups i.e. normal pregnancy compared with pre-eclampsia and PIH. Due to the low prevalence of the disorder and the retrospective diagnosis of pre-eclampsia, screening would require a large sample population. In these studies both cross-sectional and longitudinal data have been collected.

Chapter 3

Analytical methods in nitric oxide research

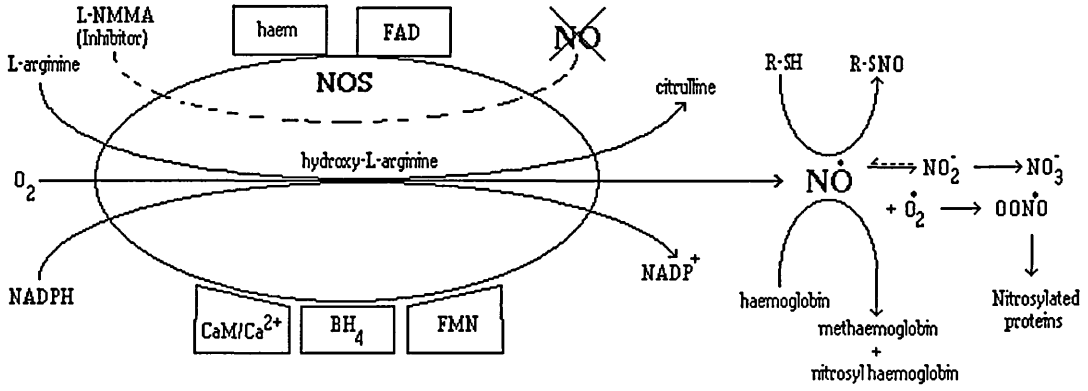


Figure 3.1 Enzymatic production and metabolism of nitric oxide.

Because of the short half life of NO in aqueous media, analytical studies on nitric oxide in biomedical research have concentrated on its known degradation products. These may arise by reaction with thiols, haemoglobin or superoxide or, more commonly by oxidation to nitrite and nitrate. Alternatively, changes in concentration of its precursors can be measured though these are of less use in whole body studies as they are not exclusive to NO generation.

Arginine, citrulline, inhibitors and enzyme activity

Methods analysing citrulline [231,232,233] and $NADP^+$ [234] are frequently used in tissue studies where the nitric oxide synthase kinetics and activity are of interest. With the citrulline assay, cells or tissue can be spiked with L-[^{14}C]arginine or L-[^{3}H]arginine (plus cofactors), which are converted into [^{14}C]citrulline or [^{3}H]citrulline. After the reaction is terminated these products are separated using a Dowex AG 50W-X8 column and the radioactivity counted [233]. If [^{15}N]arginine is used as a stable isotope then the products are separated and quantified by GC-MS [231] or electrospray LC-MS [235]. By measuring the amount of product

formed from the original radio-labelled precursor it is possible to calculate the rate of the reaction, which is a function of the activity of the enzyme. Problems associated with this assay include i) the localisation of the technique which would only take into account the NO production in a relatively small number of cells; ii) interference from the ornithine cycle [236], as citrulline can be recycled back to arginine by most cells [237]; and iii) residual endogenous L-arginine could compete within the times of incubation for labelled arginine thereby reducing the sensitivity of the assay. The requirement for biological cofactors and radio-labelled substrate makes this procedure very expensive and more applicable to research than routine laboratories.

Antibodies have been used to detect different NOS isoforms [237], including the proliferation of NOS [238,239] with nitrotyrosine [237,240] location in tissue preparations.

Diaphorase enzymes can accept electrons from donors such as reduced NADPH or NADH and then reduce soluble nitroblue tetrazolium into an insoluble diformazan dye. This is an inexpensive histochemical reagent for the study of NOS activity in tissues and cells [241, 242] which can be made more selective for NOS by use of special fixation procedures [243,244]. Care must, however, be taken as not all diaphorase activity is exclusive to NOS action [237].

Electron spin resonance

ESR (electron spin resonance) spectroscopy was first used to study the mechanism of NO trapped in a haem-protein complex to form a nitrosyl-haem-protein. This has great importance since NO elicits most of its effect through its interaction with iron containing proteins. Applications have included the detection of nitrosylhaemoglobin, nitrosylmyoglobin and nitrosyl derivatives of the cytochrome system [245,246]. These nitrosyl haem proteins are paramagnetic [247] and are usually determined at low temperature to improve signal to noise ratio [248]. Care must also be taken to avoid metallic contamination (especially by copper) [249] causing artifactual signals and oxygen [250] reducing the paramagnetic signal. Examples of biological applications of ESR include the detection of NO in ischaemic plasma [251], rat neurones [252] and in other biological fluids [253,254]. A detection limit of 1nmol L^{-1} is possible with this technique [249].

Nitroso/methaemoglobin

In venous blood nitric oxide is converted into nitrosohaemoglobin and small amounts of nitrite are produced. However in arterial blood NO is almost completely converted into nitrate and methaemoglobin [256,257]. The production of these haemoglobin derivatives can be monitored by the ESR techniques [257] discussed above or spectrophotometically. This reaction occurs with a rate constant of 3.7×10^7 M^{-1} s^{-1} which is 26 times faster than autooxidation of NO in aqueous solutions [258] with a turnover time of less than 100ms [259]. In this assay cells or tissue are incubated with pre-oxidised haemoglobin. The methaemoglobin formed is characterised by the shift in the Soret (γ) absorbance peak of haemoglobin from 433nm to 406nm or by the two weaker bands at 542nm (β) and 577nm (α) bands which are pH dependent [260,261]. Exploitation of other bands and dual wavelength detection have resulted in greater sensitivity, with a limit of detection as low as 1 nmol L^{-1} [259].

The haemoglobin concentration used in the assay has to be sufficient to trap all the NO produced, but increasing the haemoglobin also increases the background interference levels and therefore reduces sensitivity [260]. This assay does suffer from a lack of specificity as peroxynitrite also reacts with oxyhaemoglobin producing methaemoglobin. Interference is also expected from other redox active compounds. Oxidants such as hydrogen peroxide [262] and transition metals [263] are capable of producing methaemoglobin from oxyhaemoglobin, while reductants such as superoxide can convert methaemoglobin to deoxyhaemoglobin or oxyhaemoglobin [264]. Interference can also occur from L-arginine and L-NMMA [265].
It is not known whether free thiols interfere with methaemoglobin formation by NO scavenging from nitrosothiol production, but this is considered not to be a major problem as the rate of this reaction is slower than that of oxyhaemoglobin conversion [260].

Electrochemical probes

These probes have been developed to detect the release of NO from cultured cells, tissues and blood samples directly. Amperometric probes are a variation on the Clark-type electrode and more recently polymeric porphyrinic sensors have been developed. Clark-type electrodes incorporate a gas selective layer which allows NO to pass through to the working

electrode where it is oxidised resulting in a electrical current that is proportional to the concentration of NO in the sample. This reaction is dependent on pH, oxidation potential and diffusion rate through the membrane [97].

A porphyrinic sensor consists of a small needle of carbon fibre covered with a conductive selective polymeric porphyrin coating and an outer membrane that consists of a cation exchanger such as Nafion. NO is oxidised to NO^+ at the electrode surface then converted to nitrite in the presence of hydroxyl ions and repelled by the membrane into the outer solution. A ion selective membrane is used as there is only a 60-80mV difference in electrochemical oxidation potential between nitrite and nitric oxide [97,266,267] so nitrite has to be excluded to avoid interference. These porphyrinic sensors are more sensitive that the earlier Clark-type electrodes and have a faster response time, require a lower potential for NO oxidation, do not suffer from stability shifts in the counter-electrode potential and are not affected by oxygen reactions with NO [97,266]. Clark-type electrodes have been used to measure release of NO from nitrosothiols in the presence of copper ions [268] and to detect NO release from cultured cells [269]. They have been developed commercially by World Precision Instruments (Iso-NO, IsoNO30, IsoNO200), Intermedical Ltd (NO-501) and BIO-LOGICAL (EMS-100) as macro and microelectrodes. Porphyrinic sensors have been developed to investigate NO release from nitrosothiol standards [270,271,272], stimulated cultured cells [270,271,273,97,266], tissue samples [97,266], blood and for *in vivo* measurements [97] of up to 10.5 ± 2.4^{-18} M/platelet of NO [2].

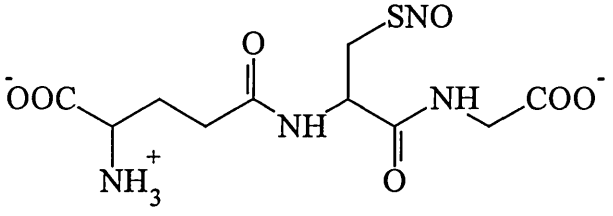
Chemiluminescence

Nitrite and nitrate can be determined by reduction to nitric oxide and analysis by chemiluminescence, first performed in biological samples by Cox [274,275]. The sealed sample contains a weak reductant for nitrite analysis, or a strong reductant to enable both nitrate and nitrite determination. In the acidic environment the NO formed is carried by a helium gas stream into a chemiluminescence detector. There the NO can react with ozone to produce oxygen and nitrogen dioxide in an excited state. This then decays to nitrogen dioxide in the ground state with emission of light in a continuum from 600-3000nm . The emission intensity is proportional to the original concentration of NO. This assay has a sensitivity down to 50ng/L and has accuracy greater than that of the Griess assay for

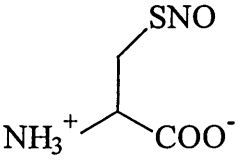
aqueous [275] and blood/urine samples [274]. The signal produced from biological samples may be subject to interference from other nitroso compounds such as nitrosothiols, either from overestimation by RSNO reduction to NO [276,277,278] or underestimation from sequestration of the NO produced from nitrite or nitrate reduction by thiols [279]. Other problems with this assay are associated with the breakdown of other compounds in complex samples by the nitrous acid intermediate to form other nitrososating species [280]. This is exacerbated if protein removal is not performed, resulting in a reduction of the assay precision [274,281]. High sodium chloride and Fe^{2+} concentrations can reduce the nitrite signal in the presence of the reductant [279, 261]. The presence of dimethylsulphoxide may also lead to a false positive results [261]. To date only one method by Sen [282] has reported selective determination of nitrite in biological samples, by coupling a reversed phase HPLC system employed a buffered solvent-free mobile phase to the chemiluminescence system discussed above. This yields a detection limits of 0.02 μmol L^{-1}.

Nitrosothiols

Any compound containing a free thiol group has the potential to produce a nitrosothiol. These are formed from thiols in the presence of N_2O_4 [283], nitrite [284] and NO [285] under appropriate reaction conditions. Two common biological molecules that have this ability are cysteine forming S-nitrosocysteine (SNC) and glutathione, forming S-nitrosoglutathione (GSNO). Proteins such as serum albumin may also form S-nitroso derivatives through their subunits.



S-Nitrosogluathione



S-Nitrosocysteine

Fig 3.2 Structure of two biologically active nitrosothiols

GSNO has greater stability than SNC thermally and at neutral/basic pH [276,286,287]. In serum, reducing the plasma pH to 6 and cooling to 4°C prevents nitrosothiol decomposition [288].

The current methods used for determining nitrosothiols fall into two categories. These are either the direct determination of the nitrosothiol or indirect detection where NO is cleaved from the stable thiol; the difference from basal to cleaved NO yielding the amount of nitrosothiol originally present.

Indirect determination

In 1963 Bell [289] produced a simple acid/alkali method for the cleavage of NO. The NO was then determined by its oxidation product nitrite using the colorimetric Griess assay. Most other methods have also used this procedure in the presence of a free transition metal ion such as mercury [289,290], copper [290,291] or iron [290] , with mercury producing the greatest release [293]. The effect of transition metals is removed by metal chelator [276]. Instead of using an electron donor, thermolysis [292] and ultra-violet light have been used for photolytic cleavage of the nitrosothiol [276,33,294].

$$RSNO \xrightarrow[h\nu \text{ or } X^{2+}]{} NO \xrightarrow[\text{in solution}]{\text{oxidation}} HNO_2 \longrightarrow H^+ + NO_2^-$$

From this reaction it can be seen that the determination of nitrite should give an indirect measurement of the concentration of nitrosothiol in the original sample.

Nitrosothiol determination in serum [292,295] has been achieved by modification of the 2,3 diaminonaphthalene (DAN) methods of Misko [296]. In this method aliquots of 2mls of sample/standard were reacted with acidified DAN and mercuric chloride to measure the nitrite that was the oxidation product from the nitric oxide cleaved from the nitrosothiols. After incubation for 10 minutes at room temperature the fluorescent product 1-[H]-napthotriazole (formed from nitrite and DAN) was made alkaline to enhance the fluorescence signal. Since endogenous nitrite would also react, neutral pH conditions were

also used to avoid effect of nitrite [293]. Alternatively the same sample was treated with EDTA and mercuric chloride instead to give the non-cleaved content. This response was then subtracted from the previous so each sample has its own blank. This assay showed linearity down to 12.5nM for SNC, GSNO and SNBSA (derivatised bovine serum albumin) standards. Problems with this assay include concentration-dependent inhibition of the signal in the presence of ascorbic acid [293,295], dithiothreitol [293,295] , mercaptoethanol [293] and other thiols [293,295,296]. This effect was also observed with the spectrophotometric Griess assay as these compounds scavenge NO/O_2 [293] .

By following these methods as described we confirmed the increase in nitrite releases from normal and spiked sera treated with mercuric chloride/DAN at neutral [293] and acid [295] pH. However we also found an inherent lack of sensitivity with SNC spiked serum samples at the normal concentration. This could have arisen from the low stability of the SNC nitrosothiol used, the effect of ascorbic acid present in serum and/or the activity of other thiols. This lack of sensitivity (limit of detection of 150nmol L^{-1}) has also been seen by Marzinzig [297] who cautioned researchers on the routine use of this assay due to varying RSNO stability and other assay factors that affect the analysis.

An alternative method of detection that avoids the interferences is chemiluminesence [292,298], when prior separation techniques such as HPLC are employed. Goldman [288] applied the Griess reaction to nitrite cleaved from plasma nitrosothiols with mercuric chloride. The chromagen was separated from interfering substances by HPLC and a limit of detection of 100 nmol L^{-1}, was reported. However there would have been interference from endogenous compounds as discussed and this was not considered by this group.

More sensitive methods using photolysis-chemiluminescence have produced limits of detection down to 1 nmol L^{-1} [276,298]. Comparison of the DAN fluorometric method and photolysis-chemiluminescence has shown good correlation with (enzymatically reduced) whole blood with NO-containing compounds [292].

The normal plasma concentration of nitrosothiols have been determined as 0.22±0.19 μmol L^{-1} [288] (HPLC+DAN), ~7 μmol L^{-1} [33] (photolysis + chemiluminesence) and below 2 μmol L^{-1} [292] (DAN). It should be noted that many of these assays cleave NO not just from

nitrosothiols but also from nitro-proteins and nitrosyl-complexes which may lead to overestimation of nitrosothiol content.

Direct measurement

The transfer of nitroso groups from one thiol to another has been used in determining activity and kinetics of nitrosothiols [299,295,294]. A GC-MS method by Tsikas [300] has been successfully used for the determination of SNC in plasma and urine. HPLC analysis of SNC and SNBSA nitrosothiols in rabbit serum has been accomplished by HPLC separation with detection by a mercury electrochemical detector that was within the nmol L^{-1} range [298]. Capillary zone electrophoresis methods have been published separating standards of thiols and nitrosothiols under acidic conditions [298,301]. While this method did produce good separation of standards the nitroso-compounds were undetectable in normal serum by this method. There has been no direct method developed for the determination of nitrosothiols in human blood. Sensitivity would be a problem where low volumes of sample are employed as detection of nitrosothiols (expected to be less than 2 μmol L^{-1}) by UV/Vis spectroscopy was not achieved by this method [298].

Peroxynitrite

The interaction of nitric oxide and superoxide produces a short lived, highly reactive radical capable of nitrating LDL, lipids, antioxidant, metalloproteins and thiols [30]. Its biochemical and analytical properties are discussed in chapters 1 and 6 respectively.

Nitrite/nitrate

The Griess-Llosvay [302,303] method is the most popular technique for nitrite determination. This apparently simple method involves the diazotization of nitrite with sulphanilamide to form a diazonium compound which then couples to napthylethylenediamine dihydrochloride under acidic conditions to form an azo dye. The pink coloured product is quantified by measuring light absorption at or approximately 540nm (fig.3.3).

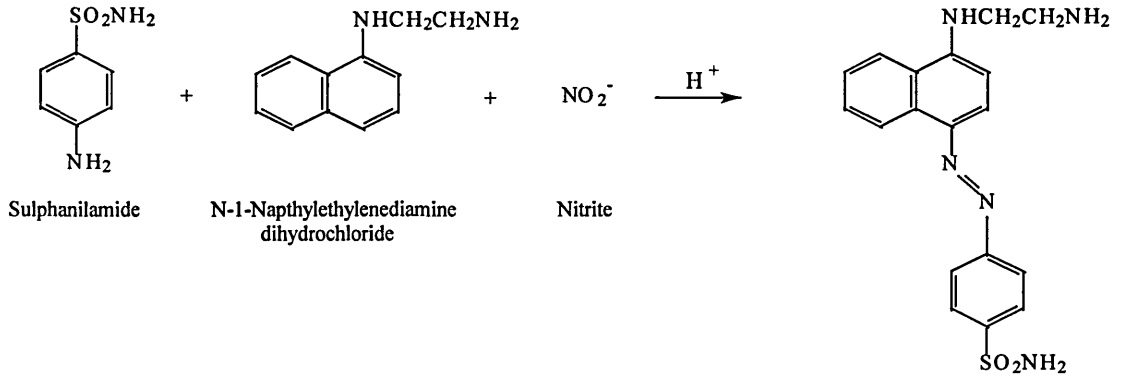


Fig.3.3 Reaction of nitrite with the Griess reagent to form a coloured product

The Griess reagent is formed by mixing equal volumes of stock A (1% sulphanilamide in 5% concentrated phosphoric acid) and stock B (0.1% napthylethylenediamine dihydrochloride) with the samples; this being left for 15 minutes in a dark cupboard to allow the colour to develop.

Problems with this assay are that any oxidiser or reducer of nitrite or the dye, (e.g. ascorbic acid [304] or formate [305]) can reduce the dye and hence lower the colour intensity [19]. The colour and turbidity of the sample may also affect the assay [19]. However, centrifuging the sample through a membrane filter removes the coloration and turbidity from large interfering molecules and improves quantification [306]. Any appreciable quantities of copper or zinc in the sample will catalyse the decomposition of the diazonium salt and bring about a reduction in the colour intensity [307,306]. For the reaction in fig 3.3 to proceed the aqueous environment needs to be acidic which has caused concern to some authors as this does favour the formation of nitrosothiols in serum samples and may therefore lead to underestimation of nitrite in biological samples [308]. This reaction is dependent on oxygen

concentration and sulphanilamide/napthylethylenediamine dihydrochloride ratio [19]. 2,3 Diaminonaphthalene may also react with nitrite in acid solution to form 1(H)-naphthotriazole, a fluorescent compound which can be detected with a sensitivity 50-100 times greater sensitive than UV absorption with the Griess reagent [296]. This alternative reagent results in a limit of detection of 10nM.

However it has been shown that serum samples left at room temperature for more than 4 hours will show almost complete oxidation of nitrite to nitrate [19], therefore for reliable results nitrate levels must also be quantified.

To quantify nitrate in the same matrix it is necessary to chemically or enzymatically reduce it to nitrite before the derivatisation. Chemical reduction is conventionally performed with copperised cadmium. The recovery of NO_3^- from spiked serum and water is almost 100%. [212,309,310], but we and other authors [297,311] have encountered major difficulties with poor response and reproducibility with this type of reduction in biological samples. This may be attributed to the further reduction of nitrate beyond nitrite [297,312, 313] or loss of the reducing capacity over time [311]. Nitro compounds such as nitro-L-arginine and nitro-L-arginine methyl ester have been shown to be reduced by cadmium to form products that react with the Griess reagent to give false positive results [311, 314]. If the column and conditions are correctly optimised this method is reportedly very useful [312]. However, this method of reduction is less desirable than the enzymatic reduction as it is time-consuming and can expose the operator to hazardous material (cadmium).

In enzymatic reduction, nitrate reductase is added to the matrix along with NADPH as a reductant to complete the reaction. However NADPH interferes with the Griess assay and has to be removed subsequently with lactate dehydrogenase and pyruvate or glucose-6-phosphate dehydrogenase [315]. This is the most expensive assay because of the co-factors and enzymes required for the reaction. This enzyme is extracted from *Aspergillus* or *Escherichia coli* species. Using an *Escherichia coli* nitrate reductase column only ~30% of nitrate was converted to nitrite [316] while this conversion in serum using *Aspergillus* reductase was ~64%. The assay was considerably improved by removal of Griess reactive substances using C18 solid phase extraction in urine [317] and meat products [318]. The yield of nitrate to nitrite conversion was reported to increase to 96% if left to incubate for 3 hours

[306]. Unfortunately even after these extra procedures in serum recovery was incomplete and unpredictable [316] especially at low concentrations [306]. Moreover, in biological samples the enzymes may be affected by nitroso products.

Combining the poor accuracy of the Griess assay with serum nitrite [308], incomplete reduction of nitrate by enzyme assay [316, 306] or over reduction by cadmium [297] has led some authors to question the reliability of biological sample analysis by this method without the use of some form of prior separation.

Chromatographic assays

Gas chromatography

The stable isotopes [^{15}N]nitrite and [^{15}N]nitrate [54,319] have been used for kinetic studies on the biological metabolism of nitrite and nitrate or as internal standards for the analysis of these anions in biological samples [320,321,55,54] following derivatisation with pentafluorobenzyl bromide [320] or nitrobenzene [321]. With the isotopic methods the derivatives can be measured by gas chromatography with high resolution, mass spectrometry. The method of Tesch [321] is prone to interferences from non nitrate sources giving rise to nitrobenzene [54] as well as co-eluting peaks from interfering substances [320]. Tsikas [308] have apparently overcome this problem by following methods by Green [309], Tsikas [320] and Wu [322] in which nitrate is first reduced to nitrite by cadmium reduction. Their method had a reported limit of detection of 22 fmol of nitrite. However, they failed to recognise the above mentioned problems. Other researchers have used GC-MS/MS to increase specificity of detecting nitro-trimethoxybenzene derivatives [323], and other derivatives of nitrite [319,324] or nitrate [325,326].

Capillary electrophoresis

Capillary electrophoresis is a very efficient separation technique [327] . A sample is placed between two electrodes that are connected by a capillary column containing a buffer solution. When a voltage is applied between the electrodes the molecules in the sample move according to both their own electrophoretic mobility and the electroosmotic flow. A detector at one end of the column detects the ions as they move past it, producing a complete analytical instrument.

Nitrite and nitrate in solution are prime candidates for analysis by this method as both are ionised at neutral pH and are UV absorbers. The first method for the determination of nitrite and nitrate in plasma by Leone [328] gave intra-assay variations at 50μmol L^{-1} of 4.6% and 1.2% respectively. This is similar to that reported by other authors [329,330,331]. The proteins and high chloride content in biological materials can affect the position of the nitrate ion peak as well as its shape and reduce column performance [332]. Recently methods have eliminated the effect of the electroosmotic flow. This can differ from sample to sample because of differences in the electrolytes which affect this flow and, in turn, mobilities of the ions. For serum analysis hydrostatic injection [330] has been favoured, with an ion-pairing reagent [331], the limit of detection being ~2.2 μmol L^{-1}.

Advantages of this method are that small sample volumes can be used, silica capillary columns are cheap, the technique is simple, short analysis times and it is of high efficiency. However, for all its advantages CE has had problems, particularly in sensitivity and quantitative reproducibility. However, the latter has been improved by using an internal standard such as bromide [331].

Ion chromatography

Nitrous and nitric acids have pKa's of 3.14 and -1.37 [333] respectively at 25°C and therefore are ionic species in aqueous solutions of pH greater then 3. From this property many early methods for biological samples used ion chromatography as the separation technique. Nitrite, being a weak base, can also be separated by reversed phase chromatography at acid pH [334]. Ion-pair chromatography involves the use of a organic counter ion that is 'dynamically' coated onto a C18 column. When the anion/cation enters the system it

associates with the ion to form a neutral salt that behaves as an organic molecule. Human [62] and rat [335] serum nitrite and nitrate have been separated by ion pairing. However, this type of technique was less successful than simple ion-exchange [336]. Chloride elutes just before nitrite from ion-exchange columns and is present in biological samples in a much greater concentration than nitrite. This can lead to problems with detection and quantification of nitrite (see also chapter 4). Since nitrite is an electroactive anion many researchers have used ion-exchange with electrochemical detection to achieve greater sensitivity and specificity for nitrite, combined with UV detection for nitrate [337,338,339,340]. Limits of detection for serum have been reported to be 0.01 μmol L^{-1} [338].

Chapter 4

Development of liquid chromatographic methods for nitric oxide products

High performance liquid chromatography

The four main groups of liquid chromatography comprise adsorption, partition, ion exchange and size exclusion [327]. Partition chromatography may be normal or reversed phase. The apparatus used consists of a pump which is capable of forcing a degassed solvent through an injector, column and detector at a set flow rate. If a solvent of fixed composition is pumped through the system this is known as isocratic separation. If the composition is changed to increase the eluent strength during the run it is known as gradient elution. An injector is used to introduce a fixed volume of the sample into the solvent mobile phase and is designed so as not to interrupt the flow. The sample is then transported along with the mobile phase into the stationary phase contained in the column. The efficiency of separation of analytes is governed by eddy diffusion, longitudinal diffusion and the mass transfer of analyte(s) to and from solid and mobile phases. Each of these physical process can contribute to broadening of the band. This can be reduced by employing a fine, uniform spherical solid phase to increase surface area, maintain optimum homogeneity and remove stagnant pools.

Once the separated analytes elute from the column they can be detected directly or can be derivatised by postcolumn addition of reagent. Absorbance, fluorescence, electrochemical, refractive index and mass spectrometric detectors are commonly used.

UV/Vis absorbance spectroscopy

Many analytes can absorb electromagnetic radiation in the ultraviolet or visible regions [327]. This electromagnetic energy is generated by a deuterium (190-400nm) or tungsten (350-2500nm) lamp and the desired wavelength is selected by passing through a monochromator before reaching the sample in a UV/Vis-transparent cell. The analyte molecules absorb some of this electromagnetic radiation and the remaining light is captured at the detector. The absorption of UV/Vis radiation at a specific wavelength results from electronic transitions, principally $\pi \rightarrow \pi^*$ for organic compounds. Absorption of energy is also affected by the solvent as its polarity can affect λ_{max} of the molecule as well as obscuring analyte absorption by direct absorbance of the solvent. The latter is mainly a problem at low wavelengths (i.e. less than 230nm). Absorption is proportional to concentration and pathlength as described by the Beer-Lambert law. Deviations from linearity may occur at high concentration.

Fluorescence

This type of detector requires a radiation source (usually xenon arc or mercury-vapour lamp), excitation monochromator, cell, emission detector monochromator and detector [327]. Molecules in the sample excited by electromagnetic energy will re-emit the energy in the form of light in all directions but is usually measured a right angles to avoid interferences from the exciting beam. The emitted light is usually at a longer wavelength than that used to excite it; the very low background signal allows highly sensitive detection. When an organic analyte is excited from a singlet ground state to a singlet excited state, vibrational relaxation allows the rapid loss of energy ($<10^{-12}$ s) down to the lower vibrational level of the electronic excited state from which decay occurs down to the ground electronic state with the release of fluorescent energy ($<10^{-5}$ s). Alternatively the molecule may lose energy with the emission of radiation by internal conversion to a triplet excited state by intersystem crossing. This results in phosphorescence which has a greater lifetime ($>10^{-4}$ s) than fluorescence. The excitation and emission spectra for a compound are usually mirror images because the vibrational energy differences within the ground and excited electronic

states are almost the same. Only a small number of molecules exhibit fluorescence, which is advantageous in reducing interferences with mixtures of compounds. Multiple conjugated double bonds and electron donating groups such as alcohol, amide and methoxy groups enhance while nitro, acids, esters, halogens and azo group inhibit fluorescence. The pH of the solution and presence of substances that act as fluorescent quenchers are also important factors. Derivatisation of non-fluorescent compounds to form fluorescent derivatives is commonly used.

Electrochemical (amperometric) detection

Electrochemical detectors require the mobile phase to be electrically conductive but since most separations require a buffer this is not usually a problem [327]. Electrodes may consist of carbon paste, mercury, platinum, gold or glassy carbon. These electrodes can be used to oxidise or reduce electroactive compounds, however in reduction mode interferences from dissolved oxygen and trace transition metals may be observed. The analytical cell contains three electrodes in close proximity: working (where electrolysis of the analyte occurs); counter (where the complementary electrolysis reaction occurs) and reference (which maintains a constant potential for measurement and setting of the working electrode potential). In amperometric mode the working electrode is held at a constant oxidation/reduction potential capable of causing the analyte to lose/gain electrons at the surface of the electrode. The potential should be great enough to cause electrolysis of the analyte only. The current signal is proportional to analyte concentration and is constantly measured between the working and counter electrodes. This mode of detection is capable of measuring small electrolysis currents and is therefore extremely sensitive, the main limit to detection being the rate of transfer/diffusion of analyte to the electrode surface and its rate of reaction there.

Ion chromatography

Ion exchange chromatography is a subgroup of high performance liquid chromatography (HPLC) used for charged analytes [327]. A solid phase is contained in a column and a liquid

mobile phase flows through it. The type of resin in the column governs the physical process of separation and it is the interaction of charges between the solid and liquid phases that principally brings about the separation of the analytes in a mixture (Fig.4.1). Ion chromatography is a specialised development of ion exchange chromatography which is designed for high efficiency separation and detection of ionic analytes.

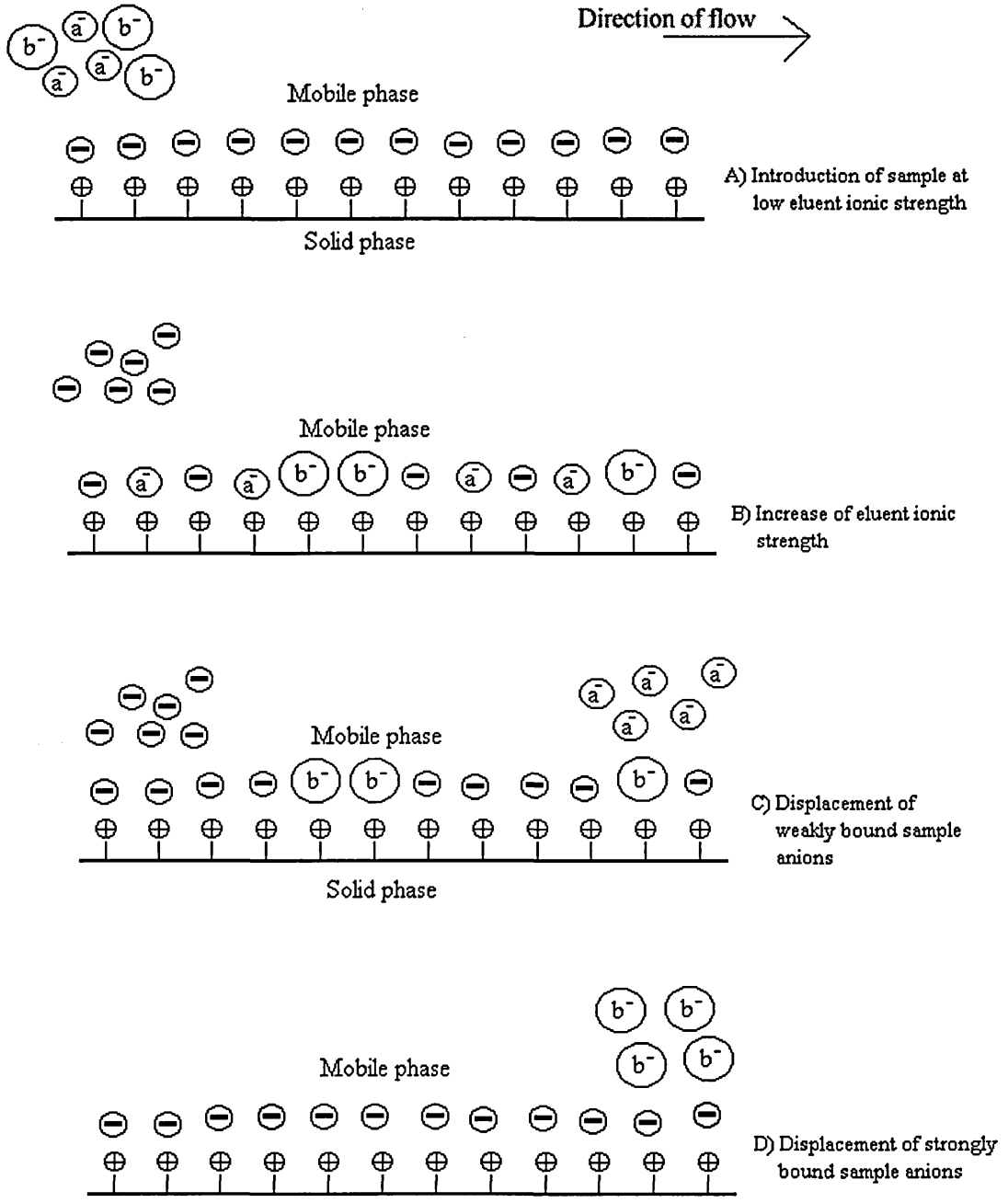


Fig.4.1 Sequence of events during the separation of monoanionic species on a cationic resin.

Electrostatic forces are the primary physical interaction in ion exchange separation.
As can be seen from Fig.4.1 the ions originally present on the anionic exchange sites are displaced by the analyte ions if the displaced ion (e.g. OH^-) has a much lower affinity for the stationary phase than other sample anion(s). This exchange must be electronically neutral so a replaced OH^- must be replaced with an ion(s) of equal net charge. As more anions enter the system competition occurs and the anions of highest free energy of binding are retained the longest in the column. Eventually when the eluent ion concentration increases the active process of exchange between analyte and eluent ions continues throughout the column until even the most strongly retained anions are eluted off. This sequential displacement results in the separation of ions in a mixture. A normal predicted elution order can be made which corresponds to charge density and size of the hydrated ion; however this is also affected by the type of resin used. The usual order of elution for common anions is F^-, Cl^-, NO_2^-, Br^-, NO_3^-, PO_4^{2-} and SO_4^{2-} where polyvalent ions are more strongly retained due to their extra charge.

Ion chromatography is divided into cationic and anionic forms, depending on the column used, and on whether the molecules to be separated are positively or negatively charged. Since nitrite and nitrate are negatively charged at neutral/basic pH they are analysed using an anion exchange column (i.e. one with cationic sites).

Anionic solid phases

Ion exchange resins with quaternary ammonium groups are used primarily as strong basic exchangers. Dionex use sulphonated styrene divinylbenzene beads giving a strong surface negative charge. Latex particles have the quaternary amine groups, which function as sites for electrostatic bonding to the bead sulphonate and as anion exchange sites for analytes.
These are formed by the reaction of partially chloromethylated styrene-divinylbenzene polymers reacted with a tertiary amine (see fig.4.2). The types of R groups affect the chromatography and these are 'tailored' to suit the type of separation necessary.
Separation with this type of resin occurs primarily via electrostatic interaction (see fig.4.1) An equilibrium is set up between the ions of the opposing sign and that of the active sites on the solid phase. Ions of positive charges will be repelled by this type of resin, neutral

polar compounds will not interact with the resin and will be carried by the flow while hydrophobic compounds may interact with the benzene rings of the resin through van der Waals forces and π-π stacking.

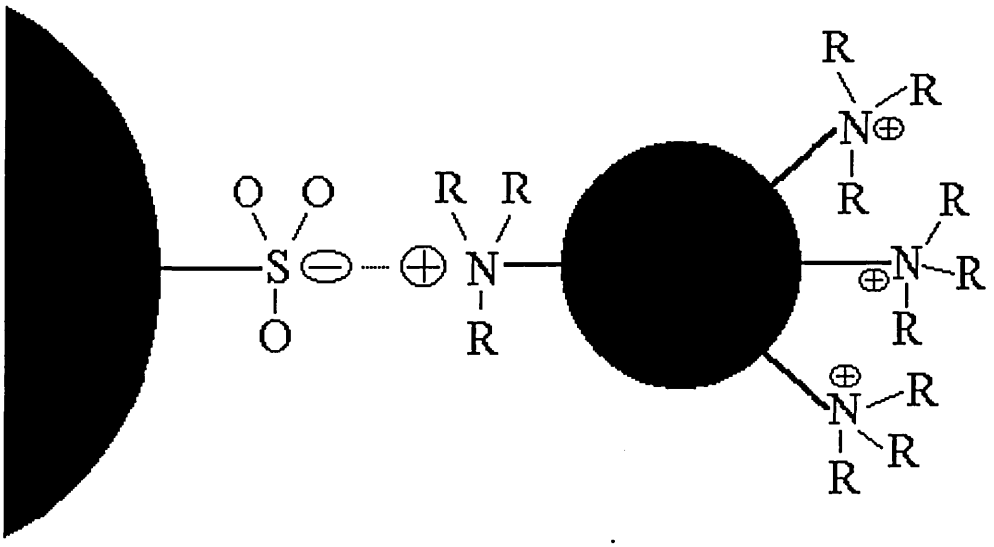


Fig.4.2 Structure of Dionex anion exchange resin.

Dionex use a pellicular bead system where the anion exchange resin beads have submicron ion-exchange latex microbeads which are held to the core bead by electrostatic and van der Waals interaction. This increases the number of active sites and creates a large surface area which greatly improves separation. The microbeads have ion-exchange surfaces where the separation occurs.

Anion exchange columns use electrostatic forces as their basic principle for separation. Another major factor which is important in selectivity and specificity is the composition of the R-groups that surround the active nitrogen on the microbeads. They may range from ionic to alkyl or mixed to create a hydrophilic or a hydrophobic resin. Increasing the chain length of these group can also create a dense molecular mesh to allow only small ionic species to the active site. Modification of the R groups allows the introduction of other

physical factors such as van der Waals forces in alkyl groups, hydrogen bonding in hydroxyl groups or steric hindrance by introduction of bulky branched groups.

The R-groups on the columns we have used are unknown as Dionex do not disclose their resin composition.

The capacity of an ion exchange resin is a quantitative measurement of the number of active sites. Capacity is governed by the physical properties of the resin and can be altered by the pH of the eluent. The higher the capacity the greater load it can take.

Conductivity detectors are commonly used with ion chromatography due their high sensitivity, linearity, simplicity and universal detection for charged species. However since high electrolyte concentrations are required to elute analyte ions they tend to swamp the detector signal reducing its sensitivity. This problem was solved by introducing a suppressor column after the analytical column. Early suppressors were a packed ion exchange column which produced a counter ion to that of the eluent ion. In anion exchange chromatography where the eluent is a sodium hydrogen carbonate buffer, the resin was a cation exchanger producing protons. The hydrogen on the resin is exchanged for sodium from the solution, the carbonate reacts with the protons to produce undissociated carbonic acid that does not significantly affect the conductivity of the solution. The suppressor resin can be regenerated continuously by forcing dilute sulphuric acid through a fiber membrane on the other side of the resin. More modern suppressors use a charge selective membrane to allow passage of the neutralising ion from the suppressor solution to the eluent.

Many anions and organic acids can absorb electromagnetic radiation in the UV region and can therefore be detected by UV spectrophotometers. They still require removal of carbonate ions as they also absorb radiation around the same wavelength range.

Materials and methods

Sample pre-treatment

Normal human serum was obtained from the Blood Transfusion Service, Northern General Hospital, Sheffield (UK).
All pre-eclampsia study samples were obtained from the Jessop Hospital for Women, Sheffield (UK) and St James Hospital, Leeds (UK). This study was approved by the local ethic committee.

