Liquid Chromatography – Mass Spectrometry Methods for Investigating Osmolytes and Related One-Carbon Metabolites in Health and Disease

A thesis
submitted in partial fulfilment
of the requirements for the degree of
Doctor of Philosophy in Chemistry
at the
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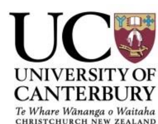

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Abstract

Organic osmolytes are methylamines, polyols, or amino acids that are essential for cell volume regulation. Some have other important biochemical roles. For example, *N*,*N*,*N*-trimethylglycine (betaine) is an osmolyte that also acts as a methyl group donor in one-carbon metabolism. Previous methods for the analysis of betaine and its metabolite *N*,*N*-dimethylglycine (DMG) used derivatization and high performance liquid chromatography with ultra violet detection (HPLC-UV). They required a long sample run time, and often lacked specificity. Liquid chromatography - tandem mass spectrometry (LC-MS/MS) methods were developed with the aim of improving the analysis of betaine and other osmolytes. The objective was to measure the osmolytes: betaine; glycerophosphorylcholine (GPC); taurine; *myo*-inositol; sorbitol; and trimethylamine-*N*-oxide (TMAO), as well as *N*,*N*-dimethylglycine (DMG), choline, sarcosine, and carnitines. The use of LC-MS/MS improved the analysis of many osmolytes (and related metabolites) compared to previous HPLC-UV methods, and the number of osmolytes that could be analyzed was increased. The analysis of polyols was challenging and no suitable method was found for the analysis of sorbitol; however, amide columns were effective at separating inositol isomers. To demonstrate applications for these methods, samples from overweight people with type 2 diabetes were analyzed to improve knowledge about how osmolytes interact with other metabolites, and their potential use as risk markers in disease. Betaine, DMG, and choline concentrations had low intra-individual variation in the plasma and urine. However, GPC, taurine, and TMAO were more variable. Most subjects with diabetes who were taking bezafibrate had extremely elevated urine betaine. A previously unknown metabolite, *N*,*N*-dimethylglycine-*N*-oxide (DMGO), was identified in plasma and urine. DMGO significantly correlated ($p < 0.05$) with DMG, betaine, and choline, suggesting that it is derived from the oxidation of DMG. The discovery of DMGO in human samples suggests that a re-evaluation of the widely accepted pathway for choline metabolism may be required.

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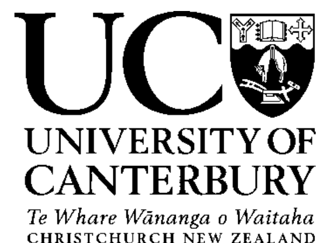

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Chris McEntyre worked with Crystal Lenky under the supervision of Dr Lever to develop an LC-MS/MS method for the measurement of betaine and other marine osmolytes in serum samples. Chris helped to prepare the paper for publication. Chris later expanded on this work to include a range of different osmolytes and other methylamines that are relevant to his thesis.

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Chris McEntyre went through the DEWL study data base and identified people who were taking bezafibrate, and found suitable controls matched for age and gender. Chris developed the methods and analyzed the metabolites in the samples. Chris assisted with the preparation of the paper before submission. Chris has carried out his own statistical analyses and interpretation of the data which is presented in this thesis.

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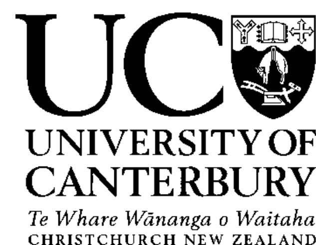

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Chapter 4. Fenofibrate causes elevation of betaine excretion but not excretion of other osmolytes by healthy adults. Published in *Journal of Clinical Lipidology* (2014, *8*, 433-40). The list of authors was: Lever M, McEntyre CJ, George PM, Slow S, Chambers ST, Foucher C.

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Chris McEntyre analyzed the samples (provided by Dr Foucher) for betaine and other metabolites using the methods developed in this thesis. Dr Lever wrote the paper with input from Chris and the other co-authors. Chris has carried out his own statistical analysis and interpretation of the data which is presented in the thesis.

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Chris McEntyre set up the methods for measuring the metabolites, and measured them in samples provided by the DEWL study researchers. Chris carried out statistical analyses with advice from Dr Lever and Dr Florkowski, wrote the paper, and after obtaining feedback from the other authors, submitted it to *Annals of Clinical Biochemistry.*

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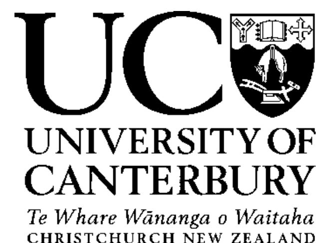

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Chapter 6. Is *N*,*N*- dimethylglycine *N*-oxide a choline and betaine metabolite?
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Please detail the nature and extent (%) of contribution by the candidate:
Chris McEntyre synthesized (*N*,*N*- dimethylglycine *N*-oxide) DMGO and validated it for use as an analytical standard. Chris carried out the work setting up LC-MS/MS methods and identifying DMGO in human samples, and helped with the preparation of the paper for submission.

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List of abbreviations

Ac Carn	Acetylcarnitine
APCI	Atmospheric pressure chemical ionization
APPI	Atmospheric pressure photo ionization
BBC	Betaine and body composition (study)
BGT1	Betaine gamma-aminobutyric acid transporter
BHMT	Betaine-homocysteine methyltransferase
BMI	Body mass index
Carn	Carnitine
CE	Collision energy
CRN	Creatinine
CV	Coefficient of variation
CVa	Analytical imprecision
CVg	Between individual variation
CVi	Within individual variation
CVt	Total imprecision
CXP	Collision cell exit potential
D	Deuterium
DEWL	Diabetes and excess weight loss (study)
DMG	*N*,*N*-Dimethylglycine
DMGDH	Dimethylglycine dehydrogenase
DMGO	*N*,*N*-Dimethylglycine-*N*-oxide
DMSO	Dimethylsulfoxide
DMSP	Dimethylsulfoniopropionate
DP	Decoupling potential
eGFR	Estimated glomerular filtration rate
ESI	Electrospray ionization
FIA	Flow injection analysis
FMO3	Flavin monooxygenase 3
GC-MS	Gas chromatography - mass spectrometry
GPC	Glycerophosphorylcholine
HbA_{1c}	Haemoglobin A_{1c}
Hcy	Homocysteine
HPLC	High performance liquid chromatography
LC-MS	Liquid chromatography - mass spectrometry
LC-MS/MS	Liquid chromatography - tandem mass spectrometry
LOD	Limit of detection
MIA	Methylisatoic anhydride
MRM	Multiple reaction monitoring
NMR	Nuclear magnetic resonance
RBC	Red blood cells
RCV	Reference change value
SCX	Strong cation exchange
SD	Standard deviation
SE	Standard error
SIM	Selected ion monitoring
TIC	Total ion count
TMA	Trimethylamine
TMAO	Trimethylamine-*N*-oxide
TMS	The metabolic syndrome
WB	Whole blood
WBC	White blood cells

1. Chapter One –Introduction

1.1. Thesis overview

Mass spectrometry is a rapidly evolving technology that can be used to improve the analysis of many metabolites in order to provide more information about their role in health and disease. Previous methods for the measurement of betaine and its metabolite, *N*,*N*-dimethylglycine, involved the use of derivatization followed by high performance liquid chromatography (HPLC) with UV (or fluorescence) detection.[1] These methods often had low specificity and were prone to interference from other components in the samples. The objective of this project was to use mass spectrometry based technologies (that were not previously available in our laboratory) to develop improved analytical methods for betaine, other osmolytes, and other one-carbon metabolites. The aim was also to use these new technologies to measure a wider range of osmolytes including ones that were difficult to measure by previous methods. The analytical methods developed in this project were applied to clinical samples in order to investigate the metabolic processes that osmolytes (and related metabolites) are involved in, and help to assess their potential for use as risk markers in disease.

Chapter 1 is a review of osmolytes, including: the structure of some important mammalian osmolytes; how these osmolytes are accumulated by cells; other biochemical roles that these osmolytes play; how osmolyte concentrations are known to change in disease; and analytical methods that have been used for the measurement of betaine, other osmolytes, and other one-carbon metabolites. The aims and hypotheses for the thesis are listed at the end of this chapter.

Chapter 2 includes the development and validation of liquid chromatography - mass spectrometry methods for the measurement of betaine, other methylamine based osmolytes and one-carbon metabolites, and taurine in human plasma and urine. Results obtained by tandem mass spectrometry (LC-MS/MS) are compared with single quadrupole mass spectrometry (LC-MS), and HPLC-UV.

Chapter 3 describes attempts to use the chemical properties of polyols to enhance selectivity and detection with the overall aim of developing a reliable method for the measurement of sorbitol and *myo*-inositol in plasma and urine.

In Chapter 4, applications for the analytical methods that have been developed are demonstrated by measuring osmolytes and related metabolites in clinical samples from a population of overweight people with diabetes. Investigations were carried out

on the metabolism and potential use of osmolytes (and related metabolites) as risk markers in disease.

The distribution of osmolytes and related metabolites in different blood components (including, whole blood, plasma, red blood cells, and white blood cells) was investigated and is discussed in Chapter 5.

The identification of a previously unknown metabolite, *N*,*N*-dimethylglycine-*N*-oxide (DMGO) and its potential metabolic significance is described in Chapter 6. DMGO was synthesized for use as a standard to enable its identification and quantification in plasma and urine samples using LC-MS/MS.

Finally, in Chapter 7, the aims and hypotheses of the thesis are re-addressed, future work is discussed, and the main conclusions of the thesis are stated.

1.2. Introduction to osmolytes

Organic osmolytes are essential for cell volume regulation in all taxa of animals, plants, and micro-organisms.[2,3] Osmolytes are abundant in the human body and can play other important biochemical roles. For example, *N*,*N*,*N*-trimethylglycine (betaine) is a major mammalian osmolyte[3] that is also involved in one-carbon metabolism, acting as a methyl group donor.[4] These roles are inextricably linked, and the metabolic processes controlling the availability of betaine can be regulated in response to osmotic stress.[4b, 5]

When under osmotic stress, cells accumulate osmolytes from the blood plasma. While these molecules have no osmotic role in the plasma, the plasma plays an important role in osmo-regulation by acting as a pool from which tissues obtain osmolytes, particularly in tissues which do not produce them. By increasing the intracellular concentrations of osmolytes, cells are able to remain isotonic in more saline environments, therefore avoiding large changes in cell volume and dehydration.[3] There are three main classes of organic compounds that are used as osmolytes in nature: methylamines,[4b] amino acids,[3] and polyols.[6] Mammals can produce osmolytes *in vivo* when they are required, but there are also many important dietary sources. For example: there are high concentrations of betaine in wheat products, shellfish, silver beet, beetroot and spinach;[7] *myo*-inositol is present in high concentrations in citrus fruits and kiwifruit[8]; and meat, eggs, and dairy products are high in taurine.[9]

Compatible solutes are compounds that are not harmful to macromolecules inside cells. In order to function as an osmolyte, a compound must be a compatible solute. Perturbing solutes, such as urea, are harmful to cells and destabilize proteins. While not harming cells, some compatible solutes are actually beneficial and are called compensatory solutes. Compensatory solutes are compounds which stabilize proteins against physiological stress such as freezing and heat, and the presence of perturbing solutes.[10] By protecting the folded structure of proteins, compensatory solutes can allow them to function normally under stressful conditions.[11] Some osmolytes also act as compensatory solutes and are beneficial to cells in both ways.[2c, 12]

Different mammalian organs use specific combinations of organic osmolytes for cell volume regulation. For example, the kidneys use: betaine (Fig. 1.1, (**1**)); glycerophosphorylcholine (GPC) (**2**); taurine (**3**); *myo*-inositol (**4**); and sorbitol (**5**).[13] Betaine is a highly water soluble zwitterionic compound which contains a quaternary ammonium group at one end and a carboxyl group at the other end of the molecule. GPC is a zwitterionic quaternary ammonium choline derivative. Taurine is an amino acid containing a sulfonate group and is not incorporated into proteins. *myo*-Inositol and sorbitol are polyols with a hydroxyl group attached to each of six carbon atoms. *myo*-Inositol contains a non-aromatic six-membered ring structure, whereas sorbitol has an open chain structure.

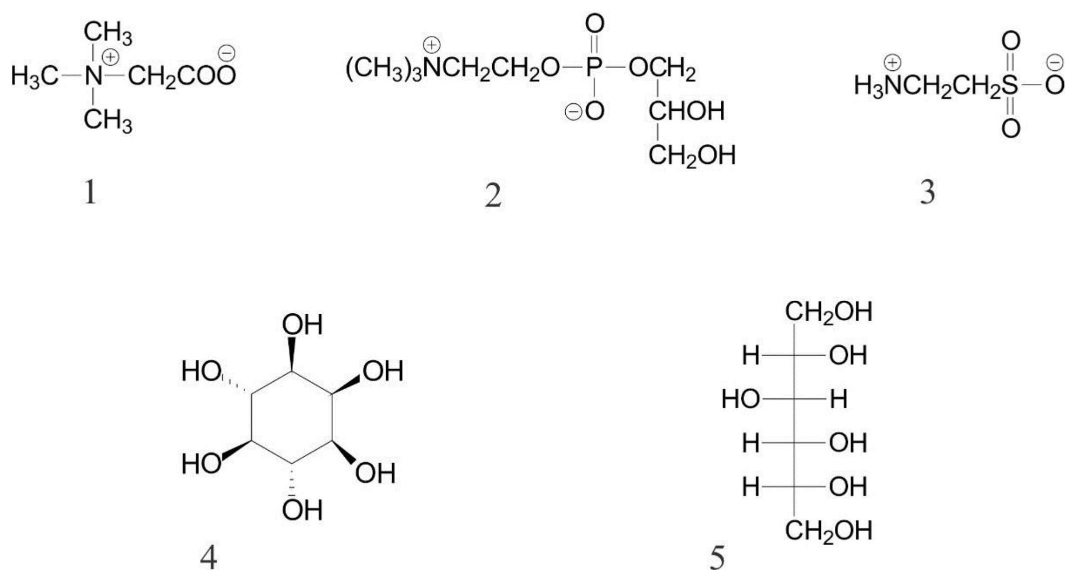


Fig. 1.1. Structure of: betaine (**1**); GPC (**2**) taurine (**3**); *myo*-inositol (**4**); and sorbitol (**5**).

Although betaine is generally retained by the kidneys, people with diabetes[14] and people on fibrate therapy[15] are known to have increased urinary excretion of betaine. As well as being an important osmolyte and compensatory solute, betaine is also involved in one-carbon metabolism.[4b] Betaine is a substrate for the enzyme betaine-homocysteine methyltransferase (BHMT), where it acts as a methyl group donor, forming *N,N*-dimethylglycine (DMG), and converting homocysteine to methionine (Fig. 1.2).[4b, 16] DMG can also donate a methyl group resulting in *N*-methylglycine (sarcosine), which can be further demethylated to glycine. BHMT expression has been shown to be upregulated under hypotonic conditions, and down regulated in hypertonic conditions in liver cells, effectively making more betaine available when it is required as an osmolyte.[17] Because the BHMT metabolic pathway is affected by the hydration state of cells, the roles of betaine as a methyl donor and an osmolyte are linked.[4b, 18] Betaine is also required for DNA methylation, and may help to protect against cancer.[19] While it is an important dietary nutrient, betaine is also synthesized in the body by a two-step oxidation of choline.[4b] Other choline derivatives that play important biological roles include: GPC which is an important osmolyte that also acts as a compensatory solute, stabilizing intracellular proteins;[13, 20] acetylcholine which is a neurotransmitter; and phosphatidylcholine which is important for cell structure and the formation of phospholipid membranes.[21]

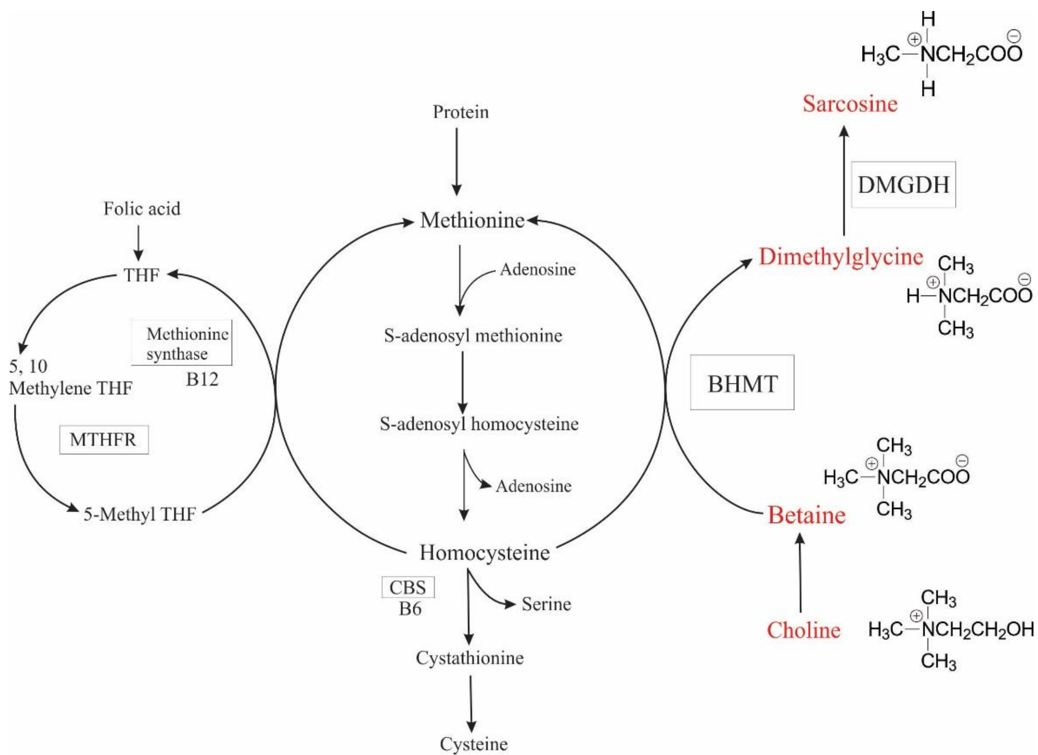


Fig. 1.2. Metabolic pathway for the metabolism of homocysteine. Abbreviations: betaine homocysteine methyltransferase (BHMT); cystathionine-β-synthase (CBS); dimethylglycine dehydrogenase (DMGDH); methylenetetrahydofolate reductase (MTHFR); tetrahydrofolate (THF).

Taurine plays many other important biochemical roles as well as acting as an osmolyte in mammals.[22] For example, taurine is used as a neurotransmitter and neuromodulator, and is involved in thermoregulation and appetite suppression.[23] Plasma taurine concentrations have been reported to be 58 ± 16 µmol/L in healthy people, and taurine is excreted in the urine at a rate of 980 ± 580 µmol/day.[9] Plasma taurine has been reported to be significantly lower in vegans (45 ± 7 µmol/L) due to a lower dietary intake.[9] High levels of taurine are added to energy drinks, greatly increasing the intake for people who consume them. While dietary taurine sources are important, it can also be synthesized in the liver from the metabolism of cysteine.[24]

Sorbitol is produced from glucose *via* aldose reductase when the body is under osmotic stress. The polyol osmolytes, sorbitol and *myo*-inositol, are accumulated in the kidney and liver.[2c] People with diabetes[25] and people with renal failure[26] have increased urinary excretion of sorbitol and *myo*-inositol. While being an important osmolyte, inositol is also a precursor of phosphatidylinositol which is an important component of neural cell membranes.[27] Tissue levels of *myo*-inositol are highest in the

brain and kidney at around 5 mmol/L, and plasma levels of *myo*-inositol have been reported in the range 17.5 – 40.7 µmol/L in healthy individuals.[28]

While carnitine is used as an osmolyte by some bacterial cells,[29] it is not known to play a role as an osmolyte in mammals. Carnitine is however necessary in mammals for carrying fatty acids through the mitochondrial membrane for energy metabolism.[30] Other biological osmolytes, such as: dimethylsulfoniopropionate (DMSP); homarine; and trimethylamine-*N*-oxide (TMAO), may be consumed in the diet, but are not used by mammals as osmolytes, and not actively retained by the kidneys.[2c]

Although osmolytes are found in all mammalian cells, they have been most widely studied in the kidney cells where the osmotic stress can be extreme. High concentrations of betaine and other osmolytes are particularly found in the kidney cells due to a need to counteract the high salt and urea levels which are found in the kidneys.[31] Mammalian kidneys have higher levels of osmolytes in the medulla than in the cortex. *myo*-Inositol concentrations average 15 mmol/Kg (wet weight), betaine and GPC concentrations are around 3 mmol/Kg (wet weight), and sorbitol concentrations average 1.4 mmol/Kg (wet weight) in the inner-medulla of the human kidney.[32] The inner-medulla cells uptake betaine, taurine, and *myo*-inositol using specific transporter proteins which are activated by a sodium gradient into the cells.[33] For example, the betaine gamma-aminobutyric acid transporter, BGT1, is a widely used membrane transporter protein for betaine.[33b, 34] As well as transporting betaine through the cell membrane, BGT1 also transports the neurotransmitter gamma-aminobutyric acid (GABA).[35] Hypertonicity increases transcription of the BGT1 transporter gene.[5] Betaine and proline have also been shown to be transported by a different transporter (SIT1) that is activated by fertilization in early mouse embryos.[36] Specific transporters for other mammalian renal osmolytes include: the taurine transporter - Na/Cl dependent taurine co-transporter (NCT), and for *myo*-inositol - the sodium/*myo*-inositol transporter (SMIT).[33] These transporters are activated by increases in sodium and chloride ions.