Samples were centrifuged through (5,000 RMM cut-off Vivaspin (Vivascience Ltd. Binbrook, Lincs.) or Centricon filters (Amicon, Stonehouse, Gloucester) for 120 minutes at 7500g prior to analysis by the High Performance Ion Chromatography method. Each filter had to be washed thoroughly before use to remove nitrate left by the manufacturing process. There was no further pre-treatment of serum.

Griess method (Nitrite determination)

Serum samples were centrifuged through 3 kDa molecular mass cut-off Centricon filters (Amicon, Stonehouse, Gloucester) then 200µl of Griess reagent was added to 200µl of sample placed in 96 plate wells and left for 15 minutes in a dark cupboard. The Griess reagent is formed by mixing equal volumes of stock A (1% sulphanilamide in 5% concentrated phosphoric acid) and stock B (0.1% napthylethylenediamine dihydrochloride) with the samples; this being left for 15 minutes in a dark cupboard to allow the colour to develop. All reagent obtained from Sigma chemicals.
Absorbance was read at 570nm on a Labsystems (Life sciences international, Basingstoke, Hampshire UK) Multiskan MS V1.5 plate reader. Principle of this assay is explained in chapter 3.

Isocratic ion chromatography method (Nitrite/nitrate determination)

Initial work involved the use of a Dionex (Sunnyvale, California, USA) QIC suppressed ion chromatography system with AS4A-SC analytical (4 x 250mm) and guard columns and conductivity detection. Physical property of column shown in table 4.4. The eluent consisted of 1.8mmoL^{-1} Na_2CO_3, 1.7mmoL^{-1} $NaHCO_3$ (BDH, Poole, Dorset). A Milton Roy Spectromonitor III - UV detector (Englewood Cliffs, N.J, USA) was connected in series after the conductivity cell outlet and a wavelength of 214nm was used for measurement as well as the conductivity signal. No other column was used with this system.

Gradient ion chomatography methods (Nitrite/nitrate determination)

The chromatography system contained of a Dionex gradient pump (Sunnyvale, California, USA) and a LDC/Milton Roy Spectromonitor III (Englewood Cliffs, N.J, USA) set at 214nm. Data was acquired using a VG 'Minichrom Data System' V1.6 (VG Fisons, Cheshire, UK). Samples were injected into the system using a Perkin Elmer ISS-100 Autosampler (Beaconsfield, Bucks. UK) with a sample volume set at 30µl. Mobile phase constitutions and gradient programs are shown in Tables 4.2 and 4.3.

Columns used

Dionex AS4A-SC, AS9A, AS12, Nucleopac-PA 100 and Carbopac-PA 100 columns (all 4 x 250mm) were tested for suitability in nitrite and nitrate determination. Physical properties of columns shown in table 4.4. Columns were kindly loaned by Dionex (UK) Ltd. The system was fully automated in that the autosampler was the chief controller for starting the Dionex gradient pump and the Minichrom data system at the start of each run. This allowed 24hr analysis of samples if required.

All the stainless steel tubing in the system was replaced with polyether ether ketone (PEEK) tubing and the apparatus was washed daily with pure water to prevent corrosion due to the use of the high concentration chloride eluent.

Reagents and samples

Stock solutions of nitrite and nitrate were prepared by dissolving the appropriate amount of BDH (Poole, Dorset) Analar sodium salt in MilliQ water that had a resistance of not less than 18 Mohms.

The eluent contained 5mM Trizma buffer (ultra-pure grade) (Aldrich Chemicals Company) and HPLC grade sodium chloride (BDH, Poole, Dorset), adjustment to pH 7.5. All the eluents were thoroughly degassed and stored under helium.

The retention times of nitrite and nitrate peaks were determined from known standard solutions in water and by superposition of spiked peaks.

Results and discussion

Method development

Ion chromatography has become one of the most powerful tools for quantitative analysis of anions and cations. The most common procedure for the analysis of anions uses an isocratic method with bicarbonate/carbonate buffered eluent, suppressed ion chromatography and conductivity detection, as used by Dionex systems. However the naturally high concentration of chloride present in serum (which is present in serum at high concentrations, approx. 10^5 ppm) obscured most of the signal from nitrite (when using conductivity detection) as chloride has a similar retention time to nitrite when using the Dionex AS4A-SC column.

Fig.4.3 shows the different traces obtained with conductivity and UV detection. The lower chromatogram (Fig.4.3) shows a typical serum sample that has been analysed on a Dionex AS4A-SC conductivity system. The largest peak (at ~2.2 mins in lower chromatogram) corresponds to chloride and it can be seen that this peak obscures the signal of nitrite. The chloride and nitrite peaks did not co-elute but had close retention times with chloride preceding nitrite. At similar low concentrations these two anions are resolved clearly but since the serum concentration of chloride is approximately 10^5 times that of nitrite it creates a broad peak that masks the relatively small signal from nitrite. Methods used to overcome this problem have included pre-treatment with a silver-loaded resin to precipitate the chloride ions in the sample prior to the chromatographic analysis [341].

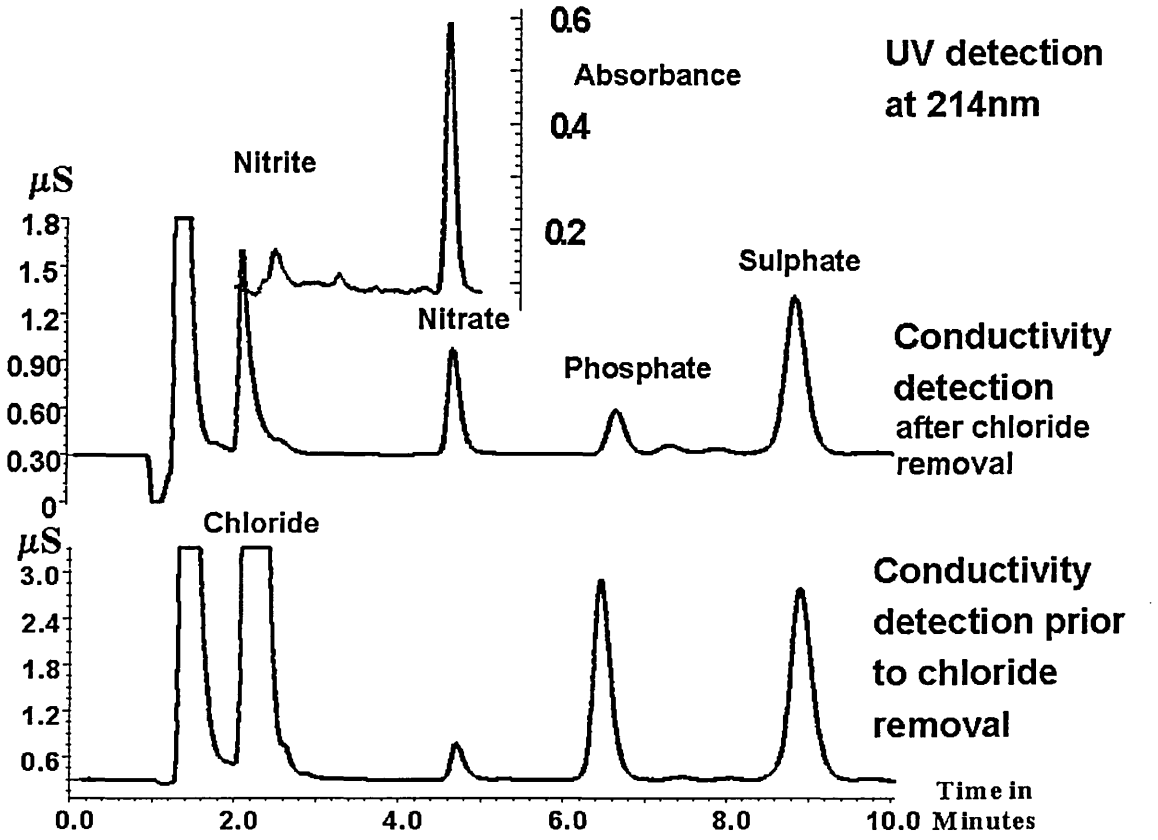


Fig.4.3 Chromatograms of diluted and chloride removed serum using an isocratic carbonate eluent, AS4A column and conductivity and/or UV detection.

The silver resin method was developed by Lippsmeyer and is shown in Fig.4.4 Once prepared the resin is added to serum that has been centrifuged through a membrane filter. The resulting precipitate is filtered off and then the sample is injected onto the column. The resulting chromatogram is the middle trace in Fig.4.3. This procedure greatly improved the separation and it is possible that nitrite could be quantified from this. Unfortunately it has been found that free silver ions will eventually damage the analytical columns [342,343]. Other disadvantages include co-elution of nitrite with organic acids (or other interfering species) producing over estimates of nitrite concentration [344,345]. This method also suffers potential problems in that the additional manipulations could introduce error.

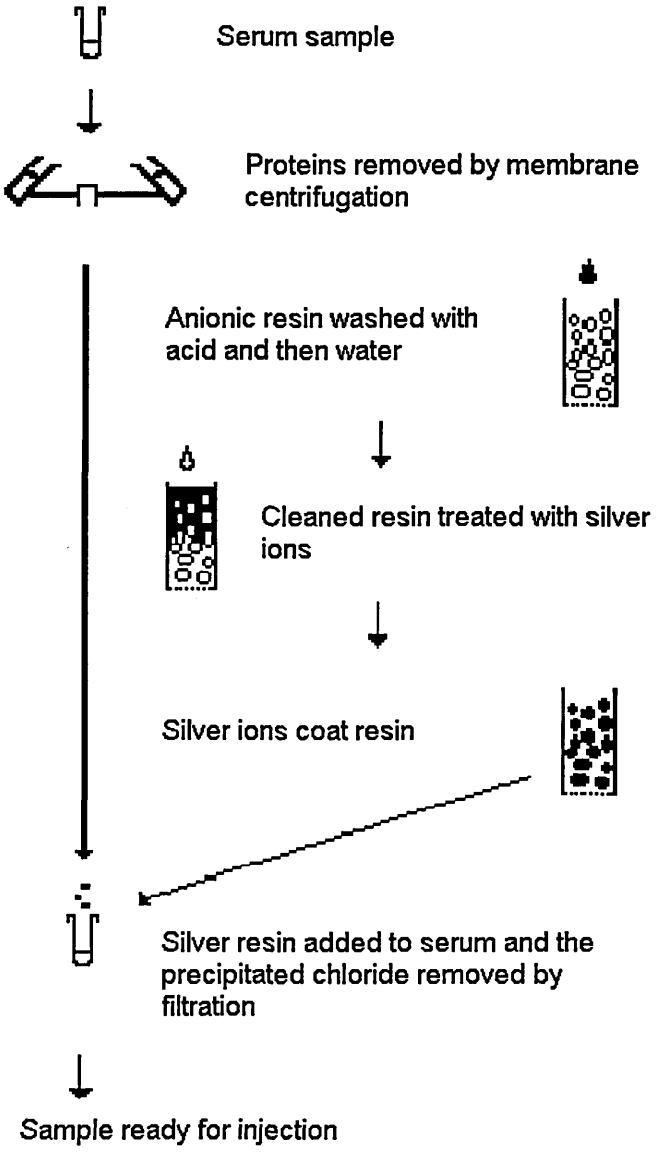


Fig.4.4 Serum chloride removal method [342]

Another possible solution to the problem of chloride interference was to use UV detection at 214nm, where chloride ions are not detected. The UV spectra in Figures 4.5 and 4.6 show the absorbance maxima for nitrite and nitrate to be at 210nm and 204nm respectively. The wavelength maximum of 214nm was selected because this would be low enough to detect these two anions but high enough to avoid interference from other substances that absorb at the lower wavelengths.

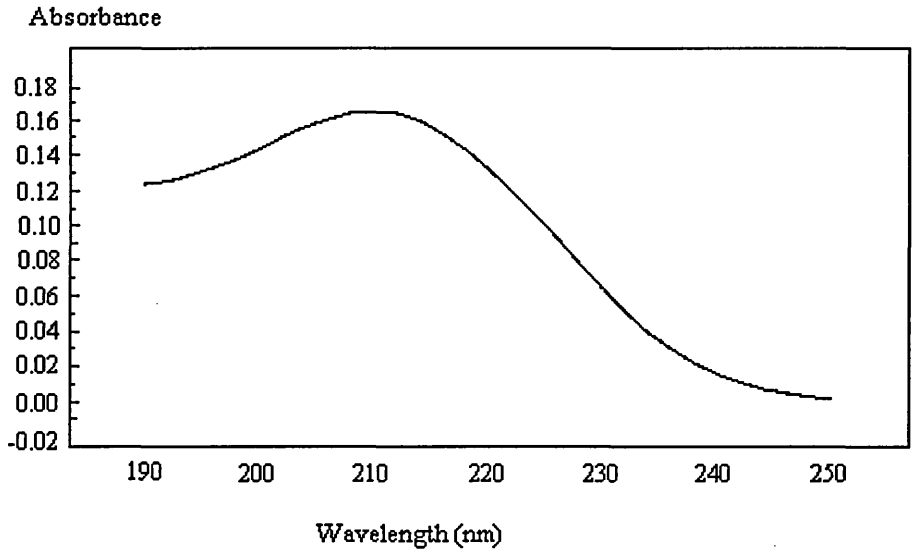


Figure 4.5 UV spectrum of nitrite

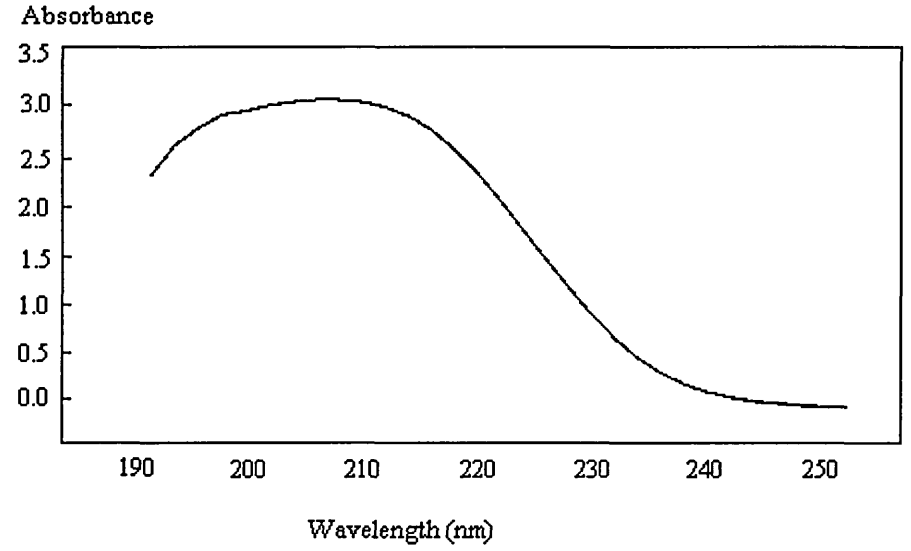


Figure 4.6 UV spectrum of nitrate

By connecting a UV detector (fig.4.3 upper chromatogram) in series with the conductivity detector (fig.4.3 middle chromatogram) it was possible to compare the chromatograms by both detection methods as a marker for possible co-elution. The UV detector response confirmed that a conducting ionic substance which was not UV active at 214nm was eluting just before nitrite.

The chloride removal step (fig.4.4) was still required because even at 214nm the effect of chloride was to produce a large negative peak at the region of the chromatogram where chloride would elute. The appearance of this negative peak is caused by the "invisibility" of

chloride at this wavelength. As these ions pass the detector the concentration of carbonate eluent anions (which absorb at 214nm) decreases as the concentration of invisible chloride ions increases thereby causing a reduction in the light absorbed. This effect has also been observed by Jackson [346] and Williams [347].

A major problem was with direct UV detection where UV absorbing co-elutants were still observed, possibly due to lactate [344] or acetate [345] which are both absorb at this wavelength.

Gradient system

Since UV detection appeared more responsive to NO_2^- and NO_3^- than to Cl^- a high performance liquid chromatography system with a gradient pump and UV detection system was constructed. This system was used to investigated the possibility of altering the retention of the organic acids and chloride by using gradient elution with the carbonate eluent. The eluent remained a mixture of carbonate/bicarbonate since it has a natural buffering effect as well as being an excellent exchanger ion. Retrospectively it might have also been possible to use a dilute hydroxide mobile phase for gradient separation however it was not considered.

Fig.4.7 (conditions in Table 4.1) shows the separation of serum by the optimised program. The serum separated into two fractions in which one was treated with the silver resin while the other was not. Even by modifications of the eluent regime it was not possible to remove the effect of the 'chloride dip'. Thus, the silver resin still had to be used. However, gradient elution did overcome the problem of co-elution of nitrite with organic acids.

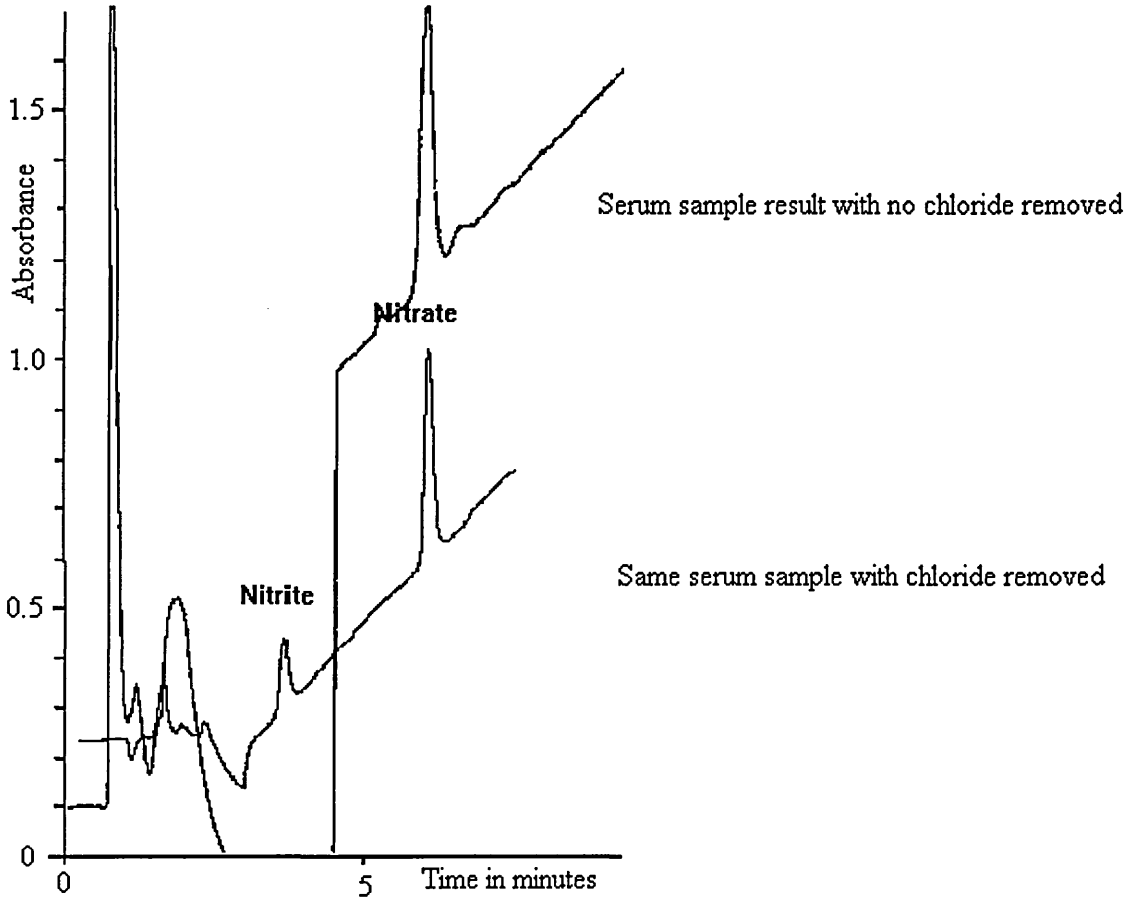


Fig.4.7 Serum and chloride removed serum analysed using a gradient carbonate eluent on a AS4A column, 214nm UV detection.

Table 4.1 Gradient carbonate eluent program employed in fig.4.5

Time (mins)	Flow rate (mls/Mins)	%Reservoir A	%Reservoir B
Initial	1.5	90	10
25	1.5	10	90

%A = MilliQ water%B = MilliQ water with 5mM $NaHCO_3$ + 5mM Na_2CO_3

Chloride as an exchanging ion

An ideal exchanger ion needs to have a relatively low affinity for the resin compared with that of the analyte anions. Chloride can be used since nitrite and nitrate will interact more strongly with the column and can be retained for longer periods than that of chloride. This effect has been exploited by Pastore [348] who reported a method that involved using the interfering matrix ion chloride as the exchanger ion. This was refined by Haddad [349] who

used a chloride eluent to determine trace amounts of nitrite in seawater, another sample having both high chloride and low nitrite levels. This procedure involved an isocratic HPLC method with 15mmol L^{-1} chloride as the eluent. The data obtained using this method is shown in figure 4.8. It appears that the negative peak found in the previous method has been eliminated, but other substances, possibly organic acids that also absorb at 214nm co-eluted with the nitrite peak.

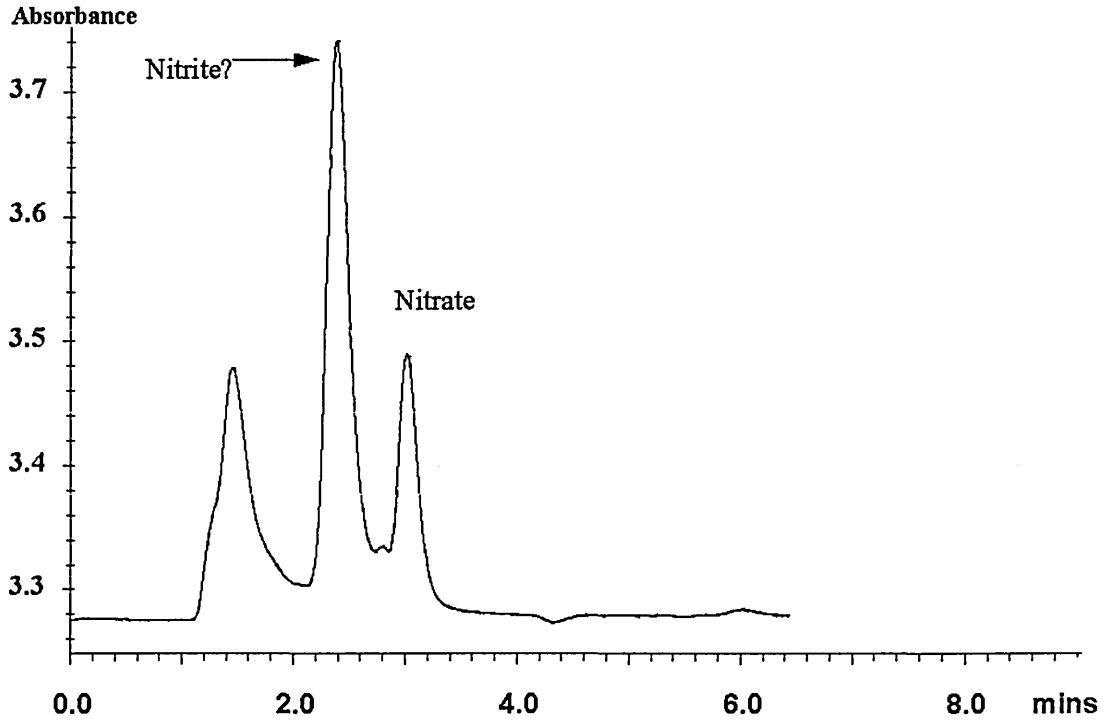


Fig.4.8 Human serum analysed on QIC system using isocratic 15mmol L^{-1} Cl^- eluent with UV detection at 214nm.

The idea for a chloride eluent came from the fact that when a matrix ion is the eluent ion, the column is coated with this ion prior to injection of the sample. Since this eluant ion is present at fixed concentration in the eluent, any added chloride in the sample (approx. 100 mmol L^{-1} [350]) would not interact appreciably with the ion-exchange resin. Therefore any of this ion in the matrix will have little if any retention on the column and will cause little disturbance upon detection.

Subsequent to the development of these methods, Santillana [351] and Radisavljevic [352] recently published similar isocratic HPLC methods employing a chloride eluent for determining serum nitrite and nitrate.

Isocratic/gradient chloride elution and column selection

From the preceding results, gradient elution with chloride appeared a logical advance. Subsequently, a range of Dionex columns were investigated for the gradient anion chromatography. Initially, the classic anion exchange column AS4A-SC for common anions (F^-, Cl^-, NO_2^-, NO_3^-, SO_4^{2-}, PO_4^{2-}) was employed for separation.

Following the method by Haddad [349] a limited range of isocratic chloride eluents were used comprising of concentrations of 15, 25 and 50 mmol L^{-1} with varying concentrations of phosphate buffer (5, 15, 50 mmol L^{-1}). Standards and two batches of filtered serum were analysed with each program (one batch with chloride removed, the other untreated). Retention times and co-elution under each set of conditions were compared with the optimum conditions of 15mmol L^{-1} chloride eluent with 5mmol L^{-1} phosphate buffer. Unfortunately co-elution could not be overcome as it was not possible to establish the purity of the nitrite peak (Fig. 4.8), which is usually much smaller than the nitrate peak. Shortly after using the new conditions these column became blocked with an unknown compound. It was found to be an acid soluble structure with absorbance maxima at 212 and 330nm and formed a red precipitate in a strong alkali solution. This problem might have been overcome by employing a higher concentration gradient to prevent the acid-soluble substance building up on the column. However, using a higher eluent strength led to poor peak resolution because of the low capacity (see Table 4.4) of the AS4A column. Therefore, it was decided to explore alternative anion exchange columns that require a gradient system for elution of the anions.

The other Dionex columns that were tested for their suitability in nitrite/nitrate analysis included. AS9A (Fig.4.9), AS12A (Fig.4.10, Nucleopac-PA 100 (Fig.4.11) and finally the Carbopac-PA 100 (Fig.4.12) columns, all supplied by Dionex UK. Optimal gradient program conditions for each column used are described in Table 4.2 and 4.3.

A range of nitrite and nitrate standards and serum were analysed on each column with eluent pH's of 5.0, 7.0 and 9.5 (unbuffered). The AS9A column showed good resolution for nitrite and nitrate but the nitrite peak emerged very close to the unknown larger peak shown in figure 4.9. The interfering peak was not seen at pH 5.0. The AS12 also gave separation of

the two anions. However, the larger peak appeared between nitrite and nitrate, shown in figure 4.10. In all three pH ranges the interfering peak was still present. The Nucleopac column produced a result similar to those of the AS9A column. However the resolution improved compared with that observed with the previous columns and so this was selected for further analysis.

Further work with the Nucleopac column showed promising results. These included modification of the conditions for gradient program, pH and identification of organic acid retention times using known standards. During this time it was evident that the large peak that eluted close to the nitrite peak would occasionally shift retention times to co-elute with nitrite (fig. 4.9) even thought, this had not occurred in the preliminary experiments. It, therefore appeared necessary to have pH buffering to stabilise each anion in the chromatogram.

One of the drawbacks of using a low-capacity column is the difficulty in using a buffer. As buffers are ionic in nature they will interact with the column as would any charged analyte in a sample mixture. Using an isocratic method removes this problem as there is a constant concentration of the eluent/buffer flowing through the system at any one time. However, when using a gradient program the buffer will accumulate on the column until a critical gradient point is reached and the chloride ions in the eluent are of sufficient concentration to displace the buffer from the stationary phase of the column. This causes marked baseline distortion and makes analysis virtually impossible. However, using a high starting concentration will ensure that the buffering ions do not interact with the stationary phase as the high eluent strength prevents accumulation of buffer ions on the column. It was, therefore, necessary to employ a higher capacity column that could accommodate the higher eluent strength necessary (table 4.4).

A change was made from the hydrophilic Nucleopac PA-100 to the hydrophobic, higher capacity Carbopac PA-100 column which has high retention capacity for anions, thereby allowing the use of a much higher eluent strength. After selecting this column a range of sodium chloride concentrations were employed to determine optimum resolution and separation for nitrite and nitrate (figure 4.12). As well as modifying the eluent concentration the gradient program was optimised to produce a minimum run-time consistent with good resolution.

Gradient programs used

Table 4.2 Optimised gradient program used to compare the retention of nitrite and nitrate in the Dionex AS9A, AS12A and Nucleopac columns.

AS9A, AS12A and Nucleopac

Time (minutes)	Flow (mls/Min)	%A	%B
Initial	1.5	99	1
6	1.5	81	19
15	1.5	81	19
30	1.5	0	100
31	1.5	99	1
40	1.5	99	1

%A = MilliQ water%B = MilliQ water with 150mM NaCl eluent Table 4.3 Optimised gradient program that was used for nitrite and nitrate analysis using the Dionex Carbopac PA-100 analytical column

Carbopac PA-100

Time (mins)	Flow (mls/min)	%A	%B	%C	Comment
Initial	1.0	60	40	0	Starting conditions
9.0	1.0	0	100	0	Linear gradient
10.0	1.0	0	0	100	Wash Cycle
13.0	1.0	0	0	100	
14.0	1.0	60	40	0	Column re-equilibration
20.0	1.0	60	40	0	Ready for next run

%A - MilliQ water with 5mM Trizma buffer set at pH 7.5%B - MilliQ water with 300mM NaCl and 5mM Trizma buffered at pH 7.5%C - MilliQ water with 1M NaCl set at pH 7.5

	AS4A-SC	*AS9A*	*Nucleopac PA-100*	*Carbopac PA-100*
Particle diameter	15 μm	13 μm	9 μm	10 μm
Latex diameter	180 nm	110 nm	140 nm	170 nm
Capacity (per column)	20 μeq	30-35 μeq	25 μeq	90 μeq

Table 4.4 Different properties of the Dionex ion exchange chromatography columns

Data obtained from Dionex product selection guide 1994-95

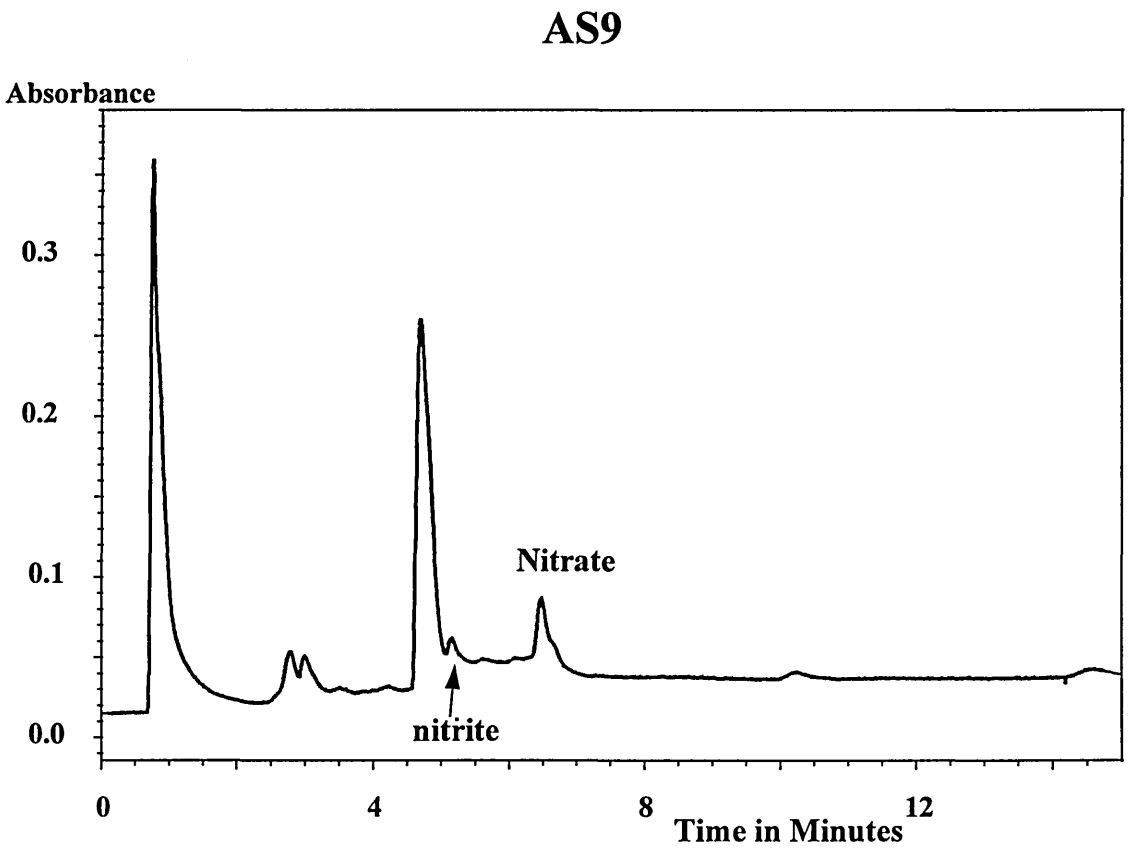


Fig.4.9 Serum analysis using chloride gradient program in Table 4.2 with the AS9 column.

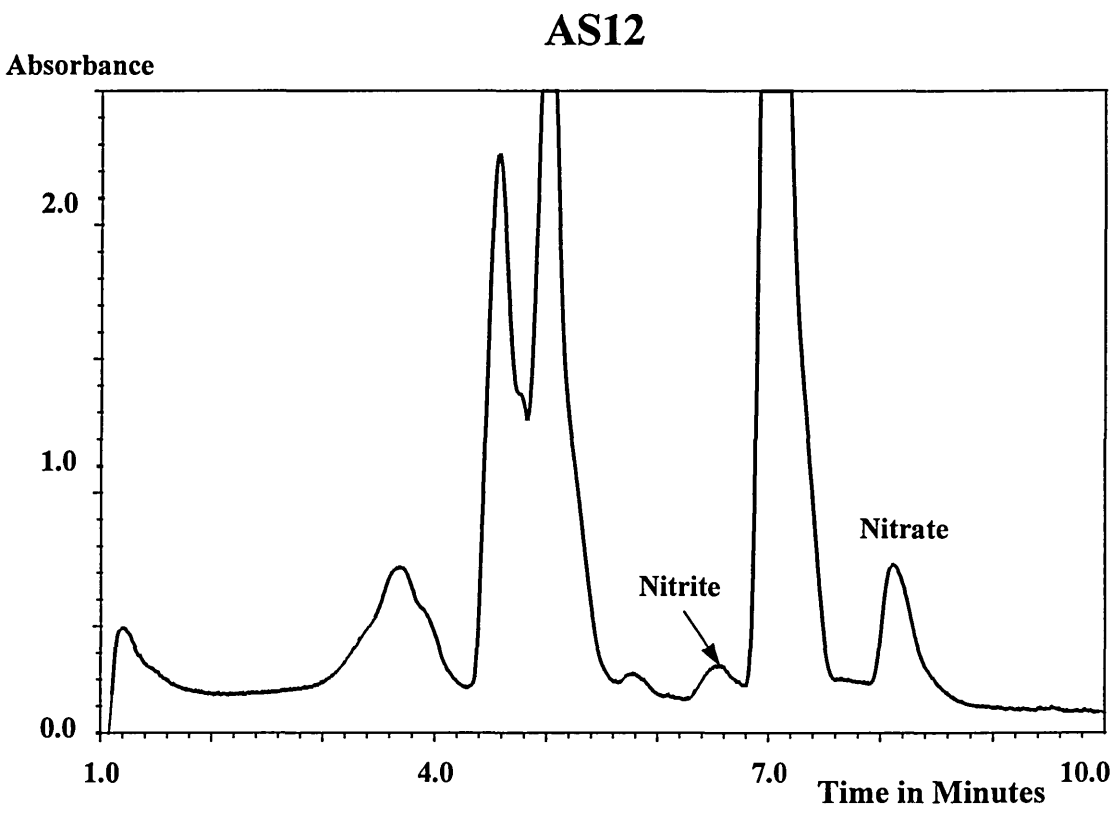


Fig.4.10 Serum analysis using chloride gradient program in Table 4.2 with the AS12 column.

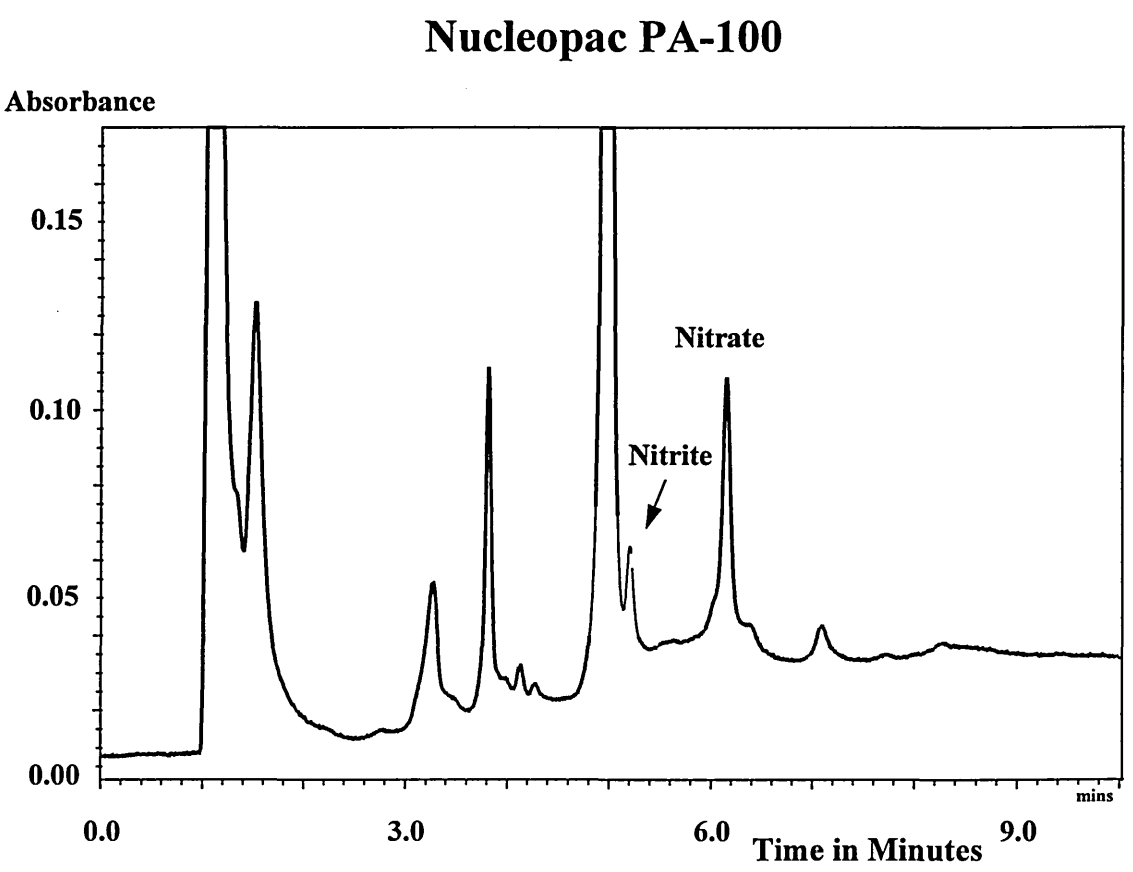


Fig.4.11 Serum analysis using chloride gradient program in Table 4.2 with the Nucleopac PA-100 column.

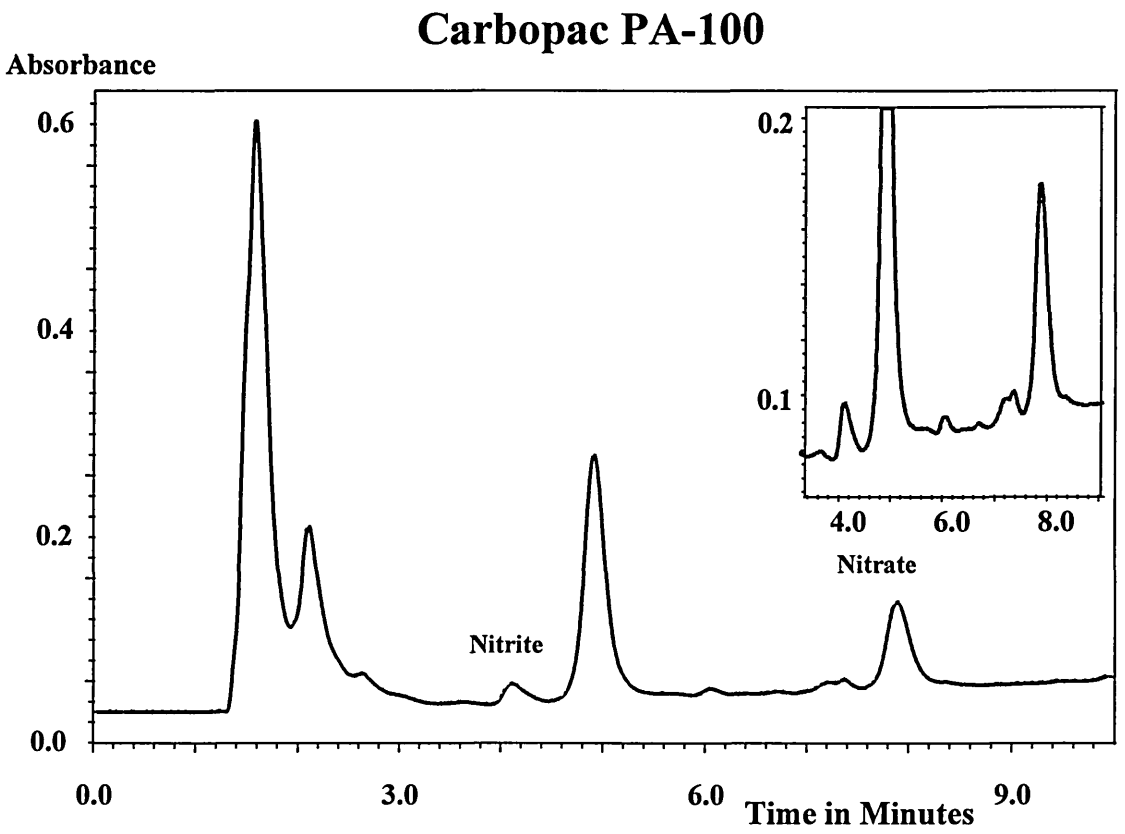


Fig.4.12 Serum analysis using chloride gradient program in Table 4.3 with the Carbopac PA-100 column.

The Carbopac-PA100 column was originally designed primarily for the determination of a range of oligosaccharides but anion exchange is the principal method of separation. Chromatograms produced using this column showed general similarity to those produced from those with Nucleopac column. The difference was that a higher concentration eluent could be used with Carbopac column because of its higher capacity and hydrophobicity, so allowing the introduction of a buffering system. Completion of the run with a wash step was possible by increasing the chloride eluent concentration sufficiently to remove tightly bound substances from the column after each sample run.

Fig.4.12 shows a typical chromatogram of a control serum sample analysed using the optimum gradient program for nitrite and nitrate analysis. It appeared that this method could be employed to successfully separate nitrite and nitrate from other anions present in serum. A short analysis time of ten minutes plus another ten minutes for a column wash and re-equilibration were all that was required for each serum sample.

Statistical data

Calibration

Calibration curves produced from aqueous standards were linear from 0.30 μmol L^{-1} to 145 μmol L^{-1} and produced excellent regression curves which the equations y = 0.012x + 0.9339 (r_2=0.9998) for nitrite and y = 0.012x + 0.1414 (r_2=0.9998) for nitrate.

Limits of detection

Limits of detection were approximately 0.20 μmol L^{-1} for both anions as calculated from; three times the signal to noise ratio. This is a comparable with the limit of detection than reported by Haddad [349] where their detection limit for nitrite was 0.17 μmol L^{-1} for sea water samples. The difference in detection limits between other methods could be due to the quality of the sample. However, technical variations with the detection system such as age of lamp and design of detector affect the signal-to-noise ratio and play a crucial role in determining limits of detection.

Spike recovery

Spike recovery averaged 106%, for nitrite value averaged at 76% whereas nitrate at 137%. Suggesting that oxidation from nitrite to nitrate was occurring during the centrifugal ultrafiltration step.

Inter and intra-assay

Inter assay for standards (n=8) was 3% (RSD) for nitrite at a mean of 13.69 μmol/L and 3% for nitrate at a mean of 23.85 μmol/L. Inter assay for serum (n=5) was 4% for nitrite at a mean of 4.46μmol/L and 3% for nitrate at a mean of 32.3 μmol/L. Intra assay for serum (n=200) was 5% for nitrite at a mean of 3.7 μmol/L and 4% for nitrate at a mean of 40 μmol/L. Each producing good reproducibility of ≤ 5% deviation.

Population study

Using this method 200 control serum samples were analysed in duplicate from serum samples obtained from the Blood Transfusion Service. Male/female samples were separated because although Michigami [62] stated there was very little difference between age and sex, Takahashi [354] claimed that males had approximately twice the concentration of nitrate of females. The results for the 200 serum samples showed that the mean nitrite and nitrate levels for males were 3.47 ± 5 μmol L^{-1} and 42.06 ± 33 μmol L^{-1} respectively while levels in females were 5.06 ± 7.3 μmol L^{-1} and 37.48 ± 28 μmol L^{-1} respectively. The variability in both groups was high, which may be due to dietary factors (see Chapter 7).
In the clinical study it was proposed to monitor patient nitrite/nitrate levels in longitudinal studies prior and through disease onset to reduce the effect (if any) of individual-to-individual variation and to establish a basal level for each subject.

Method validation

Any new methodology has to be verified by comparing it to a known established method. Since the Griess method is widely used and relatively easy to perform this techniques was selected as a comparative standard for nitrite only. Both the Griess and high performance ion chromatography (HPIC) methods compare well with spiked serum and show linearity down to 0.25 μmol L^{-1}. Since nitrite in most cases is of a much lower concentration than nitrate, nitrite was considered the most important anion to compare of the two.

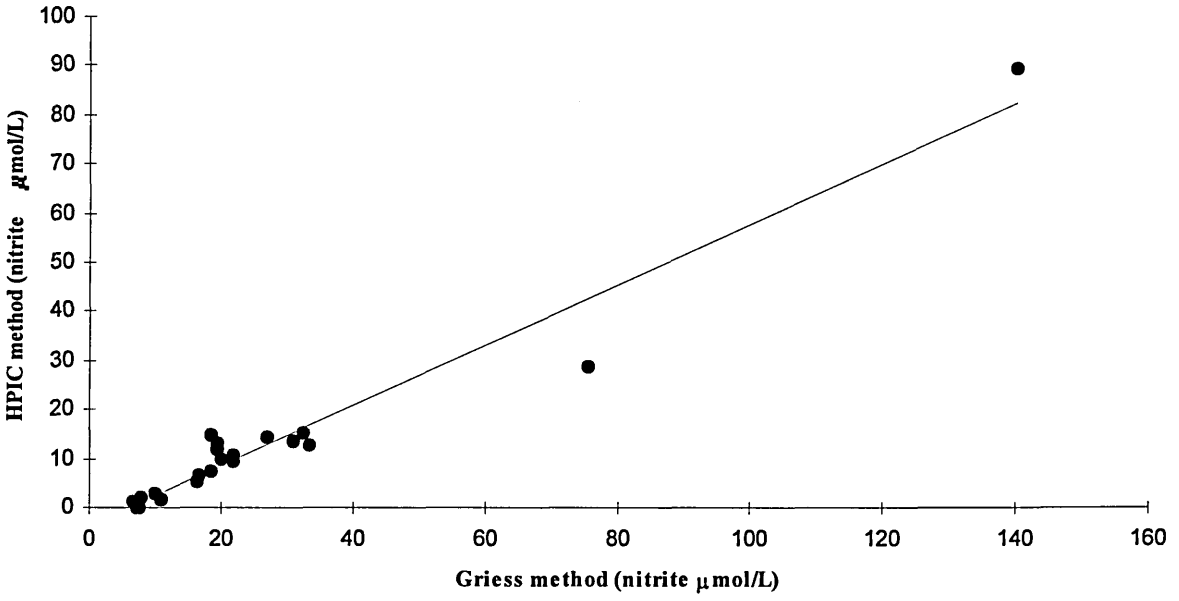


Figure 4.14a Comparison of spiked serum with nitrite by the HPIC method and the Griess assay.

The comparison between the two methods shows good correlation between the HPIC and Griess methods (Fig. 4.14a) with a correlation coefficient of 0.95. However there is evidence of bias and offset in the above graph (with an equation of y=0.65x-3.5). This bias is due to the Griess method overestimation (possible reasons for this is discussed in chapter 3) or HPIC method underestimating serum nitrite (via oxidation of nitrite to nitrate). There is also an offset where the HPIC method does not detect any nitrite while on the Griess method there is 7.67 µmol L^{-1}.

Bland-Altham plot [353]

The correlation plot shown in figure 4.14 takes into account variations in the y-axis (new method (x_1)) but not the x-axis ('gold-standard' method (x_2)) [353]. In reality the correlation plot measures how close the values of the new method compares to that of the 'gold-standard' method rather than the actual value. A better approach is to measure the difference between the methods (x_1-x_2) against their average ((x_1+x_2)/2) as shown in figure 4.14b. The 95% limits of agreement can be calculated from the mean and plotted to give the

±2 standard deviation ranges. The data should line close to zero if there is little bias. The difference between the two methods should be less than 2 SD on either side of the mean for 95% of observations if there is good agreement between the methods.

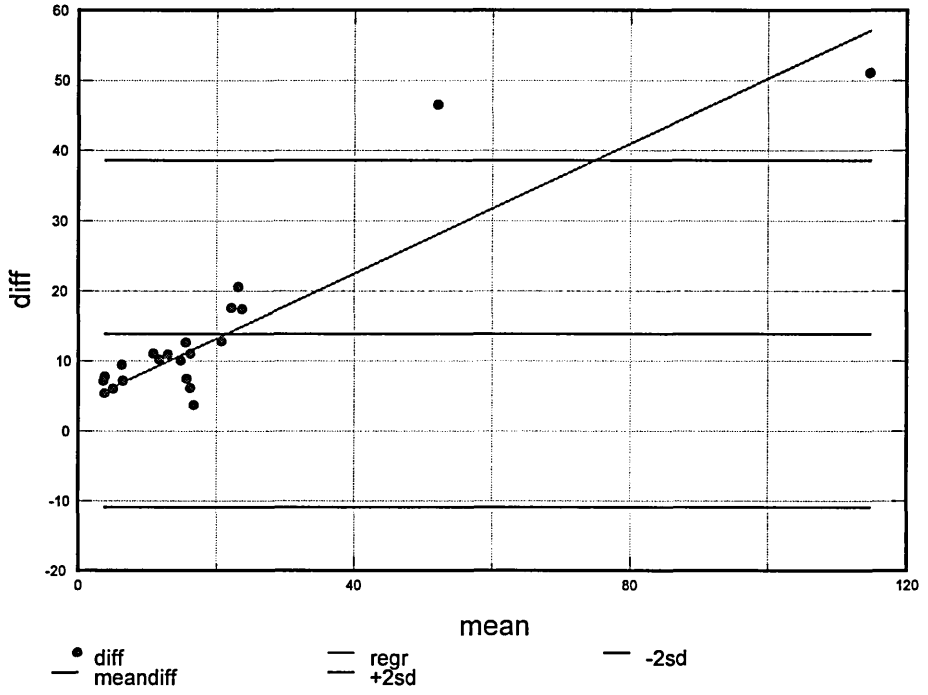


Figure 4.14b – Data from fig.4.14a replotted as Bland-Altham.

The Bland-Altman makes three assumptions

1. Errors are associated with both methods.
2. The mean of the two methods is the true value.
3. There is equality between the two methods.

From figure 4.14a it is evident that there is a unequality i.e. bias between the two methods. This is further shown in figure 4.14b as the bias shows a trend away from the mean difference. This range of this bias is 49.5 (-10.9 to 38.6 figure 4.14b). This bias can be corrected by plotting the residuals along the regression line as shown in figure 4.14c.

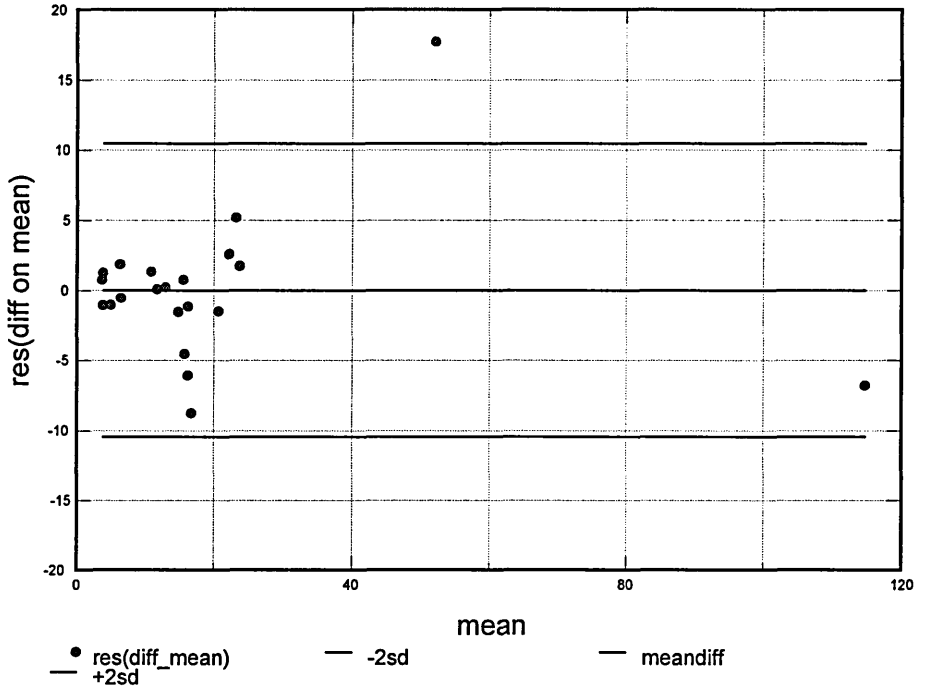


Figure 4.14c - Bland and Altman, HPIC and Griess, corrected for regression

Once the bias is corrected for in figure 4.14c most of the data points fall within the 95% limits of agreement especially the 5-40 μmol/L range. Above this range there are only two data points which can produce misleading information on how the two method compare. However the mean nitrite values of 3.47 ± 5 μmol L^{-1} and 5.06 ± 7.3 μmol L^{-1} for males and females is within the limits of agreement. The equation of the 95% agreement limits expressed in terms of the Griess assay is HPIC = (0.611 x GRIESS – 3.46) ± 8.47.

Linearity and sensitivity

Published data [355] have shown the limit of detection for the Griess method as 0.15μmol L^{-1} for aqueous nitrite. For this HPIC method, water standards are linear down to 0.25μmol L^{-1} and have a limit of detection of 0.25μmol L^{-1}. These data are comparable with the results from the present study in spiked serum samples (fig.4.14) which followed the same linear calibration.

Duplicate sample variation for the Griess reagent was a problem, possibly caused by protein contamination [19]. Excessive variation could also be explained by the performance of the analytical equipment used to read the 96 well plates. Stability of the working reagent or the individual chemicals used may also have a problem involved. These and other factors discussed in chapter 3 suggest that the Griess assay overestimates serum nitrite levels and exhibits high variability.

Other difficulties associated with poor reproducibility involved ineffective conversion of nitrate to nitrite by the cadmium reduction, which did not allow correlation with nitrate levels.

Oxidation of nitrite to nitrate

Four batches of blood were obtained from the Sheffield Blood Transfusion Service (Microbiology dept.). The first contained serum, the second contained serum which was slightly haemolysed and the third was extensively haemolysed. The final sample contained whole blood. Individual patient samples were separated before being pooled to make five stock samples of 10mls. Table 4.6 illustrates the procedure carried out to each group.

Blood sample	Preservative/ Method of preparation
1 – Serum	No preservative
2 – Slight haemolysed serum	Whole blood temporarily frozen at -20°C to cause lysis. Cellular fraction removed by centrifugation.
3 – Extremely haemolysed serum	Whole blood temporarily frozen at -20°c to cause lysis. Cellular fraction removed by centrifugation.
4 – Lysed whole blood	Whole blood frozen at -20°c to cause lysis. Cellular fraction removed by centrifugation.
5 – Whole blood	K_2 : EDTA complex

Table 4.6 Preparation of the five blood samples

To each batch of 10mls serum samples No.'s 1-4 was mixed 10mls of 30 μmol L^{-1} of nitrite standard (prepared in physiological saline). Aliquots (10mls) of whole blood (5) were

mixed with 10mls of 396 μmol L^{-1} nitrite standard. The samples were left at room temperature and open to the atmosphere. At hourly intervals from time zero a 2ml aliquot was pipetted into a fresh tube and any residual cells removed by centrifugation (15minutes). The serum was filtered through a Centricon ultrafilter (Amicon, Stonehouse, Gloucester) for 45 minutes. The filtrate was then analysed by the HPIC apparatus.

Data obtained from the serum sample and analysed by the HPIC method are shown in Table 4.7.

Amounts in μmol L^{-1}

Time (Hrs)	Serum			Haemolysed		
	Nitrite	**Nitrate**	**Total**	**Nitrite**	**Nitrate**	**Total**
0	13.2	29.0	42.1	9.85	22.5	32.3
1	12.9	17.1	30.00	14.9	25.7	40.6
2	14.4	14.2	28.6	12.0	19.3	31.3
3	**	**	**	**	**	**
3.5	13.5	14.7	28.2	15.1	25.4	40.5
4	**	**	**	**	**	**
5	13.3	15.9	29.2	11.0	19.4	30.4
24	14.6	21.2	35.8	13.8	33.7	47.4
48	16.1	18.2	34.3	14.2	53.8	68.0

Time (Hrs)	Extensively Haemolysed			Lysed Whole blood		
	Nitrite	**Nitrate**	**Total**	**Nitrite**	**Nitrate**	**Total**
0	5.36	8.71	14.1	6.67	36.8	43.5
1	9.27	16.5	25.8	BLD	27.9	>27.9
2	7.53	14.6	22.1	2.90	42.5	45.4
3	**	**	**	**	**	**
3.5	10.7	22.1	32.9	6.23	69.2	75.4
4	**	**	**	**	**	**
5	7.68	19.9	27.6	10.9	51.2	62.1
24	8.54	43.0	51.5	1.16	107	108
48	7.25	61.9	69.2	**	**	**

Time (Hrs)	Whole blood		
	Nitrite	Nitrate	Total
0	89.3	30.0	119
1	28.8	135	164
2	1.59	206	208
3	0.15	268	268
3.5	**	**	**
4	BLD	244	>244
5	**	**	**
24	2.03	234	236
48	**	**	**

** = Time not taken

Table 4.7 Oxidation study of nitrite to nitrate in serum, haemolysed serum and whole blood.

Since the sample above were diluted 1:1 with a 29.0 μmol L^{-1} solution it is assumed that the original concentration of nitrite spike should have been 14.5 μmol L^{-1}.

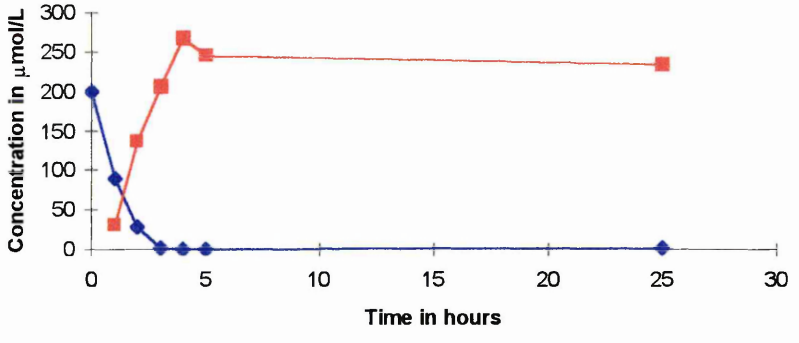


Figure 4.15 Whole blood oxidation of nitrite (decay in blue) and nitrate production (formation in pink).

Since the original blood sample had been left for over two days at 4°C it has been assumed that any nitrite present was below the limits of detection. The sample was mixed 1:1 with

398.5 μmol L^{-1} of nitrite standard which would have made a 199.2 μmol L^{-1} solution at time zero.

From Table 4.7 results it would seem that there is very little oxidation of nitrite to nitrate in the serum/haemolysed sample. Only in whole blood was there a marked decrease in nitrite over time.

These data on the conversion of nitrite to nitrate in whole blood (fig.4.15) are in agreement with those of Moshage [19] and Kelm [58] where a rapid decrease in the serum nitrite levels occurred during which time where all the nitrite appears to have been converted into nitrate after 2.5 hours. It may be that the oxidation of this anion is dependent in a variety of factors such as free radical status or oxidative enzyme concentration. However, it is known that red blood cells quickly enhance the oxidation of nitric oxide to nitrite and nitrite to nitrate via the interaction of the haem group but this is dependent on the concentration of free haem [58].

Oxidation studies on the serum/haemolysed samples were not reported by either of these research groups. It would appear that nitrite is relatively stable in serum left for two days at room temperature.

It can been seen from the other serum sample groups that there is little loss of nitrite or increase in nitrate over time. Minor variations in nitrite from the hourly intervals cannot be explained, except for analytical variation or possibly hydrolysis of nitrosothiols (Chapter 3). From this study it appears that the nitrite levels in the matrix are stable.

The haemolysed samples show a reduction in nitrite compared with those in the spiked samples, possibly due to oxidation caused by free haem or high levels of Fe^{2+}, which increase with the extent of haemolysis.

It was expected that one mole of nitrite is converted to one mole of nitrate. However, from the present results it would appear that there is a net increase in the concentration of nitrate over time. There are three possible explanations: a) there may be other sources forming nitrate during the time course reaction e.g. bacterial contamination or degradation of nitrocompounds, or b) there is an analytical discrepancy present that gives the appearance of an net increase in nitrate e.g. from differences in response factors.

Method details

Reason for a new method

There are many methods that can be used for the individual analysis of nitrite and nitrate as explained in section 2.3, however performing individual analyses first on one anion and then on the reduced/oxidised total is time consuming. Therefore, a one step method, that is analytically reliable, such as ion chromatography or capillary electrophoresis, is ideal for routine investigations.
Also other methods that rely totally on the determination of nitrite may be prone to error as nitrite is unstable and will readily oxidised to nitrate, especially if Fe^{2+} and oxygen are present [19].

Advantages of the method

The advantages of the ion chromatography method are :-
i/ Specificity - chromatography is one of the best analytical techniques for separating analytes of interest from interfering species in complex matrices.

ii/ Reproducibility - buffering of the eluent allows reliable quantification as retention times remain stable and repeats can be assured. Full loop injections give reproducible sample volumes.

iii/ Speed - In the original suppressed ion chromatography method extensive sample preparation was required before injection onto the column. We have produced a 'one-step' procedure that is simple and quick to use, with short run times and only a centrifugal filtration as sample pre-treatment.

iv/ Cost - The reagents required for the enzymatic reduction of nitrate to nitrite are expensive and many reaction steps are required. Once the ion chromatography equipment has been obtained running of the system is relatively cheap with the major expenses being

centrifugal filters and periodic replacement of the analytical column and guard columns. The wash step is an advantageous procedure for prolonging the life of the columns.

v/ Automation - In clinical studies where many samples have to be analysed it is advantageous to produce a system that can carry out batch analysis without supervision. This system has such a capability

vi/ Direct detection - From an analytical point of view this is preferred to quantify the analytes in question. The Griess reagent uses an indirect method whereby a complex reaction occurs to quantify nitrite.

Limitations of the method

Co-elution of nitrite with other components, even though normally removed by this method, is not always completely eliminated and was seen in approximately 2% of random adult samples. The Griess method was employed when the nitrite quantity is in question for such samples. A diode-array detector was connected in series with the UV detector to see if the UV scan spectra showed possible co-elution of nitrite with an unknown substance in cases where the nitrite concentration was significantly higher than the nitrate. None of the samples analysed showed any major difference in the spectra compared to the normal nitrite standard, however the diode-array detector used had a low sensitivity which may not have been able to detect slight changes in peak shape.
Serum contains a diverse array of anionic substances and molecules with a high affinity for the column packing so eventually the column will block and require comprehensive cleaning to restore. The life of the analytical column has, however, been greatly extended by the wash step in the gradient program and the use of a guard column in the HPIC system.

The aim of this work was to produce a robust, accurate and simple ion chromatographic method that had distinct advantages over the current methods available for nitrite, nitrate analysis in serum. A gradient system has been used to enable organic acid separation. Using a chloride eluent has removed the need for addition of a silver resin. The final method combines both these features to produce a reliable and simple procedure for nitric oxide

studies in mammalian systems, or indeed any method that requires the analysis of these anions in complex matrices. Examples include analysis of nitrite and nitrate in meats and vegetables which is important in regulating food preservation and also the measurement of these trace anions in sea water/brine for water quality. This method has been published as a short communication [356] and full paper [357]. Use of this assay with other clinical studies have produced publications on nitric oxide release and graft reactions [358], NOS activity and oestrogen regulation in nonpregnant women [359] and nitric oxide metabolites in human inflammatory eye disease [360]. The results of the present study have been submitted for publication [361].

Chapter 5

Lipid peroxides

Lipid peroxidation

Lipids are a diverse range of compounds that can be saturated, unsaturated, branched, cyclic, keto and hydroxylated hydrocarbon chain structures. Saturated fatty acids have the general formula CH_3-$(CH_2)_n$-COOH and in biological systems most have an even number of carbons (table 5.1). Unsaturated fatty acids contain one or more double bonds. Where double bonds are present they are in the *cis* configuration and separated by at least one methylene (-CH_2-) group and therefore are unconjugated. Differences in carbon chain length and number of double bonds alters their physical properties. Classification of the major biological fatty acids are summarise in table 5.1. Humans can synthesize most of these fatty acids with the exceptions of linoleic and linolenic acids.

In human serum most of the free fatty acids are esterified to glycerol to form mono, di and triglyceride (major form) [362]. Triglycerides, unlike free fatty acids, are neutral, nonpolar, non-ionisable and extremely hydrophobic, allowing packaging into lipoproteins for transportation and storage. Cholesterol is in a lipid class known as the steroids, it can be obtained from the diet or synthesised from acetyl coenzyme A. It can exist in the free form or esterified with fatty acids.

Carbon number:double bonds	Trivial name	Systematic name
C16:0	Palmitic	Hexadecanoic
C16:1	Palmitoleic	9-Hexadecanoic
C18:0	Steric	Octadecanoic
C18:1	Oleic	9-Octadecenoic
C18:2	Linoleic	9,12-Octadecadienoic acid
C18:3	Linolenic	9,12,15-Octadecatrienoic
C20:4	Arachidonic	5,8,11,14-Eicosatetraenoic
C22:6	--	4,7,10,13,16,19-Docosahexaenoic

Table 5.1 List of the major biological lipids

Ingested lipids are hydrolysed by lipases into free fatty acids and smaller glycerides in the stomach. As they interact with bile salts further down the digestive system they form micelles allowing absorption through the epithelial cells of the small intestine. As the long chain free fatty acids move through the cells they are resynthesised into triacylglycerides which when released with other phospholipids and apolipoproteins form chylomicrons. Lipids can be transported in this form or can be carried bound to albumin or as ketone bodies. The liver synthesises very low density lipoprotein (VLDL) which has a high abundance of triacylglycerides and this class of structures is used to distribute lipids to tissues and organs. As the TG are removed VLDL is converted into intermediate density lipoproteins (IDL) and then low density lipoprotein (LDL) where the major portion of triacylglycerides has been removed but which still contain a high amount of cholesterol. High density lipoproteins (HDL) contain a different apoprotein and act by collecting cholesterol from tissues and returning it to the liver. The functions of lipids are 1) as stores of energy, released upon β-oxidation, 2) for formation of membranes from phospholipids, 3) as surface active agents in areas such as maintenance of alveoli structures during the end of the expiration phase of respiration, 4) in membrane receptor function and protein anchoring, 5) in hormonal control by steroids (which are derived from cholesterol), 6) in paracrine control by prostaglandins, HPETEs, HETEs and leukotrienes which are derived from arachidonic acid [363].

Mechanism of lipid peroxidation

This process can occur by two separate mechanisms.

i) enzymatic oxidation with lipoxygenase or cyclooxygenase
ii) autooxidation by free radical attack

Peroxidation of unesterified arachidonic acid by cyclooxygenase and molecular oxygen forms a cyclopentane ring from C8 to C12 and a hydroperoxy group at position C15 to form the cyclic endoperoxide PGG_2 (Figure 5.1) [363]. The peroxy group is reduced by the action of glutathione-dependent peroxidase to form PGH_2 the precursor for all the thromboxanes, prostaglandins and prostacylin. The biological actions of these substances range from

contraction of smooth muscle and blood pressure regulation to thrombus formation and mediation of inflammation.

The 5,8,9,11,12 and 15-lipoxygenases catalyse the addition of hydroperoxy groups on positions 5,8,9,11,12- and 15- respectively of arachidonic acid. 5-hydroperoxy-eicosatetetraenoic acid is the most important product, stimulating an inflammatory response in most immune cells and organ. Specific signals determine which lipoxygenase enzyme is activated. Reduction of these reactive compounds leads to hydroxyeicosanoic acids derivatives and leukotrienes by the action of enzymatic epoxide formation and then reduction with reduced glutathione or water. These long lived substances bring about contraction of smooth muscle and regulation of the action of immune cells.

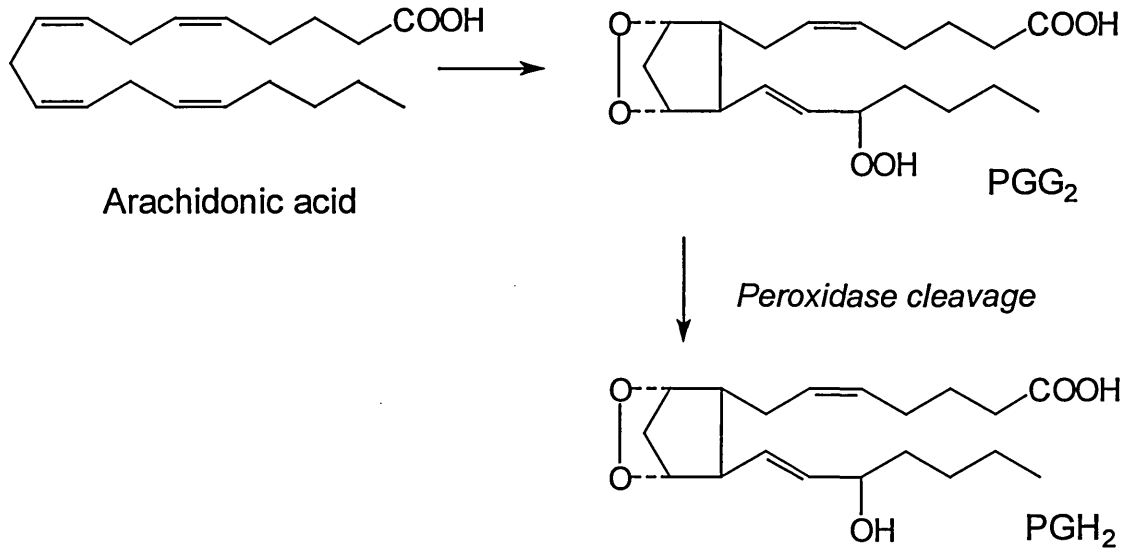


Figure 5.1 Cyclooxygenase enzymatic formation and peroxidase activity as part of the prostaglandin G/H enzyme of PGH_2 from arachidonic acid [363]

Autooxidation

It is now understood that the site of attack is close to the position of the double bond but not necessarily at the double bond. This is due to the low bond dissociation energy of C-H that exists close to the double bonds present in lipids [364]. Hydroxyl radicals formed from the Fenton reaction ($LOOH + Fe^{2+} \rightarrow LO^{\bullet} + OH^{-} + Fe^{3+}$ [362])can abstract a hydrogen from this bond leading to a lipid radical. Molecular oxygen will combine with the radical to abstract another hydrogen from a nearby lipid to form a lipid hydroperoxide and another lipid radical. The propagating reactions involved are varied and complex depending on their environment and involve the transfers from one radical to another or reaction that creates two radicals from one. Termination eventually occurs by the combination of two radicals or by stabilisation of a radical through hydrogen donation by a free radical scavenger such a α-tocopherol. The lipid hydroperoxide is a stable compound until it come into contact with transition metal ions [365] which produce more radicals by reinitiating propagation.

Frankel [366,367] found that chemical oxidation of 9-octadecenoic acid fatty acids produced peroxy- derivatives at the 8,9,10- and 11-positions. Oxidation of 9,12-octadecandienoic produced derivatives at the 9- and 13-positions [368,369,370,367]. With 9,12,15-octadecantrienoic acids; 9,12,13- and 16-peroxy derivatives were produced [172]. Oxidation of 5,8,11,14-eicosatetraenoic acid produced derivatives with hydroxy- derivatives on carbon positions 5,8,9,11,12- and 15- [369,370]. LDL is the major carrier of lipid peroxides in serum [371] and prolongs the half-life of these radicals, $t_{1/2}$ upto 3 hours [372]. It is expected that the major contribution would be derived from the stearic acids as these form the major part of LDL with the ararchidonic/docosahexaenoic acids at only a tenth of the stearic content [362]. Di and triperoxy derivatives have been observed in oxidised standards [373] and are dependent on the number of double bonds.

Production of α-hydroxy fatty acids naturally occurs in higher animals for structures of some myelin lipids. However these C-2 hydroxy compounds are usually from C_{22} and C_{24} fatty acids [363].

Methods of analysis of lipid peroxides

Methods of analysis for lipid peroxides can be separated into direct, indirect and degradation product analyses.

Direct methods - These methods detect the peroxide or reduced lipid directly by diene formation, liquid chromatography, gas chromatography , F_2-isoprostanoids analysis and less utilised techniques such as immunoassay [374] or enzymatic oxidation of glutathione peroxidase or cyclooxygenase activity [375,376,377].

When the unsaturated lipid is attacked by molecular oxygen the double bond is rearranged to form a conjugated double bond system [378]. These bonds show a strong UV absorption at 230-235nm which is useful in pure lipid oxidation studies. Analysis of biological fluids by this means has not been popular due to interference from many compounds such as DNA bases, haem proteins and polyunsaturated fatty acids themselves [379]. Corongiu [380] has used second derivative ultraviolet spectrometry to minimise this interference and increase sensitivity in the analysis of peroxidised cod liver oil.

Reversed phase liquid chromatography using UV absorption at 236nm [381,382], electrochemical [383,384,385], chemiluminescence [386] and fluorescence detection after derivatisation [387] have been used to determine phospholipids, cholesterol and free lipid peroxides. HPLC separation of reduced, methylated lipid peroxides has also been employed followed by derivatisation with GC-MS identification [388,389,390,391]. Advantages of HPLC include simplicity of sample preparation, however compared to GC-MS it is unable to determine structural isomers.

F_2-isoprostanoids are a group of F_2-prostaglandin-like compounds that are stable and are produced by a non-cyclooxygenase mechanism through bicyclic endoperoxide intermediates [392]. As well as being produced *in vivo* they can be artificially formed through autooxidation [393] if samples are left at room temperature. In animal studies of free radical tissue damage it has been shown that circulating levels of these F_2- isoprostanes are increased in plasma and urine [394,392]. They have been analysed by NICI-GC-MS of their pentafluorobenzyl esters and TMS derivatives after SPE sample cleanup [395].

Indirect methods - Iodometric, methylene blue, xylenol orange and dichlorofluorescein.

The principle of these methods is use of a reagent which can be oxidised by the peroxy group on the lipid. The most widely used indirect method is the iodometric assay, in which acidic iodide reacts stoichiometrically with the lipid/protein peroxide to form iodine which in turn reacts with the excess of iodide to form triiodide with absorption of UV radiation at 290-360nm [396,397]. Unfortunately this must be carried out under anaerobic conditions to remove interference from oxygen. Transition metals, light, ascorbate and anything that reacts with iodine or iodide will interfere, as will substances that absorb this wavelength [377]. Addition of starch to produces a blue complex absorbing at 560nm [398].

A methylene blue derivative has been used for determination of hydroperoxy standards with haemoglobin as a catalyst [399]. This method was updated by Yagi [400] but required enzymatic removal of ascorbate, complexing of holotransferrin and enzymatic hydrolysis of the lipoproteins to free the lipid peroxides before the reaction could occur. The coloured compound could be detected at 665nm.

The FOX assays consist of ferrous ions in dilute acid that contain xylenol orange. Hydroperoxides oxidise these to ferric ions which react with the reagent to form a blue-purple complex absorbing at 560nm. Sorbitol is added to the mixture as a amplification reagent, enhancing the amount of complex formed. Ascorbate in the sample interferes by affecting the absorption in a concentration dependent manner [380].

Cathcart [401] reported picomole detection of peroxides using a fluorescent oxidised derivative of dichlorofluorescein formed by the reaction of lipid peroxides and haematin with the reagent.

Chajes [402] compared the diene, methylene blue, triodide, and liberated lipids, triodide methodologies and found the latter method to be the most specific. However caution was stated as all these method produced levels in the micromolar range where methods using LC, with chemiluminesence or electrochemical detection showed much lower levels in the nanomolar range [403,404].

Degradation products - MDA, Aldehydes and hydrocarbons.

The decomposition reaction of lipid hydroperoxides with transition metals results in the formation of lipid alkoxy radicals which undergo β-cleavage reactions (homolytic scission) of the C-C bonds on either side of the alkoxy group. This is accelerated by heating under acidic conditions in the presence of a transition metal. Which side of the peroxy group is cleaved governs which aldehyde plus core aldehyde molecule or radical is produced [362,367,405,406]. Oxidised 9-octadecenoic and 9,12 octadecadienoic acid standards produce 4, 2-monohydroxy alkenals and alkanals with 8,9,10 and 11 carbon atoms [407,408]. Most research has concentrated on the determination of 4-hydroxyhexanal and 4-hydroxynonenal from the oxidation of docosahexaenoic and octadecane/eicosanoic acids [409,410] respectively. Increased interest in these products has occurred because they are not just degradation products of lipid peroxidation but have also been shown to have biological properties such as enzyme inactivation [411] , erythrocyte lysis [411,412], chemotactic activity to neutrophils and inhibition of DNA and protein synthesis [413]. Other biological and chemical actions of these substances have been reviewed by Esterbauer [414].

These compounds have been investigated by converting the aldehyde group to a pentafluorooxime derivative and analysing by GC-MS [108,407,408,415-421], GC-ECD [422], or by reacting with dinitrophenyl hydrazine and separating by LC-UV [423,424,412] or detecting by fluorescent techniques following reaction with lipids and proteins to form Schiff bases [425,379,426]. Due to the stability of these molecules the methods have become popular for biological free radical damage studies. Non-hydroxylated aldehydes and exhaled hydrocarbons such as pentane and isoprene have also been detected in exhaled air of humans and animals and analysed by headspace GC [427,428,429].