The osmolytes, sorbitol and GPC, are not known to have cell wall transporter proteins, but the metabolic pathways for their formation or degradation are regulated in response to cell volume changes. Small changes in the hydration state of cells trigger cellular metabolism and gene expression.[37] Increased aldose reductase activity is observed in the kidneys in response to increased sodium concentrations.[5, 38] GPC

production has not been shown to be increased under osmotic stress. However, the enzyme responsible for converting GPC to choline, GPC-choline phosphodiesterase, is inhibited in the presence of high salt or urea in the kidneys.[39]

Traditional HPLC methods for the measurement of osmolytes involved long sample preparation and sample run times, and often lacked specificity. In order to better investigate the role of osmolytes in health and disease, there is a need for more efficient analytical methods that can measure a greater number of osmolytes in a shorter time. The use of evolving technologies such as mass spectrometry or NMR spectroscopy is an obvious way to try and achieve this.

1.3. The measurement of osmolytes and related metabolites

Osmolytes, such as betaine, *myo*-inositol, sorbitol, GPC, and TMAO lack a significant chromophore (or fluorophore), and typically require pre-column derivatization before they can be measured by HPLC methods with ultra-violet (UV) or fluorescent detection.[1] However, osmolytes can potentially be measured more efficiently using specialized techniques such as nuclear magnetic resonance spectroscopy (NMR),[40] or mass spectrometry.[41] Osmolytes have been measured in kidneys and other tissues where they are present in high concentrations using HPLC with refractive index detection.[6] However, this technique is not sensitive enough to measure concentrations in biological fluids such as blood plasma or urine. Refractive index detection also lacks specificity, so separating analytes from other metabolites in complex samples, such as urine, is difficult. While evaporative light scattering detectors (ELSD) offer better sensitivity than refractive index detectors, they still lack the selectivity required to measure metabolites in many complex sample matrices.

HPLC with UV or fluorescence detection following derivatization

Betaine and other zwitterionic betaine analogues, such as carnitine, acetylcarnitine, proline betaine, dimethylsulfoniopropionate (DMSP), and trigonelline, were traditionally measured by HPLC following derivatization of the carboxyl group with a chromophore or fluorophore to make them suitable for UV or fluorescence detection.[1] The carboxyl groups on zwitterions such as betaine are relatively unreactive and therefore require highly reactive derivatizing reagents to

react with them. Derivatizing reagents which have been used for this purpose include: bromophenacyl bromide;[42] bromophenacyl trifluoromethanesulfonate (triflate);[1a] naphthacyl triflate; and phenanthrenacyl triflate.[43] The cationic ester derivatives formed by derivatizing carboxyls with these reagents can be measured by HPLC with UV detection, or fluorescence detection when using phenanthrenacyl triflate.[43a] HPLC stationary phases which have been used to measure these derivatives include normal phase silica, alumina, or strong cation exchange (SCX).[1b] Methods involving derivatization are more labour intensive and require long run times to separate analytes from the large number of compounds present in biological samples that may be derivatized. Attempts to measure derivatives of betaine and betaine analogues by capillary electrophoresis have only been partially successful.[1b] Insufficient sensitivity is often a problem with capillary electrophoresis due to the small path length that the detector observes through the capillary.[1b]

Storer and Lever[43a] showed that TMAO can be derivatized by triflate reagents. However, the reaction yield was low, and the limit of detection was high, which is likely to be due to the lack of a carboxyl group on the TMAO molecule. Therefore this approach is not useful for the measurement of plasma TMAO concentrations.

Polyols such as sorbitol and *myo*-inositol have been measured in tissues using derivatization of the hydroxyl groups with phenyl isocyanate.[44] This technique is not specific for polyols and many peaks can be expected in the chromatograms of biological samples due to the large number of hydroxyl compounds present that can potentially be derivatized.

While derivatization followed by HPLC with UV (or fluorescence) detection can be useful for the measurement of many osmolytes and related metabolites, a different approach is required to measure the more analytically challenging ones such as polyols, TMAO, and choline. Evolving technologies, such as mass spectrometry (or NMR), are likely to play an important role in enabling the measurement of osmolytes that have been traditionally difficult to measure.

Liquid chromatography - mass spectrometry

Liquid chromatography – mass spectrometry techniques are increasingly being used in clinical laboratories to measure analytes such as osmolytes and one-carbon metabolites in biological samples.[41, 45] Because mass spectrometry separates and

detects ions, an analyte must be ionizable in order to be detected by the mass spectrometer. Small molecules may not fragment to give mass transitions which produce adequate sensitivity or selectivity for quantitative analysis. Derivatization can sometimes be used to modify the chemistry of the analytes and overcome these problems.[46]

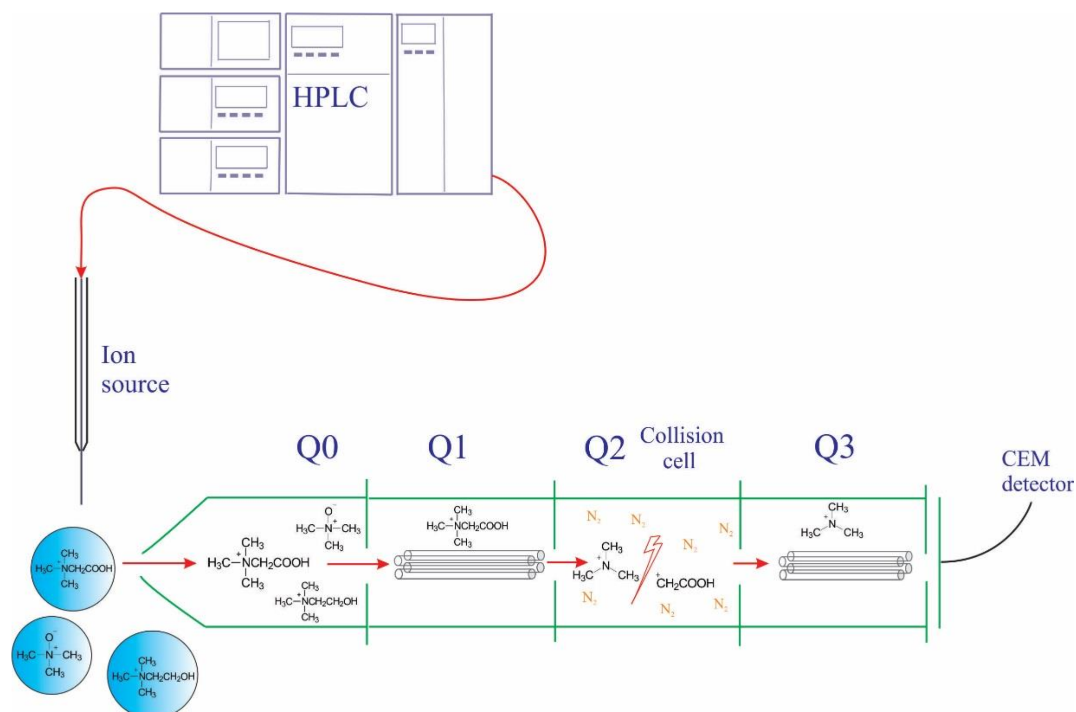


Fig. 1.3. Diagram of a triple quadrupole mass spectrometer showing the fragmentation of betaine.

Figure 1.3 illustrates how a triple quadrupole mass spectrometer acts as a detector in an LC-MS/MS system. Samples are injected into the flow of the mobile phase and separated on a chromatographic column using HPLC. The column eluent is then sprayed into the ion source of a triple quadrupole mass spectrometer where it is ionized using techniques such as: ESI (electrospray ionization); APCI (atmospheric pressure chemical ionization); or APPI (atmospheric pressure photoionization). The ions are then drawn into the mass spectrometer where they are separated in quadrupole 1 (Q1) according to their mass to charge ratio (*m/z*). The selected ions are then fragmented in the collision cell by colliding with N_2 gas molecules under a high vacuum. The fragment ions are then selected by mass in quadrupole 3 (Q3) and then detected by an electron multiplier. In MS/MS mode, the mass transitions from the parent ion mass to a fragment ion are measured because only fragment ions that have come from the selected parent ion are seen by the detector. This gives three levels of

selectivity, including: separation by chromatography; separation by monitoring the mass of the molecular ion; and separation by monitoring the mass transition from molecular ion mass to the fragment ion mass (MS/MS).[47]

While different compounds may have the same (or very similar) mass, it is unlikely that they will fragment to give the same fragment ions. There are exceptions, for example, many methylamines fragment to give a trimethylamine species. DMG and choline both have a molecular ion mass of 104 and give a fragment ion of 58, therefore a choline peak is observed in the multiple reaction monitoring (MRM) chromatogram of DMG for the mass transition 104 → 58.[41] Care must be taken in these situations to ensure that the compounds are well separated chromatographically. Interfering substances in the sample matrix such as salts can affect the ionization efficiency.[48] This may lead to differences in the detector response between the standards and the samples, therefore lowering the accuracy and precision of the method. While these substances may not show up in the chromatograms, they can still negatively affect the method by interfering with the ionization process. Ways to overcome ion suppression due to sample matrix effects include: the use of deuterated internal standards, improving the chromatography, and pre-column sample clean-up such as solid phase extraction (SPE).[48-49]

When measuring a mixture of compounds by LC-MS/MS it is ideal to separate them chromatographically, even though they may have different mass transitions and not be present in the same multiple reaction monitoring (MRM) chromatograms. The use of buffers in the chromatographic mobile phase is limited to volatile acids, such as formic acid, acetic acid, and trifluoroacetic acid, and bases such as ammonium, and triethylamine. LC-MS/MS methods for measuring hydrophilic compounds such as betaine generally use a binary gradient which starts with a low water and low buffer in the mobile phase, and moves to high water and higher buffer concentration in the mobile phase as the sample run progresses.[41] Many LC-MS/MS methods have traditionally used reversed phase columns. However, because osmolytes are highly hydrophilic they are best separated using normal phase columns, such as silica (often referred to as hydrophilic interaction chromatography (HILIC)),[1b,1a,45a] or strong cation exchange (SCX) columns.[1b]

When setting up methods using LC-MS/MS it is important to find the correct compound-specific parameters by carrying out a compound optimization on standard solutions for each analyte. This gives the optimal potentials (decoupling potential,

collision energy, and collision cell exit potential) required to obtain the highest signal intensity for a particular compound. These voltages are used to control the behaviour of ions inside the mass spectrometer. Decoupling potential is a voltage applied at the orifice plate to help prevent ions from clustering together, collision energy controls the degree of fragmentation in the collision cell, and collision cell exit potential controls the flow of ions from Q2 to Q3. Method-specific parameters which require optimization in LC-MS/MS include the curtain gas flow rate and the ion source temperature. Most modern mass spectrometers have a choice of ion source, although ESI is the most commonly used. However, an APCI ion source can be installed to improve sensitivity for neutral compounds that are difficult to ionize by ESI.[47] APCI applies a corona discharge to ionize the solvent gases which in turn transfers a charge to the analytes. Some mass spectrometers are capable of applying both ESI and APCI ion sources simultaneously in order to obtain optimum ionization and sensitivity. Another ionization technique, atmospheric pressure photo ionization, uses photons to ionize analytes in the sample. Both APCI and APPI are used to ionize neutral compounds such as free steroids, which are difficult to ionize by ESI due to a lack of an easily ionizable functional group.[47]

Zwitterions such as betaine and carnitine are easily ionized, and under acidic conditions the carboxyl group may be protonated so that they are often already present as cations when they enter the mass spectrometer. Other osmolytes, such as polyols, are not so easily ionized and may require APCI, adduct formation, or pre-column derivatization.

LC-MS/MS has revolutionized screening of new-borns in clinical laboratories, and allows for rapid screening of many inborn errors of metabolism, including amino acid disorders, organic acid disorders, and fatty acid oxidation disorders.[50] LC-MS/MS has many advantages over traditional HPLC-UV methods for the measurement of osmolytes. There is usually no need to derivatize the samples resulting in faster sample preparation, there are shorter run times, and precision and accuracy is generally improved by the use of isotopic internal standards (isotope dilution). Isotopic internal standards are chemically identical to their respective analytes except that some of the atoms have been replaced with isotopes such as deuterium. These internal standards extract and chromatograph almost identically to the corresponding analytes, but they have a different mass, and can be monitored using a different mass transition. Unfortunately, isotopic internal standards are not

always commercially available, meaning that it may not always be possible to use them for every analyte of interest. Isotopically labelled standards can sometimes be synthesized in the laboratory if required using deuterated starting materials. As well as deuterium, other stable isotopes such as ^{13}C are sometimes used in internal standards. Deuterated internal standards in mass spectrometry can improve accuracy and precision because they have very similar chemical properties to the analytes, therefore they partition the same into the extraction solvent, and ionize the same in the mass spectrometer.

Triple quadrupole mass spectrometers are most commonly used for quantifying metabolites in clinical laboratories, whereas high mass resolution instruments such as time of flight (TOF) instruments are more commonly used in academic environments.[51] Triple quadrupole mass spectrometers are useful in clinical laboratories because they offer high sensitivity and selectivity, and they can monitor many mass transitions simultaneously. However, the mass resolution of triple quadrupole instruments is generally much lower than other instruments such as TOF mass spectrometers. Therefore ions which have a mass within 1 or 2 Da of the analyte of interest can cause interference. The mass range of triple quadrupole instruments typically only detects ions up to 2000 Da. TOF mass spectrometers can measure a much greater mass range for (example 20-20,000 Da), and are therefore more suitable for detecting proteins and other large molecules. Qtrap instruments are available which can act as a traditional triple quadrupole mass spectrometer, but can also trap ions in the 3rd quadrupole, allowing for further fragmentation of the fragment ions. This can provide more structural information that can be useful for identifying unknown compounds.

A single quadrupole mass spectrometer may be adequate for measuring certain metabolites in biological samples. However, single quadrupole mass spectrometers are less selective as they do not have a collision cell and can only measure the molecular ion by selected ion monitoring (SIM). Compounds with the same (or close) mass will show up in the same chromatogram, although this is not necessarily a problem if all compounds with the same mass are separated chromatographically.

In recent years, LC-MS/MS technology has become more affordable, and instruments have become increasingly available in clinical laboratories. Manufacturers are also constantly designing LC-MS/MS systems that are more sensitive, allowing for the measurement of a greater range of metabolites that are

present at low concentrations, and may have previously been undetectable. An example of this is the measurement of low plasma concentrations of *N*,*N*-dimethylglycine-*N*-oxide shown in Chapter 6.

Gas chromatography - mass spectrometry

Gas chromatography-mass spectrometry (GC-MS) has been used to measure polyols including the osmolytes sorbitol and *myo*-inositol.[52] In order to be analyzed by GC-MS, polyols must be made volatile by using reagents such as butylboronic acid,[52b] or acetic anhydride.[52a] The sample run times required to separate polyols in biological samples are typically around 40 to 60 minutes.[52b, 53] Eades *et al*.[52b] showed a separation of sorbitol, mannitol, *myo*-inositol, and galactitol in tissues within 40 minutes after derivatization with butylboronic acid. Lee and Chung[52a] measured a range of polyols including sorbitol and *myo*-inositol in urine by GC-MS after reaction with acetic anhydride. Jansen *et al*.[54] used GC methods to measure a range of polyols in urine and plasma after formation of trimethylsilyl ethers.

NMR Spectroscopy

Many metabolites have been measured in biological samples using ^{1}H NMR spectroscopy.[55] Methylamines are particularly suited for analysis by ^{1}H NMR due to the presence of methyl groups on the nitrogen which produce an intense singlet in a ^{1}H NMR spectrum.[4b, 40] Lee *et al*.[40] described a method for measuring a range of methylamines including betaine and DMG in urine using ^{1}H NMR. However, the detection limits were high compared to mass spectrometry methods, typically around 15 µmol/L, so sensitivity would be limiting for the measurement of many metabolites, particularly in samples such as blood plasma where methylamine concentrations are often lower. Polyols can be especially difficult to measurement by NMR. The lack of identical protons creates low sensitivity, and there is often interference from other polyols and sugars in biological samples. However, Bedford *et al.*[13] were able to use NMR to measure the polyol osmolytes, inositol and sorbitol, in possum kidneys.

Proton NMR requires minimal treatment of the samples, and has the ability to measure many analytes simultaneously. However, there are problems with using NMR as a quantitative analytical tool that need to be considered. For example, the limits of detection are usually higher than other techniques. Detection limits can be

improved by obtaining more transients when collecting the spectra, although this is done at the expense of longer sample acquisition times. Using an NMR with a larger magnetic strength provides improved sensitivity. However, large NMR systems (over 500 MHz) are expensive and bulky and the technology is not usually available in clinical laboratories. Lower limits of detection can also be achieved by installing a cryoprobe on the NMR system. Analyzing aqueous samples such as urine requires the use of a water suppression technique to remove the large water signal from the spectrum. This adds to the time it takes to run samples. The analysis of blood samples by NMR spectroscopy is even more difficult. The concentrations of analytes in blood are often lower than in urine, and blood contains large amounts of protein which causes interference in the spectrum.

When using NMR to measure methylamines in aqueous samples, the samples need to be acidified in order to separate the betaine methyl group signal from TMAO. An internal standard needs to be added to each sample for calibration, and deuterium oxide (D_2O) also needs to be added as a lock standard.[40] A suitable internal standard must also be added to the samples in order to quantify analytes in the sample. Unlike UV absorbance, peak heights in NMR are arbitrary numbers, and no two spectra can be directly compared. However, the ratios of the analyte peaks to the internal standard are quantitative since the level of the internal standard in each of the samples is constant. These ratios are then compared to ratios of standards of known concentrations of the analytes to the internal standard, and this is used to calibrate the data.[56] Internal standards must be sufficiently soluble in aqueous samples such as urine. Internal standards are usually chosen which produce a ^1H singlet in a convenient place in the spectrum (typically 0-1 ppm) so as not to co-resonate with other peaks in the samples. Examples of suitable internal standards for the analysis of methylamines in urine by ^1H NMR include: tetramethylsilyl-2,2,3,3-tetradeuteropropionic acid (TSP), 4,4-dimethyl-4-silapentane-1-ammonium (DSA), acetonitrile, trimethylacetonitrile, or trimethylacetamide (pivalamide).

Many metabolites have been measured simultaneously by NMR spectroscopy in overseas metabolomics studies.[57] However, there are factors that limit the use of NMR as a diagnostic tool in clinical laboratories, or for clinical studies involving the measurement of large numbers of samples. For example, NMR instruments are expensive to purchase compared to other technologies, and the large magnets make them difficult to house in a clinical laboratory setting. The bench-work cost of sample

analysis is low for NMR spectroscopy and mass spectrometry methods, however, the main costs that need to be considered are the capital cost of the equipment, and the running costs, including service contracts to maintain the equipment.

1.4. The suitability of analytical techniques for measuring osmolytes

Consideration should be given to the chemical properties of a particular analyte when choosing an analytical method. This gives important clues as to the best way of measuring it. For example, if the analyte has a strong native absorbance or fluorescence then it might be sufficient to measure it using a simple HPLC method. If an analyte has functional groups that can be derivatized with a UV absorbing or fluorescent moeity, then pre-column derivatization followed by HPLC might be appropriate. If an analyte ionizes and fragments easily, then mass spectrometry may be the best way to measure it. If an analyte does not ionize easily and cannot be derivatized, but it does have multiple identical protons, then ^{1}H NMR may be useful for measuring it. Some osmolytes, such as polyols, are challenging to measure by any method.

Taurine is difficult to measure by conventional methods for amino acid analysis, such as the ninhydrin reaction. It has been measured by NMR[58] and by mass spectrometry,[59] but there are problems. It has no methyl groups to produce a large singlet signal in the ^{1}H NMR spectrum. It is also difficult to measure by mass spectrometry, particularly in positive ion mode, because the strongly acidic sulfonic acid group is not easily protonated. However, taurine has been successfully measured by mass spectrometry in negative ion mode.[59] A number of reagents have been used to derivatize the amine group on taurine for measurement by HPLC with UV or fluorescence detection.[60] However, these derivatizing reagents are not specific and derivatize many other amino acids in biological samples, therefore giving a complex mixture of peaks to separate chromatographically.

There are difficult analytical problems associated with the measurement of polyols, such as sorbitol and *myo*-inositol, in biological samples. They only contain relatively unreactive hydroxyl functional groups, have a negligible absorbance in the visible or UV spectrum, and have complex ^{1}H NMR spectra with multiplets of single protons causing low sensitivity. Polyol osmolytes also have multiple isomers including abundant sugars. Interference from glucose is likely to be particularly

problematic for measuring polyols because it has the same mass as inositols, and it is present in much higher concentrations (approximately 4 mmol/L) in blood samples than sorbitol (< 2 µmol/L)[61] and *myo*-inositol (approximately 20 µmol/L).[62] Polyols such as sorbitol and inositols are neutral species which may be difficult to ionize in a mass spectrometer due to a lack of readily ionizable functional groups. Potential ways to overcome these problems and measure sorbitol and *myo*-inositol in plasma and urine samples are investigated in Chapter 3.

1.5. Osmolytes in disease

Obesity, diabetes, and cardiovascular disease are an increasing problem in the New Zealand population. People with the metabolic syndrome are overweight, have high blood pressure, high blood lipids, and are at risk of developing type 2 diabetes and cardiovascular disease.[63] Relatively little is known about how changes in osmolyte concentrations may contribute to the progression of these health conditions.

Because concentrations of osmolytes are different in many disease states, it is possible that they may be useful risk markers of disease. While risk markers may be quantitatively associated with a disease, they are not necessarily causal, and changing the concentration of a risk marker by an intervention, such as supplementation, does not necessarily change the disease outcomes.[64] Risk factors are variables that are causal and directly affect the probability of a disease occurring.[64] While risk factors and risk markers are typically measured in fasting samples, concentrations can be affected by the diet and other factors which may lead to short term variations. It is therefore important to investigate the individuality of metabolites in different populations to help evaluate their diagnostic value.[65]

Diabetes

Type 2 diabetes mellitus is a growing concern in the western World and is an increasing burden on the New Zealand health system. Maori and Pacific Islanders have a disproportionately high incidence of type 2 diabetes, whereas type 1 diabetes is more common in European New Zealanders than other ethnic groups.[66] Risk factors for developing type 2 diabetes include poor diet and obesity, smoking, and physical inactivity.[67] Obesity and type 2 diabetes are more common in lower socio-economic groups because high sugar foods with low nutritional value are often more affordable

than nutritious foods such as fruit and vegetables. Many people with diabetes go on to develop further complications such as retinopathy (cataracts), nephropathy (kidney disease)[67] and cardiovascular disease.[68] Glycemic control in diabetes is monitored by measuring glycated haemoglobin A_{1c} (HbA_{1c}), a form of haemoglobin that is bound to glucose. HbA_{1c} is a more useful marker of glycemic control than glucose because it is less affected by short-term fluctuations in blood sugar levels.[69] HbA_{1c} is also a marker of protein glycation, which is a major cause of health complications in diabetes.

Because sorbitol (**5**) is produced from the reduction of glucose, sorbitol concentrations are increased in the blood when glucose concentrations are poorly controlled in diabetes.[25, 70] Although sorbitol is useful in that it is used as an osmolyte by cells, an increase in sorbitol concentration is problematic for a couple of reasons. Firstly, increased circulating sorbitol can displace other osmolytes in the body, which may be a significant cause of cell and tissue damage.[25, 71] Secondly, the conversion of sorbitol to fructose and consequent glycation of proteins is also an important factor in developing diabetic complications.[72]

Low betaine in blood plasma has been shown to be an important risk marker for developing diabetes.[73] However, lifestyle interventions and the use of the anti-diabetic drug, metformin increase plasma betaine.[73] People with diabetes are known to excrete more betaine into the urine than the normal population.[14] The urinary loss of methylamine based osmolytes from people with diabetes is likely to be detrimental, especially considering the protein stabilizing (compensatory) properties of betaine and GPC.[74]

Concentrations of various osmolytes have been shown to be different in the blood and urine of people with diabetes compared to the normal population.[14, 71] For example, the urine excretion of both *myo*-inositol and *chiro*-inositol have been reported to be greater in people with diabetes (median = 825 µg/day, and 74.0 µg/day respectively) compared to normal (88 µg/day and 2.09 µg/day respectively). The urine ratio of *myo*-inositol to *chiro*-inositol has been reported to be a sensitive marker of insulin resistance.[75] This suggests that inositol measurements may be useful as an early marker of diabetes, and may help to identify people who are at risk of developing diabetes. The concentrations of sorbitol[32] and *myo*-inositol[71] have been shown to be elevated in the urine of people with diabetes, and urinary *myo*-inositol has been reported to be a marker of glucose intolerance.[76] Supplementation with *myo*-

inositol can be beneficial and has been shown to decrease the risk of developing gestational diabetes in pregnant women.[77]

Taurine levels are lower in the eye lens of people with diabetes, and also reduce with age. A deficiency of taurine has been linked with the formation of cataracts.[2c, 78] For example, lenses exposed to high galactose concentrations (30 mmol/L) have been shown to be more likely to develop cataracts than if 0.2 mmol/L taurine is present.[78]

One-carbon metabolism and cardiovascular disease

As well as being a major osmolyte, betaine is also an important source of methyl groups in the body. People with acute coronary syndrome have been reported to be at greater risk of having a heart attack if they have low plasma betaine.[79] As has been mentioned, betaine donates a methyl group to homocysteine, forming methionine and DMG (Fig. 1.2). Homocysteine is damaging to the endothelium, leading to atherosclerosis, and has been well established as a risk factor in cardiovascular disease.[4b, 80] DMG is a feedback inhibitor of BHMT which limits the effectiveness of betaine therapy in reducing homocysteine levels, so a continual supply of betaine is important.[4b] It is likely that a depletion of betaine reserves contributes to the development of cardiovascular disease by reducing the body's ability to remove homocysteine. However, homocysteine can also be lowered by folate and vitamin B_6 (see Fig.1.2). Whether or not homocysteine is directly causative for cardiovascular events is still a major point of discussion in the literature. In 2006, the HOPE 2 study[81] reported that lowering homocysteine with folate, vitamin B_6, and vitamin B_{12} does not significantly reduce the risk of death from cardiovascular causes in patients with pre-existing cardiovascular disease or diabetes. The Norwegian vitamin (NORVIT) study also found that lowering homocysteine with folate, vitamin B_6, and vitamin B_{12} did not significantly lower the risk of recurrent cardiovascular events in people who have had a myocardial infarction (heart attack).[82] Neither of these studies used betaine as a therapy for lowering homocysteine. Betaine has other advantages including its role as an important osmolyte and compensatory solute, as well as being a methyl group donor. These studies only investigated the effect of lowering homocysteine in people who had known cardiovascular disease or diabetes, however, maintaining low homocysteine levels in healthy people may lower the risk

of developing cardiovascular disease in the first place. Homocysteine concentrations can be kept low by consuming a diet containing important nutrients including: betaine; folate; vitamin $B_{6;}$ and vitamin B_{12}.[80]

Patients with high blood lipids are often prescribed the lipid lowering drugs, fibrates. However, the fibrate drug most commonly prescribed in New Zealand, bezafibrate, has been associated with high urinary betaine excretion.[15] The mechanism for the reported loss of betaine from patients taking bezafibrate is not known. It has not yet been proven that fibrates actually cause the loss of betaine from the body, and it is not known if other osmolytes are affected in the same way.

Free choline, a precursor in the synthesis of betaine in the body, has been shown to be a useful marker for cardiovascular events such as heart attacks.[83] An increase in free choline in the blood is likely to be caused by the release of phosphatidylcholine from cell membranes when cell damage occurs. Low plasma betaine and high plasma choline have been shown to be significant risk factors for cardiovascular disease in people with metabolic syndrome.[84] Konstantinova *et al*.[84] investigated metabolite concentrations in a large study population with metabolic syndrome (>7000 subjects), and found that plasma betaine concentrations inversely correlated with components of metabolic syndrome such as non-HDL cholesterol, body mass index (BMI) and blood pressure. Whereas plasma choline positively correlated with plasma glucose, BMI, and percentage body fat.[84]

Elevated plasma trimethylamine-*N*-oxide (TMAO) has also recently been reported to be a risk factor for cardiovascular disease.[85] However, whether or not TMAO has a toxic effect in humans and causes cardiovascular disease is still a contentious issue. Other chordates (such as fish) beneficially accumulate and use TMAO as an osmolyte.[86] Methylamines such as choline and carnitine are cleaved by bacteria in the gut to form trimethylamine (TMA), which is then converted to TMAO in the liver.[87] TMAO also enters the body from the consumption of marine fish, where it is accumulated and used as an osmolyte.[86]

Taurine depletion has been shown to cause cardiomyopathy in mice,[88] and a deficiency of taurine has also been associated with liver disease, apoptosis, and loss of muscle function.[89] Taurine may also play a role in protecting against cardiovascular disease due to its anti-oxidant and anti-inflammatory properties, as well as its role in regulating blood pressure.[90] Dietary taurine can also increase levels of circulating high

density lipoprotein (HDL) cholesterol which may be protective against atherosclerosis.[24]

Osmolytes in other diseases

Changes in osmolyte concentrations have been reported to be important in many other diseases. For example, people with chronic renal failure have been shown to have lower plasma betaine, and excrete higher levels of betaine in the urine.[91] The increased urinary loss of betaine in renal patients may be an important factor in many of them going on to develop cardiovascular disease.[92]

As well as maintaining cell volume in the brain, the osmolytes taurine[22] and *myo*-inositol[93] play important roles in brain chemistry. *myo*-Inositol supplementation has been shown to help treat psychiatric disorders such as anxiety and depression.[94, 93] Patients with schizophrenia, bipolar patients, and people who have committed suicide have abnormally low concentrations of *myo*-inositol in the brain.[95] However, patients with Alzheimer's disease have been shown to have increased concentrations of *myo*-inositol and choline in the brain.[96] It is likely that tissue damage can cause the release of free *myo*-inositol and choline *via* the degradation of phosphatidylinositol and phosphatidylcholine which are present in cell membranes.

People with trimethylaminuria (fish odour syndrome) have a defect in the liver enzyme, flavin-containing monooxygenase 3 (FMO3), which converts TMA into the non-odorous TMAO.[97] Trimethylaminuria sufferers may secrete TMA in the sweat, breath and urine, and consequently have a fishy body odour which can be socially debilitating.[87a, 97] Gut bacteria convert quarternary amines such as choline, carnitine, and TMAO to TMA.[56] Therefore, the symptoms of trimethylaminuria can be lessened by cutting marine fish (high in TMAO) from their diet, eating less choline rich foods (such as eggs), as well as the use of antibiotics to reduce trimethylamine forming bacteria in the gut.[56] The ratio of TMA to TMAO concentration is important for the diagnosis of trimethylaminuria. However, the analysis of TMA can be problematic. Since plasma TMAO has been reported as a risk factor in cardiovascular disease,[87b] it might also be useful to develop a reliable method to measure the closely related metabolite, TMA, in plasma. It is possible that plasma TMA concentrations may also be an important biomarker of disease.

1.6. Research hypotheses

The overall aim of this project was to use LC-MS/MS (or NMR spectroscopy) to improve the analysis of betaine, other osmolytes, and other one carbon metabolites in order to extend knowledge about their role in health and disease, and their potential for use as risk markers. Methods were developed using the chemical properties of the analytes in combination with mass spectrometry, and were compared with traditional HPLC-UV methods. The methods developed in this project were used to measure osmolytes and related metabolites in human samples to investigate factors such as: their biological variability; how they are affected in disease; and how they interact with other biological variables.

The research hypotheses for this thesis were:

- *Hypothesis:* Tandem mass spectrometry (LC-MS/MS) can be used to measure betaine, other osmolytes, and other one-carbon metabolites in plasma and urine with greater efficiency and improved performance than conventional HPLC-UV methods.

- *Hypothesis:* Polyols (including sorbitol and *myo*-inositol) can be measured in plasma and urine using derivatization or complexation with boronic acids or transition metals along with techniques such as HPLC, mass spectrometry, or NMR spectroscopy.

- *Hypothesis:* The analytical methods developed in this thesis can be used to investigate concentrations of betaine, other osmolytes, and other one-carbon metabolites in order to provide new information on their metabolism, and the usefulness of these metabolites as risk markers in disease.

2. Chapter Two - The Measurement of Methylamines and Taurine in Biological Samples

2.1. Introduction

A reliable analytical method for the measurement of methylamine based osmolytes and related metabolites is essential for their study and monitoring in disease. Betaine (Fig. 2.1, **1**), choline (**6**), and trimethylamines-*N*-oxide (TMAO) (**10**) are useful predictors of health outcomes.[83-84, 98] The measurement of both TMAO and trimethylamine (TMA) is also important for the diagnosis of trimethylaminuria.[56] It is useful to monitor changes in the concentrations of methylamines, particularly considering the roles they play in one-carbon metabolism, osmotic control, and maintaining protein structure and function.[3] An efficient and reliable assay for the measurement of methylamines was developed here in order to investigate their concentrations in at risk groups, such as people with metabolic syndrome and diabetes. A better method for the measurement of taurine in plasma and urine was developed using negative ion mode.

Chapter Aims

This aim of the research described in this chapter was to develop and validate:

- An efficient and reliable LC-MS/MS method that can measure a greater number of methylamines that previous HPLC-UV methods, including: betaine, *N*,*N*-dimethylglycine (DMG), sarcosine, choline, glycerophosphorylcholine (GPC), TMAO, creatinine, and carnitines.
- A mass spectrometry method for measuring both TMA and TMAO using pre-column derivatization of TMA.
- An efficient mass spectrometry method for the measurement of taurine.

2.2. The measurement of betaine, *N*,*N*-dimethylglycine, choline, glycerophosphorylcholine, sarcosine, trimethylamine-*N*-oxide, creatinine, and carnitines by liquid chromatography - mass spectrometry

Introduction

HPLC-UV techniques that have traditionally been used to measure betaine, carnitine, DMG, and choline required derivatization of the samples, and separation from interfering compounds in the sample matrix was often difficult. LC-MS/MS has previously been used to measure methylamines in a range of different sample matrices without the need for pre-column derivatization.[41, 45] Tandem mass spectrometry (LC-MS/MS) has many advantages over traditional HPLC techniques, including: fast and simple sample preparation, high selectivity, lower run times, and lower flow rates. The use of compound-specific deuterated internal standards in LC-MS/MS also allows for more accurate and precise data to be obtained. However, caution needs to be taken to ensure that mass spectrometry methods are reliably quantitative over the expected biological range. Liquid chromatography - mass spectrometry methods have been described for the measurement of betaine and related one-carbon metabolites in order to assess their potential as bio-markers of disease.[41, 45a, 99] In 2002, Koc *et al*.[99] described a single quadrupole liquid chromatograph - mass spectrometer (LC-MS) to measure choline and a range of related metabolites, including betaine and GPC in tissues and foods. They showed that single quadrupole mass spectrometry produced high precision and accuracy and had considerable advantages over previous methods, including shorter sample run times, and no need to derivatize the samples, therefore allowing considerable savings in labour and reagent usage. As mass spectrometry systems became more affordable and advanced, new methods were described using more sophisticated tandem mass spectrometers (LC-MS/MS) which can achieve greater sensitivity and accuracy.[41, 45]

The aim was to develop an efficient and reliable tandem liquid chromatography mass spectrometry method which can measure the methylamines: betaine (Fig. 2.1, **1**), GPC (**2**); choline (**6**); DMG (**7**); sarcosine (**8**); creatinine (**9**); TMAO (**10**); carnitine (**11**); acetylcarnitine (**12**); and propionylcarnitine (**13**). Results using this LC-MS/MS method were compared with established HPLC methods and with single quadrupole mass spectrometry LC-MS.

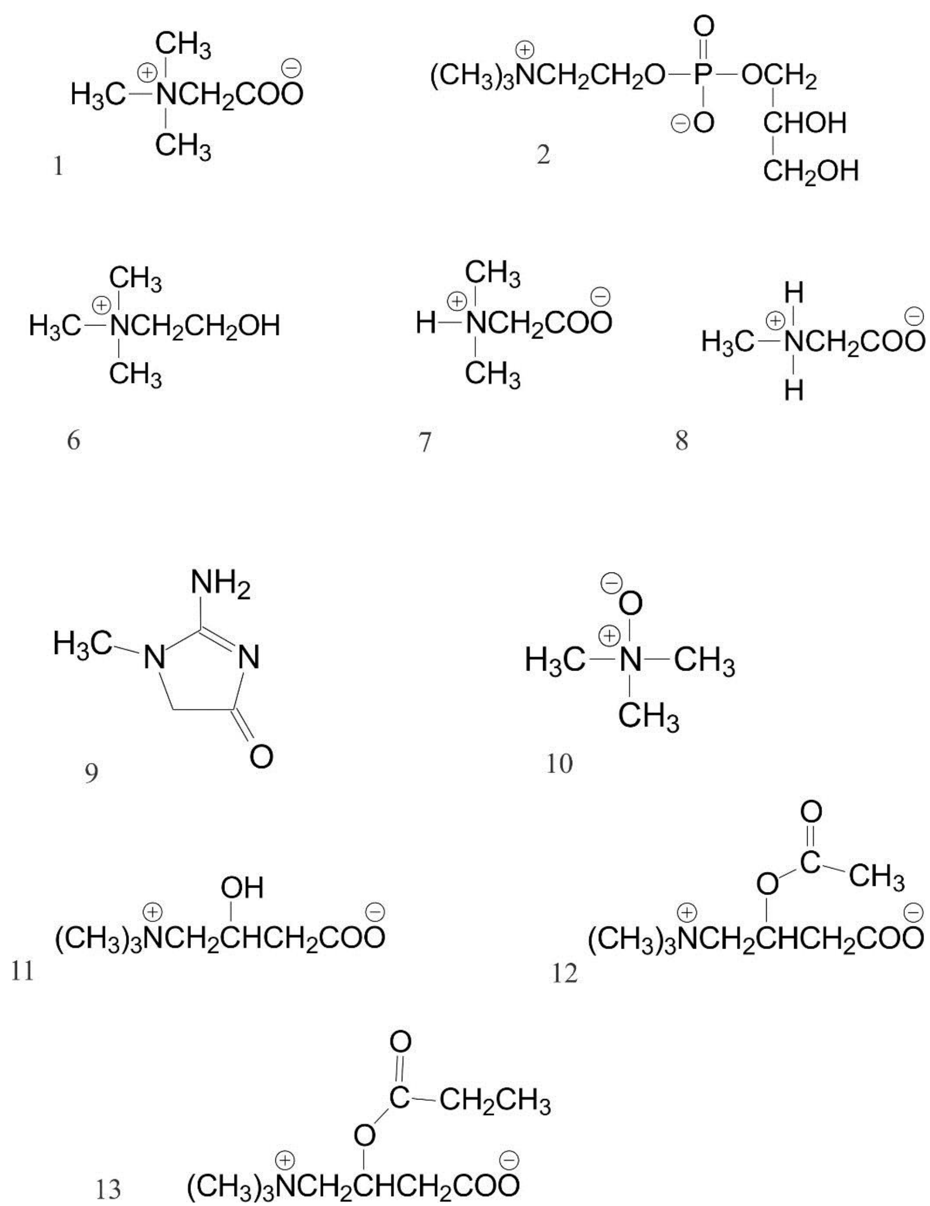


Fig. 2.1. Structures of some biologically important methylamines. Betaine (**1**), GPC (**2**), choline (**6**), DMG (**7**), sarcosine (**8**), creatinine (**9**), TMAO (**10**), carnitine (**11**), acetylcarnitine (**12**), propionoylcarnitine (**13**).