From all these compounds one degradation product, malondialdehyde (MDA), has become the most popular index of lipid peroxidation due to method simplicity and nanomolar sensitivity. Many clinical studies have used this technique and therefore it was chosen for comparative work with lipid peroxides by GC-MS. Malondialdehyde is formed from the heterolytic cleavage of cyclic endoperoxides. These are enzymatically formed from the prostaglandin pathway, being β-unsaturated aldehydes [430], dihydroperoxides and monohydroperoxides [431]. Most are derived from the decomposition of arachidonic and docosanoic acids [362,407,408]. The assay involves heating the oxidised standard or sample at

low pH in the presence of ferrous ions to catalyse the decomposition of lipid peroxides into the stable fragments of MDA. Chelators such as EDTA will inhibit this formation of MDA presumably by affecting peroxide degradation [432,433], although transition metals will also promote peroxidation of lipids. Current methods therefore use the free radical scavenger butylated hydroxytoluene (BHT) which does not interfere with the decomposition but which will stop free radical propagation [434,435]. MDA reacts with two molecules of thiobarbituric acid to form a pink chromogen which absorbs at 532nm [436]. However this reaction is not specific for MDA as biochemical and spectral interferences exist from haemoglobin, urea, glucose, cholesterol, bilirubin [437] and TBA itself [438]. Also, oxidative degradation of some amino acids [439], 2-deoxyribose [440] and polyunsaturated fatty acids [441] to MDA have led to overestimation of the peroxidation index. Attempts to overcome these inherent problems have included the use of fluorescence spectrometry [442], substitution of TBA by 1,3 diethyl thiobarbituric acid [443] which increases stability and also gives greater extraction into organic solvents [442], and the use of separation by GC-MS [439,444,445] and HPLC [446-455,438,435,444,455,396,423]. Free MDA has been detected in tissues [449] but is low (42 nmol L^{-1}) [451] to undetectable in human plasma [450]. This is expected since MDA forms complexes with proteins and amino acids [456]. All these factors have been considered by the Lepage [457] group who optimised the precipitating reagents, pH and extracting solvent before HPLC separation for serum MDA analysis. This method was followed in the present work except for the separation step, the quantification of thiobarbituric acid reactive substances (TBARS) being used instead. These have been shown to overestimate but still correlate with parallel results of the MDA-TBA_2 complex by LC [448,458,455] and GC-MS separations [439]. Calibration graphs are constructed following mild heating and acid hydrolysis of 1,1,3,3-tetraethoxypropane standard prior to analysis because MDA itself is unstable [450].

Most of analytical methods used for clinical research have used the simpler colorimetic TBARS assay. Therefore to assist in the validation of a new GC-MS method it would be useful to compare lipid peroxides determined by both techniques.

TBARS assay

Reagents - MDA

2,6 Di-tert-butyl-p-cresol (BHT) (Fluka), tungstic acid (Sigma), sodium hydroxide (Aldrich), concentrated sulphuric acid (AR, Fisons), concentrated hydrochloric acid (AR, BDH), 1,1,3,3-tetraethoxypropane (Sigma), 1,3-diethyl-2-thiobarbituric acid (DETBA)(Sigma), 1-butanol (HPLC grade) (Sigma-Aldrich) were used.

Method

Aliquots of 500µl of 0.5% BHT in methanol and 2mls of H_2O were added to 500µl of serum or plasma. Acid hydrolysis of MDA from protein was accomplished by the addition of 200µl of 0.33mol L^{-1} H_2SO_4 and addition of 150µl of 0.3M Na_2WO_4, while vortex mixing. The protein fraction was pelleted by centrifugation at 1000xg for 10 minutes. The supernatant was mixed with 1ml of a 1% DETBA solution in water which had been heated to 60°C for 30 minutes to dissolve. The mixture was placed in a Reacti-vial and monitored to have a pH between 2.5-4.5 because TBA_2-MDA complex can dissociate at high pH and the reaction between MDA and TBA must occur at no less than pH 2.0 or the colour development will be inhibited.

Each tube was sealed with a Teflon-lined cap and heated to 100°C for 60 minutes. After cooling 500µl of 5mol L^{-1} HCL was added and the mixtures extracted twice with 1ml of 1-butanol (which has the best sample recovery [457] and lowest fluorescence [442]). Precipitation of the proteins and solvent extraction greatly reduced background interferences. Fluorescence from the samples was measured in a Hitachi F-2000 fluorescence spectrometer with excitation at 532nm and emission at 558nm and results were quantified by comparison with standards of known concentration.

Comparison of results by other groups summarised by Wade [459] and Wong [444], has shown discrepancies, with normal reference ranges going from 0.94-47.2 µmol L^{-1} using

spectrometry but less variation of 1.7-3.74 μmol L^{-1} with fluorometry. This overall difference has been attributed to different procedures carried out by each group. Yagi [460] reported the closest TBARS method to that is used here; their normal reference range was quoted as 1.8-3.9 μmol/L.

Gas chromatographic techniques for analysis of lipid hydroperoxides

Gas chromatography - basic principles

A sample mixture in a suitable solvent is injected into a heated injector port where the solvent and the samples are vaporised and mixed with the flow regulated carrier gas (N_2, H_2 or He [327]). From here the remaining components pass into the temperature controlled column. These can be high capacity packed or high efficiency wall-coated open tubular columns with an immobilised liquid incorporating polar or a non-polar stationary phase. Most of these contain polymethyl siloxane with different substituent groups that are used to adjust the polarity of the column to the species that are separated. As the mixture is passed over the stationary phase the separate analytes will have varying retardations. Separation is governed by the boiling points of the analytes (and the oven temperature) and their relative affinities for the stationary phase (polarity). When the analyte elutes from the column it is passed into a detector.

Mass spectrometry, atomic emission and electron capture detection

Mass spectrometry and atomic emission detection are useful complementary techniques where structure can be determined by mass spectrometry and elemental composition can be confirmed by atomic emission spectra, especially when modifying with halogen or silicon containing derivatives. Electron capture detection allows a very sensitive analysis for electronegative groups [327].

Mass spectrometry detector

In electron ionisation mass spectrometry sample molecules are bombarded by electrons at 70eV in a vacuum [327]. When ionised the now unstable positively charged sample molecules can fragment to form smaller positive ions and neutral fragments. The charged ions are repelled into a quadrapole mass analyser which consists of four electrically charged rods. Two opposing rods are positively charged while the other two are negatively charged. Alteration of the electrical field by changing the radio-frequency and DC voltage between the four rods creates an oscillating electrical field causing oscillation of the ions. Under these conditions only those of a specific mass/charge will track through to reach the detector. Fragmentation is characteristic of molecular size, composition and the functional groups present from the parent molecule.

Atomic emission detection

The separated volatile compounds from a GC are carried into a microwave-energised helium plasma [327]. The high energy plasma atomises the compounds and excites the electrons in each element of the compound. As the electrons return to the ground state they emit radiation at specific wavelengths that correspond to the originating element. The radiation is detected by a diode-array spectrometer. The use of a diode array allows the detector to monitor up to four elements at any one time provided they all fall within the detection window that is being monitored. Replicate injections at different wavelengths allow elemental determination from a wide range of volatile substances.

Electron capture detection

The nitrogen carrier gas is ionised by β particles emitted from a Ni^{63} source [327]. This forms low energy electrons and positively charged nitrogen gas. The electrons quickly move to an anode before they can recombine with N_2^+. In the absence of any analyte a constant current is created between the electrodes via the ionised carrier gas. When an electronegative compound from the column enters the electron stream it will combine with electrons to

form a negative species which in turn can react with the ionised nitrogen to form two neutral species. This reduces the electron flow to the anode resulting in a (negative) concentration dependent response of the analyte. This sensitive detector is used for any molecules containing electronegative functional groups such as halogens, however this detector has a limited linear response.

Gas chromatographic method development for lipid peroxides

GC-MS techniques for lipid hydroperoxide determination have evolved from the analysis of methyl esters of straight chain fatty acids, a method which has acceptable detection limits for dietary lipids but poor sensitivity for lipids of low abundance. Kuijk [461,462] produced a GC-MS method for lipid peroxides in tissues, which reduced the extracted peroxides to hydroxyl groups with sodium borohydride. The treated phospholipids were then transesterifed into methyl esters (analysed by EI-MS for structural information) [461] or pentafluorobenzyl esters (analysed by CI-MS for picomolar detection) [462] using pentafluorobenzyl alcohol, although this reagent is not very effective for derivatising free lipids. The hydroxyl group was then transformed into a trimethylsilane ester with N,O-Bis (trimethylsilyl) trifluoracetamide (BSTFA). The lipids were separated on a short DB-5 column which could resolve hydroxyl lipids by chain length but not by hydroxyl position. This method did not use an internal standard and so the information produced was only qualitative.

The same group [463] improved the original method by using a platinum oxide/H_2 catalyst to reduce the double bonds of the lipids as well as reducing the peroxide into a hydroxyl group. This greatly simplified the chromatograms and also reduced autooxidation of the lipids in the work-up procedure [464] and possible artifactual formation of peroxides during sodium borohydride reduction [465]. A chemical saponification step was introduced that involved heating the sample with potassium hydroxide to liberate free lipid peroxides from triglyceride and cholesterol. This greatly improved the conversion but had to be monitored by TLC separation since any water in the sample would deactivate the sodium pentoxide catalyst. A more reactive transesterification reagent, pentafluorobenzyl bromide, was therefore used for derivatising both free and esterified acid groups. This esterification had

already been successfully used for determination of free fatty acids by GC-ECD [466] and for analysis of prostaglandins [467,468,469] and leukotrienes [470] by GC-NICI-MS.

Short chain acids eluted first and of those with hydroxyl groups closest to the acid group eluted earlier than those with the hydroxyl group nearer the methyl side. The identity of the hydroxyl C18 and hydroxyl C20 acids was determined by the molecular ion using SIM-NICI. This method is, however, semi-quantitative. Lipid peroxide methyl esters had been determined by a much early method using SIM-NICI-MS [471].

This method was finally improved by the addition of a deuterated internal standard [472]. However, it was discovered that during the hydrogenation method these standards may be changed to undeuterated molecules [473]. This could be overcome by using stable isotope dilution [474] or by using standards with hydroxyl groups at odd numbered positions [473].

Recent methods have concerned problems associated with the water reactive derivatising agents used in previous methods. A method by Nikkari [475] involved hydrogenation, saponification and esterification of fatty acids into methyl esters. The lipids were separated by TLC and the hydroxyl groups were acetylated and detected by GC-FID. Unfortunately this method could not completely resolve the different positional isomers of C18 and C20 hydroxyl fatty acids. The most recent GC lipid peroxide publication by Wilson [473] followed the accepted work-up procedure as previously discussed [463,471] except the fatty acid methyl esters (FAME) were purified on a silica column. The extracted hydroxyl FAME's were then methylated and analysed by GC-EI-MS. The concentration of the LPO were quantified by the used of hydroxy-C17 and hydroxy-C19 prepared at their laboratory.

The aim of the present work was to produce a practical method that could accurately determine the lipid peroxides present in normal and pre-eclamptic serum.

Chemical derivatives for GC lipid peroxide analysis

Derivatisation of the carboxyl group is required to give sample stability at high temperature, better separation and increased volatility for gas chromatographic analysis. Lipids are generally converted into their methyl esters with boron trifluoride:methanol [476] or with diazomethane [477]. These derivatives are employed extensively because of the simplicity of their formation and detection. Trimethylsilyl-esters of saturated lipids have been analysed [477] but more recently pentafluorobenzyl-esters of compounds containing carboxylic acids

have been studied [466,470] giving sensitivity with GC-ECD and MS detection. Derivatives of hydroxyl groups are more diverse. In early investigations into the locations of double bonds [478] unsaturated fatty acids were treated with osmium tetroxide or potassium permanganate to form two hydroxy groups where the double bonds were present, these functional groups then being converted into TMS esters. Fragmentation between the two ester groups leads to two charged fragments that can be used to calculate the original position of the double bond. This has been used to locate the position of hydroxy groups from reduced lipid peroxides. Other derivatives including acetyl [475], *tertiary* butyldimethylsilyl [479,480], methoxy [473,481] and pentafluorobenzoic [482,483] esters have been used to overcome the problem of TMS ester hydrolysis [484]. Recently a method by Turnipseed [485] used the direct formation of TMS derivatives of peroxy groups with MSTFA for analysis of standards and liver microsomal fractions. This derivative would be unlikely to survive the workup procedure necessary for serum lipid peroxide analysis.

Using the derivatisation method of Thomas [472], general assumptions can be made on what will be seen by each detector. Mass spectrometry shows that the PFB group from the lipid molecule is easily ionised in the ion source and fragments giving an ion with mass/charge of 181 [470,486]. However, there is conflicting evidence over what occurs with the remaining portion of the molecule. Since the authors of the original method used chemical ionisation they did not use fragmentation as a marker for identification but only the molecular ion [462,463,472]. It is known that the TMS group will ionise to form mainly a fragment of m/z 73 with other less abundant fragments such as 129 [487,488].

In this work, parallel experiments with mass spectrometric and atomic emission detection were planned, with different heteroatoms used for derivatisation of the carboxy and hydroxy groups of reduced lipid peroxides. Atomic emission data will show unoxidised lipids containing the elements carbon (hydrocarbon chain), oxygen (acid group) and fluorine (pentafluorobenzyl ester) while the oxidised lipids should contain carbon, oxygen, fluorine and silicon (TMS hydroxyl derivative) as shown in figure 5.2. Comparison with electron capture detection will allow greater sensitivity for PFB esters which may be useful with real samples.

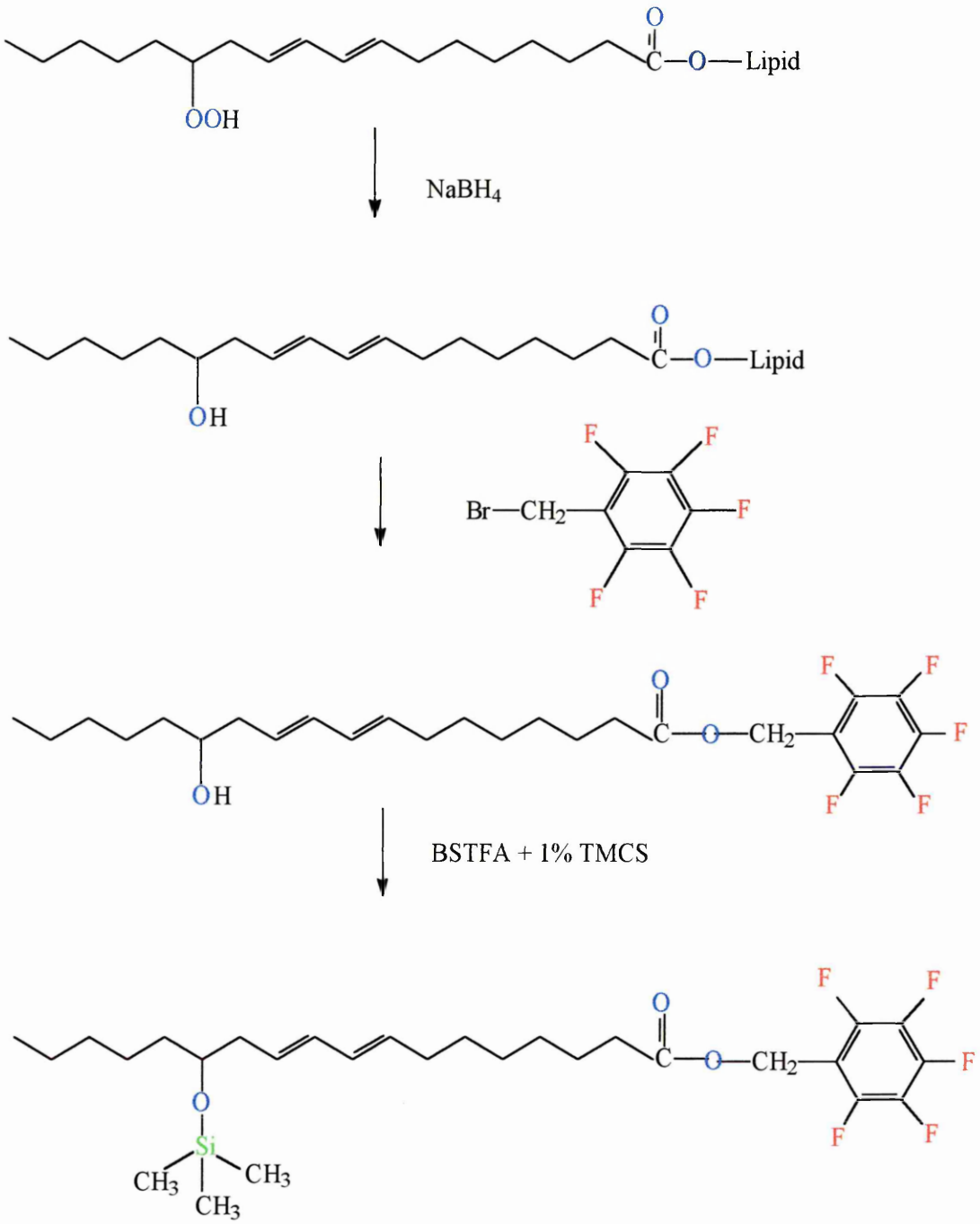


Figure 5.2 - elemental detection of lipid peroxides

Reagents

Methanol and dichloromethane were of HPLC grade and were dried using molecular sieves, 3A (Aldrich).
Standards of heptadecanoic, hexadecanoic, 2-hydroxyeicosanoic, 5,8,11,14 eicosatetraenoic (from porcine liver) and 9-octadecenoic acids were obtained from Sigma. 9,12-octadecenoic acid and cholestan-3β-ol were purchased from BDH. 12-hydroxystearic and 2-hydroxyhexadecanoic acid were from Lancaster. Raney nickel (50% slurry in water), N,N-diisopropylethylamine, pentafluorobenzyl bromide, sodium hydroxide were obtained from Aldrich. bis(trimethylsilyl)trifluororacetamide with 1%TMCS was bought from Sigma. Magnesium sulphate (GPR) was obtained from BDH.

Experimental for Gas chromatographic method

The method is a modification of procedures by Thomas [472] and Wilson [473].

Extraction/hydrogenation

Aliquots of 100µl of pure heptadecanoic acid internal standard was added to each glass sample vial and mixed thoroughly. Lipids were extracted from serum samples (500µl-1000µl) into 2mls of dichloromethane:methanol 2:1 (v/v) mixture twice [473]. The extracted lipids in the solvent were removed and pipetted into a Reacti-vial followed by 100µl of 50% Raney nickel (in water) and 300µl of methanol to solubilise the water/DCM phases into one homogenous layer. The solvent was purged for several minutes with H_2 gas in a fume cupboard under a slow flow, then capped with a hydrogen headspace and heated to 60°C. After 1 hour the vials were allowed to cool before being centrifuged to pellet the Raney nickel. Then the solvent was decanted off into a fresh vial and the liquid was evaporated under a stream of nitrogen until only water remained.

Saponification

An aliquots of 200µl of ethanol with 100µl of 1M sodium hydroxide (in water) was added to the water layer and the mixture was heated at 60°C for 1 hour. Once cooled the solution was neutralised by addition of 300µl of 1.1M HCl solution then 0.5mls of water and 1ml of DCM were added. The layers were mixed and the water layer removed, after which two more DCM extractions were performed. The DCM fractions were pooled, dried with magnesium sulphate and centrifuged. The dry DCM lipid solution was pipetted into a clean, dry Reacti-vial and evaporated to dryness.

Derivatisation

To each Reacti-vial was added 40µl diisopropylethylamine, then 50µl of dry DCM with 50µl of pentafluorobenzyl bromide and the vial left for 1hr at 80°C. After cooling, 50µl of BSTFA containing 1% TMCS was added and the vial incubated at 60°C for 30 minutes.

Conditions

Equipment used - HP5890 Series II GC-MSD (HP5971A), GC-AED (HP5921A) and HP5880 - GC-ECD each with a Restek Rtx-5 column, length 30 meters with ID of 0.25mm and film thickness of 0.25mm. The injector temperature was set at 275°C with the transfer line temperature (except for GC-ECD) maintained at 280°C. Helium was used as the carrier gas for the GC-AED and GC-MSD. The detector temperature for the GC-ECD was set at 300°C and nitrogen was used as the carrier gas. The flow rate for all three gas chromatography systems was set at 1 ml/min. The oven temperature started at 150°C and was held isothermally for 10 minutes and then increased by 10°C a minute to 260°C when the rate was reduced to 2°C a minute until it finally reached 300°C. This temperature was then maintained for 5 minutes. 1-2µl of sample was injected in splitless mode.

Oxidised serum

Oxidised human serum and unsaturated lipid standards were used to establish the fragmentation patterns by GC-MS, and to provide model responses for GC-AED detection and retention/resolution optimisation of the column and temperature program. The oxidation method was that described by Esterbauer [489,408]. The serum sample was diluted 1:1 with phosphate buffered saline (PBS), while lipid standards (oleic and linoleic acid) were dissolved in 1ml of methanol and 1ml PBS. To each sample was added 200µl of a 20mM ascorbate/0.8mM iron sulphate solution and the samples left for approximately 24 hours (ascorbate is used to regenerate the Fe^{2+} from Fe^{3+} after it has donated a electron). After this time 1 drop of 30% H_2O_2 was added and the mixture left for 36 hours. Both reactions were at room temperature The samples were then treated as previously described in the method section using Raney nickel or sodium borohydride reduction.

Results and Discussion

In initial studies sunflower oil was used as a lipid sample because it contains a variety of unsaturated lipids. These were oxidised using a mixture of hydrogen peroxide and iron/copper to form the reactive hydroxyl/oxygen radicals to produce the lipid peroxides. At the beginning of method development the method of Thomas [463] was followed but since platinum oxide/hydrogen reduction would be too costly for the large sample numbers anticipated for the clinical study. Raney nickel was applied as a cheaper alternative to platinum oxide or rhodium. This is capable of reducing double bonds [490,491] under mild conditions even in the absence of hydrogen [490]. It was necessary to saturate the double bonds to eliminate the formation of peroxides during the work-up procedure and simplify the resulting chromatograms.

Reduction of the peroxy-lipid is necessary to stop the peroxide fragmenting during sample preparation. Replacing pentafluorobenzyl alcohol with pentafluorobenzyl bromide in the derivatisation reaction resulted in a greater response by revealing more esterified lipids. In an attempt to reduce the number of peaks seen in the chromatogram silica SPE cartridges were used to separate the hydroxy fatty acids from the free fatty acids. It was still not possible to detect lipid peroxides but aliphatic fatty acids (hexane eluent) were separated from the more polar lipids such as cholesterol in 50:50% hexane:ethyl acetate eluent. However, SPE would prove too costly to use on each sample and was abandoned. The results showed no peaks that could accurately be determined as lipid peroxides by GC-MS or GC-AED with samples oxidised by the Fenton reaction, so a modified method using a mixture of iron sulphate, ascorbic acid and lipid standard/sample [408,489] left several days (instead of hours) was tried. This was successful in revealing lipid peroxides and led the way to forming a theoretical fragmentation chart which could be used to predict the major major fragment ion(s) found in hydroxy hexadecanoic, octadecanoic and eicosanoic acids (table 5.2a, 5.2b). For mild reduction sodium borohydride was used as the presence of double bonds allowed further confirmation of structural peroxide identity when combined with retention data. In subsequent clinical samples it was necessary to saturate the double bonds from these C18 fatty acids to simplify the chromatograms.

The use of hydroxy acid standards enabled approximation of the retention time of derivatised reduced lipid peroxides, as well as confirming the fragmentation pattern by GC-

MS and completion of the derivatisation. When Raney nickel was first used the resulting water/methanol phase was removed from the dichloromethane/methanol phase after hydrogenation. The recoveries of 2-hydroxyhexadecanoic and 12-hydroxyoctadecanoic were 4 and 19% respectively. When using 1M NaOH in 95% methanol for saponification there was evidence of methyl ester formation [492] by MSD/AED data, but this was eliminated by using 1M NaOH in water.

18-CH3	17	16	15	14	13	12	11	10	9	8	7	6	5	4	3	2	1-COO
103	117	131	145	159	173	187	201	215	229	243	257	271	285	299	313	327	
15	29	43	57	71	85	99	113	127	141	155	169	183	197	211	225	239	

Table 5.2a Theoretical mass to charge ratio expected for OTMS group added on to octadecanoic acid

2nd column = oxidised fragment ion + OTMS group moving from position 8-2
3rd column = unoxidised mass fragment group moving from position 1-17

20-CH3	19	18	17	16	15	14	13	12	11	10	9	8	7	6	5	4	3	2	1-COO
103	117	131	145	159	173	187	201	215	229	243	257	271	285	299	313	327	341	355	
15	29	43	57	71	85	99	113	127	141	155	169	183	197	211	225	239	253	267	

Table 5.2b Theoretical mass to charge ratio expected for OTMS group added on to eicosanoic acid

2nd column = oxidised fragment ion + OTMS group moving from position 20-2
3rd column = unoxidised mass fragment group moving from position 1-19

Hydroxy standard fragmentation

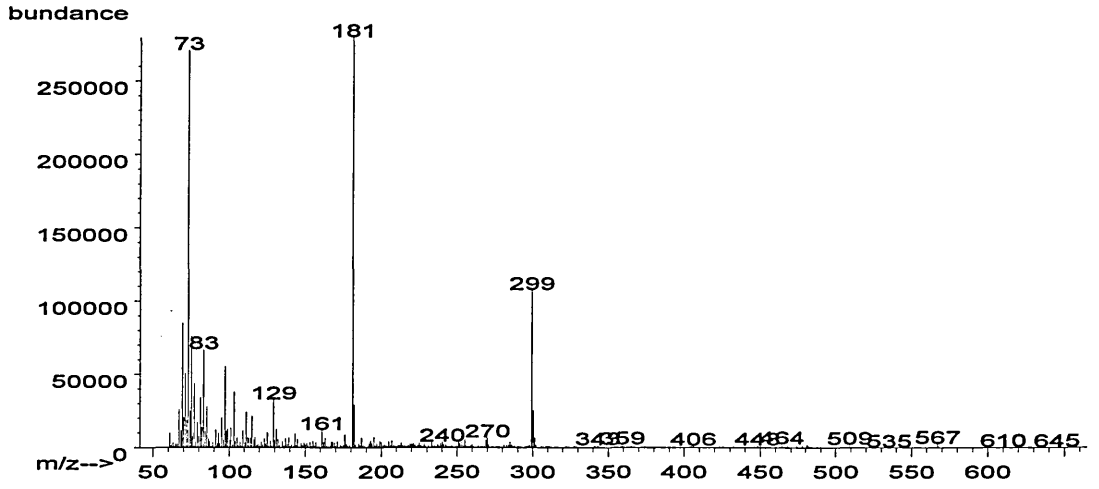


Figure 5.3 EI Mass spectrum of 2-hydroxy hexadecanoic acid (PFB/TMS) derivative

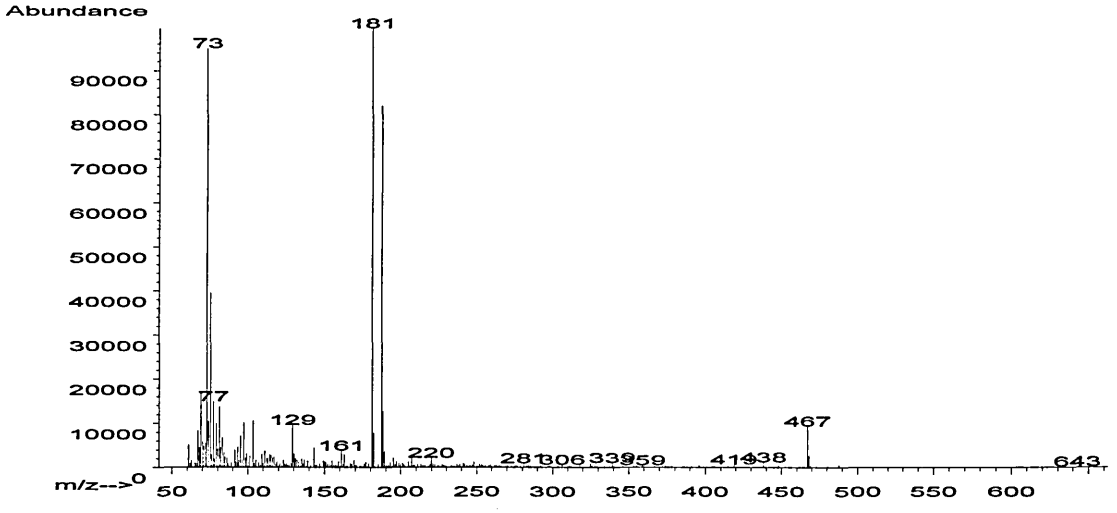


Figure 5.4 EI Mass spectrum of 12-hydroxy octadecanoic acid (PFB/TMS) derivative

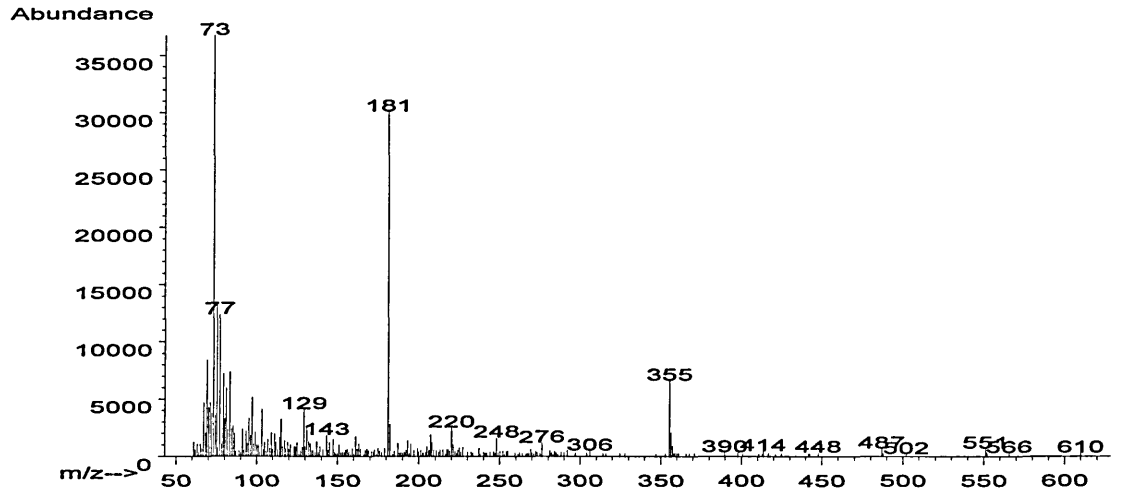


Figure 5.5 EI Mass spectrum of 2-hydroxy eicosanoic acid (PFB/TMS) derivative

Figures 5.3-5.5 show the mass spectra of commercial hydroxy acid standards derivatised as described. The total RMMs for derivatives of 2-hydroxyhexandecanoic, 12-hydroxyoctadecanoic and 2-eicosanoic acids were 525, 553 and 581 respectively, although the molecular ions were not seen. The ions of most importance are the base peak 181 (C_6F_5-CH_2^+) from the tryponium ion formed from the cleavage of the pentafluorobenzyl group, both 73 ($(CH_3)_3Si^+$) and 129 ($(CH_3)_3SiO^+$=CH-CH=CH_2)[121] from the trimethylsilyl group. Other major fragment ion(s) are the fragments 299 (fig.5.3), 187 (fig.5.4) and 355 (fig.5.5) formed from the cleavage of the OTMS group and the methyl end of the lipid portion as illustrated in fig.5.6. It is also observed that the base peak ion shows triplets of m, m+1, m+2 with abundances of approximately 100:5:3 corresponding to the natural abundance's of ^{28}Si, ^{29}Si and ^{30}Si respectively [502]. The expected fragments from the OTMS group and the carboxyl end [486] were not seen, although two ionised fragments are normally seen when TMS esters are formed from lipid methyl esters [493-500,389,487,488]. Whether the loss of this ester group could lead to the formation of the charged methyl-OTMS group and an uncharged fragment is unknown, or alternatively detector insensitivity at high masses might be the reason for the lack of this ion. Insensitivity seems unlikely as since the fragment ion of 2-hydroxyeicosanoic (355) is clearly seen, so it therefore seems that under these condition the carboxyl ion is not formed in appreciable abundance.

Using the fragmentation patterns of the standards as a guide it is possible to predict the methyl-OTMS+ fragment for each lipid and determine the position of the original hydroxy group as shown in tables 5.2a and b.

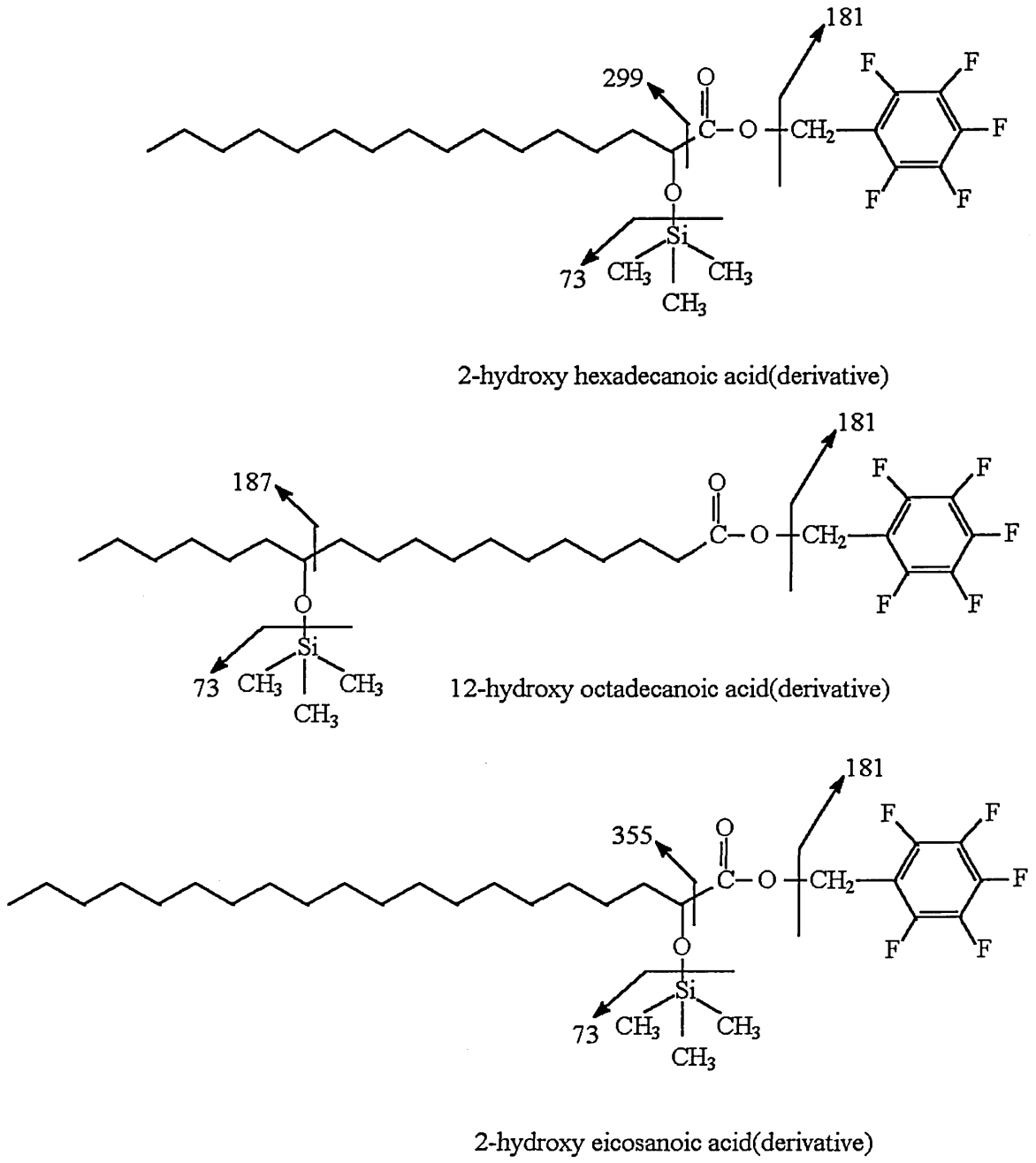


Figure 5.6 Major fragmentation patterns of hydroxy standards

Standards for normal lipids were also analysed by GC-MS (figs 5.7-5.11) and show the base peak to be 181. There is also a smaller peak with a mass of M-PFB-18 which is surrounded by two other ions of M-PFB and M-PFB-36 produced when the PFB group has been cleaved off in the ion source. These are possibly formed from cyclisation of one of the free oxygens with two hydrogens followed by the other oxygen with two hydrogens. However

the M-PFB-18 is more abundant then M-PFB or M-PFB-36. The actual composition of these fragments is unknown. This fragmentation is very different to the methyl esters which show M^+, M-31 and M-43 followed by subsequent losses of 14 mass units [477,501].

The random bond breaking along the hydrocarbon chain of the structually identical molcules produces the sequential loss of CH_2 (m/z 14) to produce the series shown in the spectra. In the octadecanoic series the fragment loses masses of 2 from oleic to 4 in linoleic suggesting that the fragment retains the double bond information from the central region of the lipid but the position is uncertain because the double bond migrates when the molecular ion is formed [477]. The molecular ion (i.e. fragment +18 +181= molecular ion) is seen in some cases (such as hexadecanoic acid) but not all. Using this main ion (M-181 (PFB)-18 (?)) it was possible to predict lipid identification as shown in table 5.3.