Methods

Reagents and chemicals

Carnitine (HCl), acetylcarnitine (HCl), betaine (HCl), *N*,*N*-dimethylglycine (HCl), choline iodide, glycerophosphorylcholine (cadmium chloride complex), sarcosine, and creatinine (anhydrous) were obtained from Sigma (St Louis, MO, USA). *N*,*N*,*N*-Trimethyl-D_9-glycine (HCl, D_9-betaine) and choline-trimethyl-D_9 chloride were obtained from Isotec (Miamisburg, Ohio, USA), D_3-creatinine, D_9-trimethylamine-*N*-oxide, and D_3-carnitine were obtained from Cambridge Isotope Laboratories (Andover, MA, USA), and *N*,*N*-dimethyl-D_3-glycine (HCl, D_3-DMG) was obtained from CDN isotopes (Pointe-Claire, Quebec, Canada). Ammonium formate and formic acid were purchased from Sigma-Aldrich (St Louis, MO, USA). HPLC grade acetonitrile was obtained from Mallinckrodt (Paris, KY, USA), and methanol was obtained from Merck (Darmstadt, Germany).

Sample preparation

Plasma, urine, or standard (50 µL) was added to 1.0 mL of extraction solvent which contained 90% acetonitrile and 10% methanol with 5.0 µmol/L of each of the deuterated internal standards added (D_9-betaine, D_9-choline, *N*,*N*-dimethyl-D_3-glycine, D_3-creatinine, D_3-carnitine, and D_9-TMAO). Samples were vortexed for 5 minutes and centrifuged at 13000 × *g* for 5 minutes, then transferred to HPLC vials and capped for analysis.

Chromatography

A number of normal phase (HILIC) columns were investigated for the separation of methylamines in biological samples, including: silica (Kinetix HILIC 100 × 2.1 mm, 2.6 µm, Phenomenex, CA, USA), silica hydride (Cogent Silica C 100 × 2.1 mm, 4 µm, Microsolv Technologies, NJ, USA), silica hydride with carbon (Cogent diamond hydride 100 × 2.1 mm, 4 µm, Microsolv Technologies), a strong cation exchange (Epic SCX 12.5 × 3 mm, 3 µm, ES Industries, NJ, USA), and an XBridge Amide column (100 × 2.1 mm, 3.5 µm, Waters). A mixed aqueous standard (containing 100 µmol/L of each of betaine, choline, DMG, sarcosine, TMAO,

creatinine, carnitine, acetylcarnitine, and propionylcarnitine) was injected onto each column and their selectivities were compared. All columns were operated with the same conditions as described in the next section.

LC-MS/MS

HPLC was performed on a Shimadzu Prominence (Kyoto, Japan) system. The flow rate was 0.3 mL/min. The injection volume was 10 µL. For the validation studies, a Cogent 100 mm × 2.1 mm, 4 µm diamond hydride silica column (Microsolv Technologies, NJ, USA) was used with a silica guard column, and the oven temperature was 40ºC. The gradient used for the analysis is described in Table 2.1. Line A contained 10 mmol/L ammonium formate and 10 mmol/L formic acid in 50% distilled water and 50% acetonitrile. Line B contained 10 mmol/L ammonium formate, 10 mmol/L formic acid, 73% acetonitrile and 27% water.

Table 2.1. Gradient used with Diamond Hydride column.

Time (min)	%A	%B
0.0	0	100
6.0	100	0
6.1	0	100
8.0	0	100

Samples were detected using an MDS Sciex API 4000 (Applied Biosystems, VIC, Australia) tandem mass spectrometer with an electrospray ionization (ESI) probe. Methylamines were measured in positive ion mode using tandem mass spectrometry with multiple reaction monitoring (MRM). Mass transitions used for MRM are shown in Table 2.2. The dwell time was 150 ms for all compounds. Instrument dependent parameters were investigated using betaine as a model compound for the method. Ion source temperature was 350ºC, source gas (nitrogen) 1 was set to 40 L/min and source gas 2 was set to 45 L/min. The collision gas was also nitrogen which was produced in a nitrogen generator. In order to find the optimum parameters for each compound, a compound optimization was performed by infusing 2 µmol/L standards of each analyte in mobile phase into the mass spectrometer using a syringe pump at a flow rate of 10 µL/min. The optimum compound-specific MS/MS mass transitions and potentials are shown in Table 2.2 for each analyte. There were at

least four fragments produced in the collision chamber for most of the analytes. However, the mass transition which produced the greatest signal was usually chosen for quantitation. The structure of the parent ions and the MS/MS fragment ions used for detection are shown in Fig. 2.2. The HPLC system and mass spectrometer were controlled using Analyst software (Applied Biosystems). Data was calibrated using peak area ratios of external standards to internal standards as compared to peak area ratios in the samples to the internal standards. The internal standards used for each compound are shown in Table 2.2.

Table 2.2. Mass transitions and potentials used to measure methylamines by LC-MS/MS.

Analyte	Mass transition	DP	CE	CXP	Internal standard
Betaine	118.2 → 59.0	56.0	27.0	4.0	D_9-Betaine
Choline	104.1 → 60.2	16.0	25.0	4.0	D_9-Choline
Dimethylglycine	104.1 → 58.1	51.0	21.0	4.0	D_3-Dimethylglycine
Sarcosine	90.1 → 44.0	6.0	21.0	6.0	D_3-Dimethylglycine
Creatinine	114.1 → 44.0	6.0	29.0	4.0	D_3-creatinine
Acetylcarnitine	204.3 → 85.2	81.0	31.0	8.0	D_3-carnitine
Propionoylcarnitine	218.2 → 85.0	51.0	29.0	4.0	D_3-carnitine
Carnitine	162.1 → 85.0	71.0	31.0	16.0	D_3-carnitine
Trimethylamine-*N*-oxide	76.1 → 58.1	16.0	27.0	10.0	D_9-TMAO
Glycerophosphorylcholine	258.2 → 104.0	66.0	23.0	8.0	D_9-Betaine

Internal standard	Mass transition	DP	CE	CXP
D_9-Betaine	127.2 → 68.0	61.0	27.0	4.0
D_9-Choline	113.2 → 69.0	61.0	27.0	2.0
D_3-Creatinine	117.2 → 47.1	51.0	33.0	4.0
D_3-Dimethylglycine	107.2 → 61.0	46.0	29.0	4.0
D_3-Carnitine	165.2 → 85.0	61.0	29.0	6.0
D_9-Trimethylamine-*N*-oxide	85.2 → 66.0	91.0	29.0	2.0

Abbreviations: Declustering potential (DP), collision energy (CE), and collision cell exit potential (CXP). All potentials are given in electron volts (eV).

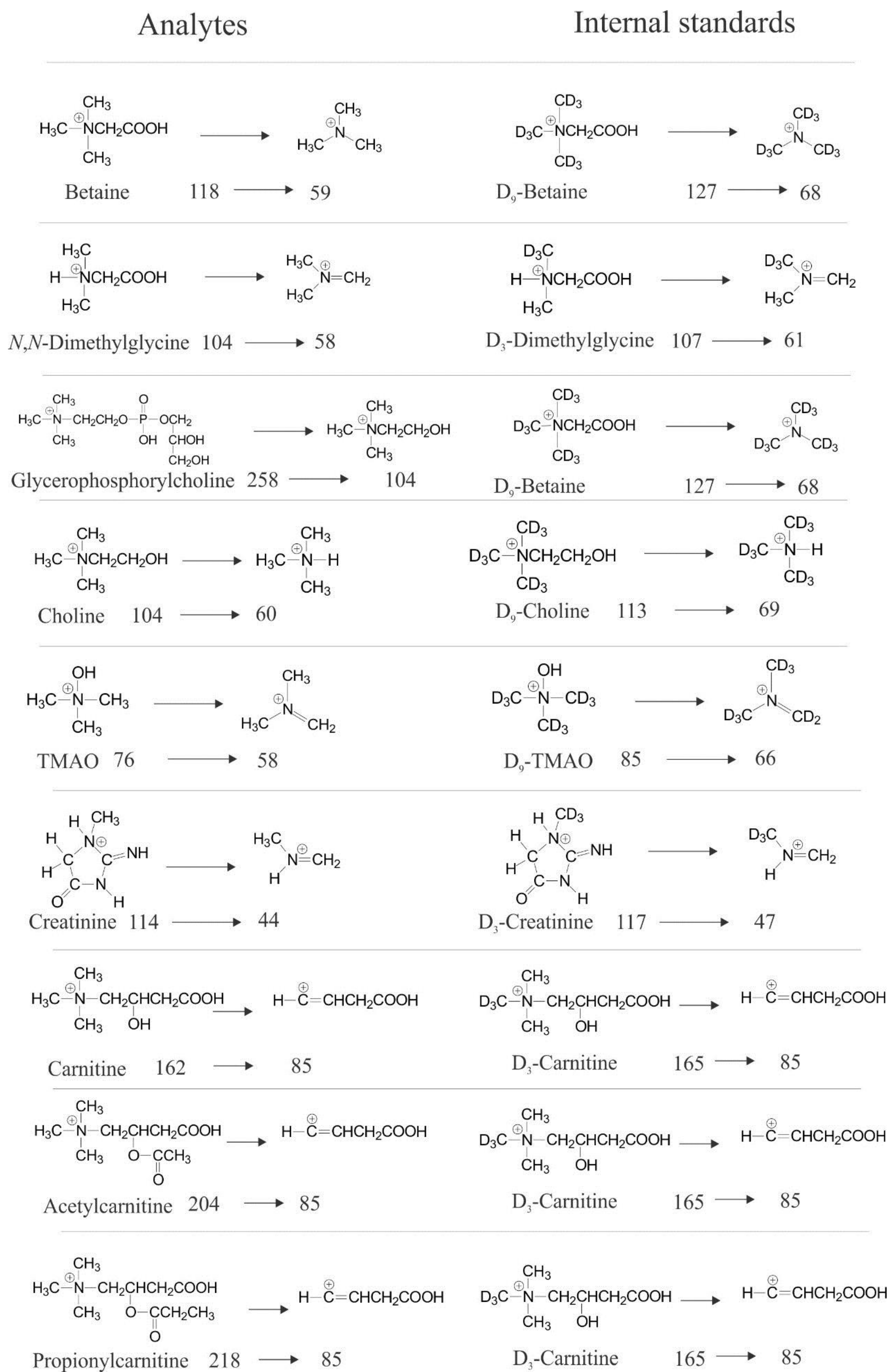


Fig. 2.2. Mass transitions used for MRM detection of methylamines by LC-MS/MS.

Single quadrupole LC-MS

A method for the measurement of methylamines was also developed using an Agilent 6120 single quadrupole mass spectrometer with an ESI source connected to an Agilent 1200 Series HPLC system (Agilent, VIC, Australia). Methylamines were measured by monitoring the parent mass in positive ion mode $[M+H]^+$. Mass spectrometric parameters (energies and temperatures) were optimised using flow injection analyses (Table 2.3). It was expected that single quadrupole mass spectrometry (LC-MS) and triple quadrupole mass spectrometry (LC-MS/MS) would produce similar results for these low molecular weight methylamine compounds. The LC-MS method was validated by comparison with the LC-MS/MS method.

Table 2.3. Optimized parameters for analytes detected on the Agilent 6120 single quadrupole mass spectrometer.

MSD 6120	Mass to charge ratio (*m/z*)	Fragmentor (V)	Drying gas Flow rate (L/min)	Nebulizing pressure (PSIG)
Betaine	118	115	7	35
TMAO	76	85	7	35
Choline	104	90	7	35
DMG	104	90	7	35
Creatinine	114	110	7	35
Carnitine	162	85	7	35
Acetylcarnitine	204	100	7	35

	Drying gas temperature °C	Capillary voltage (V)
Betaine	275	1500
TMAO	275	1500
Choline	275	1500
DMG	275	1500
Creatinine	275	1500
Carnitine	275	1500
Acetylcarnitine	275	1500

Note: Taurine and inositol were measured in negative ion mode. Inositol was detected with an APCI source, and the other analytes were detected with electrospray ionization.

Validation studies

Validation studies were carried out on pooled EDTA plasma, and urine samples. Samples were stored at -80 ºC before analyses.

Precision and accuracy

Six batches of four replicates were run to investigate the precision of the assay for betaine, DMG, choline, carnitine, acetylcarnitine, propionylcarnitine, TMAO, and creatinine in a plasma sample containing high and low levels of methylamines. The within batch and between batch coefficients of variation (CVs) were calculated for each compound.

Method comparison

Plasma samples were analyzed for betaine, DMG, carnitine, acetylcarnitine, and creatinine using the LC-MS/MS method developed here, and the results were compared with those obtained using established HPLC-UV methods using Passing-Bablok regression analysis carried out with Acomed Statistik V3.[100]

Betaine, DMG, carnitine and acetylcarnitine were measured by HPLC-UV using the method described by Storer *et al*.[1b] For betaine, DMG, carnitine, and acetylcarnitine 22 plasma samples were derivatized with 2-naphthacyl triflate. The samples were separated on a Phenosphere SCX (250 × 4.6, 5 µm) column (Phenomenex) and measured by UV detection at 249 nm.

Creatinine was measured by HPLC-UV without derivatization. Fifty µL of sample was extracted into 1.0 mL of 80%:20% acetonitrile and methanol. Samples were then vortexed, and centrifuged at 13,000 × *g* for 5 minutes. Creatinine was separated using a Sachtopore titania 150 × 3 mm, 3 µm column (Sachtleben Chemie, Duisburg, Germany). The mobile phase contained 50 mmol/L H_2SO_4, 8% water, and 92% acetonitrile. The flow rate was 0.8 mL/min and the injection volume was 50 µL. The oven temperature was set to 40 ºC and detection was by UV at 214 nm. The run time was 15 minutes.

Methylamine results from 48 plasma samples obtained by tandem quadrupole mass spectrometry (LC-MS/MS) were compared with single quadrupole mass spectrometry (LC-MS) using Passing-Bablok regression analyses (Acomed Statistik

V3).[100] The LC-MS/MS instrument used was an AB Sciex API4000, and the LC-MS single quadrupole instrument was an Agilent 6120.

To compare the method performance for methylamine concentrations obtained by the different methods, a plasma sample and the same plasma with 50 µmol/L of added betaine, DMG, choline, sarcosine, TMAO, GPC, carnitine, acetylcarnitine, and creatinine were analyzed by LC-MS, LC-MS/MS, and HPLC-UV. Precision was evaluated by calculating the coefficient of variation over 10 replicates. Accuracy was evaluated as the recovery of analytes added to plasma

A urine sample and the sample urine with 100 µmol/L of betaine, DMG, choline, sarcosine, TMAO, GPC, carnitine, and acetylcarnitine were measured by LC-MS, LC-MS/MS, and HPLC. Precision was evaluated for the different methods by calculating the coefficient of variation over 10 replicates. Accuracy was evaluated as the recovery of analytes added to urine.

Linearity

To investigate the linearity of the method aqueous standards containing 50, 100, 250, 500, 1000, 1500, 2000 µmol/L of betaine, DMG, choline, carnitine, acetylcarnitine, TMAO, and glycerophosphorylcholine were measured by LC-MS/MS and calibration curves were generated.

To investigate the linear range in plasma for DMG, choline, GPC, acetylcarnitine, and propionoylcarnitine, concentrations ranging from 0 to 50 µmol/L were added to plasma. Concentrations of betaine ranging from 0 to 100 µmol/L were added to plasma, and concentrations of TMAO, creatinine, and carnitine ranging from 0 to 200 µmol/L were added to plasma.

To investigate the linear range for urine, concentrations ranging from 0 to 50 µmol/L of choline and GPC were added to urine. Concentrations ranging from 0 to 500 µmol/L of DMG, carnitine, acetylcarnitine were added to urine. Concentrations of betaine ranging from 0 to 1000 µmol/L were added to urine, and concentrations of TMAO ranging from 0 to 2000 µmol/L were added to urine.

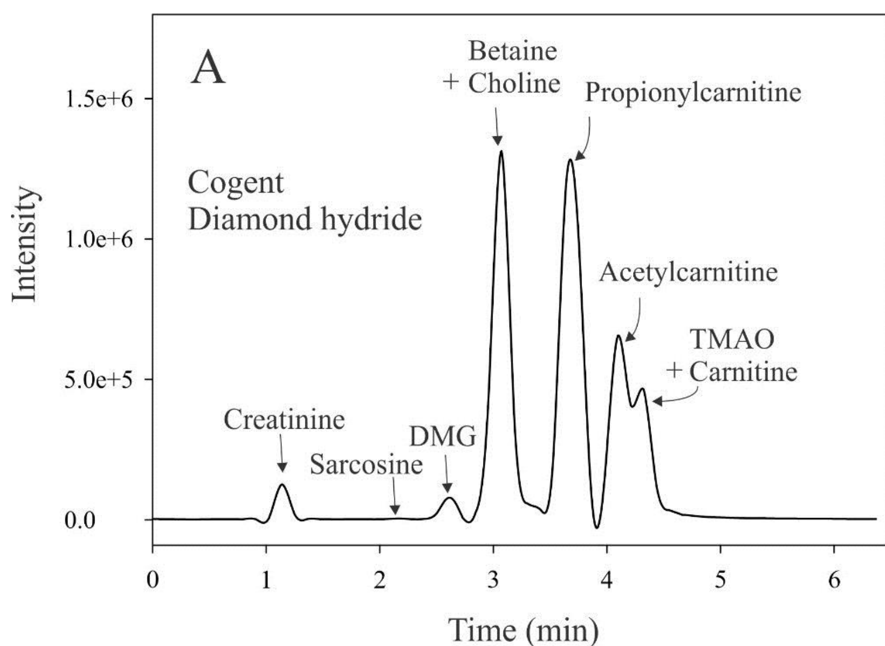
Each concentration level was analyzed in triplicate for each analyte in both plasma and urine.

Results

Comparison of LC columns

The chromatographic separation of methylamines on different columns are shown in Figs. 2.3 and 2.4. Only the silica hydride columns were effective at separating choline and DMG chromatographically. The diamond hydride had a different selectivity to the Silica C column with choline co-eluting with betaine on the diamond hydride column, and choline co-eluting with acetylcarnitine on the Silica C column. The main difference between these columns is that the diamond hydride column has some carbon on the stationary phase. The Cogent columns both have a silica hydride surface on the mobile phase so that no silanol groups are present. The silica hydride columns generally gave broader peaks for compounds containing hydroxyl groups, such as carnitine and choline. Ammonium formate buffer was required in the mobile phase, otherwise much broader peaks were observed.

While the strong cation exchange (SCX) and Kinetex silica columns are useful for separating methylamines, they are not suitable for measuring DMG. The DMG and choline peaks co-elute, and choline causes interference in the DMG chromatogram.



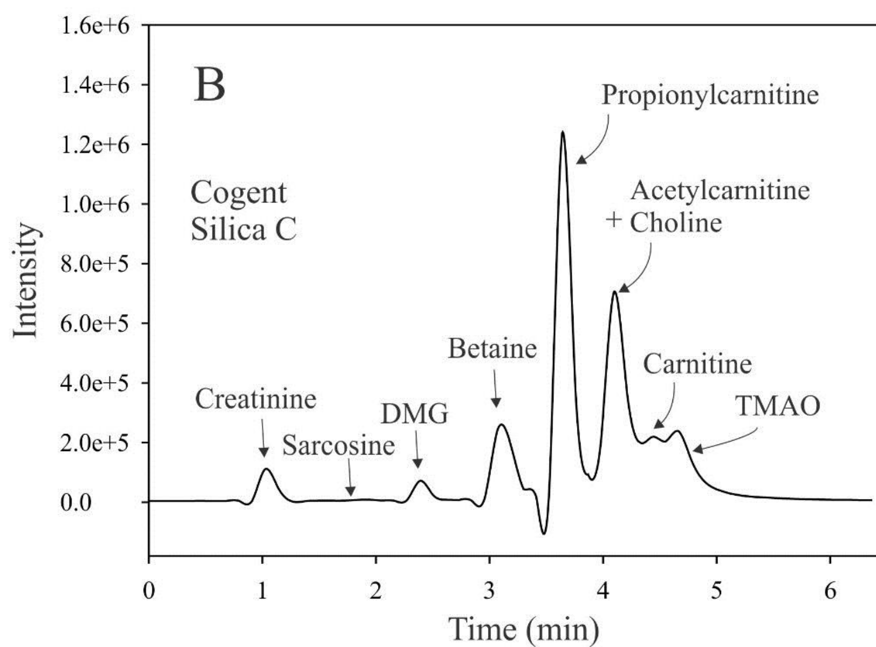


Fig. 2.3. Total ion count (TIC) chromatograms of methylamines separated on a Cogent diamond hydride column (A), and a Cogent Silica C column (B). Abbreviations: *N*,*N*-Dimethylglycine (DMG), trimethylamine-*N*-oxide (TMAO).

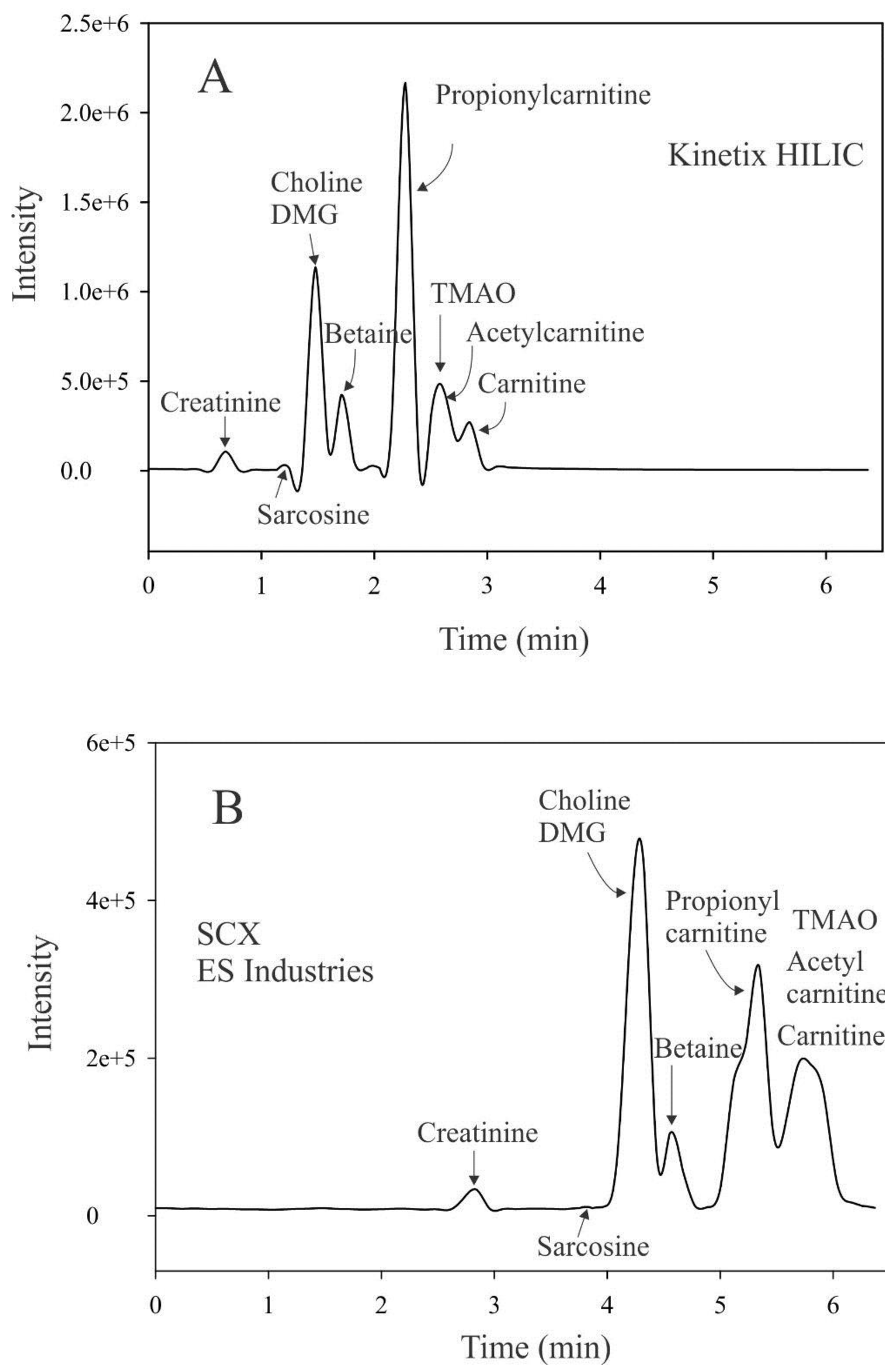


Fig. 2.4. Total ion count (TIC) chromatograms of methylamines separated on a Kinetex HILIC silica column (A), and an ES industries SCX column (B). Abbreviations: *N*,*N*-Dimethylglycine (DMG), trimethylamine-*N*-oxide (TMAO).

LC-MS/MS

All methylamine analytes were separated by a combination of chromatography and their mass transition (Fig. 2.5). Choline produced a minor fragment ion at *m/z* = 58 which caused interference in the DMG chromatogram. It is therefore important to separate these compounds chromatographically for reliable quantitation. Fortunately, the silica hydride columns were effective at separating DMG and choline. The lowest sensitivity was observed for sarcosine.

Carnitine, acetylcarnitine, and propionoylcarnitine fragmented in the tandem mass spectrometer to produce the most intense mass transition signal for the *m/z* = 85 fragment ion, which is the butanoic acid ion fragment. Betaine, choline, DMG, and TMAO all gave fragment ions of trimethylamine, whereas sarcosine and creatinine gave a fragment ion of dimethylamine. Choline and DMG both have a mass at 104 Da (although they are not isomers) and produce a fragment ion at 58 Da. Consequently, choline appears on the same chromatogram as DMG.

When detecting nine analytes as well as internal standards, a maximum dwell time of 150 ms was required to obtain 15 data points across the peaks. With a dwell time of 200 ms, there were only 4 data points across the peak, which was insufficient to reliably define and quantify the peak.

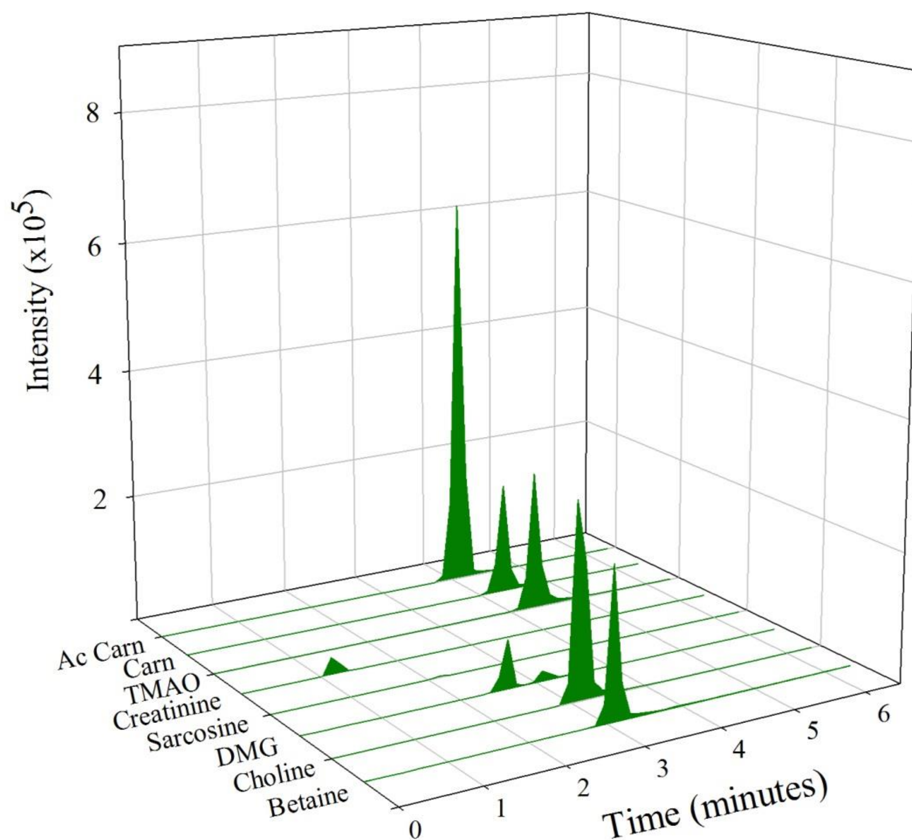


Fig. 2.5. MRM chromatograms showing separation of some methylamines in plasma separated on a Cogent diamond hydride silica column. Abbreviations: *N*,*N*-dimethylglycine (DMG), trimethylamine-*N*-oxide (TMAO), carnitine (Carn), acetylcarnitine (Ac Carn).

Precision and accuracy for the LC-MS/MS method

The within batch and between batch CVs were below 8% for most methylamines in plasma (Table 2.4), and below 6.6% for all methylamines in urine (Table 2.5). The limit of detection ranged from 0.01 to 0.5 µmol/L for methylamines in plasma. Sarcosine measured by this method had a high detection limit, and poor precision as indicated by within batch and between batch CVs over 7.8%.

Table 2.4. Method validation data for plasma methylamines measured by LC-MS/MS.

Analyte	Mean (μmol/L)	Within-batch CV	Between batch CV	Recovery (%)	LOD (μmol/L)
Betaine					
Normal	54	3.6	2.4		
High	155	4.3	3.1	101	0.44
Choline					
Normal	19.8	3.1	3.6		
High	70.9	4.4	4.3	102	0.11
Dimethylglycine					
Normal	8.1	6.4	6.6		
High	25.3	7.7	8.0	86	0.5
Sarcosine					
Normal	7.7	17	8.5	67	3.0
High	41.3	7.9	11		
Carnitine					
Normal	54.9	2.5	1.1		
High	107	2.6	1.2	104	0.26
Acetylcarnitine					
Normal	5.1	3.6	1.2		
High	20.6	2.6	1.4	103	0.03
Propionylcarnitine					
Normal	4.9	3.3	4.9		
High	20.3	2.8	1.8	103	0.01
TMAO					
Normal	20.9	6.1	5.1		
High	77.3	3.6	6.3	113	0.13
Creatinine					
Normal	63.6	4.7	3.6		
High	122	4.3	5.0	117	0.54
GPC					
Normal	10.9	2.7	2.1		
High	25.9	2.8	4.2	100	0.04

Table 2.5. Method validation data for methylamines in urine measured by LC-MS/MS.

Analyte	Mean (µmol/L)	Within-batch CV	Between batch CV	Recovery (%)
Betaine				
Low	91.6	1.55	1.32	
High	268	1.63	1.47	88
Choline				
Low	14.1	1.82	1.70	
High	22	1.42	1.10	80
Dimethylglycine				
Low	43.8	0.84	3.76	
High	133.4	4.38	3.60	90
Carnitine				
Low	125	1.27	1.23	
High	200	1.07	2.21	75
Acetylcarnitine				
Low	34.6	1.75	1.90	
High	129	1.34	2.32	94
GPC				
Low	1.7	2.18	2.13	
High	12.1	2.67	1.54	104
TMAO				
Low	183	1.9	8.1	
High	1147	5.7	6.5	96

Linear range

The linear range for the LC-MS/MS method was found to be limited at the higher concentrations. The calibration curves show that there was a lower than expected signal response at concentrations over 250 µmol/L for most methylamines (Fig. 2.6).

The plasma samples with added levels of all methylamines were linear over the expected biological ranges with r^2 of 0.976 for DMG, and r^2 above 0.985 for all other metabolites in the plasma (Fig. 2.7). The r^2 values for urine with added methylamine concentrations were 0.998 and higher. The linearity in the urine was greater than for standards, whereas the urine results were still linear at concentrations of 500, 1000 or 2000 µmol/L (Fig. 2.8).

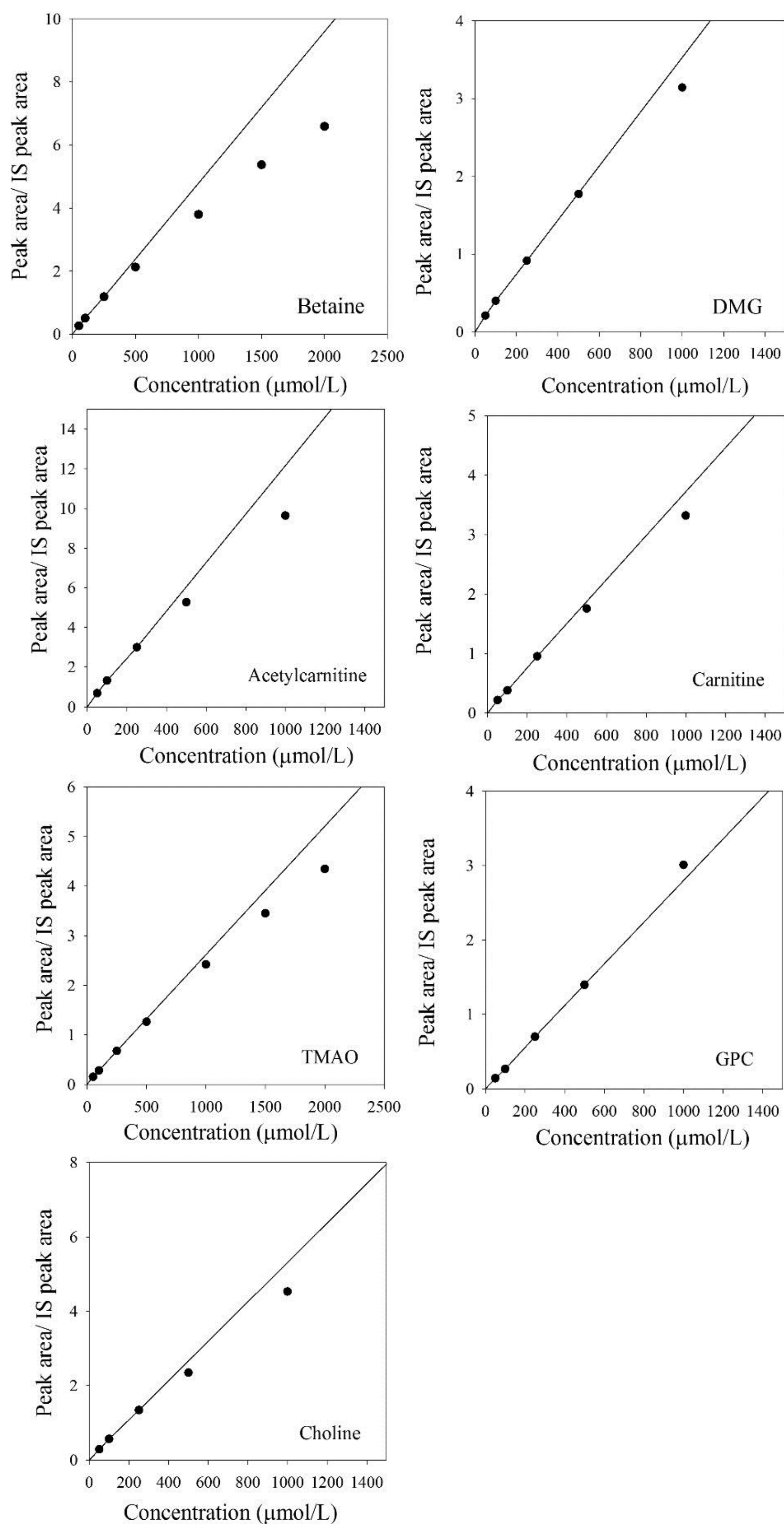


Fig. 2.6. Calibration curves of aqueous standards showing data with peak area ratios to the internal standard peak area. Linear regression lines were calculated using concentrations up to 250 µmol/L only. Abbreviations: *N*,*N*-dimethylglycine (DMG), trimethylamine-*N*-oxide (TMAO), glycerophosphorylcholine (GPC).

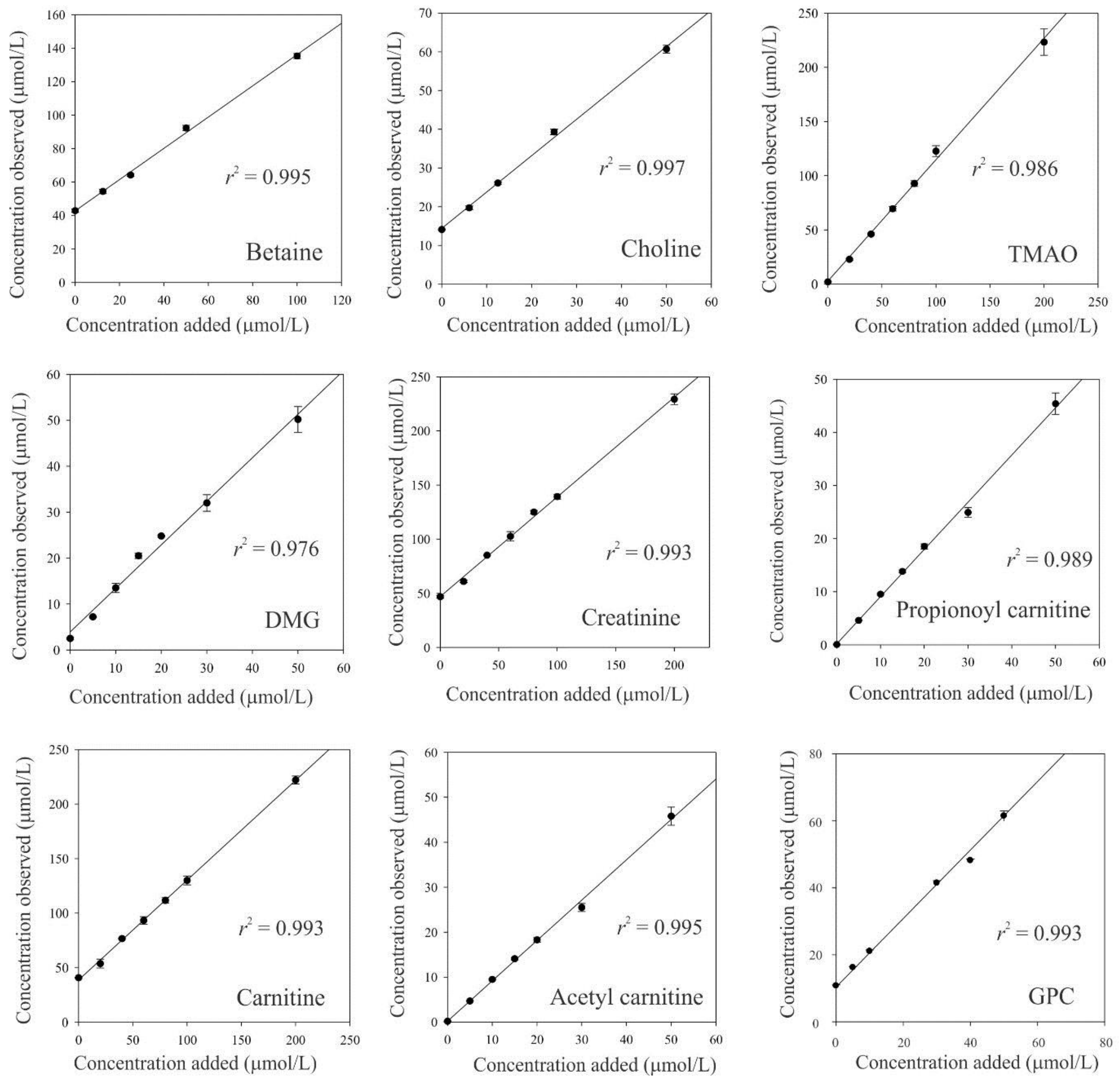


Fig. 2.7. Linearity of methylamines added to plasma and measured by LC-MS/MS (error bars represent standard errors). Abbreviations: *N*,*N*-dimethylglycine (DMG), trimethylamine-*N*-oxide (TMAO), glycerophosphorylcholine (GPC).