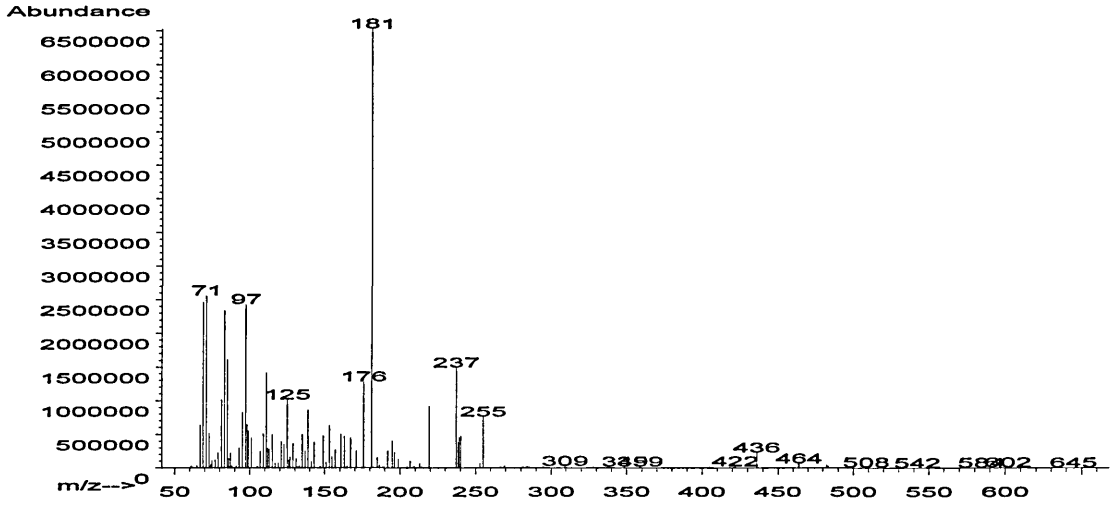


Figure 5.7 EI Mass spectrum of hexadecanoic acid (PFB ester) derivative

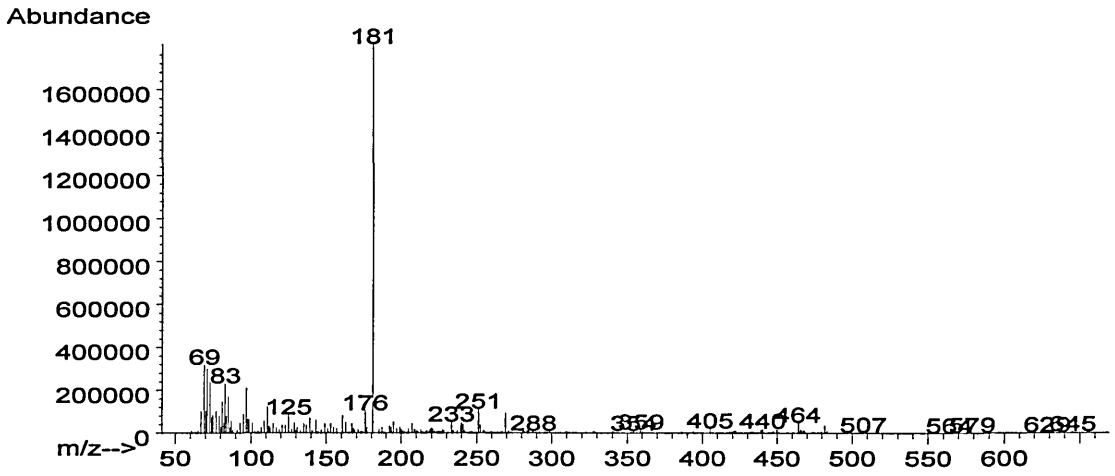


Figure 5.8 EI Mass spectrum of heptadecanoic acid (PFB) derivative (internal standard)

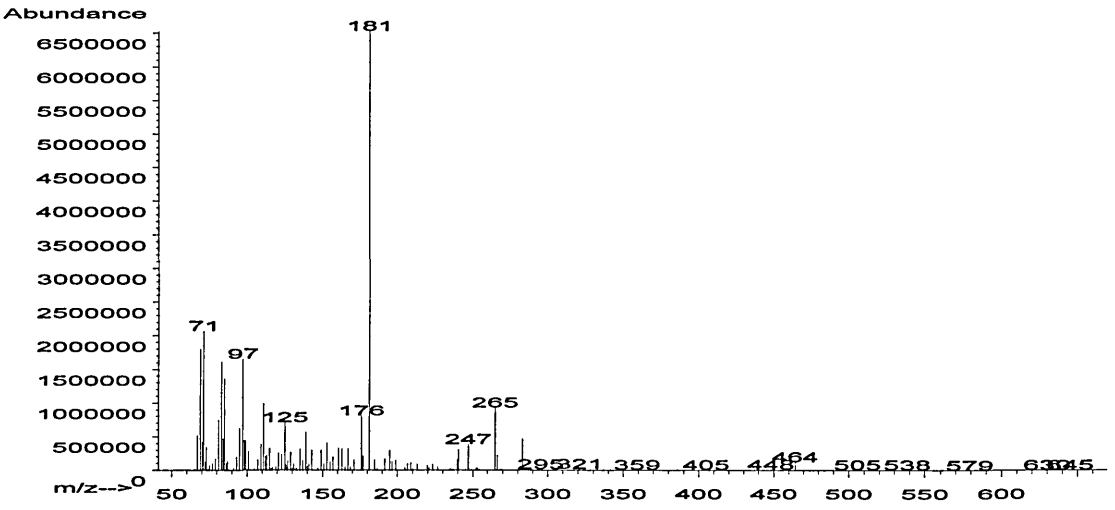


Figure 5.9 EI Mass spectrum of octadecanoic acid (PFB) derivative

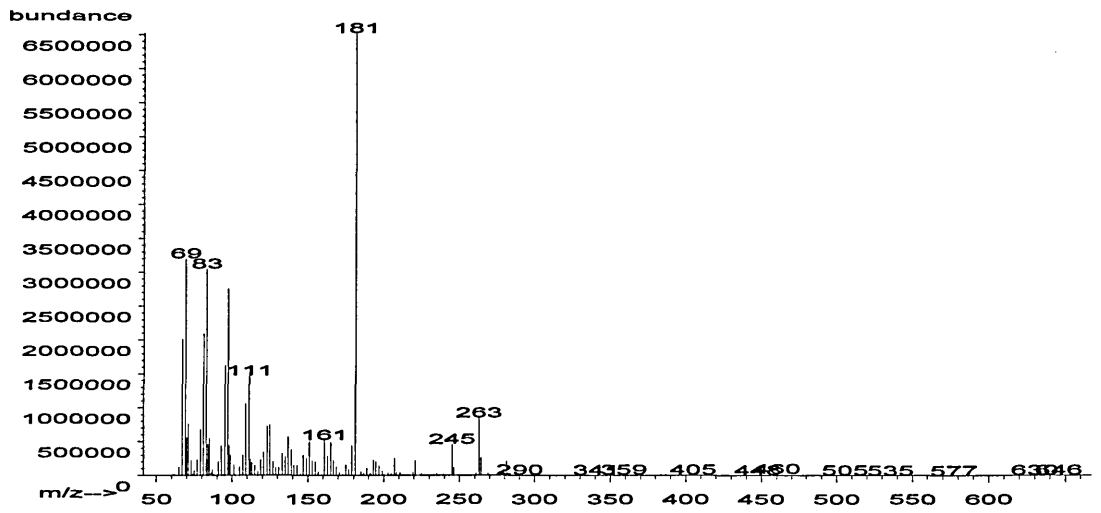


Figure 5.10 EI Mass spectrum of octadecenoic acid (PFB) derivative (location of double bond unknown)

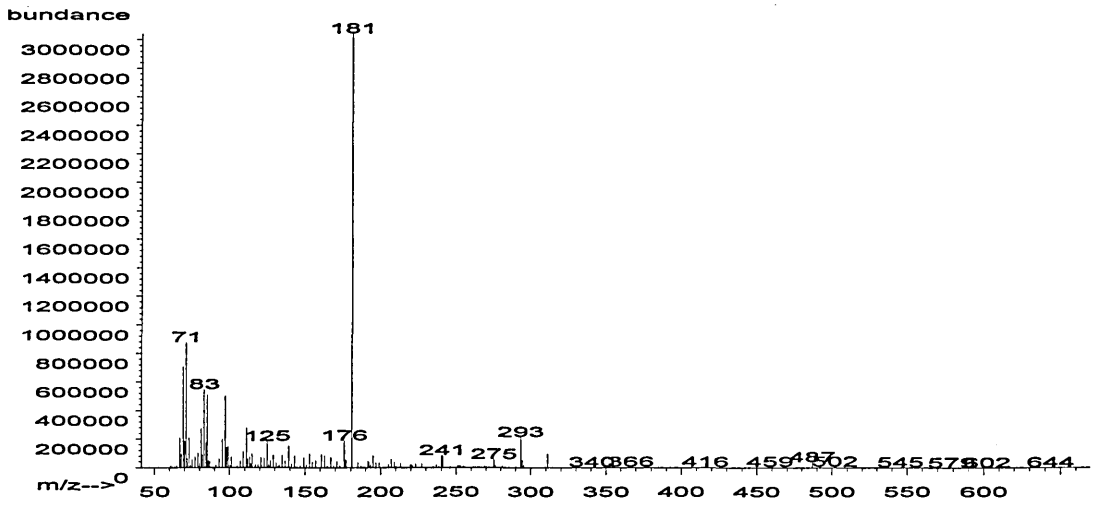


Figure 5.11 EI Mass spectrum of eicosanoic acid (PFB) derivative (from human serum)

Oxidised serum

Having thus established a table (5.3) of the ions that would be expected in the hydroxy lipids, 9-octadecenoic (oleic) and 9,12-octadecadienoic (linoleic) acid standards were oxidised and analysed using the three gas chromatographic detectors.

Following the oxidation of 9-octadecenoic acid from standards two unresolved peaks were observed even under isothermal conditions (spectra in figures 5.12 and 5.13).

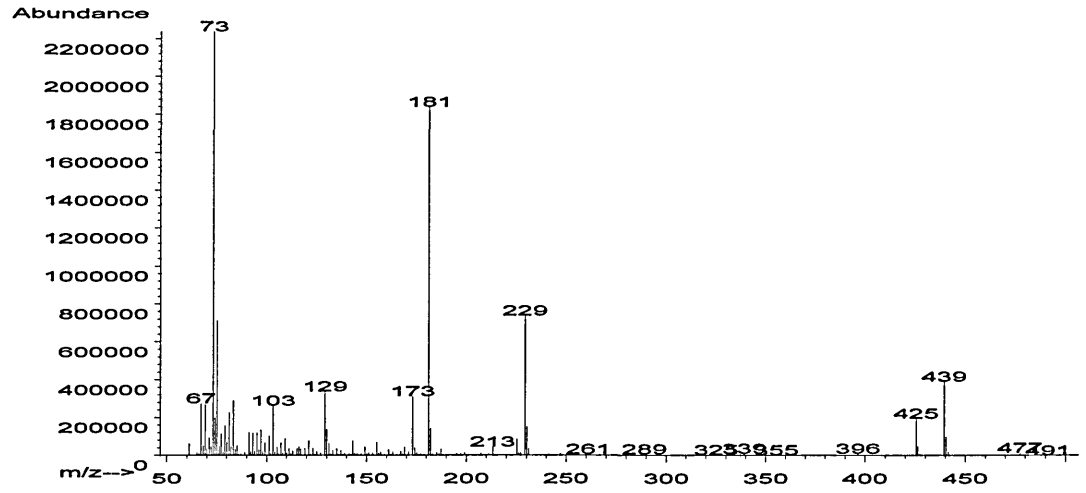


Figure 5.12 EI Mass spectrum of a suspected 9-hydroxy octadecanoic acid (PFB/TMS) derivative containing a fragment ion of m/z 229 with the probable formula $C_{10}H_{20}OTMS^{+}$.

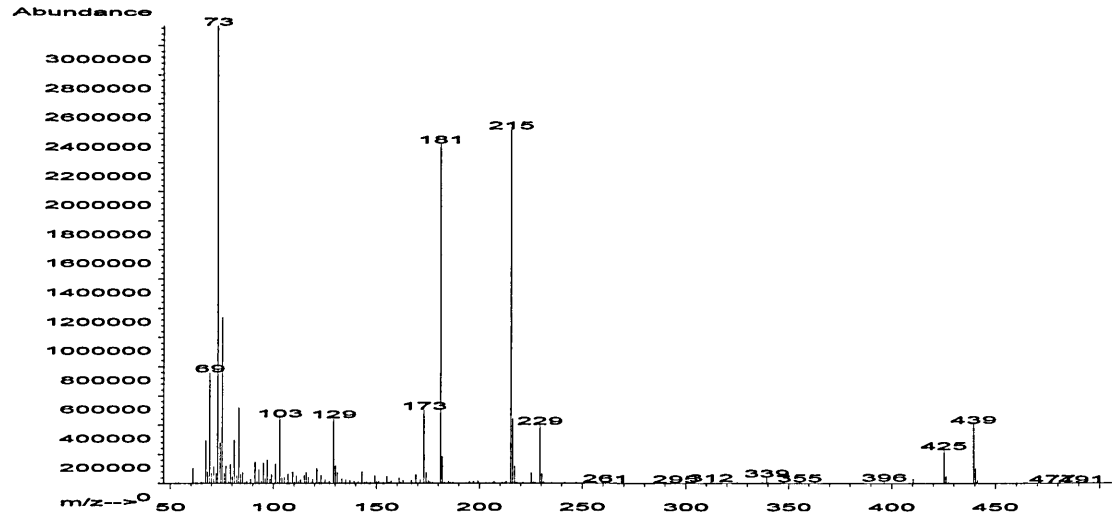


Figure 5.13 EI Mass spectrum of a suspected 10-hydroxy octadecanoic acid (PFB/TMS) derivative containing a fragment ion of m/z 215 with the probable formula $C_9H_{18}OTMS^{+}$.

The other peak showed a fragment from the adjacent carbon of $C_9H_{18}OTMS^{+}$ with a mass of 215. This indicated the formation of lipid peroxides on either side of the double bond. Whether diols were formed is unknown since the peaks were unresolved but the absence of mass 147 would suggest that they had not been produced.

Reduction with Raney nickel and sodium borohydride was then used to see, from the fragmentation pattern, if double bonds were retained.

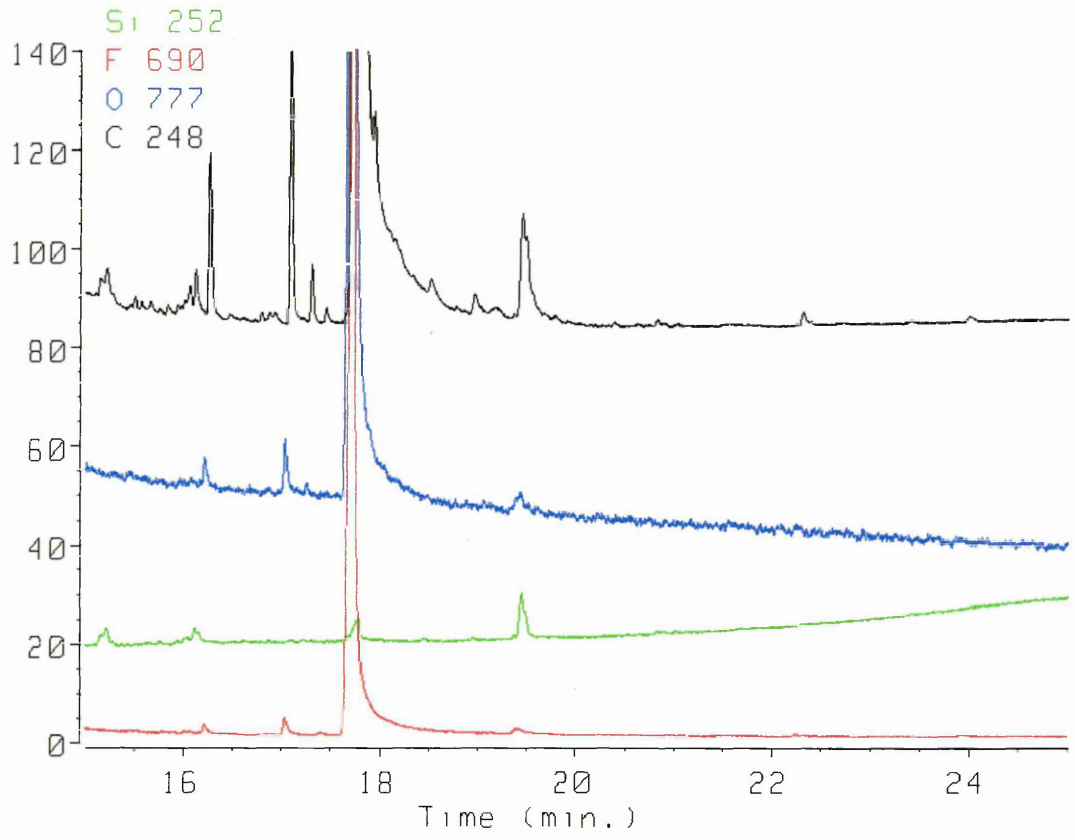


Figure 5.14a Elemental chromatogram of oxidised oleic acid standard by GC-AED

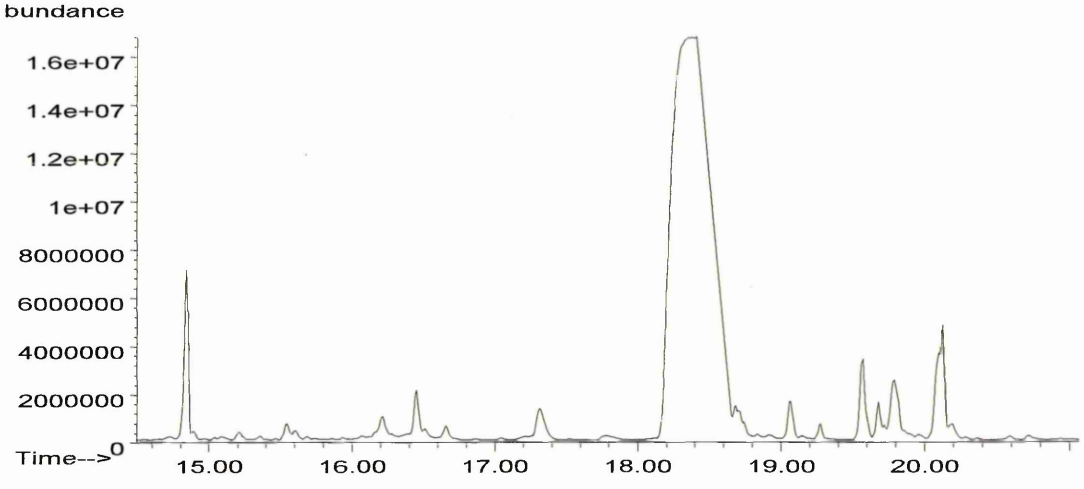


Figure 5.14b TIC chromatogram of oxidised oleic acid standard by GC-MSD

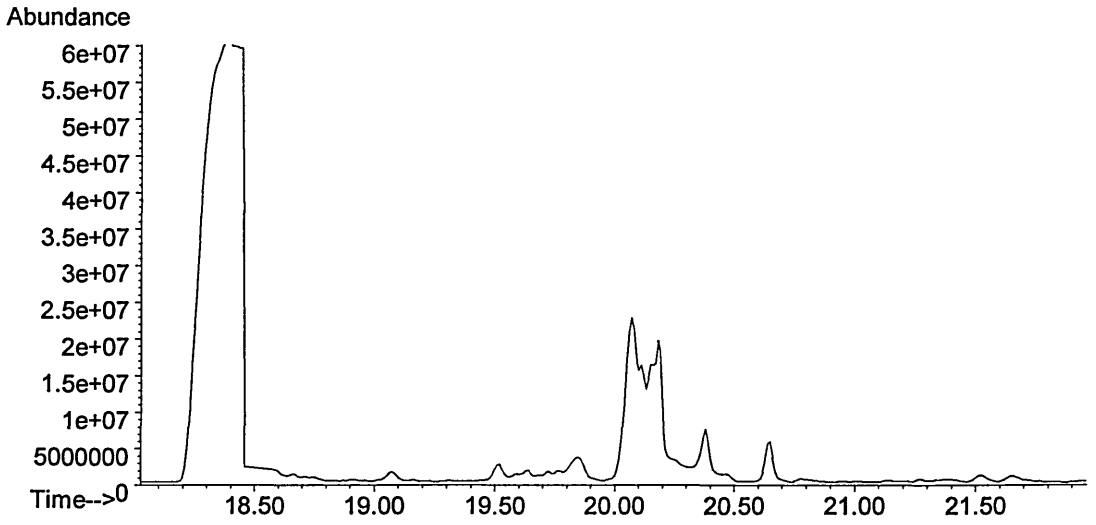


Figure 5.15 TIC chromatogram of oxidised linoleic acid standard by GC-MSD

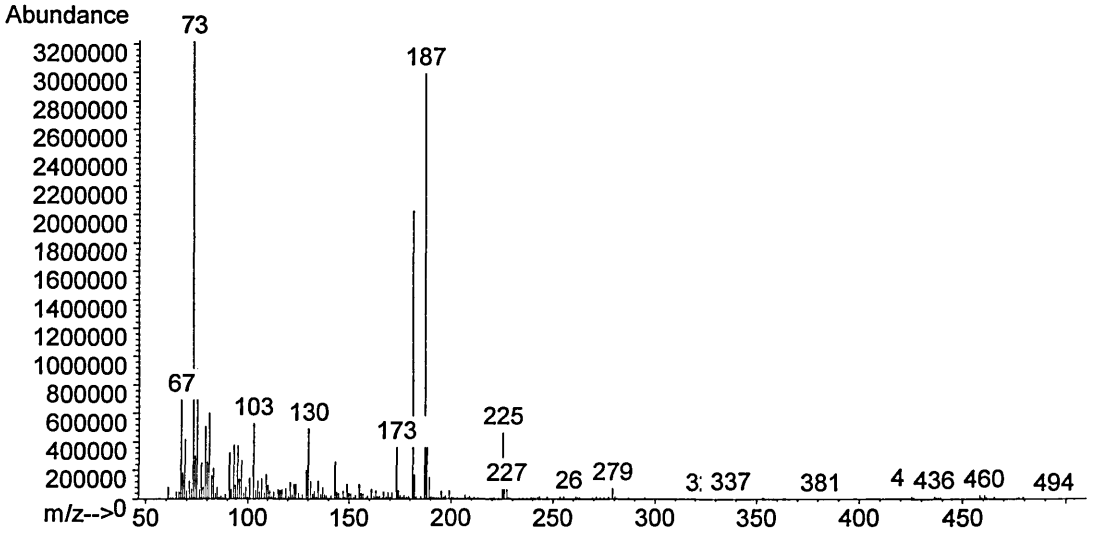


Figure 5.16 EI Mass spectrum of a suspected 12-hydroxy octadecanoic acid (PFB/TMS) derivative containing a fragment ion of m/z 187 with the probable formula $C_7H_{14}OTMS^+$.

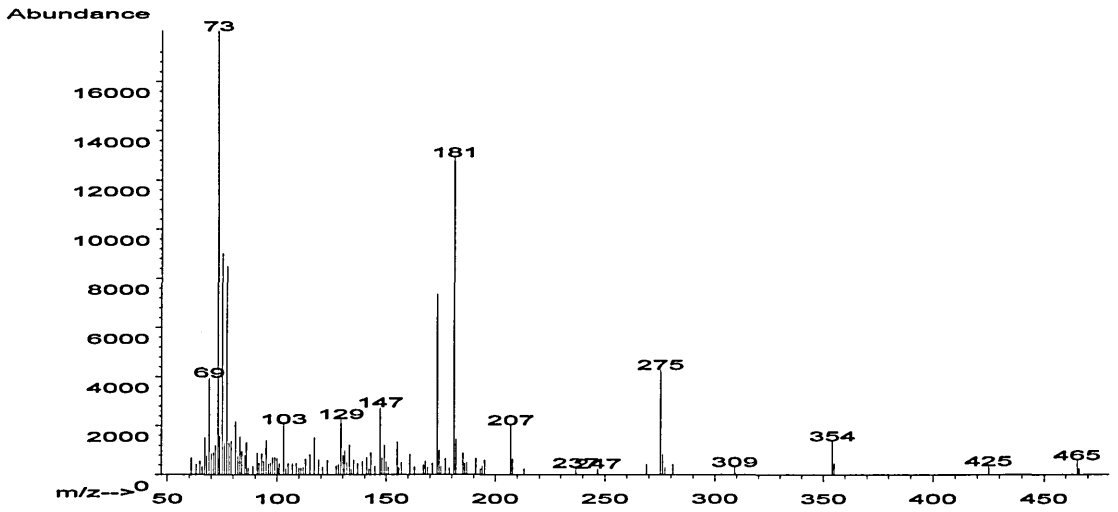


Figure 5.17 EI Mass spectrum of a suspected 13-hydroxy octadecanoic acid (PFB/TMS) derivative containing a fragment ion of m/z 173 and 275, with the probable hydroxy groups on C13 and C6 respectively.

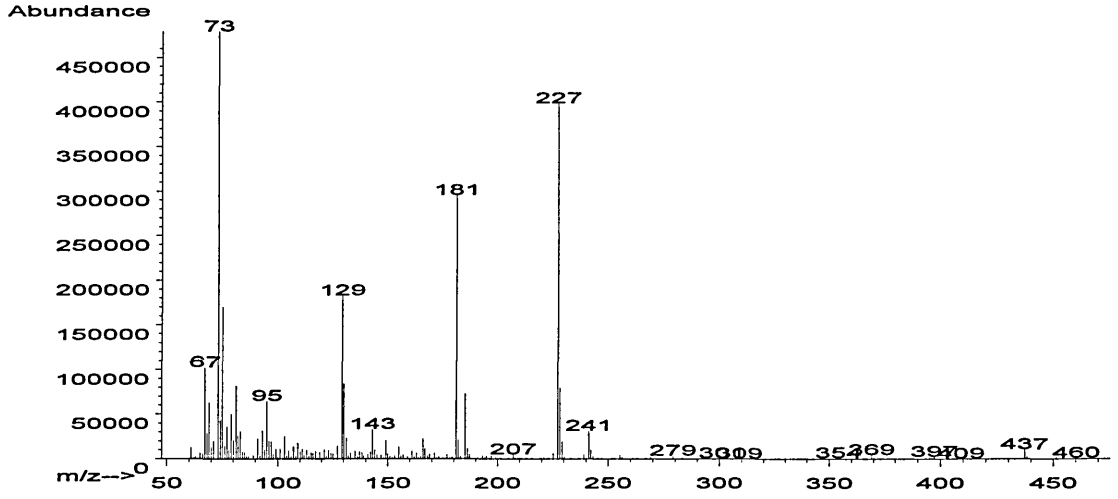


Figure 5.18 EI Mass spectrum of a suspected 9-hydroxy octadecenoic acid (PFB/TMS) derivative containing a fragment ion of m/z 227 with the probable formula $C_{10}H_{18}OTMS^{+}$.

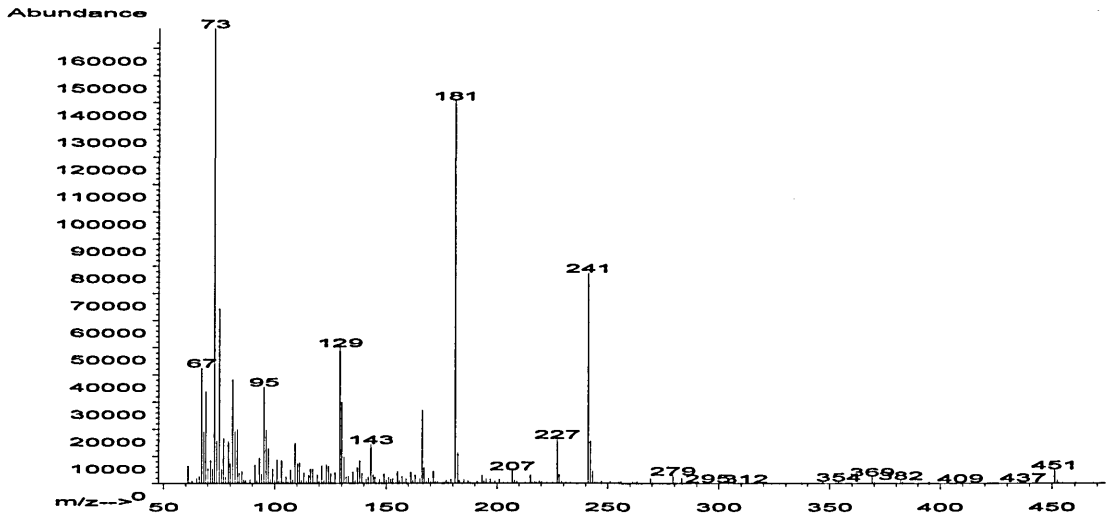


Figure 5.19 EI Mass spectrum of a suspected 8-hydroxy octadecenoic acid (PFB/TMS) derivative containing a fragment ion of m/z 241 with the probable formula $C_{11}H_{20}OTMS^{+}$.

Figures 5.14a and 5.14b show the chromatograms of an oxidised oleic acid standard by GC-AED and GC-MSD respectively. In the oxidised oleic acid two unresolved peaks with mass spectra indicating 9 and 10-hydroxyoctadecanoic acids (figures 5.12 and 13) were seen at 19.5mins (atomic emission chromatogram figure 5.14a) and 20.10mins (mass spectrometry TIC chromatogram figure 5.14b). The peak at ~17.5mins (figure 5.14a) and ~18.5mins (figure 5.14b) corresponds to an oleic acid derivative. The large peak is that of 9-octadecadienoic acid which is surrounded by smaller peaks containing fragment mass to charge ratio m/z 117 (20.70mins), 131 (20.60mins), 241 (19.60mins) with 73,129 and 181. This indicates that the majority of peroxy groups were formed at the original site of the double bond.

The main problem encountered with the serum samples and fatty acid standards was the number of PFB- and TMS-containing compounds that make identification very difficult using atomic emission data. However, this form of detection in conjunction with MS data was useful in confirming the success of the derivatisation in producing hydroxy lipids containing fluorine and silicon for comparison with the mass spectral data.

Oxidised linoleic acid (figure 5.15) produced hydroxy derivatives at positions 12 (figure 5.16), 13 (figure 5.17), 10 (figure 5.13), 9 (figure 5.12) and 9 with double bond retained (figure 5.18). From the TIC of oxidised linoleic acid (figure 5.15) C13-OH is seen at

20.38mins, while C9=OH, C9-OH, C10-OH and C12-OH are unresolved in the peaks seen from 20.00 to 20.20mins. As with oxidized oleic acid most of the peroxy bonds were formed at the original site of the double bonds in the 9 or 12-positions.

The main fragments above are $C_7H_{14}OTMS^+$ (m/z 187) (Figure 5.16) and $C_6H_{12}OTMS^+$ (m/z 173) (Figure 5.17) as well as peaks with fragments 215 and 229. The mass spectrum in figure 5.17 contains fragment 147 which indicates this may be a diol which could only be present if this acid had two double bonds to produce fragments 173 at C13 and 275 at C6. The hydroperoxy groups formed would need to lie close to the original location of a double bond. Therefore it is possible in this case that this may have been derived from linoleic acid although this is unlikely due to this distance. When the double bond is retained at the 12 position the mass spectra showed fragments of m/z 227 and 241, being $C_{10}H_{18}OTMS^+$ (Figure 5.18) and $C_{11}H_{20}OTMS^+$ (Figure 5.19) respectively. The atomic emission detector was useful in the determination of lipid peroxides only if their concentration was sufficiently high to be detected at the relatively insensitive fluorine and oxygen wavelengths.

There was additional evidence of other diol formation with two methyl fragments being formed. Given more time it would be interesting to investigate the formation of peroxy cholesterol derivatives.

A problem with the assay is the availability of a suitable internal standard. Stable isotope and deuterated standards are expensive and odd numbered hydroxyl fatty acids are not commercially available. The alternatives were i) to prepare hydroxyl standards, ii) use heptadecanoic acid or iii) use hydroxy hexadecanoic acid. Odd carbon numbered hydroxy fatty acid were not commercially available. To prepare hydroxy standards would be time consuming but not too difficult with the correct reagents. Using heptadecanoic acid would ensure there is no overlap with endogenous fatty acids (which are all even numbered) but it will not behave in the same way as hydroxyl fatty acids during extraction and derivatisation and so is not ideal. Hydroxy-hexadecanoic acid is a good matching molecule but we cannot have complete certainty that this acid is absent in serum. Using a smaller hydroxy acid

(<14C) would be unreliable because of interference from other compounds detected below at retention times lower than that of hexadecanoic acid.

Analysis of samples

To increase the sensitivity of the GC-MS experiment, a selected ion monitor (SIM) experiment was set up to monitor 30 mass ions.

m/z of fragment	Corresponding lipid(s)
181	All esterified acid compounds
73	All silylated hydroxy compounds
129	All silylated hydroxy compounds
103	18-hydroxy-C18, 20-hydroxy-C20
117	17-hydroxy-C18, 19-hydroxy-C20
131	16-hydroxy-C18, 18-hydroxy-C20
145	15-hydroxy-C18, 17-hydroxy-C20
159	14-hydroxy-C18, 16-hydroxy-C20
173	13-hydroxy-C18, 15-hydroxy-C20
187	12-hydroxy-C18, 14-hydroxy-C20
201	11-hydroxy-C18, 13-hydroxy-C20
215	10-hydroxy-C18, 12-hydroxy-C20
229	9-hydroxy-C18, 11-hydroxy-C20
243	8-hydroxy-C18, 10-hydroxy-C20
257	7-hydroxy-C18, 9-hydroxy-C20
271	6-hydroxy-C18, 8-hydroxy-C20
285	5-hydroxy-C18, 7-hydroxy-C20
299	4-hydroxy-C18, 5-hydroxy-C20
313	3-hydroxy-C18, 4-hydroxy-C20
327	2-hydroxy-C18, 3-hydroxy-C20
209	Tetradecanoic acid (C14:0)
237	Hexadecanoic acid (C16:0)
251	Heptadecanoic acid (C17:0)
263	9-Octadecenoic acid (C18:1)
265	Octadecanoic acid (C18:0)
293	Eicosanoic acid (C20:0)
321	Docosenoic acid (C22:0)
349	Tetracosanoic acid (C24:0)
147	Di-TMS lipid [136]
329	Dihydroxy cholesterol (from MSD library)

Table 5.3 SIM ions used identifying each lipid in the study

For calibration of samples and identification of retention times of lipids a stock solution of standards was made containing 2.63μg/ml of C16(1), 2-OH-C16(3), C18(4),12-OH-C18(5), cholestanol(7), 1.32 μg/ml of 2-OH-C20(6) and 26.3 μg/ml of C17(2) as shown in figures 5.20 and 5.21.

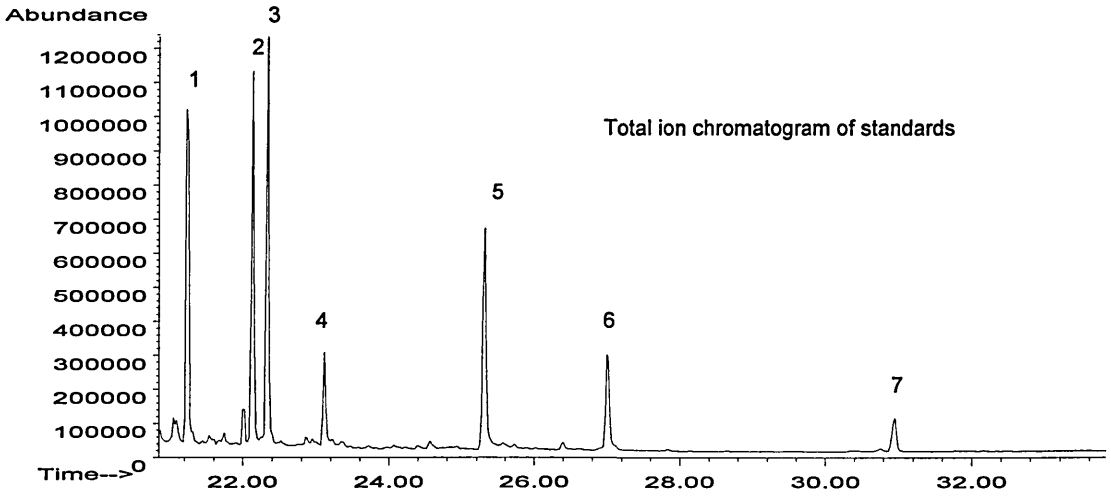


Figure 5.20 TIC of all lipid standards by GC-MSD

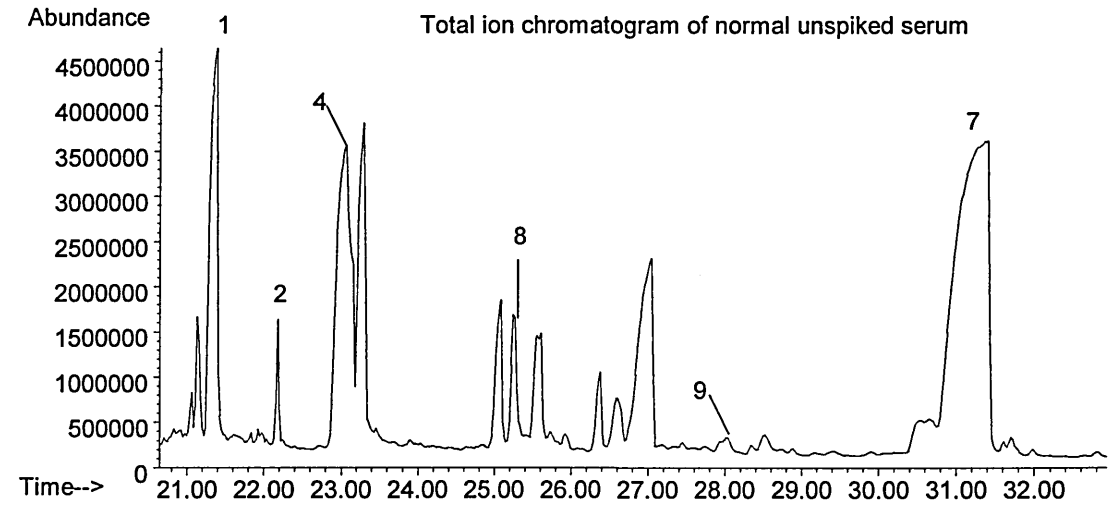


Figure 5.21 TIC of normal (typical) serum.

Peak 8 contained a fragment with m/z 173 indicating 8-hydroxy octadecanoic acid and peak 9 contains m/z 229 indicating 11-hydroxy eicosanoic acid. The standards 2-hydroxy hexadecanoic, 12-hydroxy octadecanoic and 2-hydroxy eicosanoic acids had retention times of 22.34, 25.32 and 27.02 minutes respectively.

There is a natural progression of retention as the chain length increases, and the hydroxy derivatives were retained longer than the parent acids when comparing C16 with 2-OH-C16 and C18 with 12-OH-C18. The closer the hydroxy group is to the carboxylic acid the less retained they were using this column. This is shown by the lipid peroxide results in fig.5.22 from C20 monohydroxy lipids where, as the fragment size increases (i.e. the position of the OTMS group gets closer to the PFB group), the polarity is reduced and thereby a shorter retention time is seen on the column.

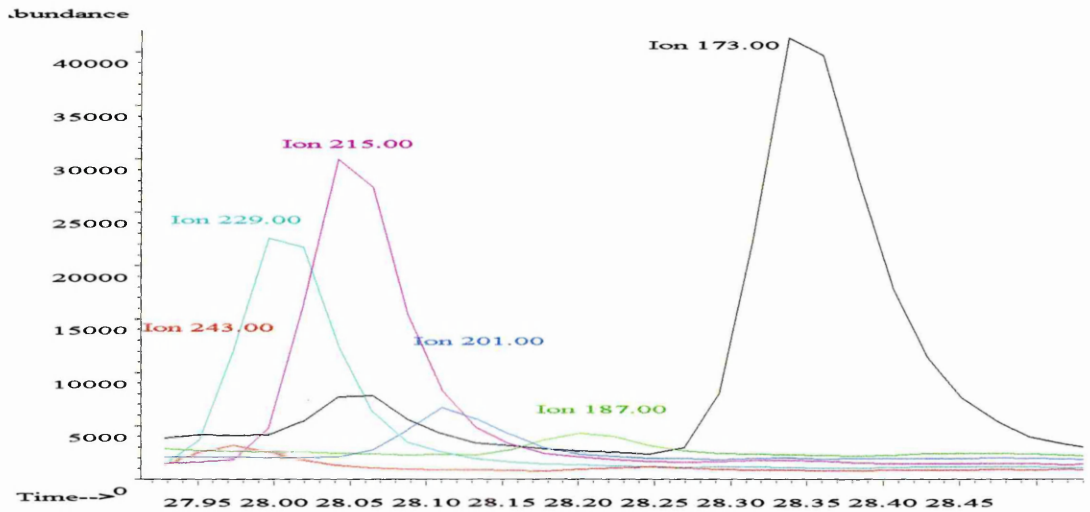


Figure 5.22 Chromatogram of separated C18-OH lipids showing order of elution

This method has the advantage over previous GC-MS methods of being cheaper, less time consuming and detecting unsaturated lipids as well as hydroxy lipids.

Statistics

Reproducibility

5 x 1ml samples of serum were spiked to a concentration of 6.58µg/ml with 2-hydroxyhexadecanoic and 6.58µg/ml of 12-hydroxyoctadecanoic acids and worked up as previously described. Prior to derivatisation, heptadecanoic acid was added to 6.58µg/ml in each vial and the contents analysed by GC-MSD using the SIM method.

	% recovery 2-OH-C16	% recovery 12-OH C18	
	70.41	90.00	1st spike
	48.50	101.11	2nd spike
	57.00	105.54	3rd spike
	50.99	105.39	4th spike
	75.27	107.08	5th spike
Mean	60.43	101.82	
SD	11.87	6.98	
% RSD	19.63	6.86	

Table 5.4a Recovery of hydroxy-hexadecanoic and hydroxy-octadecanoic acids.

The large variation of the % RSD has been attributed to variable extraction differences from step 1 of the method. Consistently lower recovery of C16-OH indicate loss of shorter chain hydroxy fatty acids possible in the solvent extractions. However C18-OH had a recovery close to 100%. Position of the hydroxy position would also have affected the molecules polarity.

Sensitivity

The all three standards were analysed using a SIM method with ions 181,299,187,355 where they each showed linearity to 0.01μg/ml. The sensitivity would be less with real samples as thirty ions would be monitored instead of four.

Chapter 6

Peroxynitrite

The chemical and biological properties of peroxynitrite have previously been discussed in chapters 1 and 3 respectively.

Analytical methods have focused on nitrotyrosine because it is a stable end product of peroxynitrite reaction with proteins and therefore is a marker for its production in biological fluids. Sample preparation involves centrifugation to remove cells following which the supernatant is acid hydrolysed to break down the proteins into their amino acids and increase the yield of tyrosine derivatives for detection by UV. These have been separated and determined using isocratic liquid chromatography from human plasma [503,504], cultured cells [505,506], human [507,508] and rat tissue [504].