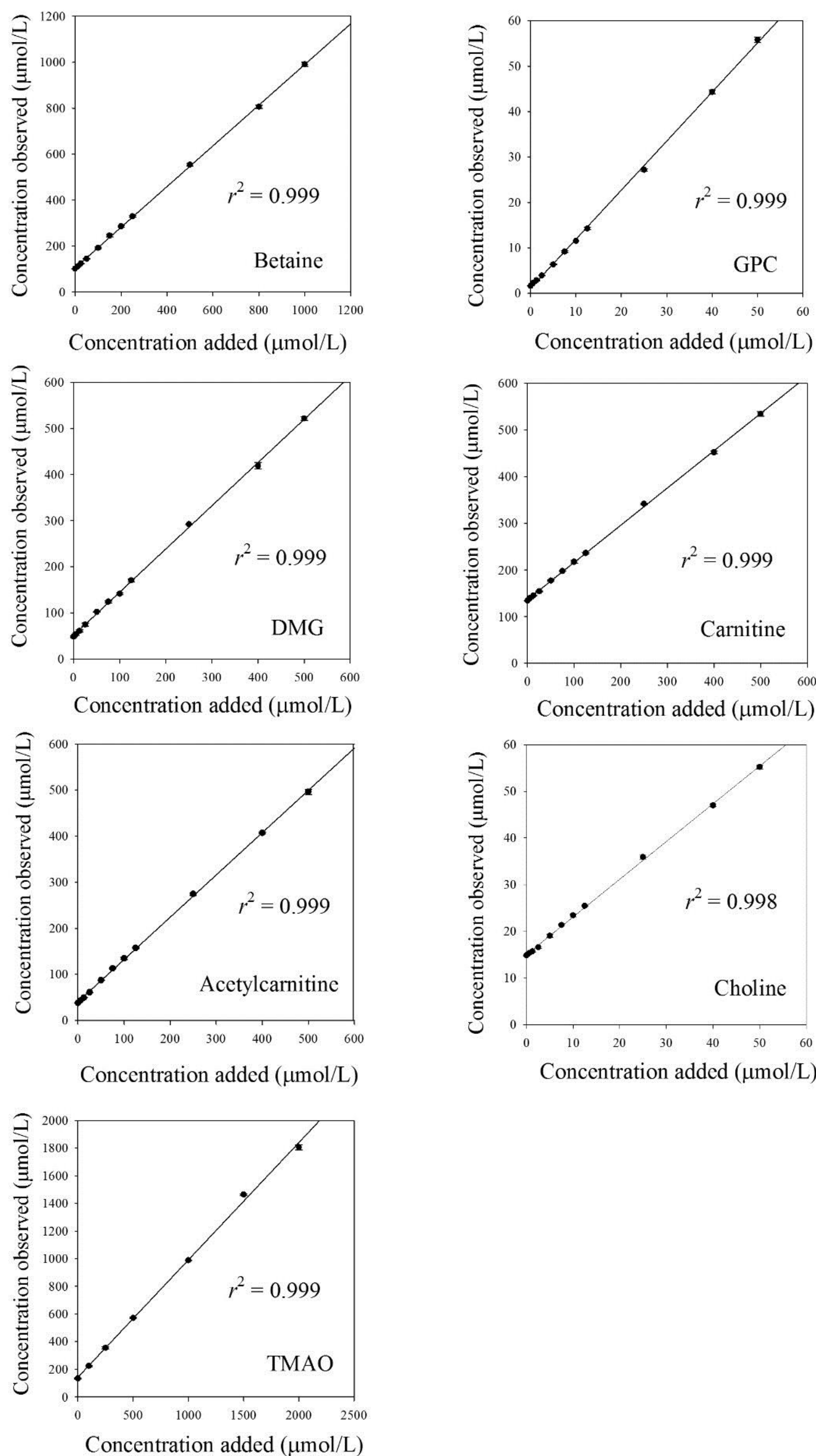


Fig. 2.8. Linearity of methylamines added to urine and measured by LC-MS/MS (error bars represent standard errors). Abbreviations: *N*,*N*-dimethylglycine (DMG), trimethylamine-*N*-oxide (TMAO), glycerophosphorylcholine (GPC).

Comparison of LC-MS/MS and HPLC methylamine concentrations

Comparisons of the LC-MS/MS method with established HPLC methods for measuring methylamines in plasma are shown in Fig. 2.9.

The agreement of LC-MS/MS with HPLC was best for creatinine with r^2 = 0.999. Both creatinine and betaine methods were not significantly different with Passing-Bablok analysis showing a slope not significantly different to 1, and an intercept not significantly different to zero. The LC-MS/MS method for DMG had the lowest correlation with the HPLC assay (r^2 = 0.748). The carnitine results showed a significant bias of 7% with results tending to be higher when they were determined by LC-MS/MS. This is consistent with some carnitine contamination observed in the reagent blanks in the LC-MS/MS method (of approximately 2 µmol/L), which was identified as coming from the deuterated carnitine internal standard. D_9-Carnitine was later purchased from a different supplier and the contamination problem was solved.

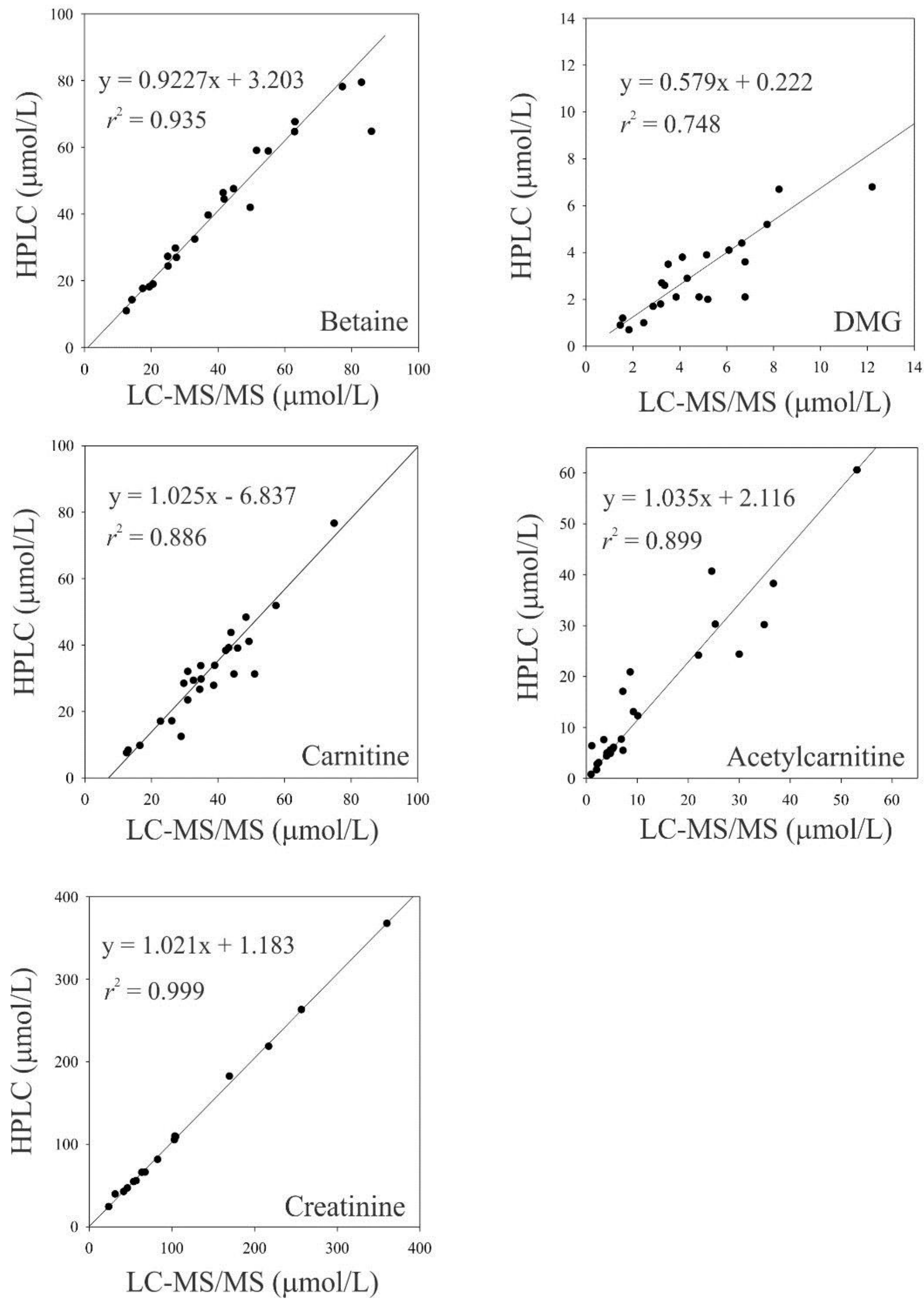


Fig. 2.9. Methylamines in human plasma comparing the current LC-MS/MS method with established HPLC methods. Regression equations are from Passing-Bablok analyses.

Comparison of tandem mass spectrometry (LC-MS/MS) with single quadrupole mass spectrometry (LC-MS)

The comparison of data obtained by LC-MS and LC-MS/MS show that similar results can be obtained by both methods with the slope of the Passing-Bablok analyses within 15% of 1 for all analytes (Fig 2.10). Correlations (r^2) were above 0.8 for betaine, DMG, TMAO, and carnitine. However, choline (r^2 = 0.693) and acetylcarnitine (r^2 = 0.635) had lower correlation coefficients.

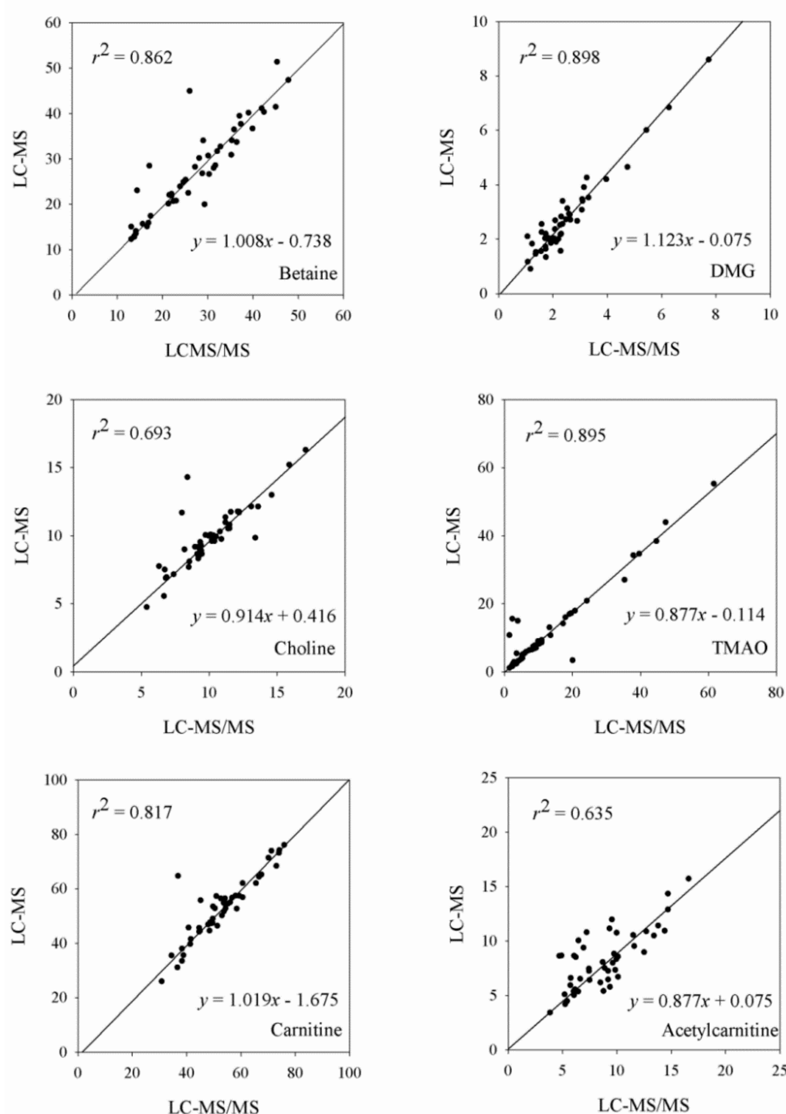


Fig. 2.10. Single quadrupole mass spectrometry results versus tandem mass spectrometry for 6 different analytes in plasma. All units are in μmol/L. Regression equations are from Passing-Bablok analyses.

Method performance for methylamine concentrations obtained by LC-MS, LC-MS/MS, and HPLC-UV

Plasma methylamine results for the different methods are shown in Table 2.6. The concentrations calculated by the three different methods were similar for all methylamines in plasma. Precision and accuracy were similar for the different methods with CVs below 3% for all analytes and recoveries 85% and higher.

CVs for urine methylamine concentrations were below 3% for LC-MS and LC-MS/MS methods, and below 5% for HPLC-UV methods. Recoveries were over 88% for methylamines measured by all methods. The HPLC-UV method appears to considerably over-estimate the concentrations of betaine and acetylcarnitine in these urine samples compared to LC-MS and LC-MS/MS (see Table 2.7).

Table 2.6. Method comparison data for some methylamines in plasma.

	Betaine			DMG			Choline		
	LCMS	LC-MS/MS	HPLC	LCMS	LC-MS/MS	HPLC	LCMS	LC-MS/MS	HPLC
Plasma									
Mean (µmol/L)	30.24	32.75	33.7	2.00	2.78	3.28	36.1	40.6	ND
SD	0.44	0.50	0.56	0.13	0.19	0.19	0.39	0.48	
CV%	1.47	1.52	1.65	6.61	6.72	5.86	1.09	1.19	
Plasma + 50 µmol/L added									
Mean (µmol/L)	75.3	79.7	79.7	48.9	50.8	47.5	82.3	90.3	ND
SD	0.70	1.16	1.39	0.72	1.27	1.01	0.72	0.45	
CV%	0.92	1.44	1.74	0.88	2.50	2.14	0.88	0.50	
Recovery %	90	95	92	94	96	88	92	99	
LOD	0.08	0.44	0.2	0.28	0.5	0.12	0.29	0.11	
	Carnitine			**Acetylcarnitine**			**Creatinine**		
	LCMS	LC-MS/MS	HPLC	LCMS	LC-MS/MS	HPLC	LCMS	LC-MS/MS	HPLC
Plasma									
Mean (µmol/L)	30.03	32.75	27.4	2.28	1.08	0.66	62.4	65.0	65.4
SD	0.30	0.50	0.79	0.03	0.04	0.29	0.63	1.36	1.15
CV%	0.99	1.52	2.87	1.17	3.89	43.7	1.01	2.09	1.76
Plasma + 50 µmol/L added									
Mean (µmol/L)	76.83	83.4	71.3	44.8	49.6	46.4	107.4	109.2	111.4
SD	0.72	2.06	1.46	0.48	0.85	0.97	1.14	2.20	2.48
CV%	0.94	2.47	2.05	1.06	1.72	2.10	1.06	2.50	2.22
Recovery %	94	101	88	85	97	91	90	96	92
LOD	0.08	0.25	0.24	0.12	0.03	0.24	0.31	0.04	0.32

Note: Means and standard deviations were calculated on 10 replicates. HPLC data was collected using derivatization with 2-naphthacyl triflate, strong cation exchange chromatography and UV detection at 249 nm. LOD is the limit of detection (where $S/N = 3$). ND = not determined.

Table 2.7. Method comparison data for some methylamines in urine.

	Betaine			DMG			Choline		
	LCMS	LC-MS/MS	HPLC	LCMS	LC-MS/MS	HPLC	LCMS	LC-MS/MS	HPLC
Urine									
Mean (µmol/L)	31.1	33.0	63.2	17.98	19.24	22.7	6.42	7.48	ND
SD	0.30	0.74	6.97	0.565	0.60	3.2	0.08	0.28	
CV%	0.95	2.23	11.0	3.14	3.11	14.1	1.17	3.72	
Urine + 100 µmol/L added									
Mean (µmol/L)	122.7	129.5	163.8	114.2	113.1	150.8	101.1	110.4	ND
SD	0.93	2.07	8.05	1.65	2.47	7.2	0.66	1.17	
CV%	0.76	1.60	4.9	1.44	2.18	4.8	0.65	1.06	
Recovery %	92	97	101	96	94	128	95	103	

	Carnitine			Acetylcarnitine		
	LCMS	LC-MS/MS	HPLC	LCMS	LC-MS/MS	HPLC
Urine						
Mean (µmol/L)	25.79	28.74	34.4	5.14	6.56	41.0
SD	0.29	0.60	0.52	0.22	0.16	6.6
CV%	1.12	2.09	1.5	4.28	2.48	16.2
Urine + 100 µmol/L added						
Mean (µmol/L)	121.3	126.3	150	94.4	101.1	146
SD	0.75	1.57	4.6	1.27	2.38	7.6
CV%	0.62	1.24	3.1	1.34	2.35	5.2
Recovery %	96	98	116	89	95	105

Note: Means and standard deviations were calculated on 10 replicates. ND = not determined.

Discussion

Sample preparation is important for ensuring that the samples are clean enough for injection into an LC-MS/MS system, and good chromatography is obtained. The sample preparation as described in Holm *et al*.[41] (1:3 sample:acetonitrile) could leave a high salt content and some protein in the injection solvent which can have negative effects on the column and the chromatography. The method described here uses a greater dilution of 1:20, *i.e.* 50 µL of sample in 1000 µL of extraction solvent. There is still enough sensitivity to measure most analytes with this greater dilution. However, the sensitivity was not sufficient to reliably measure sarcosine in plasma, where concentrations are reported to be typically less than 2 µmol/L.[61b] Improving the sensitivity for sarcosine may require pre-column derivatization, or a more sensitive mass spectrometer. The inflated concentration of 7.7 µmol/L sarcosine in plasma observed here suggests that there may be interference from other compounds such as L-alanine or β-alanine.

The linear range for the detection of methylamines by mass spectrometry has been shown to be limited. It was observed that the calibration curves for many methylamines were not linear over 250 µmol/L. Therefore, any results above this concentration may require the sample to be diluted at least ten-fold, and re-analyzed to be within the linear range. It may be necessary to analyze biological samples at different dilutions because some analytes are present in low concentrations when others are present in high concentrations, meaning some analytes may be diluted below the detection limit. As well as external standards, it is also important to ensure that the internal standard concentration is within the linear range of the mass spectrometer. The linearity of methylamine added to urine was greater with concentrations of many analytes still linear at concentrations over 1000 µmol/L, possibly because ion interference from the sample may allow less ions to reach the detector. The use of deuterated internal standards helps to correct for this effect. There are a number of reasons why the signal drops off at higher concentrations in the mass spectrometer leading to a non-linear calibration curve. The detector may become over-loaded (saturated with ions), the electrospray droplet surface may become saturated with the analytes, or singly charged dimers $[2M+H]^+$ may be formed by two analyte molecules at higher concentrations.[101] The linear range can sometimes be improved by using APCI instead of ESI.[101]

The accuracy of the method was initially poor for DMG (typically recoveries were around 70%) when using D_9-betaine as the internal standard. However, recoveries improved after obtaining D_3-dimethylglycine and using it as the internal standard for DMG. There were three peaks observed in the MRM for DMG in plasma, as was reported by Holm *et al.*[41] DMG and choline both have mass transitions of 104 → 58, therefore they need to be well separated chromatographically. While unmodified silica and strong cation exchange (SCX) columns did not separate choline and DMG, the silica hydride columns were found to have a good selectivity for separating then. Kirsch *et al.*[45a] showed a different selectivity on a Waters Acquity BEH HILIC column, with choline eluting before DMG. For methods that do not require the measurement of DMG, silica columns or SCX may perform as well. For example, the Kinetex HILIC silica column was found to be useful in an LC-MS/MS reference method which just measures creatinine. The Kinetex column is a core-shell column which is a technology that reduces peak broadening and gives good resolution in a short run time.