Detection of nitrotyrosine is performed by UV absorption or electrochemical means as it does not have appreciable fluorescence [509]. Its absorption wavelength and oxidation potential are affected by the pH of the solution. Both λ_{max} [510] and cell potential [505] decrease as the pH decreases with reported optimum pH of 3.5 . Reported levels of free 3-nitrotyrosine are 31±6 nmol L^{-1} [503] which require a more sensitive mode of detection than UV and therefore electrochemical methods have been used.

3-Nitrotyrosine as a marker for peroxynitrite production

NO_2• has been shown to react with the aromatic amino acids including tryptophan and tyrosine [511,512], resulting in the formation of the phenoxy radical which can dimerize e.g. to dityrosine or combine with NO_2• to form the stable molecule 3-nitrotyrosine.

NO_2^+ is thought to attack the aromatic ring by electrophilic aromatic substitution [513] through radical and non-radical mechanisms. It has also been shown that under acidic conditions nitrite can also form nitrotyrosine, i.e. in the stomach [514]. Therefore caution must be made when attributing all of the dityrosine/3-nitrotyrosine to the peroxynitrite nitration.

Reagents

3-nitro-L-tyrosine (Sigma), L-tyrosine (Sigma), KH_2PO_4 (AR, Fisons), methanol HPLC grade. MilliQ water used for dilutions and pH adjusted with HCL (Aristar, BDH).

Results

An isocratic Philips PU4015 pump with PU4020 UV detector were used with a Spherisorb ODS2 column and manual injector with a 20µl loop. A UV spectrum of a 500µmol/L standard of 3-nitrotyrosine showed three peaks at <200nm, 216nm and 278nm and therefore the detector was set to 278nm to minimise interference. Electrochemical detection of tyrosine and 3-nitrotyrosine was performed using an ESA Coulochem electrochemical detector (model 5100A) set to potentials of guard cell (+0.45), detector 1 (+0.40), detector 2 (+1.00) with both detectors at x20 gain. Voltage output was the result of subtraction of detector 2 response from detector 1 response. A tyrosine spectrum showed main absorbances at 190nm and 275nm, so 275nm was chosen. A range of eluents containing differing concentrations of methanol with 50mmol/L KH_2PO_4 were tested, adjusted to pH 3.0. The results are shown in Table 6.1.

% Methanol	Retention time of 3-nitrotyrosine in minutes
80	2.42
60	2.71
40	3.69
20	4.26
10	9.37

Table 6.1

An ideal retention time would be long enough to allow resolution from other substances in the filtered serum but short enough so band broadening is not a problem. The 10% solution of methanol was therefore selected. It was discovered that the limits of detection by UV_{278} were 1 µmol L^{-1} and by electrochemistry at the above oxidation potentials 100 nmol L^{-1} with the equipment used.

To determine the optimum oxidation/reduction potentials for each analyte 500 μmol L^{-1} solutions of 3-nitrotyrosine (Sigma) and L-tyrosine in 50mmol/L KH_2PO_4 adjusted to pH 3.0 with 10% methanol were made. Eluent containing the dissolved standard was pumped through the electrochemical detector at 1.0 ml/minute. The oxidation/reduction potential was incremented from 0±1.3 V for both detectors at the same time and the response recorded, with the results shown in figures 6.1-4.

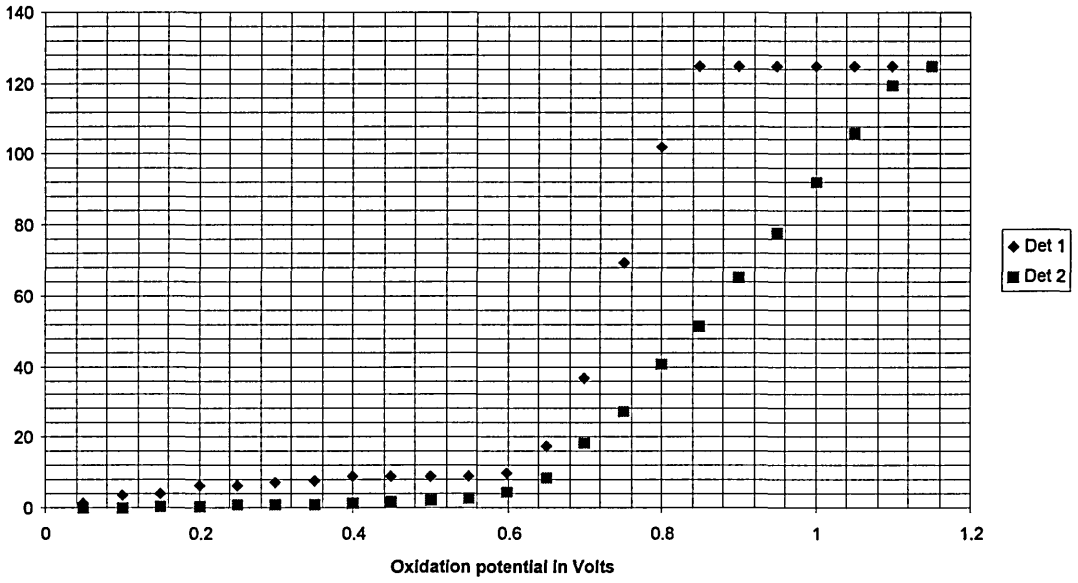


Figure 6.1 - Oxidation potential plot for 3-nitrotyrosine solution

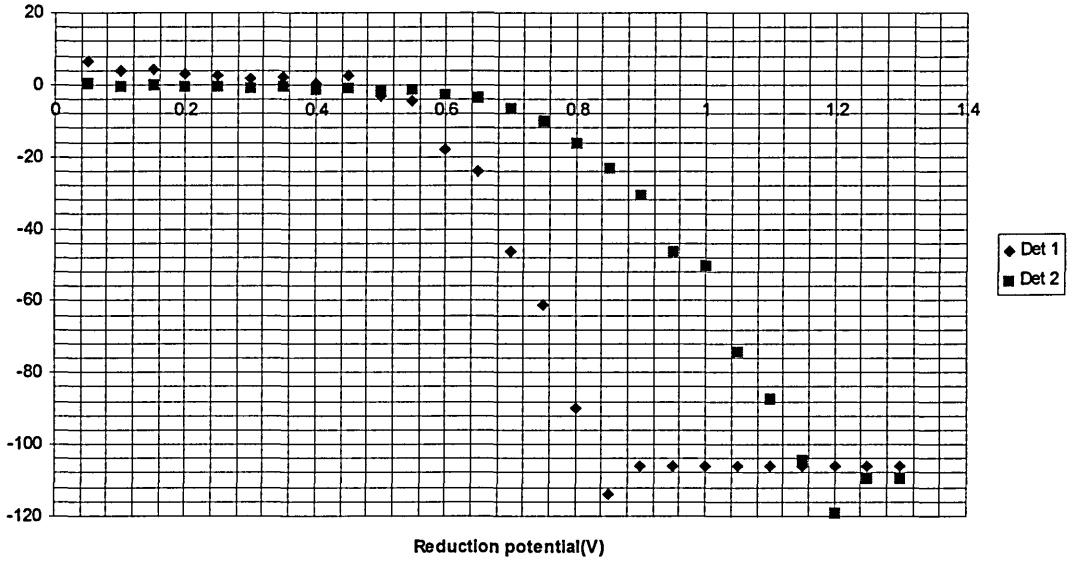


Figure 6.2 - Reduction potential plot for 3-nitrotyrosine.

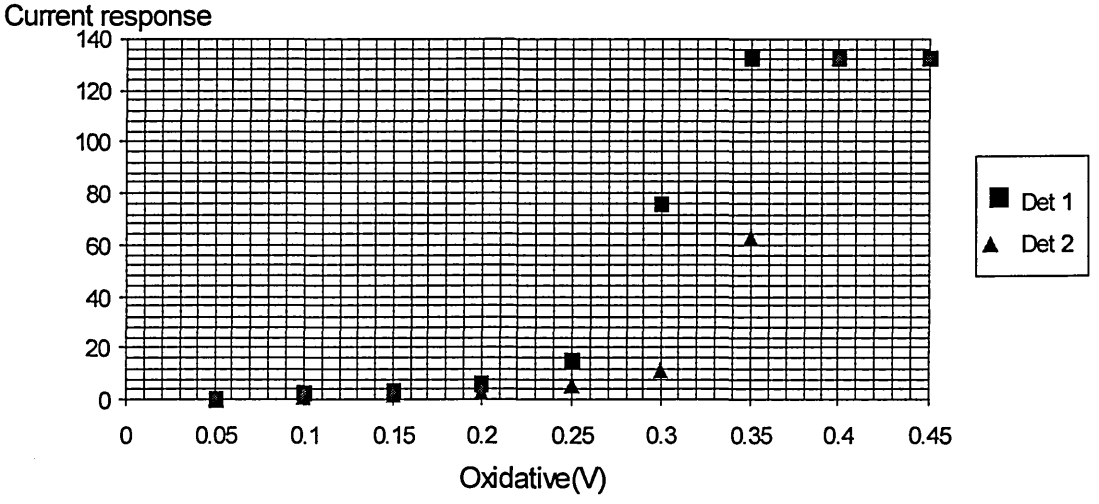


Figure 6.3 - Oxidation potential plot for L-tyrosine (500 μmol L^{-1})

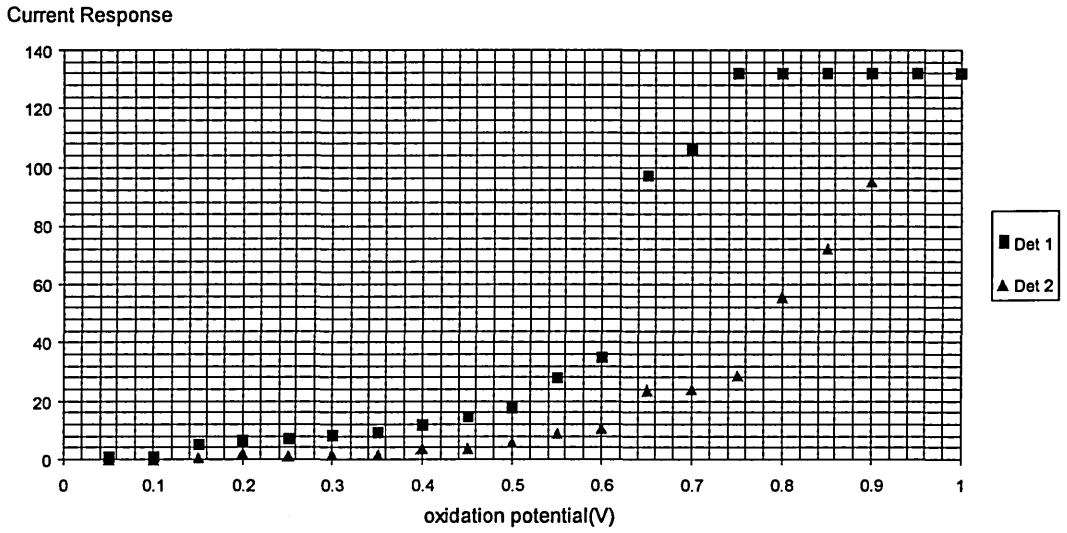


Figure 6.4 - Oxidation potential of the eluent without analyte.

The voltammogram of 3-nitrotyrosine in figure 6.1 shows optimum oxidation potentials of 830mV (det. 1) and 1.16V (det. 2). This result compares with that of Hensley [515] who found +900mV to be the optimum operating condition. It was also found that using a potential greater than +1V caused an unacceptable increase in the baseline noise, probably due to oxidation of the eluent (fig 6.4). L-tyrosine requires a much smaller potential (+400mV) for its detection. Since this detector has two analytical cells it is possible to use different detection modes, of which two were tried. In screen mode, the first detector is set to a potential slightly lower than that of the analyte (in this case <+250mV for tyrosine) and its response is subtracted from that of the second detector which is set just above the highest oxidation potential of the analyte (which is +1V for 3-nitrotyrosine) [516]. There is a large difference in the two potentials which make this mode less sensitive than other modes such as the redox mode used by Althaus [517] where the analytes are reduced at detector 1 and oxidised at detector 2 giving a selective and sensitive response. This method was tried using det. 1 at -2.0 Vs and det. 2 at +450mV but the response produced was less than that of the screen mode.

Serum samples were prepared from a 1:1 dilution with MilliQ water and filtered through Vivaspin membrane filters as described in chapter 4.

Chromatography of typical serum

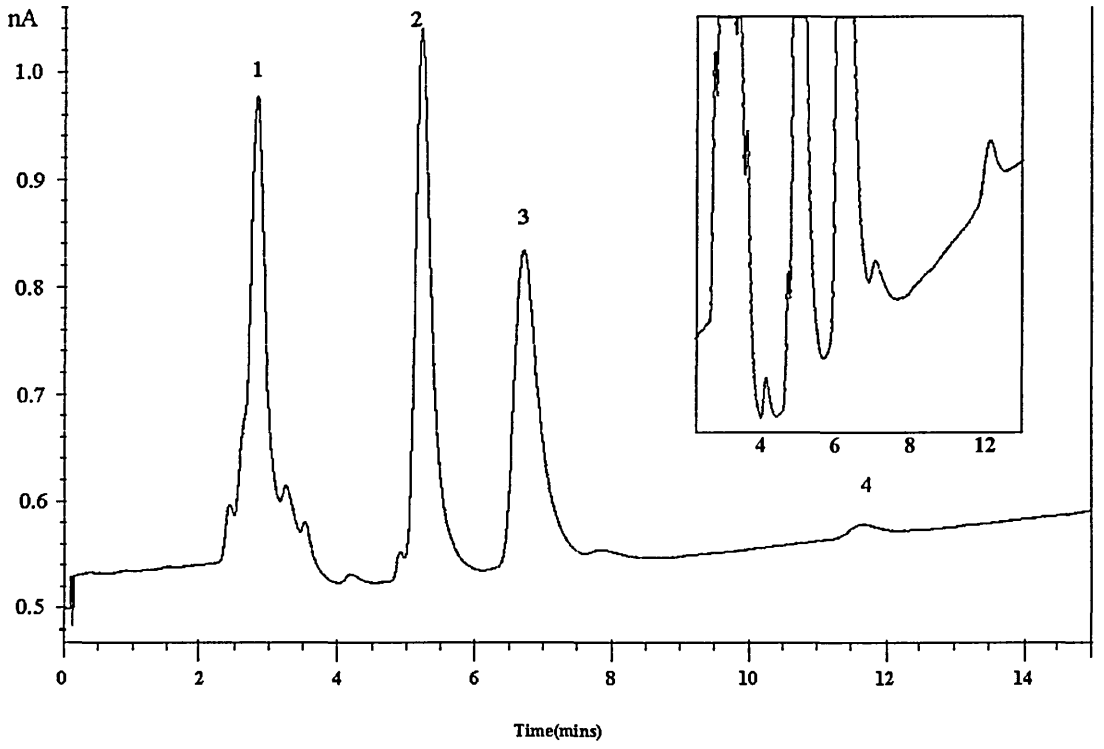


Figure 6.5 shows a chromatogram of 30µl of filtered normal serum (x2 dilution) using the screen mode showing an inset of the 3-nitrotyrosine peak with detector 1 at +0.40 Vs and detector 2 at +1.00 Vs.

Peak 1 (2-4mins)- unretained substance(s)

Peak 2 (4-6mins)- L-tyrosine

Peak 3 (6-7mins)- Unknown

Peak 4 (10.9mins)- 3-Nitrotyrosine

The identity of L-tyrosine and 3-nitrotyrosine were determined from the retention of known standards and by spiking of samples, where the superposition of peaks was exact, with no evidence of fronting or tailing.

A calibration curve from spiked serum is shown in figure 6.6 which compares the electrochemical (red line) and UV (blue line) responses. The inset shows spiked concentrations from 10-50000 nmol/L.

Inter assay for serum (n=12) was 4% for 3-nitrotyrosine at a mean of 1.5 mmol/L. Intra assay for serum (n=6) was 5% for 3-nitrotyrosine at a mean of 1.5 mmol/L. Producing good reproducibility of ≤ 5% deviation.

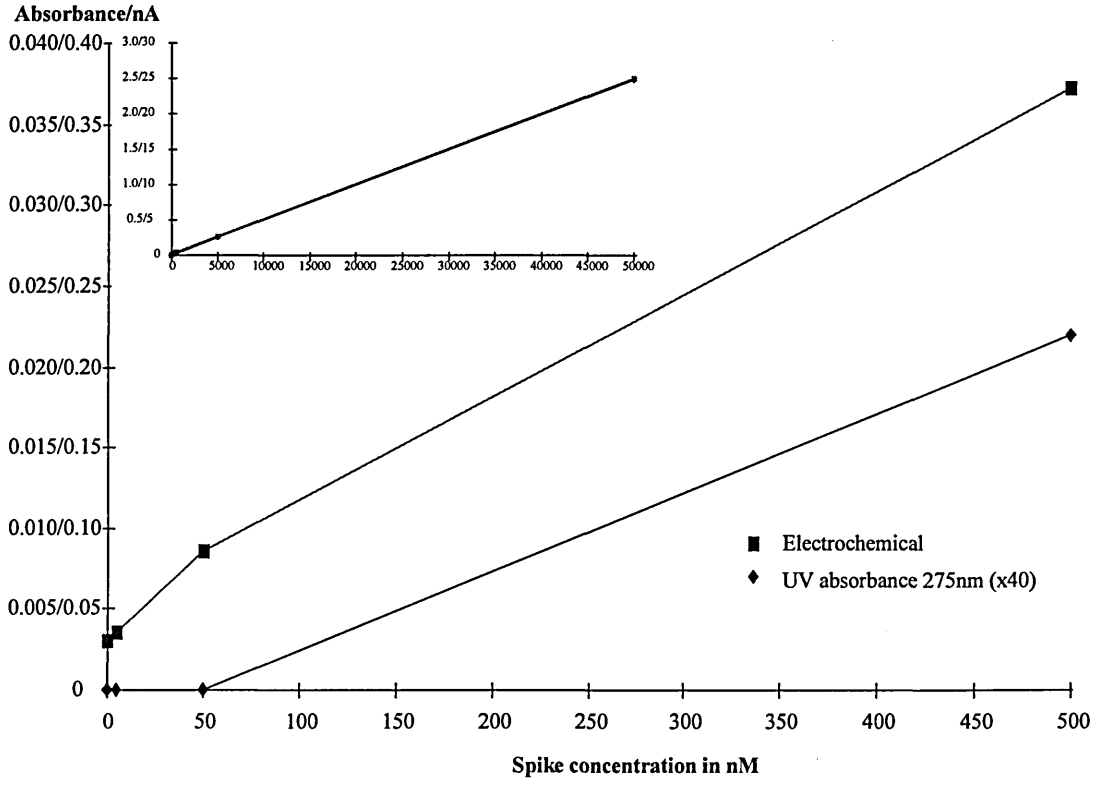


Figure 6.6 Calibration graph for serum spiked with 3-nitrotyrosine using UV (absorbance units) and electrochemical (nA signal) detection.

These results show that it is possible to detect the presence of free nitrotyrosine in filtered human serum by the simple method described above. After each sample run the methanol gradient was increased from 10 to 90% for 3 minutes to clean the column. This method was used to analyse sera from normal, pre-eclamptic and pregnancy induced hypertensive patients for free 3-nitrotyrosine and tyrosine.

Chapter 7

Results from patient samples

Definitions

Hypertension - By convention hypertension is defined as a rise in blood pressure to 140mmHg systolic and 90mmHg diastolic or greater. Hypertension may arise *de novo* in pregnancy or be pre-existing i.e. chronic hypertension. Chronic hypertension may be due to essential (idiopathic) hypertension or due to underlying renal or adrenal disease.

Pregnancy induced hypertension (PIH) sometimes also referred to as pregnancy associated hypertension is defined as a rise in blood pressure to 140/90mm Hg or greater presenting after 20 weeks of pregnancy without proteinuria. There should be evidence of normotension pre-pregnancy and in the first 20 weeks of pregnancy.

Pre-eclampsia (PE) is defined as a rise in blood pressure to 140/90mm Hg or greater accompanied by proteinuria. Pre-eclampsia usually presents after 20 weeks of pregnancy and characteristically occurs in primigravid women. There should be evidence of normotension pre-pregnancy and in the first 20 weeks of pregnancy.

Pre-eclampsia is a syndrome i.e. a collection of signs and symptoms. If the central nervous system is affected then generalised seizures may occur - a complication known as **Eclampsia**. "Superimposed" pre-eclampsia may develop in women with chronic hypertension [66].

Samples

Classification of the diseased subjects from which the samples of plasma were obtained followed the well-established criteria. The normal group was defined as having normal blood

pressure, absence of proteinuria and no complications during pregnancy. The blood samples obtained from severe PE and PIH patient were a mixture of cross-sectional and longitudinal however from normal subjects all the samples were cross-sectional. The identity of each sample was unknown until all the analyses were completed and concentration reported to avoid operator bias. Fresh whole blood samples were taken from pregnant subjects into EDTA coated tubes, then stored at 4°C until centrifuged to remove the cellular fraction. The plasma was then frozen at -20°C, where nitrite and nitrate are stable for at least one year [19] and were analysed within this time period. The time taken from removal of the cellular fraction and freezing was variable. Since the oxidation results in chapter 4 reveal that the conversion of nitrite to nitrate occurred rapidly (especially in the presence of haemoglobin) the separation/freezing time need to be kept constant. Since this was not possible in each case both nitrite and nitrate were added to produce a NOx value. Each sample was analysed in duplicate and the mean of the two values taken, a variation greater than 25% for nitrite or 5% for nitrate was repeated or not included in the study data (variation range calculated from control sample group).

Statistics

To compare each group the Fishers-test [519] was first performed to compare the variation between the normotensives and PE or PIH group. Depending if the variations were equal or unequal the respective Student t-test was carried out to see if there was a statistical difference seen between the means of the two groups. The two tests are only valid if the results produce a binomial normal distribution when plotted. As can be observed in figure 7.12 the results for the distribution of NOx between the three groups is not binomial and is also skewed. In this event a non-parametric test (Mann-Whitney) and log-transformed analysis was also performed if sufficient numbers were available. Statistical significance was set at $\leq 0.05\%$.

	Normotensive			PE			PIH			
	Mean	Standard deviation	n	Mean	Standard deviation	n	Mean	Standard deviation	n	
Males μmol L^{-1}	45.4	38.3	105	--	--	--	--	--	--	--
Non pregnant females μmol L^{-1}	42.2	40.0	95	--	--	--	--	--	--	--
Amniotic fluid NOx μmol L^{-1}	44.0	31.5	21	--	--	--	--	--	--	--
Maternal NOx μmol L^{-1}	46.0	38.3	71	55.1	72.6	28	51.1	32.9	11	ns‡
1st trimester	45.4		1	--	--	--	--	--	--	ns
2nd trimester	47.0	47.1	41	--	--	--	--	--	--	ns
3rd trimester	45.6	32.5	29	--	--	--	--	--	--	ns
Cord NOx μmol L^{-1}	--	--	--	111.0	168.6	11	109.4	137.0	5	ns
%age of samples where nitrotyrosine was detected	78	--	23	75	--	13	100	--	3	ns
C16 μg L^{-1}	121	218	17	129	236	8	57.4	30.1	8	ns
C18 μg L^{-1}	152	263	17	103	157	8	73.2	51.5	8	ns
C20 μg L^{-1}	7.63	8.88	17	4.00	2.72	8	2.95	2.35	8	ns
C22 μg L^{-1}	2.49	2.4	17	1.47	0.9	8	1.44	1.01	8	ns
LPO by GC μg L^{-1}	2.08	2.33	15	3.66	4.99	8	4.54	6.40	6	ns
TBARS in μmol L^{-1}	359	320	30	355	354	10	384	563	6	ns

Table 7.1 Summary of results, NOx levels in males, non-pregnant females, amniotic fluid and throughout gestation in normal, PE and PIH groups. Nitrotyrosine, fatty acids and lipid peroxides levels in all three pregnant groups is also compared.

ns = not significant when α=0.05 using Student t-test, ‡ = not significant when α=0.05 using log-transformed and Mann-Whitney U- tests. All samples were gestationally matched.

Lipids

Each lipid chain length will have contributions from other fatty acids e.g. C18 is derived from steric (C18:0), oleic (C18:1), linoleic (C18:2) and linolenic (C18:3) fatty acids. The data for the lipids show only the relative instead of the absolute concentration as the response factor for each lipid was not calculated. However it was the proportion between

the three pregnant groups that we hoped to investigate. It is important to note that in clinical biochemistry and in the published studies fasted samples are required to remove the effect of recently ingested lipids. In the samples from this study fasting was not carried out.

Hubel [91], Lorentzen [90] and Franz [93] each observed an significant increase in free fatty acids and triglycerides in PE patients compared to their normal controls. The actual lipid groups were investigated by Lorentzen [530]. In their 17 PE patients palmitic (C17:0), oleic (C18:1) and linoleic (C18:2) levels were significantly increased in early (10-20wks) and later pregnancy. The esterified lipids in our study did not show any difference between the diseased and normal groups in early pregnancy. Lorentzen et al [530] gave several plausible explanations for this including biochemical alteration of lipoprotein composition, differing diet, preferential sequestering of linoleic by the placenta or and increased degradation of linoleic acid by the maternal circulation. The last explanation has been connected with the formation of lipid peroxides that preferentially attack polyunsaturated lipids and result in alteration of membrane stability via composition change and peroxidation as discussed in Chapter 2. A larger study by Monique [525] observed altered lipid composition in 52 patients with PE and respective controls. They found that there was no difference in esterified lipid composition between the two groups throughout pregnancy except after 32 wks and after delivery when linoleic and linolenic (C18:3) were shown to be significantly decreased while cervonic acid (C22:6) was increased in the diseased group. They attributed this change to a consequence of the disease rather than a contributory factor.

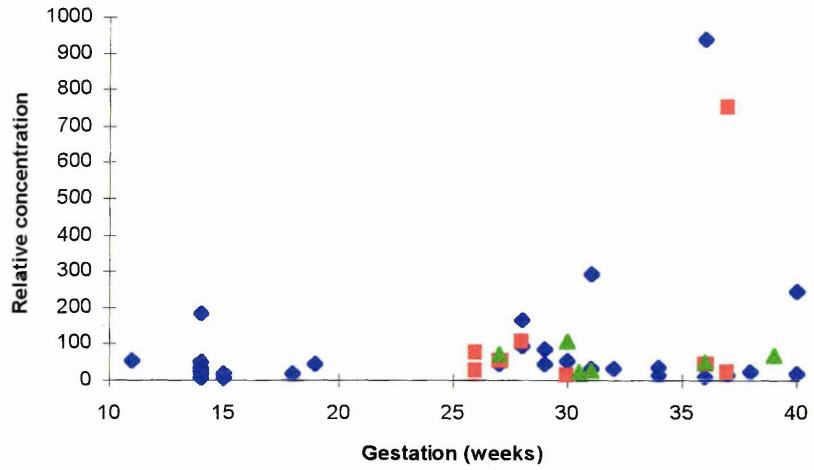


Figure 7.1 Changes in relative concentration of total plasma C16 lipid levels through gestation in normal (blue diamonds), PE (red squares) and PIH (green triangles) pregnant patients.

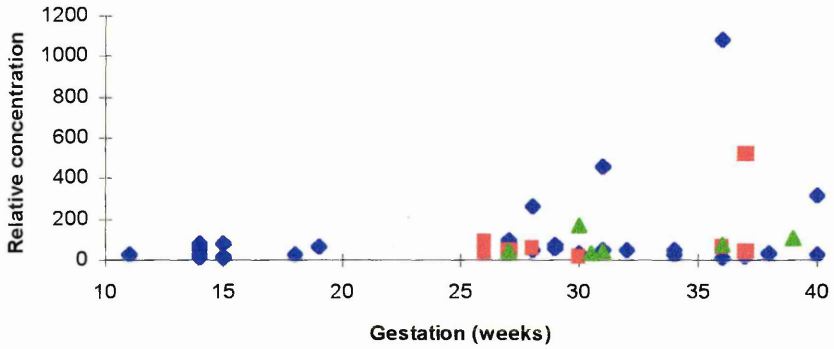


Figure 7.2 Changes in relative concentration of total plasma C18 lipid levels through gestation in normal (blue diamonds), PE (red squares) and PIH (green triangles) pregnant patients.

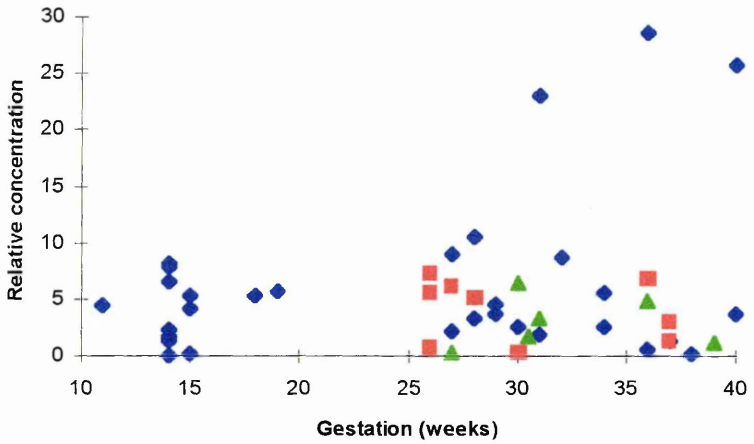


Figure 7.3 Changes in relative concentration of total plasma C20 lipid levels through gestation in normal (blue diamonds), PE (red squares) and PIH (green triangles) pregnant patients.

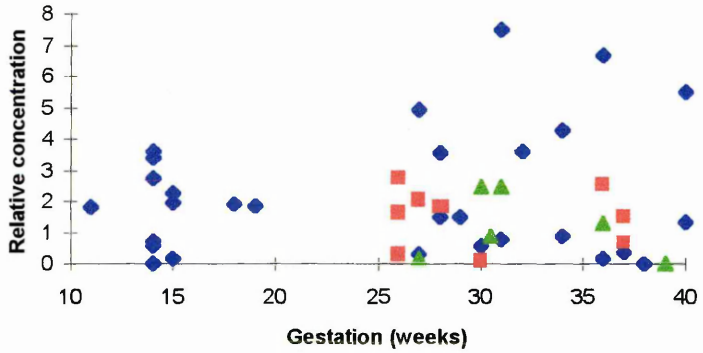


Figure 7.4 Changes in relative concentration of total plasma C22 lipid levels through gestation in normal (blue diamonds), PE (red squares) and PIH (green triangles) pregnant patients.

Figures 7.1-7.4 represent the total plasma lipid for each chain length in μg/L as calculated from the internal standard heptadecanoic acid and plotted against gestation. As is evident from the sample chromatogram in figure 5.18 and the relative concentrations from figures 7.1-7.4 the C18 acids were the most abundant closely followed by C16, C20 and C22 respectively. In certain circumstances it was also possible to detect C24 acids. Cholesterol was detected in most but not all occasions. This occasional absence may have been due to losses throughout the workup procedure and was therefore excluded from the study.

Comparison of the general trend through gestation does not support the idea of total lipids being increased even though there is a dispersal of points after 25 wks in all lipid groups. A spread of results is seen with the C20 (fig. 7.3) and even more so with the C22 acids (fig. 7.4), which could be related to random variation as the lower concentrations produce a lower response. Interestingly the relative abundance of each lipid appears to be of constant proportion from sample to sample producing a graph that is almost repeated from lipid to lipid. This proportional response is seen not only in the normal population but also in both the diseased groups. Statistical analysis after gestation was matched did not reveal any difference between the two diseased groups and the normal samples for each lipid. This is not unexpected when comparing these results to that of Lorentzen [530] since they observed a difference in the ratio of esterified oleic and linoleic acids. The method used in the present study detected only the total C18 fatty acids so it would be expected that these differences would cancel each other out to produce only small differences that could be seen as sample variation. The contribution made from altered free fatty acid compositions is small where the percentage of free fatty acids to total is 0.01% [362]. Since the number of results for the diseased groups in this study after 32 weeks was small (PE n=3, PIH n=2) it was not possible to deny or confirm the observation made by Monique [525]. However, visual comparison of the results in figure 7.3-7.4 shows a lower concentration of C20 and C22 in both diseased groups prior 32 weeks in support of their observations.

Lipid peroxides

As previous discussed in chapter 2 it is well established that lipid peroxides are increased in pre-eclampsia. The results of the GC-MSD assay for lipid peroxides were disappointing as there were relatively few samples with detectable amounts of lipid hydroperoxides. This poor response might be due to the instability of the trimethylsilyl ester (chapter 5) even though every effort was made to analyse the samples as soon as they were prepared. Interestingly the mean is much greater in the diseased groups, though this difference is not statistically significant given the errors involved. More samples would be needed to see if this variation could be reduced and if the difference is real. The TBARS test also did not show any significant difference between the two groups and did not correlate with the GC-

MSD assay. Extraction of the TBA_2-MDA complex into butanol was variable and led to major differences between replicate standards.

NOx and age and gender

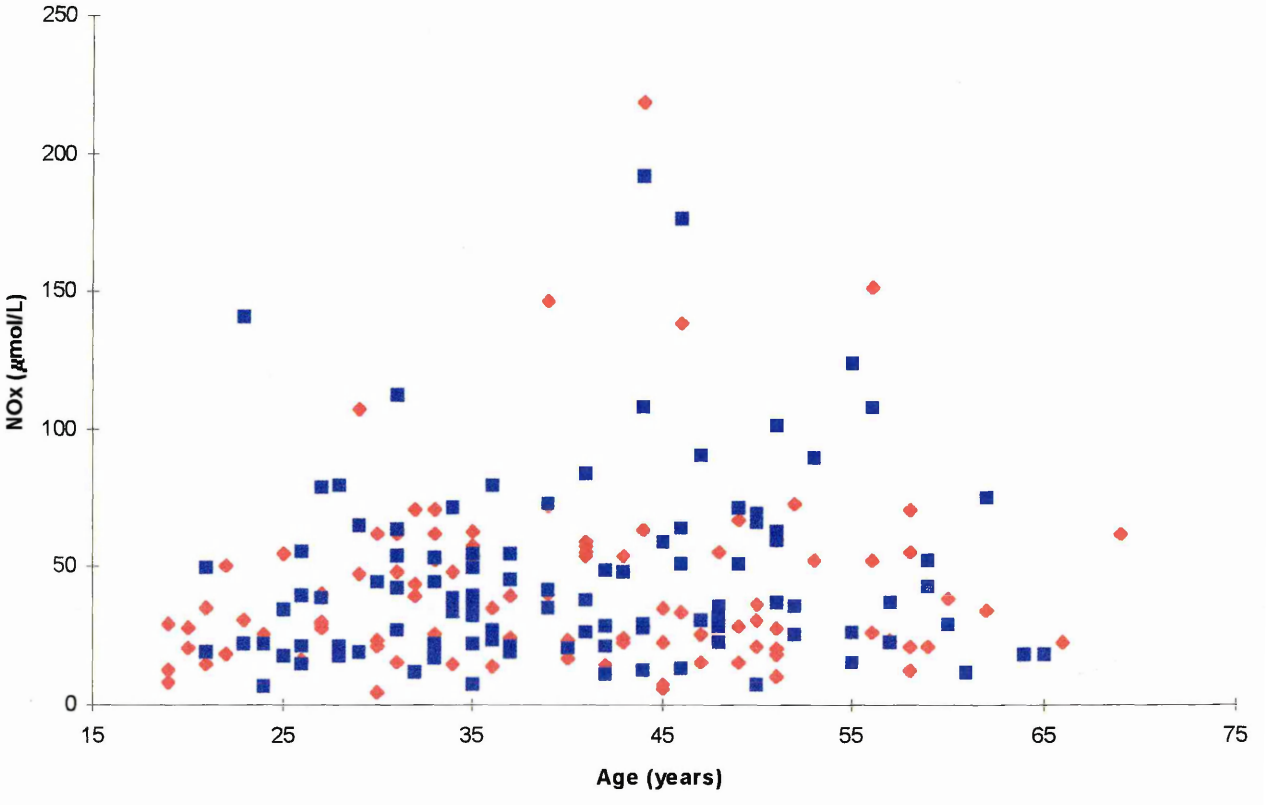


Figure 7.5 NOx levels relationship with age in males (blue squares) and non-pregnant females (red diamonds)

When the samples means of the male (45.4 ± 38.3 µmol L^{-1}) and non-pregnant female (42.2 ± 40.0 µmol L^{-1}) groups are compared, the normal male group appears to have a greater levels of NOx. However statistically there is no real difference between the two groups after the F- and t-test analyses. Interestingly, the amount of variation is greater than half of the sample mean. This maybe accounted for by the skewed effect that is often seen in biological data and can be observed in figures 7.6a and 7.6b.

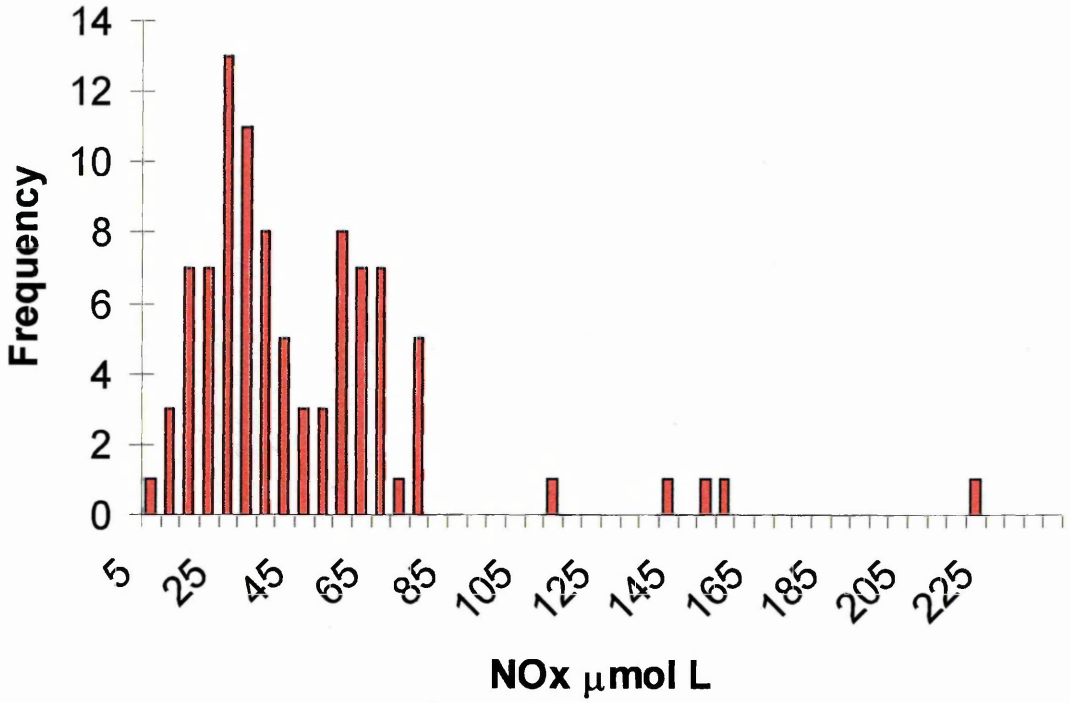


Figure 7.6a Variation of NOx levels in non- pregnant females from 20-65 years of age.