The lack of a commercially available isotopic standard for GPC could be problematic. While recoveries of 100% were initially observed using D_9-betaine as an internal standard, a slight change in the chromatography could allow ion interference to occur and affect the accuracy of the results. Koc *et al.*[99] synthesized a deuterated GPC internal standard from a hydrolysis of 1,2-dipalmitoyl-sn-3-glycerophosphocholine-[*N*,*N*,*N*-trimethyl-D_9]. However, 1,2-dipalmitoyl-sn-3-glycerophosphocholine-[*N*,*N*,*N*-trimethyl-D_9] is not commercially available in sufficient quantities for synthesis, so this was not considered to be an affordable or practical option.

The comparison of the present LC-MS/MS method with HPLC techniques generally showed good agreement for plasma. Comparison with LC-MS/MS shows that DMG concentrations are under-estimated by around 40% when measured by HPLC-UV using the Phenosphere SCX column. The lower plasma DMG results observed by the HPLC-UV method compared to LC-MS/MS are likely to be caused by interference in the HPLC assay. The DMG peak was often co-eluting with an interfering peak in the HPLC-UV method. There was high agreement for the HPLC creatinine method with LC-MS/MS, with no significant difference in the results detected by Passing-Bablok analysis. This shows that HPLC and LC-MS/MS are both suitable as reference methods for plasma creatinine. Comparison of data for

methylamines measured in urine using the different methods showed that HPLC-UV is prone to significant interference with large overestimates of concentrations for betaine and acetylcarnitine compared to mass spectrometric detection.

The tandem mass spectrometry (LC-MS/MS) methylamine results generally agreed well with single quadrupole mass spectrometry (LC-MS). However, the choline and acetylcarnitine results did not correlate as well ($r^2 < 0.7$). This is likely to be caused by sample instability after the samples were re-frozen and thawed between runs using the different methods. There were some unexpected outliers for many compounds, which may be due to sample stability, or it may be caused by interference in either the LC-MS, or LC-MS/MS assays. Metabolites such as acetylcarnitine and choline[41] are known to have relatively low stability in plasma samples. Because these samples were stored for several months between analyses at -20°C, sample stability is likely to be a factor in the poor agreement of the concentrations of some metabolites between methods.

The LC-MS, LC-MS/MS, and HPLC data were in agreement for plasma methylamines (Table 2.6). The precision was generally greater when using mass spectrometric detection, particularly in urine. However, the main advantage of mass spectrometry compared to HPLC is the improved selectivity. This was apparent in the urine where interferences can potentially go unnoticed. Spiked controls may not necessarily provide a good estimate of the accuracy of a method due to the presence of endogenous co-eluting peaks which are not separated chromatographically. Urine betaine results can be considerably higher in the HPLC results compared to LC-MS and LC-MS/MS in the urine (Table 2.7). Despite this, the HPLC gave a calculated recovery of 101% for betaine. Without a comparison with mass spectrometric data it would not be possible to know the degree of interference. The HPLC method often has co-eluting peaks which affect the accuracy and precision. However, it is not always obvious when there is a peak directly underneath the analyte of interest. The higher specificity that mass spectrometry provides is especially useful when measuring analytes in urine. Other advantages of mass spectrometry methods include relatively fast and efficient sample preparation, high specificity, and the ability to measure a range of methylamines with a lower flow rate and a shorter run time than traditional HPLC techniques. Problems encountered when measuring methylamines by mass spectrometry include ion suppression and non-linearity at higher concentrations. If urine methylamine concentrations are above the linear range, it is

necessary to further dilute samples before injection. Non-linearity was found to be less of a problem when using single quadrupole mass spectrometry. Tandem mass spectrometry has greater selectivity than single quadrupole mass spectrometry. However, in some cases, such as DMG and choline, the chromatographic separation of analytes is still important.

2.3. Methods for the measurement of both trimethylamine and trimethylamine-*N*-oxide

Background

Trimethylaminuria is a condition where the enzyme (flavin monooxygenase 3, FMO3) which converts trimethylamine (TMA) to trimethylamine-*N*-oxide (TMAO) is dysfunctional. Trimethylaminuria sufferers accumulate high levels of TMA in their urine and sweat. The incidence of trimethylaminuria is thought to be as high as 1 in 1000 people. However, it often goes on for many years undiagnosed, when people may avoid socialising because of the fishy smelling TMA present in the sweat.[102] While there is no cure for trimethylaminuria, the condition can be effectively managed by antibiotics, and a diet which avoids foods high in TMAO (*i.e.* seafood) and choline (*e.*g. eggs).[56, 102] Diagnosing trimethylaminuria (or fish odour syndrome) requires the reliable measurement of trimethylamine and trimethylamine-*N*-oxide in urine. An oxidising ratio $< 84\%$ indicates that a patient has trimethylaminuria (whereas, the oxidising ratio is defined as: $([TMAO]/([TMAO]+[TMA]) \times 100)$.[56]

TMA and TMAO have previously been measured by gas chromatography (GC),[103] NMR spectroscopy,[40, 104] and by LC-MS/MS.[105] NMR spectroscopy can lack the sensitivity to measure TMA in samples from patients who are not sufficiently loaded with TMAO (after being asked to consume a meal of marine fish), meaning there is potential for a false negative result to be obtained for trimethylaminuria diagnosis.

While TMAO can be measured directly by LC-MS/MS,[45b] TMA is more problematic. TMA is a small molecule which does not fragment sufficiently for MS/MS detection, and a noisy baseline signal provides low sensitivity with selected ion monitoring or single quadrupole mass spectrometry. Acetonitrile can form adducts with ammonium ions to produce a signal at $m/z = 59$ which may interfere with the TMA signal at $m/z = 60$. TMA also has a similar mass to acetamide which may be produced when acetonitrile in the mobile phase hydrolyzes (Fig. 2.11, **14**). The hydrolysis of acetonitrile to acetamide is a slow reaction that requires a catalyst under normal conditions.[106] However, even a small concentration of acetamide in the mobile phase could help explain the high background signal observed when monitoring the molecular ion expected for TMA at $m/z = 60.1$.

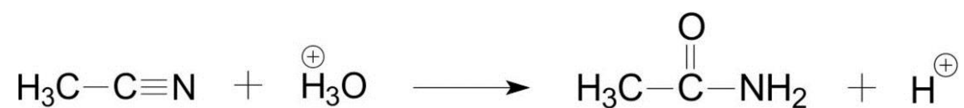


Fig. 2.11. Hydrolysis of acetonitrile to give the acetamide (**14**) product with a similar mass to TMA.

Derivatization of TMA can be used to overcome the problem of high baseline interference when measuring TMA by mass spectrometry. TMA can be converted to a quaternary ammonium compound which readily ionizes and produces high sensitivity for mass spectrometric detection.

The aim of this section is to develop a method that can measure both TMA and TMAO in urine for diagnosis of trimethylaminuria. However, because TMAO is reported to be an important risk marker in cardiovascular disease, it is also an aim to see if its precursor TMA can be reliably measured in blood plasma, where the concentrations are expected to be low. TMA concentrations have not been well studied in plasma, which is likely to be because it is analytically challenging to measure the expected low concentrations.

Derivatization of TMA with alkyl halides

Introduction

The aim here is to derivatize TMA with an alkyl halide to form a quaternary ammonium compound that can be measured reliably by tandem mass spectrometry (LC-MS/MS) or single quadrupole mass spectrometry (LC-MS). Results obtained by this method will be compared to those obtained by ^{1}H NMR.

Quaternary amines such as proline betaine have been synthesized by reaction of tertiary amines with alkyl halides in the presence of silver oxide.[107] TMA has previously been derivatized with alkyl halides such as: ethyl bromoacetate[105] or trideuteromethyl iodide,[108] and the TMA derivative measured by LC-MS/MS. Here, the substitution reaction of TMA with the alternative derivatizing agent iodoacetonitrile was investigated. Iodide is a much better leaving group than bromide, meaning the reaction proceeds more easily. Iodoacetamide was also considered as a possible derivatizing reagent for TMA. However, the derivative formed by the reaction of TMA with iodoacetamide forms a quaternary amine compound with a mass close to D_3-creatinine (117.2 Da), and only one mass unit from the betaine molecular ion (118.2 Da). The MS/MS transition from a quaternary amine to a trimethylamine species fragment is usually the most intense signal. The species formed by the reaction with TMA (Fig. 2.12, **15**) and iodoacetonitrile, cyano-*N*,*N*,*N*-trimethylmethanaminium (**16)** has not been reported to exist in nature and is therefore unlikely to be present in biological samples. Whether silver oxide (or another silver salt) is necessary to catalyze the reaction was investigated.

Fig. 2.12. Reaction of trimethylamine (**15**) with iodoacetonitrile to form the derivative (**16**).

Methods

Materials and chemicals

TMA (HCl) and TMAO (dihydrate) were obtained from Sigma. D_9-TMA (HCl) was purchased from CDN Isotopes, and D_9-TMAO (free base) was purchased from Cambridge Isotope Laboratories. Disodium hydrogen phosphate (anhydrous), acetonitrile, and methanol were obtained from Merck (Darmstadt, Germany).

Reaction conditions

To investigate the optimal reaction conditions, a standard containing 100 µmol/L TMA and 100 µmol/L TMAO was reacted with iodoacetonitrile. The effect of iodoacetonitrile concentration, reaction time, and reaction temperature were investigated. The presence of: silver oxide; silver carbonate; or silver sulfate in the reaction mixture was also investigated. A control with no silver salt added was used as a comparison. Each silver salt was made up with Na_2HPO_4 (10:90 w/w), and a spatula (approximately 300 mg) added to the samples separately. For the samples without a silver salt, just Na_2HPO_4 was added. The samples were analyzed in triplicate.

A 1 mmol/L aqueous standard containing just TMAO was placed through the reaction procedure with various concentrations of iodoacetonitrile to investigate if TMAO also reacts with the derivatizing reagent.

Sample preparation

Samples and standards were diluted ten-fold in distilled water. An extraction / derivatizing solution was made up containing 40 mmol/L iodoacetonitrile (Sigma), 20 µmol/L D_9-TMA, 20 µmol/L D_9-TMAO, in 90% acetonitrile and 10% methanol. Fifty µL of diluted sample or standard was added to 500 µL of the extraction / derivatizing solution, and a spatula of Na_2HPO_4 (approximately 300 mg) was added as a drying agent. The samples were then placed on a shaker for 5 minutes and centrifuged (13,000 × *g*, 3 minutes). Fifty µL of the supernatant was added to 500 µL of acetonitrile and 250 µL was transferred to HPLC vials and crimp capped for analysis by LC-MS or LC-MS/MS.

Aqueous calibration standards were run with each batch containing known levels of TMA and TMAO. Standard 1 contained 100 µmol/L TMA and 500 µmol/L

TMAO; Standard 2 contained 200 µmol/L TMA and 1000 µmol/L TMAO; Standard 3 contained 500 µmol/L TMA and 5000 µmol/L TMAO.

LC-MS/MS

The chromatography was performed using a 100 × 2.1 mm, 4 µm Cogent Diamond Hydride silica column (Microsolv Technologies, NJ, USA). A gradient was used with a Shimadzu Prominence binary pump system. Solvent A contained 10 mmol/L ammonium acetate, 10 mmol/L formic acid and 50% water and 50% acetonitrile. Solvent B contained 90% acetonitrile and 10% water. The gradient started with 60% solvent A, going to 100% solvent A over 7 minutes, then back to starting conditions at 7.1 minutes. Detection was carried out using an AB Sciex API4000 tandem mass spectrometer. The mass transitions used for detection are shown in Table 2.8. The data was quantified using peak area ratios of the analytes to their respective deuterated internal standards using Analyst (ABSciex) software.

Table 2.8. LC-MS/MS mass transitions and potentials used.

	Mass transition	DP (V)	CE (V)	CXP (V)
TMA-IACN derivative	99.1 → 58.1	51	35	10
D_9-TMA-IACN derivative	108.1 → 66.1	51	39	4
TMAO	76.1 → 58.1	16	27	10
D_9-TMAO	85.2 → 66.0	91	29	2

Abbreviations: decoupling potential (DP); collision energy (CE); collision cell exit potential (CXP).

Single quadrupole LC-MS

An Agilent 1200 Series HPLC system connected to an Agilent 6120 Quadrupole mass spectrometer were used for the analysis. A 100 × 2.1 mm, 4 µm Cogent Diamond Hydride silica column (Microsolv Technologies, NJ, USA) was used for the separation. Solvent A contained 10 mmol/L ammonium acetate, 10 mmol/L formic acid and 50% water and 50% acetonitrile. Solvent B contained 90% acetonitrile and 10% water. A gradient was used starting with 60% solvent A, and going to 100% solvent A over 7 minutes. The flow rate was 0.3 mL/min, the injection volume was 10 µL, and the oven temperature was set to 40ºC. Samples were diverted to waste post-column for one minute after injection, and the total run time was 8 minutes. The mass

to charge *m/z* values used for selected ion monitoring (SIM) were: TMAO = 76.1, D_9-TMAO = 85.1, $TMA_{(iodoacetonitrile\ derivative)}$ = 99.1, D_9-$TMA_{(iodoacetonitrile\ derivative)}$ = 108.1. The capillary voltage was set to 3500V. The data was quantified using peak area ratios of the analytes to their respective deuterated internal standards using Agilent Chemstation software.

Method validation

Precision

A normal urine sample, and a urine sample with 500 µmol/L TMA and 1000 µmol/L TMAO added, were analyzed by tandem mass spectrometry LC-MS/MS using an AB Sciex API4000. A normal urine sample, and a urine sample with 1000 µmol/L TMA and 2000 µmol/L TMAO added, were analyzed by single quadrupole mass spectrometry (LC-MS) using an Agilent 6120 mass spectrometer. Six batches containing four replicates of the normal urine, and urine with elevated concentrations, were analyzed to determine the intra-assay and inter-assay co-efficient of variation (CV) for both TMA and TMAO.

Accuracy

Recoveries were calculated from TMA and TMAO added to urine samples. The limit of detection was calculated as the concentration when the signal to noise ratio equalled three.

Linearity

Various concentrations of TMA (50, 125, 250, 500, 750, and 1000 µmol/L) and TMAO (100, 250, 500, 1000, 1500, and 2000 µmol/L) were added to urine, the samples were measured in triplicate by the current LC-MS/MS method, and the linearity of the assay was investigated using linear regression analysis of the data.

Results

Reaction conditions

The use of a silver salt decreased the yield of the TMA derivative (Fig. 2.13). The yield of the TMA derivative decreased with reaction time whether a silver salt

was present or not. However, allowing the samples to react for 5 minutes gave the drying agent some time to work, and the standard error of the measurements were lower. Addition of silver carbonate produced the lowest yield of derivative after 5 minutes of reaction.

The peak area of the TMA iodoacetonitrile derivative was optimized at around 40 mmol/L iodoacetonitrile (Fig. 2.14A). Increasing the concentration of iodoacetonitrile above this improved the yield slightly more, but also increased the cross reaction, where TMAO reacts with the derivatizing reagent (Fig. 2.14B). This reaction was increased with increasing concentration of iodoacetonitrile with the ratio of apparent TMA present in a TMAO standard over 6% at 100 mmol/L of iodoacetonitrile. A concentration of 40 mmol/L or less iodoacetonitrile is required to ensure that the cross reaction is under 1%.

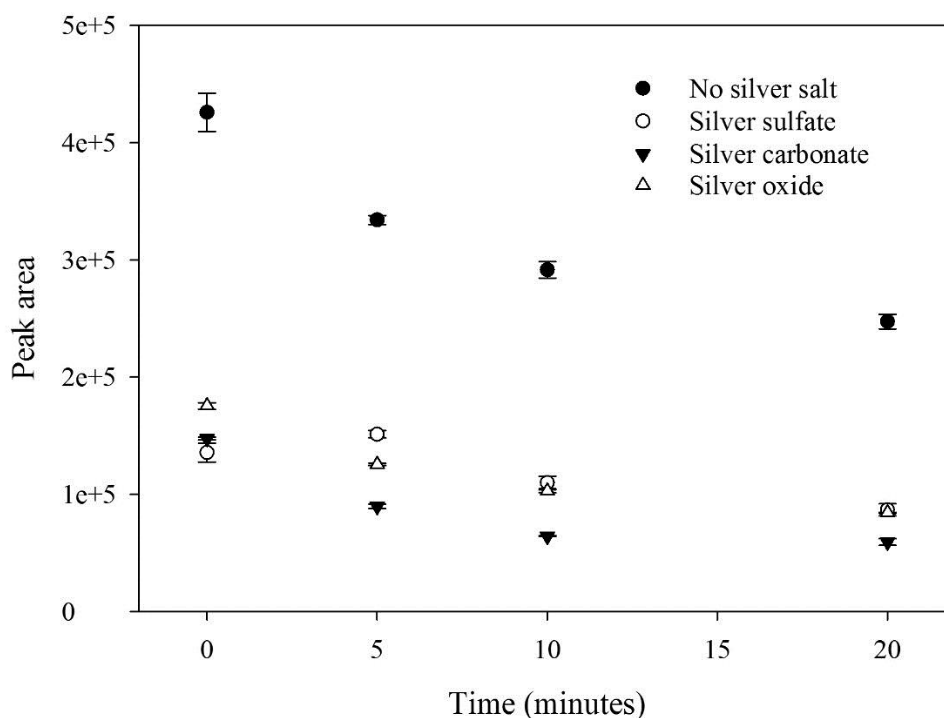
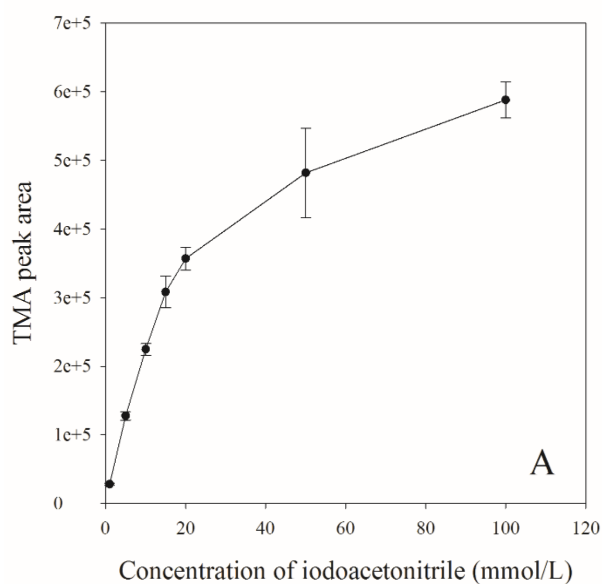


Fig. 2.13. The effect of silver salt and reaction time on the TMA (iodoacetonitrile derivative) peak area. Error bars represent standard errors.



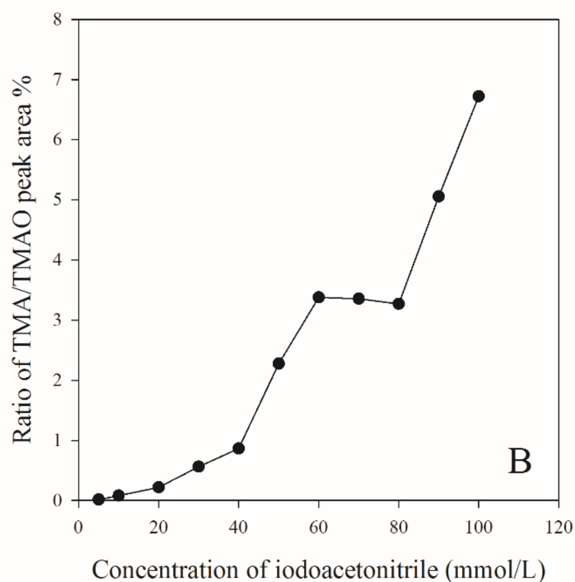


Fig. 2.14. The effect of iodoacetonitrile concentration on the TMA derivative using an aqueous standard containing 100 µmol/L TMA (A). The mean of three replicates ± SE are shown for each concentration of derivatizing reagent. The ratio of TMA/TMAO peak area in a pure aqueous 1 mmol/L TMAO standard over different concentrations of derivatizing reagent (iodoacetonitrile) (B).