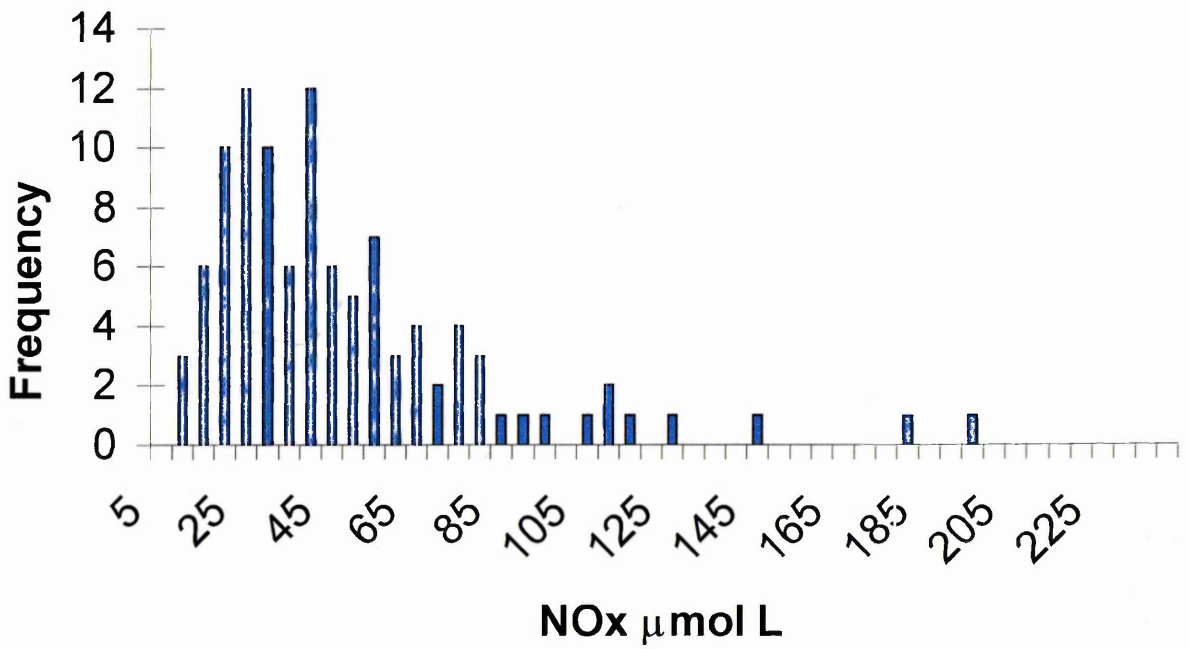


Figure 7.6b Variation of NOx in males from 20-65 years of age.

Here most samples in both groups fall between 0 and 85 μmol L^{-1} although some higher concentration skew the results. Michigami [62] also observed no difference between NOx levels and age. Work by Takahashi claimed that males were had approximately twice the concentration of females [354] such an effect was not seen in this study. The data trend in figure 7.5 suggests a small increase upto the age of 45 years after which it falls in old age. Whether there is a real effect or if this is caused by a small number of higher but normal NOx levels is unknown.

NOx and pregnancy

Comparison of NOx levels over gestation is shown from cross-sectional data in figure 7.7

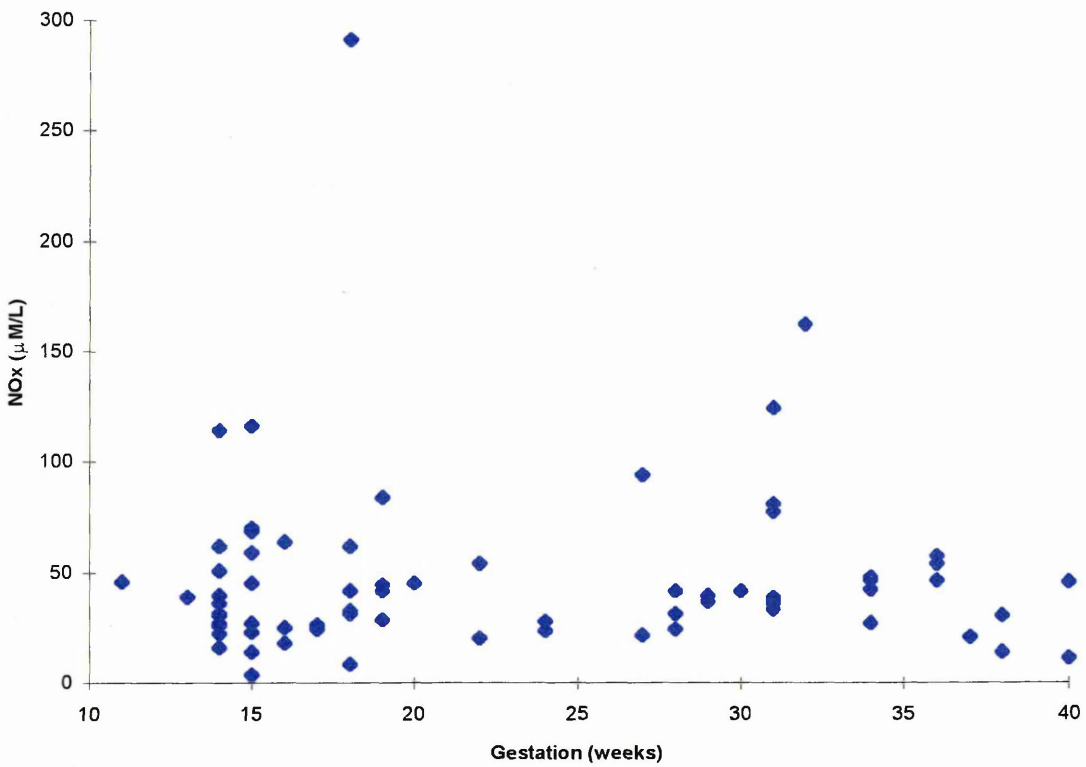


Figure 7.7 Correlation between plasma NOx levels and gestation in normotensive patients.

Unfortunately there was only one data point in the first trimester. The abnormal trophoblast invasion associated with pre-eclampsia is though to originate in mid-trimester so levels from the 2nd trimester onwards are more relevant to this study. When all the sample data means from the three trimesters were compared there was very little difference between them (of

3%). This similarity in NOx levels in the three trimesters from the plasma of pregnant women was observed by Curtis [64] and by Conrad [210]. However when comparing each group there appears to be a much greater amount of variation between the 2nd trimester compared to the 3rd trimester even though it is the larger of the sample groups (2nd tri 47±47 n=41, 3rd tri 46±33 n=29). If the outliner in the 2nd trimester is removed the mean and standard deviation is reduced to 41.0 ± 24.6 μmol L^{-1}. Comparison of the means and variations shows no statistical difference. This does not provide any evidence of the continuing decrease in NO production and activity over gestation that has been reported in human placental studies (chapter 2).

Comparison of the NOx level means of the non-pregnant females to the normotensive group showed no difference. When the age range 19-40 was considered the mean was 40.34 ± 26.27 μmol L^{-1} (n=47). The data from this study are in accordance with those of Conrad [210] and Curtis [64] who observed no difference between non-pregnant and pregnant groups. However Norbunga [205] and Seligman [203] reported that the non-pregnant females had a significantly lower NOx levels compared to the pregnant group. Even though the mean for non-pregnant females in the present study was lower then the pregnant group this was not significantly different (table 7.1).

The normotensive group were also split by parity to see if the effect of previous successful pregnancies affected NOx levels.

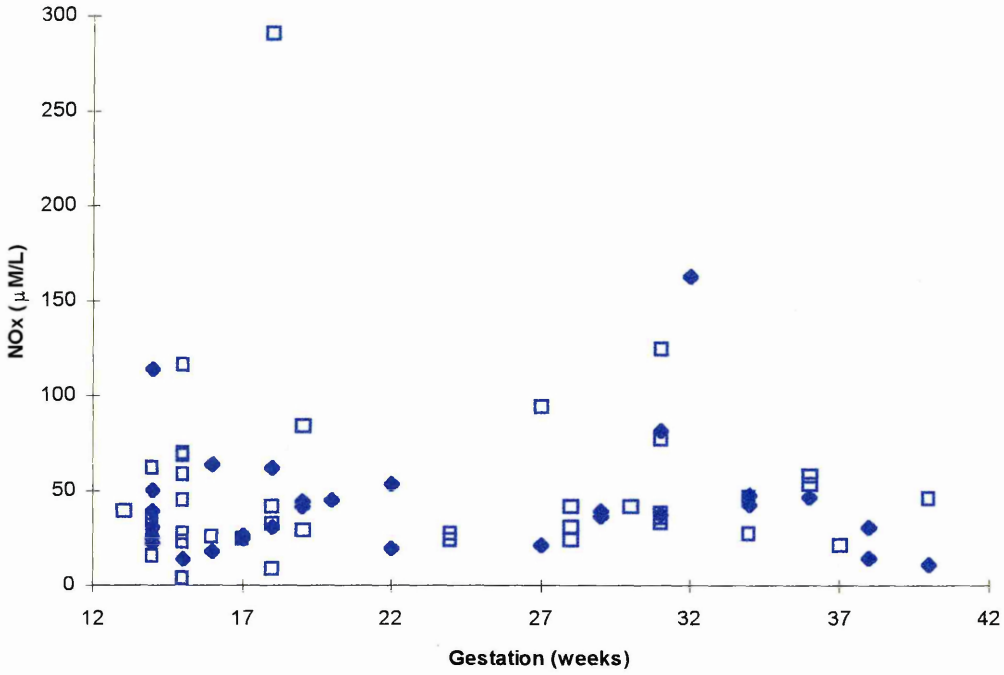


Figure 7.8 Effect of parity on plasma NOx levels throughout gestation in normotensive patients. First pregnancies (blue diamonds), those who had one or more previous pregnancies (empty squares).

As can be seen from figure 7.8 there was no difference visually or statistically (table 7.1) between the two groups.

Amniotic fluid

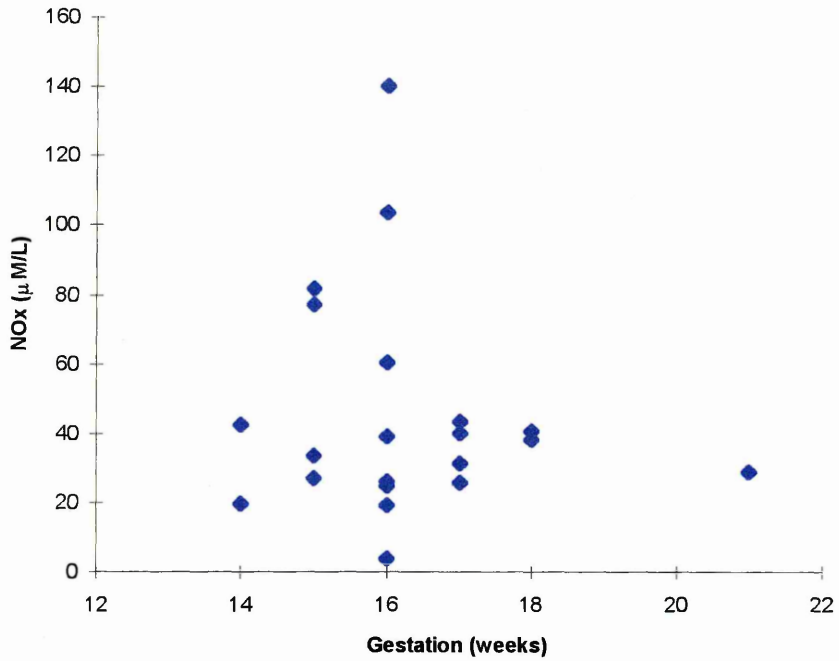


Figure 7.9a relationship between NOx levels and gestation in normal amniotic fluid.

Figure 7.9a shows the NOx concentrations present in amniotic fluid as a function of gestation. There is no clear trend or obvious pattern emerging from the amniotic NOx levels over the 2nd trimester. Also as expected these levels do not differ significantly from the normal plasma by their means or variations (figure 7.9b).

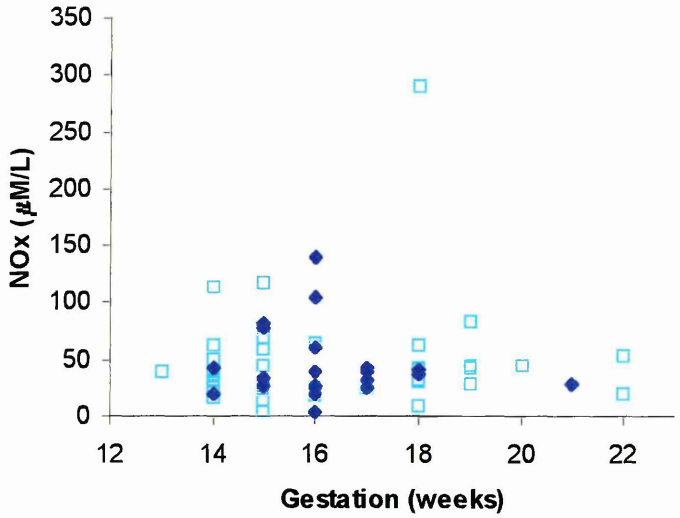


Figure 7.9b Comparison of normal plasma (clear squares) and amniotic fluid (solid triangles) over gestation.

Out of the 14 samples measured for nitrotyrosine, only 2 had detectable levels (14%). In the plasma samples from the pregnant groups this ratio was much larger (75-100%) tentatively suggesting that the amniotic fluid is relatively resistance to peroxynitrite oxidation.

None of the amniotic fluid samples came from diseased patients. It would have been interesting to see the relationship between gestation, NOx levels and disease states because Di Iorio [518] recently reported a correlation of uterine artery resistance index to amniotic NOx, and that these levels were significantly reduced in intrauterine growth retardant pregnancies. Their data were expressed as µmol/mg creatinine. Amniotic creatinine levels were not measured in this study and so data cannot be compared. They did not detect any changes over gestation.

NOx and PE/PIH

The severe pre-eclamptic (PE) and pregnancy induced hypertensive (PIH) patients were gestationally matched to first-pregnant normotensive controls as shown in figure 7.10.

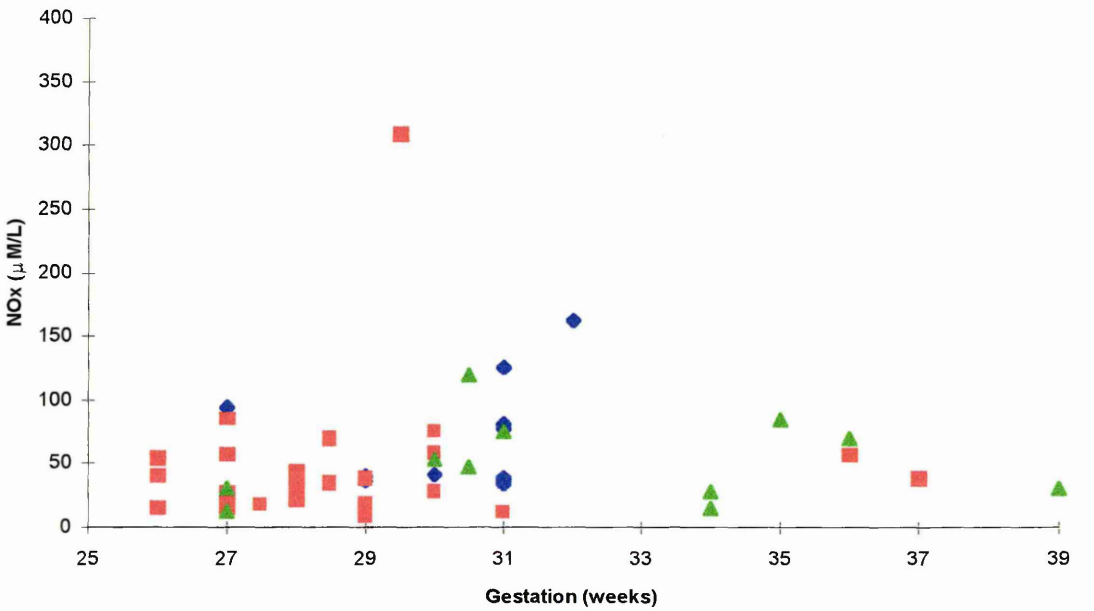


Figure 7.10 Comparison of plasma NOx levels in normal (blue diamonds), PE (red squares) and PIH (green triangles) patients throughout gestation.

The overall results produced means of the diseases groups that were greater than that of the normal patients. However the amount of sample variation between the samples in these groups eliminated any statistical difference (figure 7.11). A frequency distribution for each group is shown in figure 7.12.

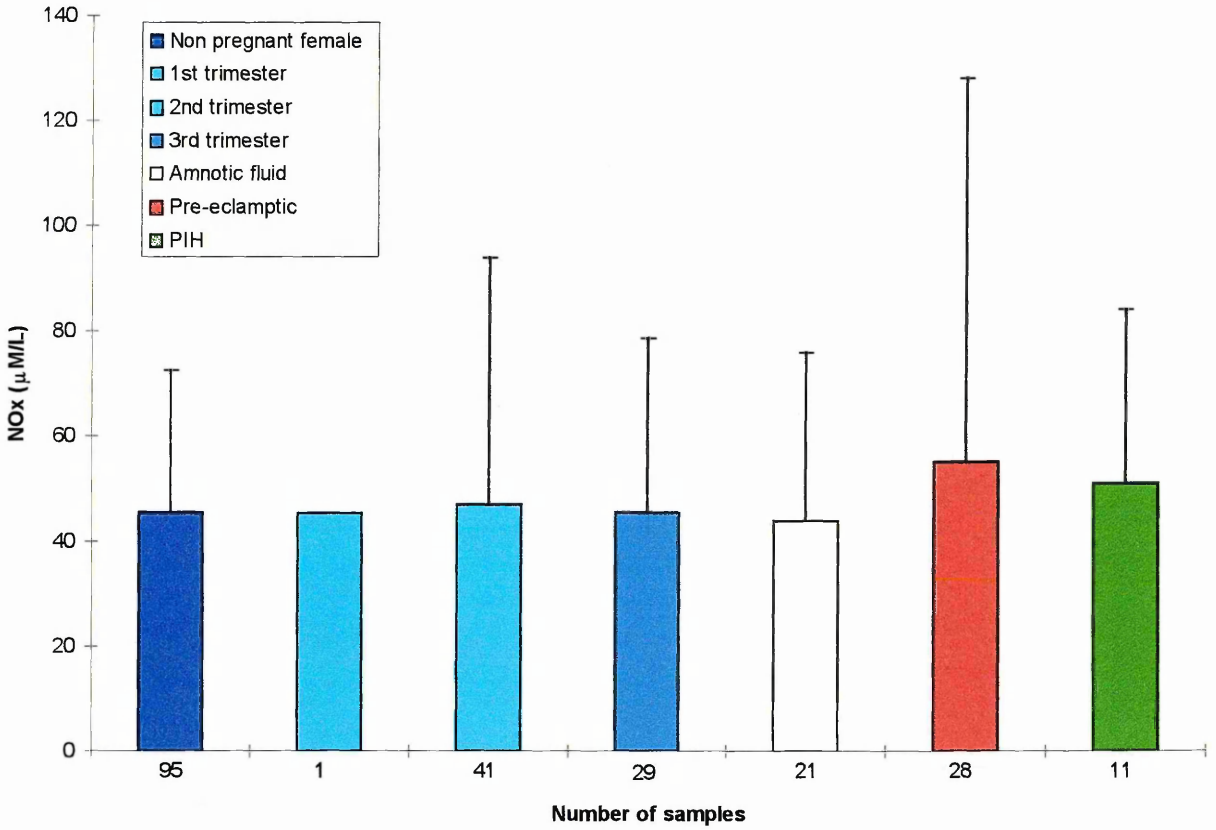


Figure 7.11 Summary of all NOx data showing variations in each group.

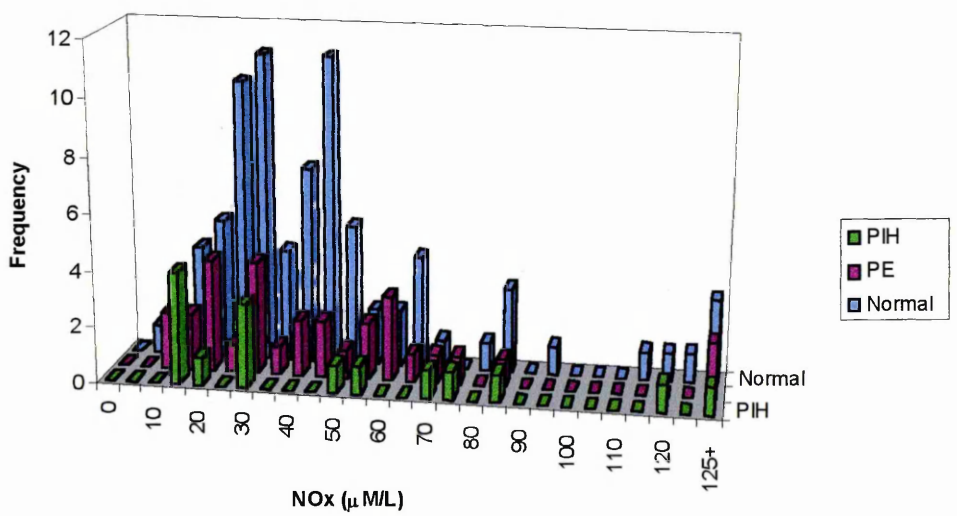


Figure 7.12 Distribution frequency of plasma NOx levels in normal, PE and PIH pregnancies.

The data for all three groups do not follow normal distributions and are skewed with possible evidence of two underlying populations (outliers at high concentrations). Unfortunately there is insufficient data for the two diseased groups to see if the pattern for the normal group is also evident in them. Comparison of the normotensive group distribution and those of the normal non-pregnant females (figure 7.6a) show remarkable similarities. Since the t-test is only significant when the distribution is binomial a Mann-Whitney non-parametric test was also performed. Neither test produced any significant differences between normal pregnancies and severe PE, and normotensives and PIH patients. This is in accord with Di Iorio [193], Davidge [200], Silver [202], Curtis [64], Lyall [63] and Conrad [210] who all found no difference between PE and normotensive serum NOx levels. Smarason [201], Norbunga [205], Seligman [203] and Garmenda [204] detected statistically significant differences. However, Smarason reported an increase when the assay was conducted by chemiluminescence, but when the samples were analysed by the Griess method after using nitrate reductase, they reported the same decrease in serum NOx in PE patients as Seligman [203]. Many of the groups used the Griess method (Table 2.1) and reported either no change [193, 200, 63], an increase [205] or a decrease [203,204] between the two groups. Curtis et al [64] were the only other group to analyse nitrate by ion chromatography and nitrite by the Griess reaction. They also detected no difference between normal and PE pregnancies but their concentrations were approximately twice the reported values in this study. Reported serum NOx concentrations in normal pregnancy range from 0.98 to 99 μmol L^{-1} (table 2.1) with most between 10 and 40 μmol L^{-1}. The normal values from this study are in accord with the majority of other studies. A possible explanation for the wide ranging results could be international and regional variation in diet and environmental factors [65]. Clinical treatment with hypertensive drugs and magnesium sulphate has also been offered as a cause of differing conclusions [201] , although disputed by others [204]. An alternative proposal by Buhimschi [135] suggested that patients with pre-eclampsia each have differing abilities to cope with the disease. Therefore a person may present with some factors of the disorder but be able to compensate, while others are unable to do so and proceed to a more severe stage in the disease . In this case the diagnosis between PE, severe PE, PIH and eclampsia becomes blurred and could result in biochemical differences.

The statistical power calculation is used to determine the sample size necessary to reduce the probability of rejecting a false null hypothesis [519,520]. Taking α as 0.05, power of 0.80, the smallest difference as 9.1 (Mean PE NOx – Mean normotensive NOx) and the standard deviation obtained for the normotensive group, the calculated result states that this study would require 280 individuals in both groups to have significant power. This is ten times the number of patients actually included, highlighting the difficulties of this work, and showing that NOx levels from individuals would be of little use diagnostically.

NOx in serial samples and cords

Cord blood was only taken from the two diseased groups and not normal patients. However the means for these groups is more than twice the maternal plasma values (table 7.1). Unfortunately umbilical blood from normotensive gestationally age-matched women was not obtainable. Therefore, these results cannot be compared with those of Lyall et al [63] who detected a decrease in the disease group compared to the normal group in umbilical but not maternal blood. From immunstaining for NOS, Di Iorio [193] and others (chapter 2) suggested that there is an increase in the overall placental NO production. However, Lyall found a increase in circulating NO metabolites in umbilical blood compared to maternal serum.

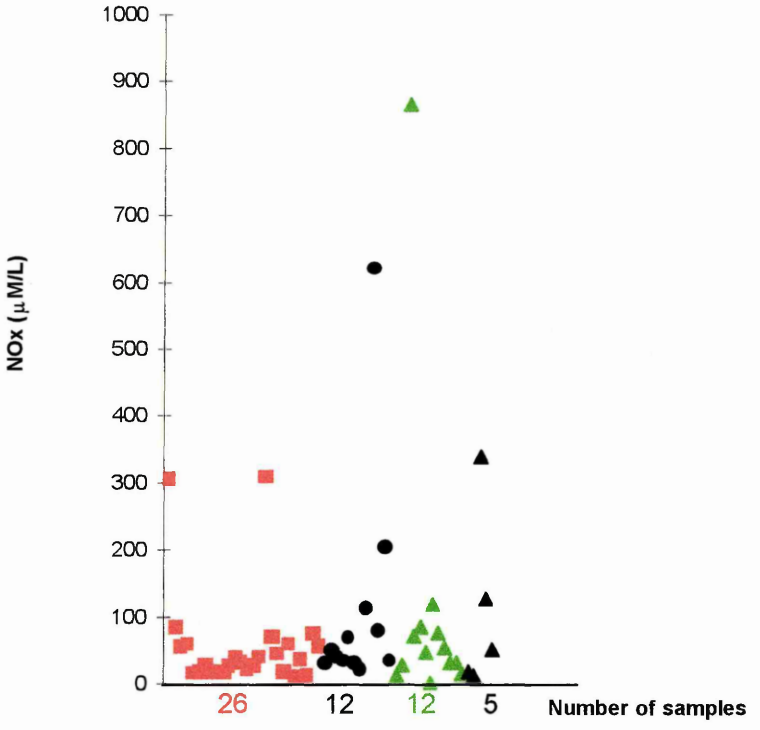


Figure 7.13 Comparison of PE maternal NOx levels in plasma (red squares), PE cord NOx levels in plasma (black circles), PIH maternal NOx levels in plasma (green triangles) and PIH cord NOx levels in plasma (black triangles)

This is further illustrated when the data are compared to gestation as shown in figures 7.14 and 7.15.

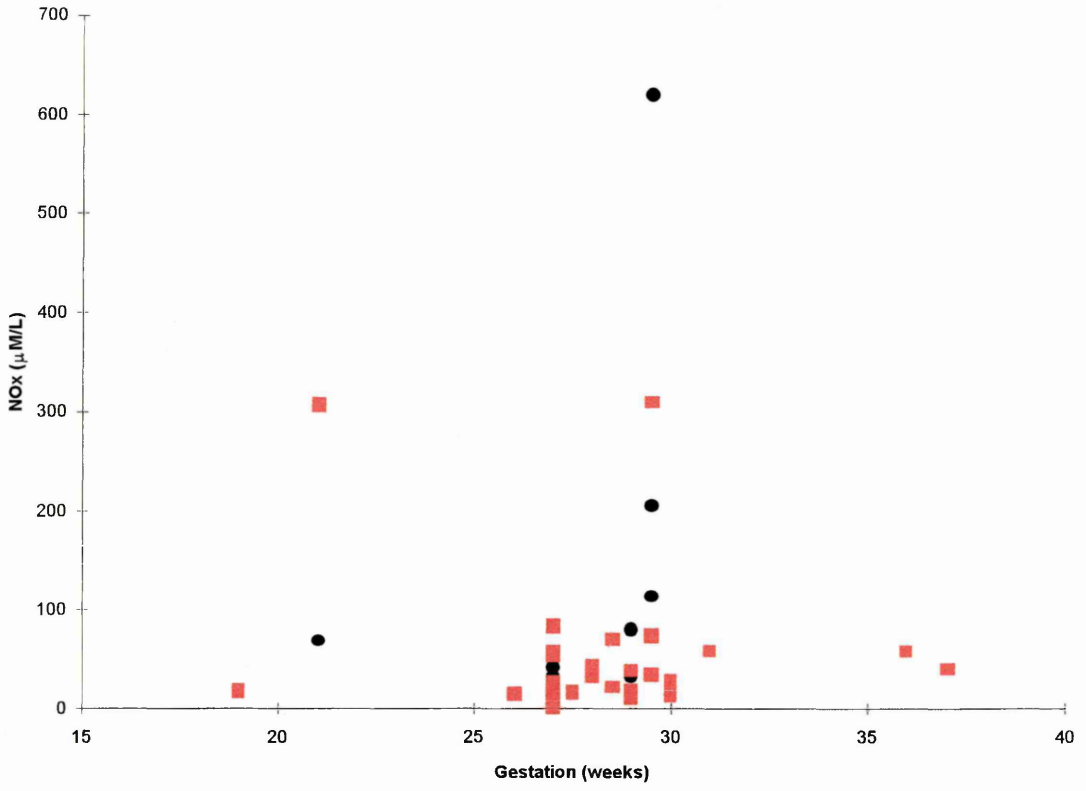


Figure 7.14 PE maternal (red squares) and umbilical (black circles) NOx levels throughout gestation.

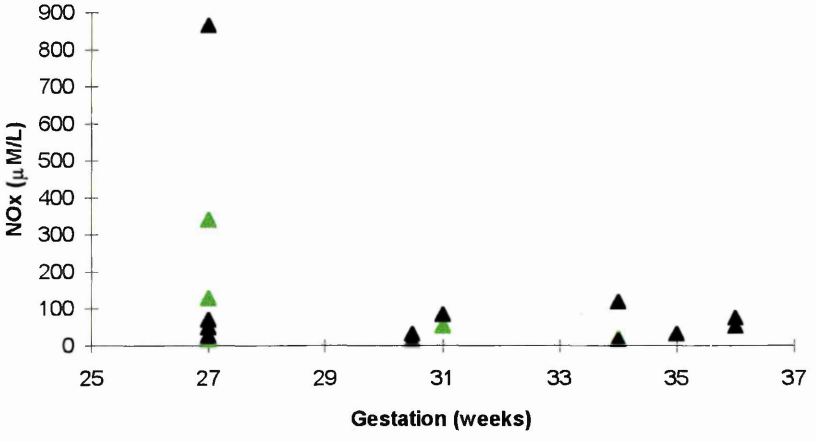


Figure 7.15 PIH maternal (green triangles) and umbilical (black triangles) NOx levels throughout gestation.

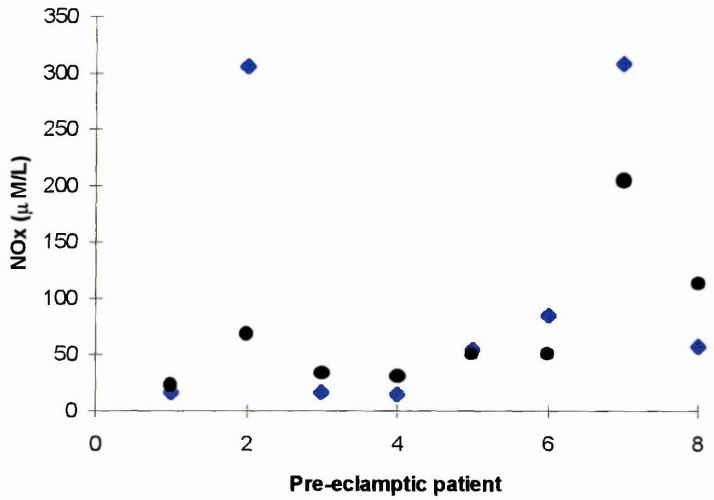


Figure 7.16 Comparison of maternal (blue diamonds) and umbilical (black circles) NOx levels from maternal and matched cord blood for eight subjects.

Some of the cord samples were taken on the same day as the mothers blood sample.

Figure 7.16 shows eight different women's plasma and cord NOx. It can be seen that there is no direct evidence of any trend between the maternal blood NOx levels and that of the foetal blood. Changes in the maternal plasma levels in three patients is shown in figure 7.17. Unfortunately, there were insufficient cord samples to determine if intra-individual and inter-individual variations were different.

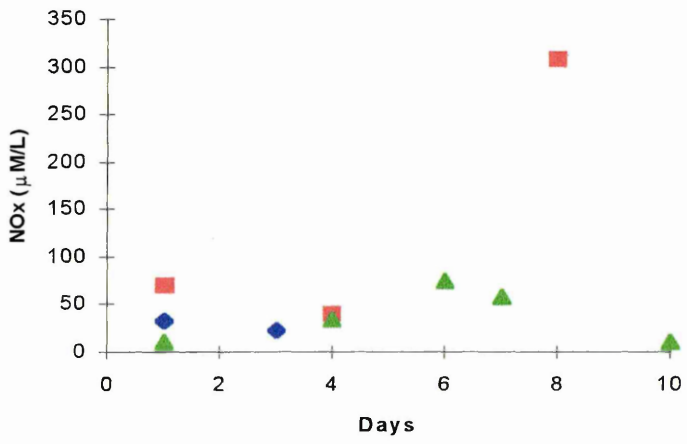


Figure 7.17 Changes in NOx levels from three different women over several days.

It can be seen that NOx levels can change by as much as 300 μmol L^{-1} over seven days (red squares).

NOx and systolic BP

As previously reported [203,204,205,206] there does appear to be a negative trend between systolic blood pressure and NOx levels in PE patients (fig 7.18), although this has not been seen by all groups [200].

No correlation was observed in the PIH group (figure 7.18b). The systolic blood pressure data were not recorded for the normal group.

NOx levels were also compared to plasma urate, platelets and proteinuria within the PE group but no correlation with any of these factors was found. Systolic blood pressure, urate and platelets were also compared to NOx levels in the PIH group but did not correlate with any of them. Data on plasma urate, platelets and proteinuria were provided by the Jessop Hospital for Women's clinical chemistry department. Information on systolic blood pressures was obtained from Dr Fiona Fairlie (Jessop hospital for women, Sheffield).

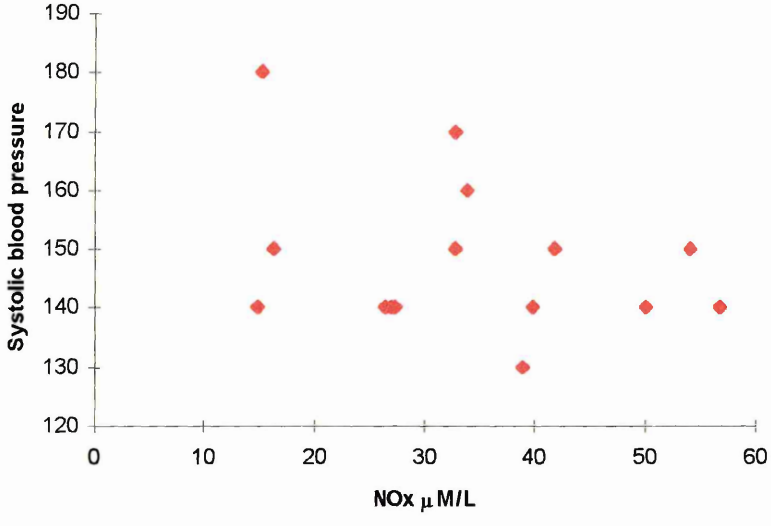


Figure 7.18 Correlation between systolic blood pressure and plasma NOx levels in PE patients.

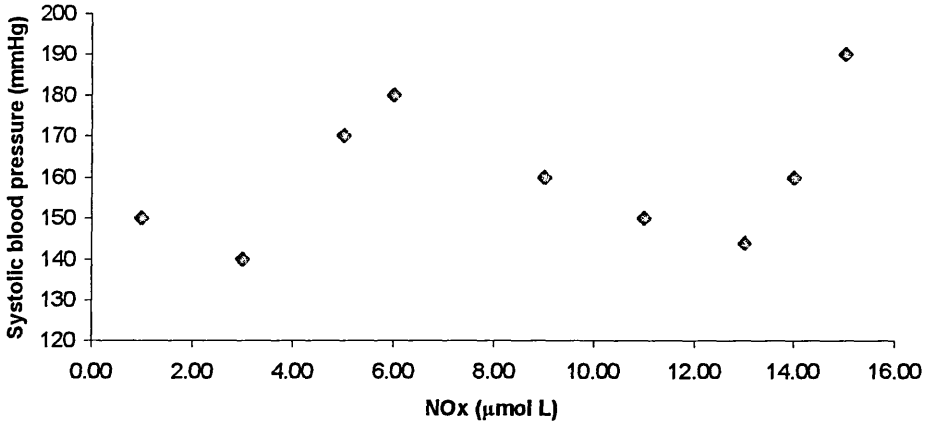


Figure 7.18b Comparison of NOx levels and systolic blood pressure in women with PIH.

Peroxynitrite and PE/PIH

Figure 7.19 shows the ratio of 3-nitrotyrosine/L-tyrosine in normal (blue), pre-eclamptics (red squares) and PIH (green triangles) pregnancies. Statistical analyses using the Mann-Whitney and Student t-tests revealed no significant difference between any of the groups. Figure 7.20 shows the results for the pre-eclamptic cord blood levels. Because many of the samples were below the limits of detection table 7.1 reports the percentage of samples where 3-nitrotyrosine was detected. Even though there was no difference between the disease groups and normal the cord blood levels appear to be much lower than the maternal levels, possibly due to the cytoprotective nature of the placental and foetal system (chapter 2). However, these results must be treated with caution as major difference can be detected between the total and free fractions that are dependent on plasma binding proteins. This is especially true for PE cases since these present with proteinuria. Allowance would have to be made for this, but is improved by using ratio measurements of the two amino acids rather than total 3-nitrotyrosine.

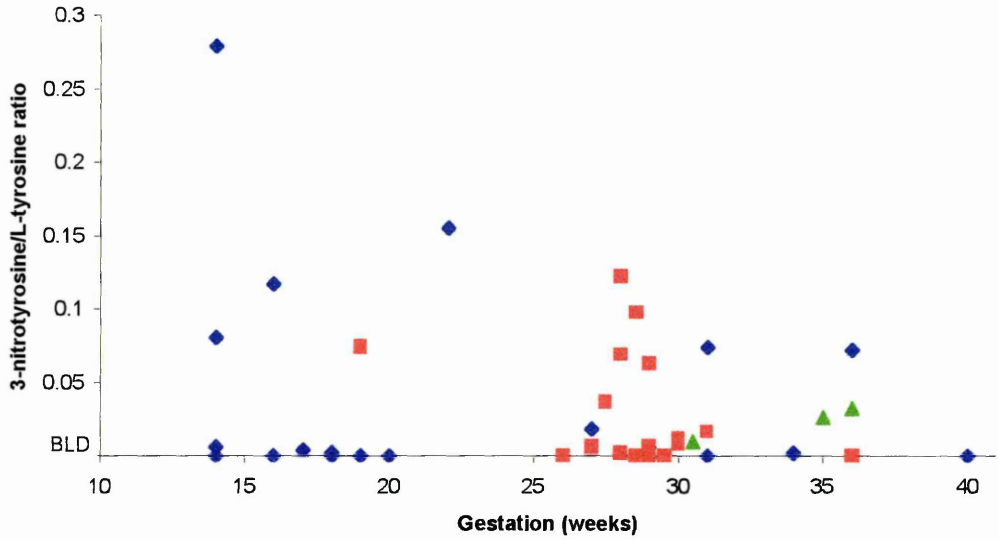


Figure 7.19 Comparison of plasma tyrosine/nitrotyrosine ratio levels in normal (blue diamonds), PE (red squares) and PIH (green triangles) patients throughout gestation. BLD - below limits of detection.

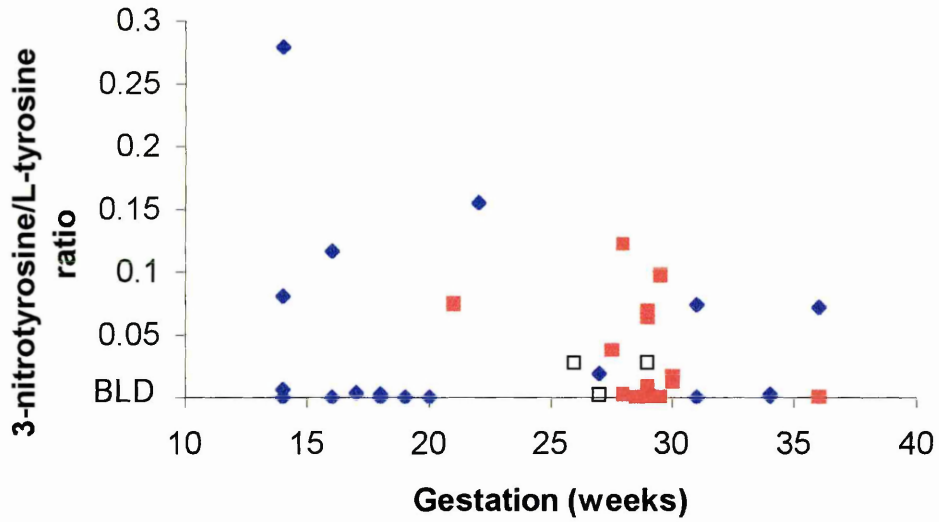


Figure 7.20 Relationship between plasma tyrosine/nitrotyrosine ratio in normal (blue squares), PE maternal (red squares) and PE umbilical blood (clear squares) over gestation. BLD below limits of detection.

Summary

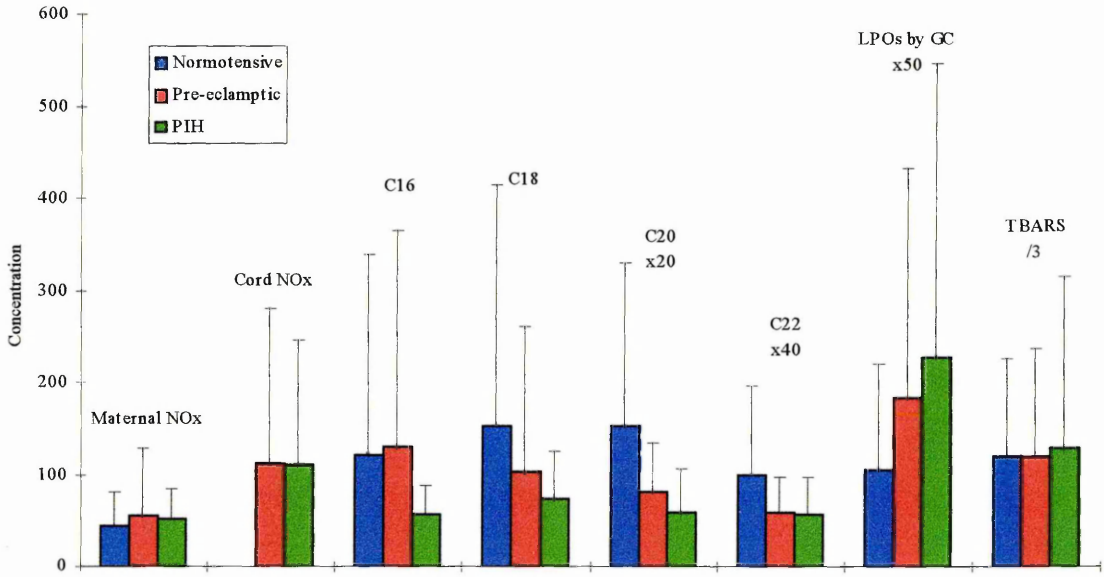


Figure 7.21 Shows the summary data from this study and compares it to each group. NOx and TBARS in μmol L^{-1}, fatty acids and lipid peroxides are expressed in μg L^{-1}. The coloured block show the mean levels, and the error bars indicate the range.

Figure 7.21 summarises all the data from this study. Inter-individual variation was in some assays was almost two times that of the mean. This large spread of results for each analyte removed any significant change in their means and therefore no statistical difference between the pregnant study groups could be deduced from the sample numbers available. Each group of analytes (lipids, lipid peroxides, nitrotyrosine and NOx) was compared to the three pregnant study sets independently of each other.

The lipid peroxides show an overall increase in the diseased groups compared to normals but this was not statistically significant. The only human study into the activity of peroxynitrite came from Myatt et al [218]. There is no evidence from this study of increase or decrease nitrotyrosine and therefore peroxynitrite in PE/PIH. It is possible that there is a reduction in foetal peroxynitrite, but due to the small sample number this cannot be substantiated. The total nitrite and nitrate levels in women with PE or PIH failed to show any statistical difference over gestation when compared to normotensive pregnant women.

Chapter 8

Overall discussion and conclusions

Nitric oxide is a vital second messenger used in neural communication, regulation of vessel homeostasis and control of the immune system. Its proposed roles in normal pregnancy include maintenance of low vascular resistance and anti-aggregation of platelets in the feto-placental circulation, relaxation of the uterine myometrium and blunting the effect of vasopressors.

Studies of NO metabolites in normal pregnancy and PE have produced conflicting results. Researchers [203, 204] who have found decreases in NOx levels or NOS activity in pre-eclamptic patients compared to normal women have suggested that down-regulation of the enzyme and/or endothelial damage occurs in the development of the disease, with the consequent reduction in NO synthesis contributing to the disease. Other groups [201,205] observing an up-regulation or over-production of NO in the disease group explain this as a compensated mechanism for endothelial damage and an effort to correct the hypertensive disorder. The results from this study and many others [193, 200, 202, 64,63,210] have not discovered any difference in plasma NOx levels in PE and normal pregnant women. This is not to say that there is no difference relating to the PE disease at the placental bed, but that cannot be illustrated in serum measurements. Determination of NO production via nitrite/nitrate involves problems compounded by dietary intake, smoking, pollution and other external factors (chapters 1&2). Increased serum nitrite/nitrate has been seen in a variety of diseases such as toxic shock [7,1,8] and tissue rejection [358] where large production of NO is produced by stimulated iNOS. In disease states where the difference is smaller it may not be possible to notice this change over an uncontrolled daily variation. It is also possible that activation of platelets in serum may produce a different result than with plasma. However, Moshage [19] did not detect any difference.

The aim of this study was to develop reliable methods for the determination of plasma lipids, lipid peroxides, nitrotyrosine, nitrite and nitrate that was superior to methods previously

available. Once completed these methods would be used to determine the role of nitric oxide in pregnancy, pre-eclampsia and pregnancy induced hypertension. If there was a difference detected between the normal and diseased groups it could have an important clinical use in early detection of the disorder. Lipid peroxides measurements would be used in conjunction with clinical information to confirm the presence of PE/PIH and to suggest the severity of the disorder.

Unfortunately this index could not be used as there was no evidence in our data of statistically significant differences in the levels of these metabolites between normal and afflicted patients.

The role of nitric oxide in pre-eclampsia is still not understood as there is no clear consensus on its activity throughout normal and diseased pregnancy. It is known that this syndrome is multi-factorial. As with the research on prostacyclin many groups like to consider one variable's as the ruling factor in this disease. Undoubtedly more research will eventually confirm or deny a role of nitric oxide in the disorder and probably come to the same conclusion, that dysfunction in the synthesis of this molecule may answer some of the questions in the presentation of the clinical symptoms. However the ultimate cause, be it genetic, environmental or immunological, that affects the production of nitric oxide and the other biochemical factors remains to be solved. Future work on the comparison of urinary excretion, placental tissue production and serum nitric oxide metabolites in normotensive and women with PE may help to clarify the discrepancies seen.

Future research

As shown in Chapter 7 a larger sample size would improve the statistical probability of their finding a difference in the serum concentration of nitrite and nitrate in all the study groups. Futher sub-grouping of the normal sample population could help distinguish the effect of environmental factor discussed in Chapter 1 i.e. smoking and diet. A useful study would be to assess the changes in serum NO_2^- and NO_3^- compared to its excretion in urine as markers of NO production.

It would also be of interest to perform enzymatic studies on the placentae of PE and normotensive patients and compare the results with serial serum/urine nitric oxide metabolites to obtain comparative data.

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Appendix

Conferences attended

Capillary chromatography training seminar (Restek corporation) - York - June 14th 1995

RSC and SAC/R&D topics in analytical chemistry - University of Hull - 11-15th July 1995 [b]

Reproductive cardiovascular workshop (2nd meeting) - Amsterdam - 26-27th April 1996 [a]

RSC R&D topics in analytical chemistry - Nottingham Trent University - 22-23rd July 1996 [b]

International ion chromatography symposium - University of Reading - 16-19th September 1996 [a]

[a] podium presentation
[b] poster presentation

Ethical approval statement and publications

Dr P R Jackson
Chairman
South Sheffield Research Ethics Committee
Ethics Office: Tel & Fax No: (0114) 271 2394
PRJ/KAK

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4 August 1995

Dr F M Fairlie
Consultant Obstetrician & Gynaecologist
Jessop Hospital for Women

Dear Dr Fairlie

95/47 - Does nitric oxide deficiency play a role in the pathophysiology of pre-eclampsia

Thank you for submitting your protocol to the Ethics Committee. It was considered at the meeting on the 3 August. The protocol was approved in principle although there was some concern about the evidence that doppler ultra-sound might inhibit fetal growth. The Committee would welcome you comments about that possibility and would also like you to consider the following alterations to the information sheet:-

1. There should be separate information sheets for control and pre-eclamptic patients.

2. It needs to be made crystal clear to subjects that this is altruistic or non-therapeutic research and that there is no chance of them personally benefitting from this particular investigation.

3. It needs to be made clear particularly to the control subjects that the number of anti-natal visits will indeed be increased. We do not know of any centre which sees normal subjects every 3 days during the final weeks of pregnancy. Clearly there will also be extra samples for these patients and the number and nature of samples will need to be crystal clear.

4. At least one member of the Committee felt that some patients might be concerned that the cord be cut before it stops pulsing. Whilst this might not matter to many subjects it could be crucial to a small minority and it would not harm to include it in the information sheet.

5. Some of the responses to the questions in your information sheet are rather brief and require expansion. Thus the bounds of confidentiality will need to be explained. Confidential within the Hospital could mean limiting it only to the two primary investigators or alternatively it could mean that everyone from the Chief

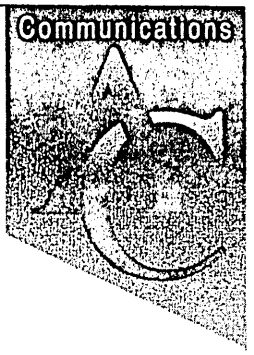
Executive to the ward cleaner are advised of the results. The ability to withdraw without giving reasons must also be stated.

6. Finally, there is required a paragraph in relation to harm. Even if you cannot envisage the way your protocol could harm patients undoubtedly one will trip and break a leg at one of the extra visits. The Committee advises the following paragraph, headed by "What if I am Harmed".

'If you are harmed by your participation in this study, there are no *special* compensation arrangements. If you are harmed due to someone's negligence, then you may have grounds for a legal action. Regardless of this, if you have *any* cause to complain about your treatment whilst taking part in the trial, the normal complaints mechanisms available to anyone receiving care in the National Health Service are available to you and are not compromised in any way because you have taken part in a clinical research study.'

Yours sincerely

P R Jackson
Chairman



itrate and Nitrite Determination in Complex atrices by Gradient Ion Chromatography

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imultaneous determination of nitrate and nitrite in uman serum has been accomplished using gradient ion romatography and direct UV detection, with centrifugal ltrafiltration as the only pretreatment. The method has vantages over previous techniques in both ease of use d cost per assay. The sensitivity is adequate for use as a onitor of nitric oxide induced changes in serum nitrate d nitrite levels.

simple and reliable method for determination of nitrate and trite in human serum is needed because of the immense terest in the role of nitric oxide as a controller of vascular tone, d the implication of faulty NO balance in many diseases.[1]

Determination of nitrate and nitrite in simple aqueous media ay be carried out by any of several methods,[2] but is most quently performed by spectrophotometric assay of nitrite. In is method, a diazonium salt formed by reaction of nitrite with, r example, sulfanilamide is coupled with a chromophore such *N*-(1-napthyl)ethylenediamine to yield an azo dye of high olar absorptivity. The method has high sensitivity and works ll in simple matrices such as water. For detection of nitrate, duction to nitrite must be carried out before the spectrophoto- etric steps, and the result from a separate nitrite determination btracted from the total. The reduction is most often performed th cadmium powder.

In complex matrices such as biological extracts, however, the dmium reduction step is often subject to interference. One dard method for wastewater[3] advocates the use of hydrox- nine as an alternative reductant, but we have found this to be ffective in serum. Several strategies have been proposed to ercome the difficulty of nitrate reduction in serum, either olving activation of the cadmium surface[4,5] or use of an ymic reduction step.[6] Pretreatment to reduce the interference also advocated.[4] These approaches, however, add con- erably to the cost and complexity of the determinations.

The investigation of alternative methods for this matrix is refore of considerable interest. Among the possible tech- ues, ion chromatography has gained considerable acceptance the analysis of simple anions and cations. In relatively ple matrices, simultaneous determination of a range of mmon anions including nitrate and nitrite can be achieved th a run time of a few minutes. Determinations of nitrite and ate in serum and other biological media by ion chromatog- hy have been reported,[6–11] but have been subject to several blems. The standard method of detection for ion chromatog- hy is conductivity, which gives response to all eluted ions. e of this in serum, however, requires pretreatment to reduce chloride concentration which, at approximately 0.1 mol l^{-1}, some four to five orders of magnitude higher than typical els of nitrite. Nitrite elutes close to the chloride, and is often amped by the chloride response if the latter is not removed by pretreatment. Detection by direct UV absorption, which will not record chloride, is an alternative to chloride removal, but the column packings commonly used for anion analysis are designed for high selectivity at the expense of capacity. The result is that, with sample loadings sufficient to give a reasonable response from nitrite, peak shapes and positions are distorted by the effects of column overload from the (invisible) chloride peak.

We have developed a method which overcomes these problems by use of: (*i*) direct UV detection to avoid recording of chloride; (*ii*) a chloride concentration gradient for elution to eliminate the distortion due to the sample chloride peak which is seen with other eluent ions; and (*iii*) a high capacity column to prevent overload.

This combination of factors is novel and results in a determination for which the only pretreatment necessary is a simple centrifugal ultrafiltration to remove macromolecules.

Experimental

Fig. 1 shows the chromatogram obtained from a typical sample of human serum. The serum was centrifuged through a 3 kDa molecular mass cutoff filter (pre-washed Centricon-3; Amicon, Stonehouse, Gloucestershire, UK) before being loaded onto a Dionex Carbopac PA-100 guard column (Dionex UK, Camberley, Surrey), followed by a 4 × 250 mm analytical column of the same material. Peaks were identified by comparison of retention times with standards. Samples spiked with nitrite and nitrate showed superposition of the spike peak with the native peak, indicating that the peak identities were correct.

The elution regime was a linear gradient of chloride concentration from 0.12 to 0.30 mol l^{-1} over 9 min, followed by

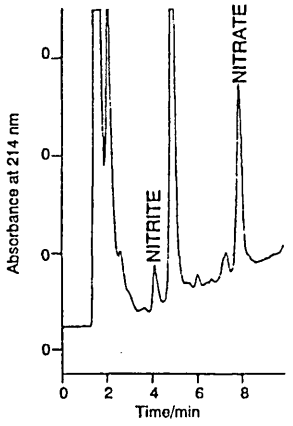

Fig. 1 Ion chromatogram of ultrafiltered human serum. Detection by direct UV absorption at 214 nm. Conditions as in text.

4 min washing with 0.3 mol l^{-1} chloride then re-equilibration with 0.12 mol l^{-1} chloride for 6 min, provided by a Dionex 3000 quaternary gradient pump. The flow rate was 1.0 ml min^{-1}. TRIS buffer (5×10^{-3} mol l^{-1}, pH 7.5) was present in all eluents. The water used was obtained by distillation then de-ionisation (Milli-Q, Millipore, Watford, UK), with a minimum resistance of 18 MΩ. HPLC grade sodium chloride (Merck, Poole, Dorset, UK) was used to produce the chloride solutions. All tubing was polyether ether ketone (PEEK) and metal contact was reduced to a minimum to avoid corrosion problems. The system was flushed with water at the end of each session.

Ultraviolet detection was at 214 nm (Spectromonitor III, Thermo-LDC, Warrington, Cheshire, UK), and chromatograms were recorded, processed and displayed by a Minichrom data station (VG Data Systems, Altrincham, Cheshire, UK). Peak areas were calculated from a baseline drawn to either side of the relevant peak.

Results and Discussions

Limits of detection for both nitrate and nitrite were approximately 2×10^{-7} mol l^{-1} (10 and 13 ppb, respectively). The calibration graph was linear up to 2×10^{-4} mol l^{-1} (the highest concentration used in the calibration set). The relative standard deviation for nitrite, as determined by replicate injections of serum samples, was 5% at the mean concentration (3.7×10^{-6} mol l^{-1}) found for a set of 200 random adult serum samples, and for nitrate was 4% at the mean adult level (4.0×10^{-5} mol l^{-1}). Spike recoveries, calculated from additions of equal masses of nitrite and nitrate, averaged 106%. In these, however, the nitrite value averaged 76% whereas the nitrate value averaged 137%, suggesting oxidation of nitrite to nitrate by a serum component; further investigations of this are under way. The average nitrite to nitrate molar ratio in the random adult serum set was 0.09.

In contrast to previously described methods for determination of nitrite and nitrate in serum and similar complex matrices, this method requires minimal pretreatment, gives separate readings for each ion from one experiment, and is quick and inexpensive to perform. Sensitivity is adequate and selectivity is good. Work is in progress in applying the method to the investigation of nitric oxide function in various diseases.

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Determination of nitrite and nitrate in human serum

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Abstract

A simple and effective assay for nitrite and nitrate in human serum has been developed using ion chromatography. Initial experiments using isocratic carbonate–bicarbonate elution with conductivity detection on a Dionex QIC system with an AS4A-SC column showed promise but were unsatisfactory because of co-elution problems with nitrite. Carbonate and chloride were investigated as eluents using a gradient system, and direct UV detection at 214 nm was used in place of conductivity detection. Dionex AS4A, AS9A, AS12, Nucleopac PA-100 and Carbopac PA-100 columns were compared for selectivity and resistance to overload. The final method, using a chloride concentration gradient, pH buffering and direct UV detection with a Carbopac PA-100 column, shows good resolution, does not suffer from chloride overload and is simple to use. The method is being used in an investigation of the role of nitric oxide in pre-eclampsia, a hypertensive disorder during pregnancy.

Keywords: Nitrate; Nitrite; Inorganic anions; Nitric oxide

1. Introduction

Nitric oxide (NO) is a vital messenger in many cellular communication and control systems. Because of the short half life of NO in aqueous media, assay of NO is performed on its metabolites, nitrite and nitrate. Classically, these have been determined by the Griess method, in which nitrite is diazotised with sulphanilamide and then reacted with N-1-naphthylethylenediamine to form a coloured product. However, this approach does not work well in the complex matrix of serum, where the reduction step from nitrate to nitrite is difficult to achieve chemically or expensive if enzymatic reduction is used. Redox buffers such as ascorbic acid may be responsible for the reduction problem, but other factors can also affect the assay. Colour and turbidity in the sample will cause interference and the presence of copper in the sample will catalyse the decomposition of the diazonium salt and bring about a reduction in the results [1]. Assay of nitrite alone, without accompanying nitrate, is unsatisfactory because serum samples left at room temperature for more than 4 h may show oxidation of nitrite to nitrate [2].

Ion chromatography has become one of the most powerful tools for the quantitative analysis of anions and cations. The most common procedure for the analysis of anions is an isocratic method with bicarbonate–carbonate buffered eluent, suppressed ion chromatography and conductivity detection, as used by Dionex systems. Ion chromatographic methods for nitrate and nitrite determination have been reported previously using such systems (see Sah [1] for a review). However, the naturally high concentration of chloride present in serum compared to nitrite and nitrate (the concentration of chloride is approx. 10^5 times that of nitrite) swamps out the

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al from nitrite when using conductivity detection
ause nitrite elutes shortly after chloride from most
n-exchange columns. This has led previous
kers [3] to pretreat samples with silver-loaded
n in order to reduce the chloride concentration.
h an approach is effective but time-consuming,
nsive and likely to reduce precision. Silver ions,
ch may leach from the resin, also ultimately
age the ion-exchange columns.
irect UV detection at about 214 nm avoids the
e positive signal from serum chloride and, if an
nt is chosen that has low absorption at this
elength, a cleaner trace for double bond-con-
ing ions can be obtained. Ideally, for ion chroma-
phy, the eluent ion should have lower affinity
the column than most of the analyte species and
ould have low toxicity. Hydroxyl ions are good
nts, and chloride can also be used.
e use of chloride as an eluent ion for nitrate and
te in samples containing high levels of chloride
been reported by Pastore et al. [4]. Haddad et al.
eported a working method to quantify low nitrite
s in sea water and Radisavljevic et al. [6] have
ished an isocratic chloride method for serum
les. The benefit of using chloride as the eluent
s that the column is coated with this ion prior to
tion of the sample and displacement artifacts
to the passage of a large slug of sample-derived
de through the system are greatly reduced.
like the more commonly used carbonate–bicar-
te system, however, chloride has no inherent pH
ring capacity and so another ion must be added
w concentration to provide buffering. Phosphate
been used as a buffer [6] but obviously this
udes the determination of sample phosphate.
atic elution with sufficient resolution to separate
e from interferences also resulted in a long run
[6]. Gradient elution is an answer but at low
t strength it is possible for buffer ions to
ulate on the column; these are displaced at
r eluent strength as the gradient elution pro-
es, producing an artifactual peak. This work
sses the problem by use of an eluent strong
to prevent buffer accumulation and a high
ity column with unusual selectivity. A pre-
ary account of this work has been published
here [7].

2. Experimental

2.1. Reagents

Stock solutions of nitrite and nitrate were prepared by dissolving the appropriate amount of AnalaR sodium salt (BDH, Poole, UK) in Milli-Q water that had a resistance of not less than 18 MΩ. Trizma buffer (ultra-pure grade, Aldrich, Gillingham, UK) was dissolved in the eluent reservoirs to 5.0 m*M* and the pH was adjusted to pH 7.5 with AnalaR hydrochloric acid. HPLC-grade sodium chloride (BDH) was used to make the eluent. All eluents were thoroughly degassed and were stored under helium.

The location of nitrite and nitrate peaks were determined from known standard solutions in water and were confirmed, where necessary, by spiking serum samples.

2.2. Samples

All control serum samples (prepared from fresh blood) were obtained from the Blood Transfusion Service (Northern General Hospital, Sheffield, UK). Serum samples were diluted four-fold with deionised water and then centrifuged through 3000 M_r cut-off Centricon filters (Amicon, Stonehouse, UK) for 120 min at 7500 *g* prior to analysis. Each filter was washed thoroughly before use with deionised water to remove nitrate left by the manufacturing process. There was no further pre-treatment of serum.

2.3. Griess method

A 200-μl volume of Griess reagent was added to 200 μl of deproteinised serum in a 96-well plate, which was left for 15 min in a dark cupboard and then the absorbance was read at 570 nm on a Labsystems Multiskan MS V1.5 plate reader.

2.4. Isocratic ion chromatography method

A Dionex (Camberley, UK) QIC suppressed ion chromatography system was used with AS4A-SC analytical and guard columns and conductivity detection. The eluent consisted of 1.8 m*M* Na_2CO_3 plus 1.7 m*M* NaHCO*3*. An LDC/Milton Roy Spec-

tromonitor III (Thermo Analytical Systems, Stone, UK) UV detector set at 214 nm was connected in series to the conductivity cell outlet for comparison of UV and conductivity traces. No other column was used with this system.

2.5. Gradient ion chromatography methods

The chromatographic system contained a Dionex gradient pump and an LDC/Milton Roy Spectromonitor III set at 214 nm. Data were acquired using a VG Minichrom data system, with v1.6 software (VG Data Systems, Altrincham, UK). Samples were injected into the system using a Perkin-Elmer ISS-100 Autosampler (Beaconsfield, UK) with a sample volume of 30 μl. All the stainless steel tubing in the system was replaced with polyether ether ketone (PEEK) tubing and the apparatus was washed after every use with pure water to minimise corrosion due to the use of a high concentration of sodium chloride as the eluent.

Dionex AS4A-SC, AS9A, AS12, Nucleopac-PA 100 and Carbopac-PA 100 columns (all 250×4 mm) were kindly loaned by Dionex (UK). Equivalent guard columns were placed before the analytical columns. The system was fully automated in that the autosampler was the chief controller for starting the Dionex gradient pump and the Minichrom data system at the start of each run, allowing 24 h analysis of samples, if required.

For the AS4A-SC, AS9A, AS12 and Nucleopac PA-100 columns, the chloride gradient programme was as follows: isocratic 1.5 m*M* chloride for 6 min, gradient from 1.5 to 30 m*M* chloride for 9 min, isocratic 30 m*M* chloride for 15 min, isocratic 150 m*M* chloride wash for 1 min and then re-equilibration over 9 min with isocratic 1.5 m*M* chloride. The flow-rate was 1.5 ml min^{-1} throughout and no buffer was present.

For the Carbopac PA-100 column, the short gradient programme used (no resolution of organic acids) was: gradient from 120 to 300 m*M* chloride over 10 min, isocratic 1000 m*M* chloride wash for 4 min and then re-equilibration over 6 min with 120 m*M* chloride. 5 m*M* Tris buffer was present throughout and the pH value of all eluents was adjusted to 7.5. The flow-rate was 1.0 ml min^{-1} throughout.

The longer programme with the Carbopac PA-100 column for separation of organic acids was: isocratic 30 m*M* chloride for 1 min, gradient from 30 to 300 m*M* chloride over 15 min, a 4-min was with 1000 m*M* chloride and then re-equilibration over 10 min with isocratic 30 m*M* chloride. The buffer and the flow-rate were as for the previous programme.

3. Results and discussion

Fig. 1 shows the traces obtained with conductivity and UV detection from an isocratic elution with carbonate eluent and a Dionex AS4A-SC column.

The lower chromatogram shows a typical serum sample with conductivity detection. The largest peak is from chloride and clearly obscures the signal from nitrite, which elutes just after chloride. The result of pretreatment with silver-loaded resin, as advocated by Lippsmeyer et al. [3], is shown in the upper two traces of Fig. 1. Once prepared, the resin is added to serum that has been centrifuged through a membrane filter. The resulting precipitate is filtered off and then the sample is injected onto the column. The resulting conductivity chromatogram (Fig. 1, middle trace) shows that this procedure greatly reduces the chloride content, but does not always reduce it sufficiently to allow quantification of nitrite. The use of

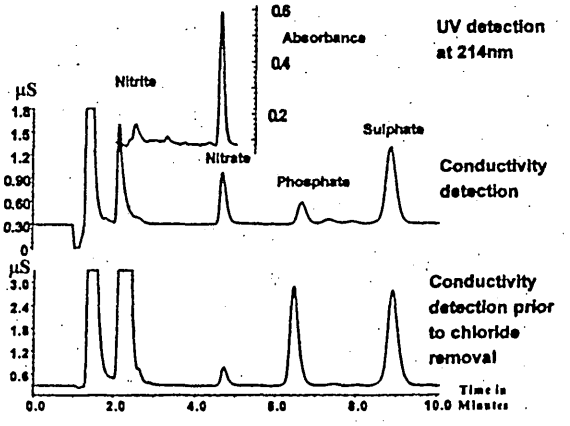

Fig. 1. Chromatograms of ultrafiltered human serum obtained using an AS4A-SC column with isocratic carbonate elution. The amount of chloride was decreased by pretreatment with silver resin (middle and upper traces).

detection at 214 nm (Fig. 1, upper trace) e chloride invisible and allows quantifica- e nitrite peak. However, there are many absorbing components in serum (particu- ic acids), which, under these conditions, close to the nitrite peak. Reducing the ngth to improve resolution in the early part omatogram results in an unacceptably long time for nitrate. Additionally, the use of eatment in this method creates additional ons which are possible sources of error. fied elution protocol is needed to improve tion of the early-eluting species (including ile retaining a reasonable elution time for for late-eluting species. A concentration f carbonate–bicarbonate can be used to e resolution of early-eluting species, how- val of chloride is still necessary, even if on is used, because the amount of chloride uses a large negative peak in the UV trace s is probably due to displacement of UV- eluent ions by the chloride slug as it wn the column. The effect is to render rmination impossible.

of chloride as an eluent ion has been previously as a means of overcoming duced artifacts without resorting to its m each sample [4–6]. Haddad et al. [5] trace amounts of nitrite in seawater in a signed to solve essentially the same prob- re, namely a high concentration ratio of nitrite. Use of this procedure, an isocratic th 15 m*M* chloride as the eluent (data not d remove the negative peak due to chlo- e UV trace, but organic acids co-eluting itrite peak caused interference.

de gradient was then used to resolve the problem, using a range of anion-exchange ith a gradient from 1.5 to 30 m*M* chloride y a 150 m*M* chloride wash before re- on. An AS4A-SC column (capacity ca. 20 r column) produced reasonable results (not t became blocked after several runs with required extensive cleaning. A higher olumn that was designed for gradient e AS9A (capacity 30–35 μequiv. per gave improved results but still did not clearly separate the nitrite peak from interfering species (Fig. 2).

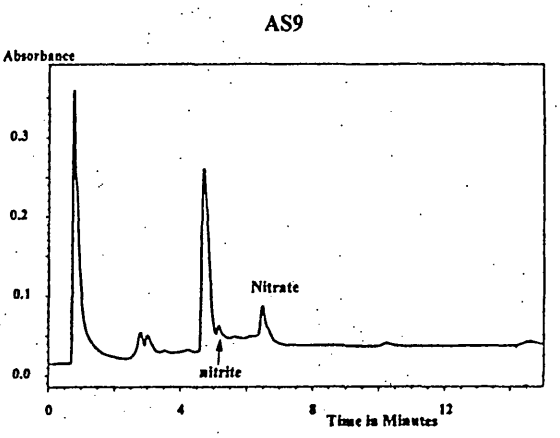

Fig. 2. Chromatogram of ultrafiltered human serum obtained using an AS9A column with low concentration chloride gradient elution and UV detection.

An AS-12 column (Fig. 3) showed better separation of the nitrite peak from interferences in the serum samples but at the expense of overall resolution.

Because the co-elution problem seemed to be with organic acids, where elution behaviour might also be affected by hydrophobic interactions with the stationary phase, column packings designed for the separation of organics were also investigated. Fig. 4 shows the chromatogram obtained with a Dionex

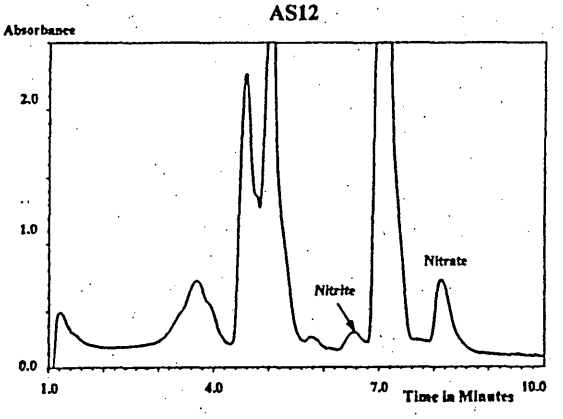

Fig. 3. Chromatogram of ultrafiltered human serum obtained using an AS12 column with low concentration chloride gradient elution and UV detection.

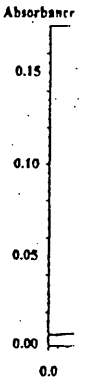

Fig. 4. Ch
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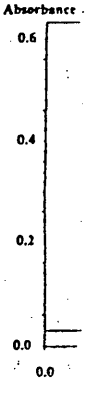

Fig. 5. Chr
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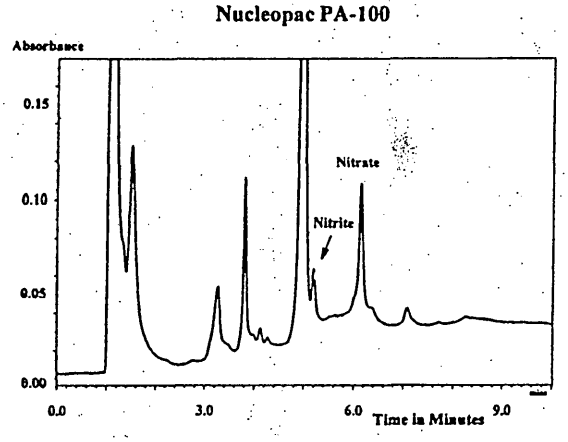

Fig. 4. Chromatogram of ultrafiltered human serum obtained using a Nucleopac PA-100 column with low concentration chloride gradient elution and UV detection.

Nucleopac PA-100 column (capacity 25 μequiv. per column), where reasonable resolution is evident but where nitrite still elutes on the tail of a larger unidentified peak.

Change of column type to a Dionex Carbopac PA-100 (a high capacity column, of 90 μequiv. per column) and use of a higher concentration of chloride as the eluent yielded the best results (Fig. 5). This method showed good separation of nitrite from adjacent peaks, good overall resolution and good resistance to overload. An analysis time of 10 min plus another 10 min for washing and re-equilibration

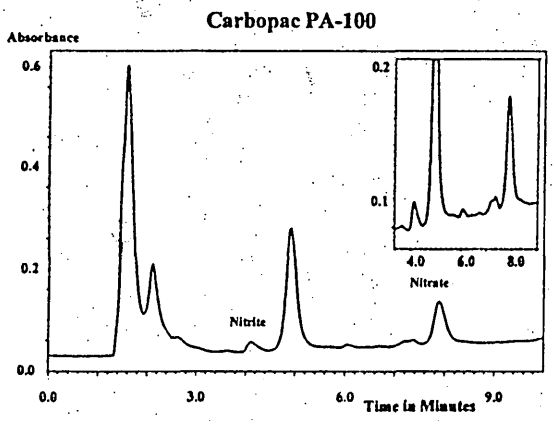

Fig. 5. Chromatogram of ultrafiltered human serum obtained using a Carbopac PA-100 column with high concentration chloride gradient elution (short run) and UV detection.

of the column were adequate for nitrate and nitrite quantification in each serum sample.

With this 20 min cycle time, organic acids in the sample elute early and are not fully resolved. A modified elution programme, starting at a lower eluent strength and with a 1-min isocratic step before a longer gradient time, was found to allow separation of some of the organic acids present in serum (Fig. 6), with a full cycle time of 30 min. Organic acids indicated (identification probable, by retention time comparison only) include lactate, pyruvate, formate, acetate, malonate, hippurate, fumarate, citrate and maleate. Further work is needed to investigate the efficiency of separation of the serum organic acid complement, which can prove difficult to separate fully, but the method appears promising.

All of the columns used are of the pellicular type, where small latex particles bearing quaternary ammonium ion-exchange groups are electrostatically bound to larger modified styrene–divinylbenzene beads. The nature of the organic side chains of the quaternary ammonium groups forms a secondary selectivity mechanism in ion chromatography. Here, the AS-12 and Carbopac PA-100 columns have essentially hydrophobic groups while the AS9A and

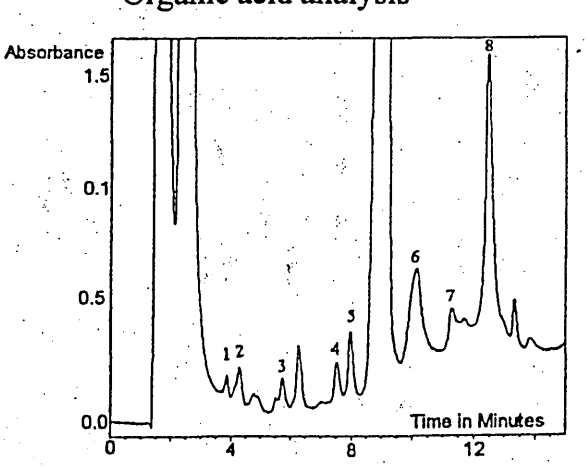

Fig. 6. Chromatogram of ultrafiltered human serum obtained using a Carbopac PA-100 column with wide concentration range chloride gradient elution (long run) and UV detection. 1=Lactate, 2=acetate, 3=pyruvate, 4=malonate, 5=nitrite, 6=fumarate, 7=citrate and 8=nitrate.

and previously published normal range values for nitrite and nitrate in human serum (or plasma, where indicated). Deviations resent the full population variation observed, not experimental variations

	Mean nitrite concentration (μM)	Mean nitrate concentration (μM)	Total NO_x (μM)	n
i et al. [10]	4.4±2.8	61.1±36.8	65.5±39.6	34
Burggraaf [12]	–	36.0±19.0[a]	–	20
. [13]	–	–	28.8±1.07[a]	
al. [14]	9.5±0.6[a]	85±8.0[a]	94.5±8.6[a]	59
vic et al. [6] (plasma)	3.1±0.4	10.3±0.3	13.4±0.4	22
al. [15] (plasma)	0.45±0.2	41±15	41±15	5
s and Delsenne [16] (plasma)	not detectable (<0.5)	92±19	92±19	10
(this work)	4.2±3.9	39.9±22.0	44.1±22	200

ted for females

ac PA-100 columns have hydrophilic groups. ference in selectivity may account for the change in elution order of the nitrite peak large unidentified peak seen in Figs. 2–5.

the Carbopac PA-100 column with the gradient programmes, calibration curves eous nitrate and nitrite standards were linear '. The limits of detection for both anions proximately 0.25 μM (S/N=2:1) with the ctor used. Improvement of these limits of should be possible with a UV detector with S/N ratio. Comparison with the Griess for nitrite showed a linear correlation over ·ng range of the method.

the quick Carbopac PA-100 method, 200 l serum samples (in duplicate) from the od Transfusion Service were analysed. Male le samples were separated because there are g reports in the literature concerning sex ifferences in serum nitrate and nitrite cons [10,11]. Results for the 200 serum samshown in Table 1, along with previously values. It is clear that there is a wide l-to-individual variation in levels and that y be systematic (technique dependent) or ulation variations as well. Sample storage s may play a large part in these variations. n of nitrite to nitrate will occur in whole in partially haemolysed samples.

lusions

im of this work was to produce a simple, versatile and robust ion chromatographic method that had distinct advantages over previously published methods for analysis of nitrite and nitrate in serum. A gradient system has been used with a selected anion-exchange column to enhance resolution of nitrite and, as a bonus, to enable separation of a number of organic acids. The use of a chloride eluent has removed the need for silver resin pretreatment of samples and UV detection removes the need for a conductivity suppressor. The method developed combines these features to produce a reliable and simple procedure for studies of nitric oxide in mammalian systems. It should also be suitable for any study that requires the analysis of UV-absorbing anions in complex matrices, such as foodstuffs, waters (including brine, sea water and waste waters) and environmental samples.

The advantages of the method are:

1. Specificity: The method shows good resolution for the target analytes in nearly all samples investigated.
2. Reproducibility: Buffering of the eluent allows reliable quantification because retention times remain stable and repeats can be assured. Full loop injections gives reproducible sample volumes.
3. Minimal sample pretreatment, yielding faster and more reproducible assays. A "one-step" centrifugal ultrafiltration is all that is required.
4. Cheapness: The reagents required for the enzymatic reduction of nitrate to nitrite are expensive and many reaction steps are required. Once the ion chromatography equipment has been obtained, the system is relatively cheap to run.

The wash step is advantageous for prolonging the life of the columns, periodic replacement of which is the principal running cost.

5. Automation: The automated system is advantageous in clinical studies where many samples can be analysed in batch mode without operator attendance.
6. Direct detection of target analytes: Unlike the Griess method, no chemistry is performed on the analytes and thus there are no problems with inhibition of essential reactions.

The method described has clear benefits over previously described methods and, in one year of use, has shown only two problems.

Firstly, co-elution of nitrite with other UV-absorbers (even though normally removed by this method) is not always completely eliminated. So far, two serum samples of over 300 analysed have shown an unidentified interfering peak that partly co-eluted with nitrite and made quantification difficult. Inspection of chromatograms is always recommended and the Griess method should be employed for the nitrite component, either on the original sample or as a post-column derivatisation, if such a problem is noticed.

Secondly, serum contains a diverse array of anionic substances and molecules with high affinity for the column packing. The result is eventual column blockage, which requires comprehensive cleaning with organic solvents to remove. The life of the analytical column is, however, greatly extended by the wash step in the gradient program and by the use of a guard column.

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