LC-MS/MS

Multiple reaction monitoring (MRM) chromatograms are shown for the TMA iodoacetonitrile derivative and TMAO in Fig. 2.15. The TMA derivative elutes at around 2 minutes in the chromatogram.

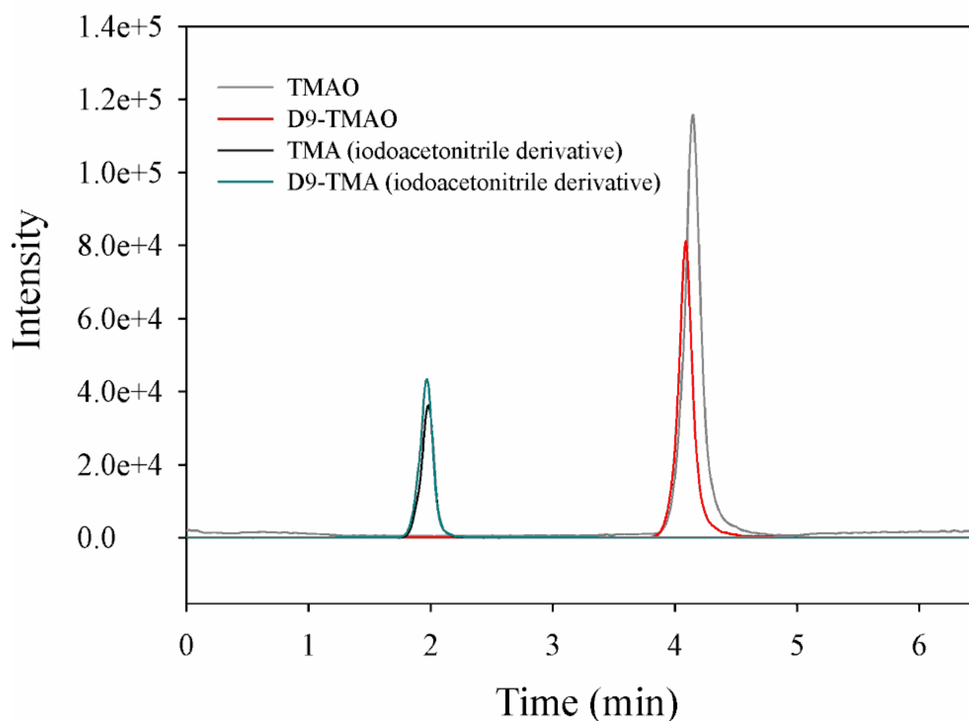


Fig. 2.15. LC-MS/MS chromatograms showing TMA and TMAO after derivatization of TMA with iodoacetonitrile.

Precision and accuracy

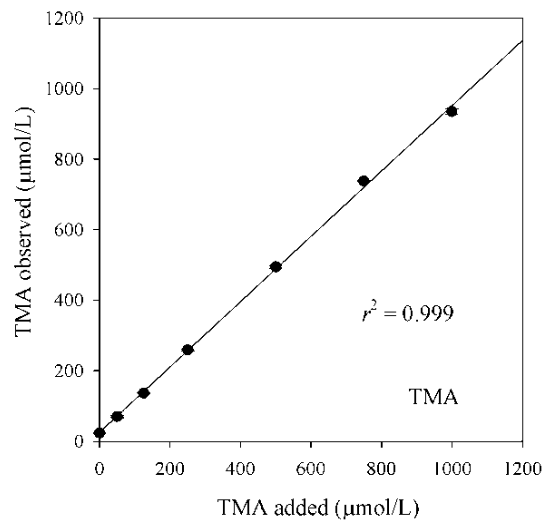
The CVs were below 8.3% for TMA and TMAO, except for the lower level of TMA where the CV was 16%. The recoveries were over 95% for TMA and TMAO in urine (Table 2.9). The limit of detection was 0.4 μmol/L for TMA and 5.2 μmol/L for TMAO.

Table 2.9. Method performance for the measurement of TMA and TMAO.

	TMA		TMAO	
	Normal level	High level	Normal level	High level
Mean (µmol/L)	8.3	525	183	1147
Within batch CV%	16.0	2.1	1.9	5.7
Between batch CV%	8.2	7.6	8.1	6.5
Recovery		103%		96%

Linearity

The linearity of TMA and TMAO added to urine is shown in Fig. 2.16. TMA added to urine and measured as the iodoacetonitrile derivative gave high linearity up to 1000 µmol/L with r^2 = 0.999. TMAO also showed high linearity up to 2000 µmol/L with an r^2 of 0.998. The assay was non-linear at higher concentrations, with the response in aqueous standards showing significant tailing off at 2000 µmol/L for TMA, and 3000 µmol/L for TMAO.



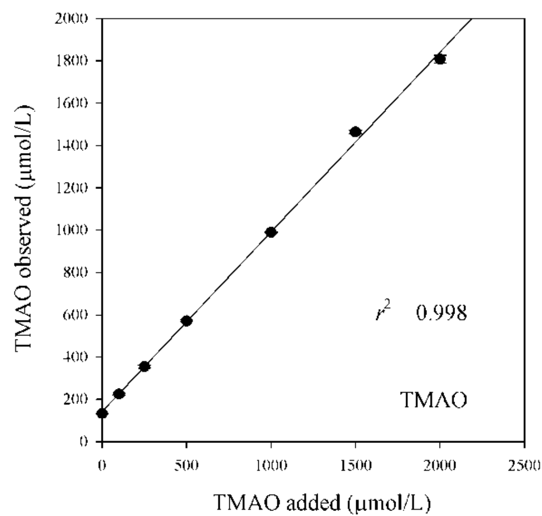


Fig. 2.16. Linearity of the TMA and TMAO added to urine and measured by LC-MS/MS. Mean concentrations are shown with error bars representing standard errors.

Single quadrupole LC-MS

Extracted ion chromatograms (EIC) are shown for the TMA iodoacetonitrile derivative and TMAO in Fig. 2.17. The TMA derivative elutes at around 2 minutes in the chromatogram.

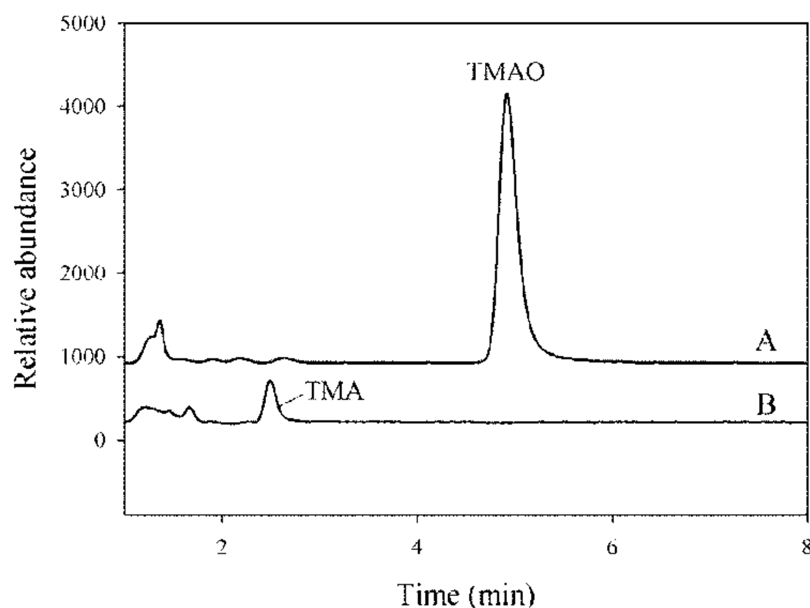


Fig. 2.17. A urine sample showing extracted ion chromatograms (EIC) at A, $m/z = 76$, and B, $m/z = 99$ after derivatization with iodoacetonitrile.

Precision and accuracy

The CVs were below 7.9% for TMA and 2.9% for TMAO. The recoveries were over 91% for TMA and TMAO in urine (Table 2.10). The limit of detection ($S/N = 3$) was 1.3 µmol/L for TMA and 1.0 µmol/L for TMAO.

Table 2.10. Method imprecision and accuracy for the measurement of TMA and TMA by single quadrupole LC-MS.

	TMA		TMAO	
	Normal level	High level	Normal level	High level
Mean (µmol/L)	17.4	939	197	2109
Within batch CV%	5.0	1.6	2.8	1.4
Between batch CV%	3.5	7.8	2.8	1.6
Recovery		92%		94%

Linearity

The TMA added to urine and measured as the iodoacetonitrile derivative showed high linearity up to 1000 µmol/L with $r^2 = 0.999$. TMAO also showed high linearity up to 2000 µmol/L with an r^2 of 0.999 (Fig. 2.18).

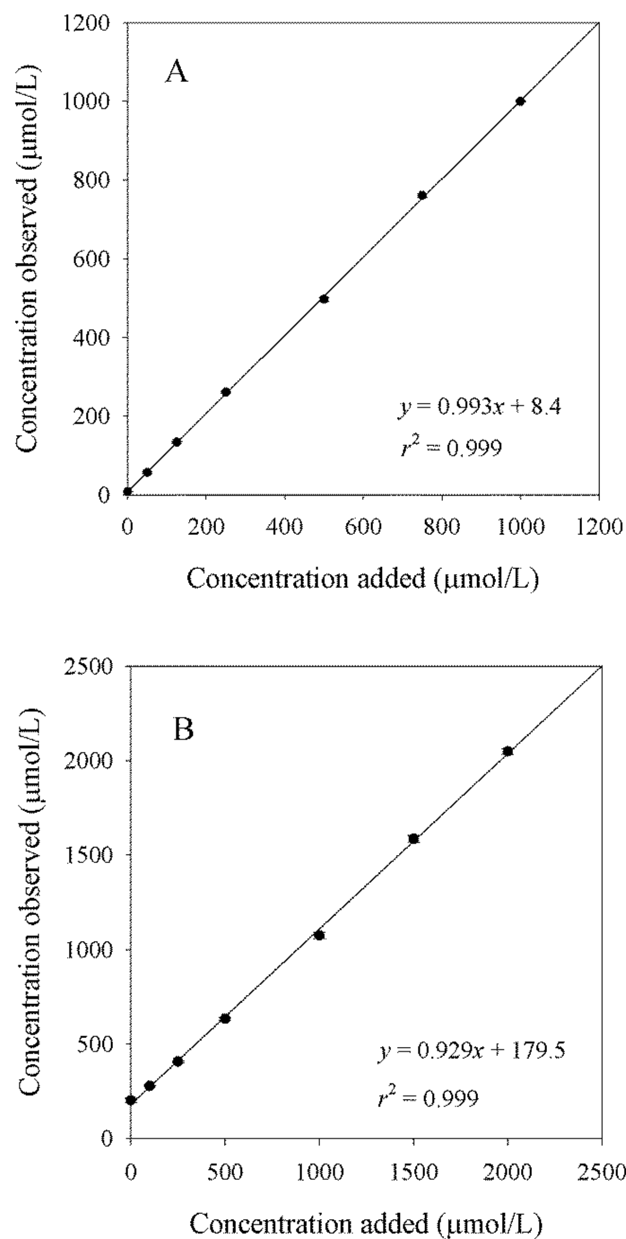


Fig. 2.18. Linearity of: A, TMA, and B, TMAO added to urine and measured by LC-MS. Mean concentrations are shown with error bars representing standard errors.

Discussion

Alkyl halides are useful for the derivatization of tertiary amines such as trimethylamine. Iodo-compounds have previously been used to quaternize secondary and tertiary amines for the synthesis of betaine analogues such as proline betaine[107]

and homarine.[45b] Here, iodoacetonitrile is used to derivatize TMA for analysis by LC-MS or LC-MS/MS. The addition of the acetonitrile group to the TMA molecule makes it easily fragment in the mass spectrometer and produce good precision, accuracy, and linearity for the assay. The observation that the yield of the TMA-iodoacetonitrile derivative decreased with reaction time suggests that an undesirable secondary reaction may be occurring which destroys the derivative. The methylene group between the amine nitrogen and the nitrile group may under-go further reaction. This appears to be exacerbated by the use of silver salts which produced considerably lower derivative yields (see Fig. 2.13). The TMA derivative was least stable in the presence of silver carbonate, suggesting that the degradation reaction may be base catalysed. After centrifuging the samples and removing the Na_2HPO_4 and silver salts, the TMA derivative was stable, and the assay was quantitative.

The limit of detection for trimethylamine using tandem mass spectrometry is much lower than for NMR methods,[109] meaning a clinically meaningful result for a TMA/TMAO ratio can be achieved for trimethylaminuria, even on a sample from a patient that was not well TMAO loaded. The sensitivity is much improved by using LC-MS (or LC-MS/MS) compared to NMR. However, the linear range was found to be much narrower with tandem mass spectrometry. The single quadrupole mass spectrometer had a greater linear range than the tandem mass spectrometer. While it may be necessary to dilute samples with concentrations greater than 2 mmol/L TMA or TMAO, the advantage of tandem mass spectrometry is the higher specificity associated with measuring mass transitions compared to monitoring the parent mass (SIM), meaning there is less chance of having interfering peaks from other compounds in the chromatograms.

The observed cross reaction resulting in the formation of the TMA derivative from TMAO (likely *via O*-alkylation mediated deoxygenation) was problematic, but could be kept to less than 1% by using a concentration of derivatizing reagent of 40 mmol/L. This was considered sufficient for the reliable diagnosis of trimethylaminuria from the analysis of urine. Urine samples analyzed for the diagnosis trimethylaminuria often contain high TMAO levels, unless the patient is homozygous positive and contains more TMA than TMAO. To adequately diagnose trimethylaminuria, patients are required to consume a marine fish meal before sample collection.[56]

Unfortunately, the unwanted reaction of TMAO with alkyl halides is problematic for the measurement of TMA in plasma. Plasma TMA concentrations are reported to range from 0.29 to 1.66 μmol/L[110] and are considerably lower than plasma TMAO concentrations, so any interference from TMAO will affect the accuracy of the method. Therefore this method was not considered sufficiently reliable to measure TMA in plasma samples.

Derivatization of TMA with alkenes

Introduction

Tertiary amines such as trimethylamine can undergo reactions with alkenes such as acrylic acid, acrylamide, or acrylonitrile (Fig. 2.19) to form cationic quaternary amines.

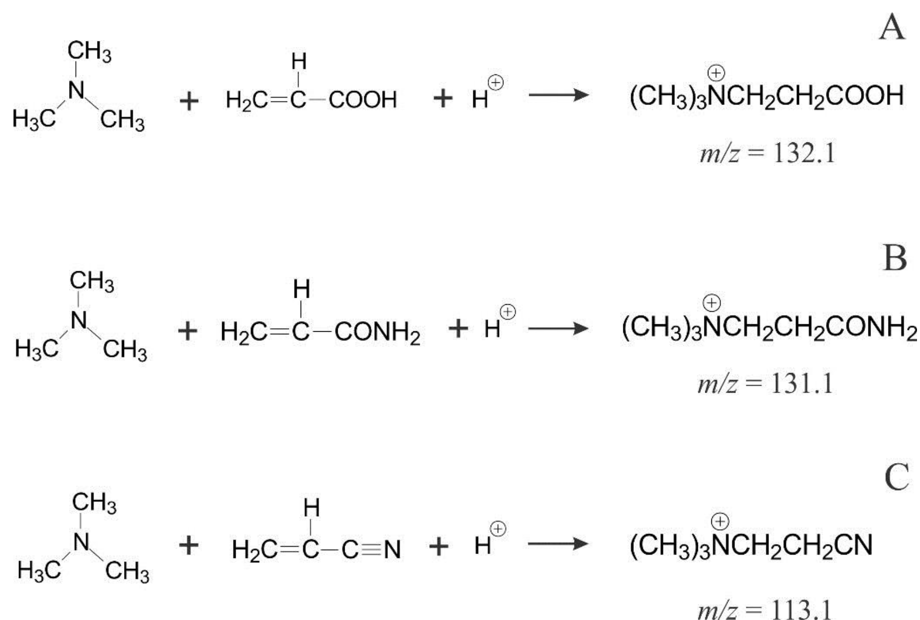


Fig. 2.19. Reaction of TMA with acrylic acid (A), reaction of TMA with acrylamide (B), and reaction of TMA with acrylonitrile (C).

The aim here is to investigate whether these alkenes can react with TMA. It is hypothesized that TMAO will not react with these reagents to form the same derivative. This may allow for a more reliable method to be developed for the measurement of TMA in plasma.

Methods

A 100 µmol/L aqueous standard of TMA was derivatized with either: acrylic acid, acrylamide, acrylonitrile, or methacrylic acid (Sigma). To find the optimum concentration of reagent for the reaction, various concentrations of alkene reagent were added to acetonitrile, 50 µL of the TMA standard was added, and a spatula (approximately 250 mg) of disodium hydrogen phosphate (anhydrous) was added. The samples were mixed on a vortex mixer for 5 minutes, then centrifuged (13000 ×

g, 5 minutes). The effect of concentration of these reagents on peak area was investigated. Alternatives to disodium hydrogen phosphate were investigated including: no drying agent, sodium dihydrogen phosphate (monohydrate), tripotassium orthophosphate, sodium sulfate, sodium carbonate (anhydrous).

The TMA derivatives were detected using an Agilent 6120 single quadrupole mass spectrometer in positive ion mode with electrospray ionization. The acrylic acid TMA derivative (propiobetaine) was monitored at $m/z = 132.1$, the acrylamide derivative (trimethylammonium propylamide) was monitored at $m/z = 131.1$, and the acrylonitrile derivative was monitored at $m/z = 113.1$. The optimized mass spectrometer parameters were: fragmentor voltage 100 V, capillary voltage 2000 V, drying gas temperature 325°C, drying gas flow rate 9 L/minute, and nebulising pressure 25 psig. Chromatography was performed on a Cogent diamond hydride with a gradient of 10 minutes going from 50% A to 100% A. Solvent A contained 10 mmol/L ammonium formate, 10 mmol/L formic acid, and 50 % water and acetonitrile. Solvent B contained 10% water and 90% acetonitrile. The flow rate was 0.3 mL/min.

Results and discussion

The separation of TMA alkene derivatives on the diamond hydride silica column is shown in Fig. 2.20.

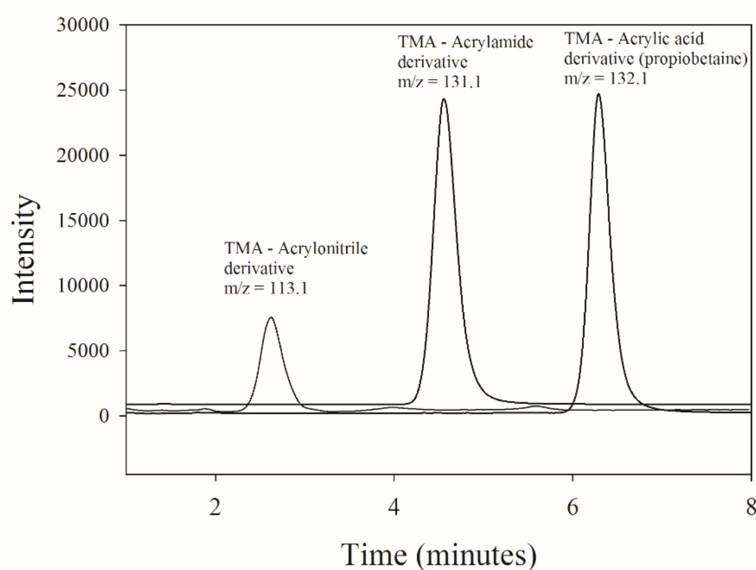


Fig. 2.20. Ion chromatograms showing the chromatography of the acrylonitrile, acrylamide, and acrylic acid derivatives of TMA. Derivatized 100 µmol/L aqueous TMA standards are shown.

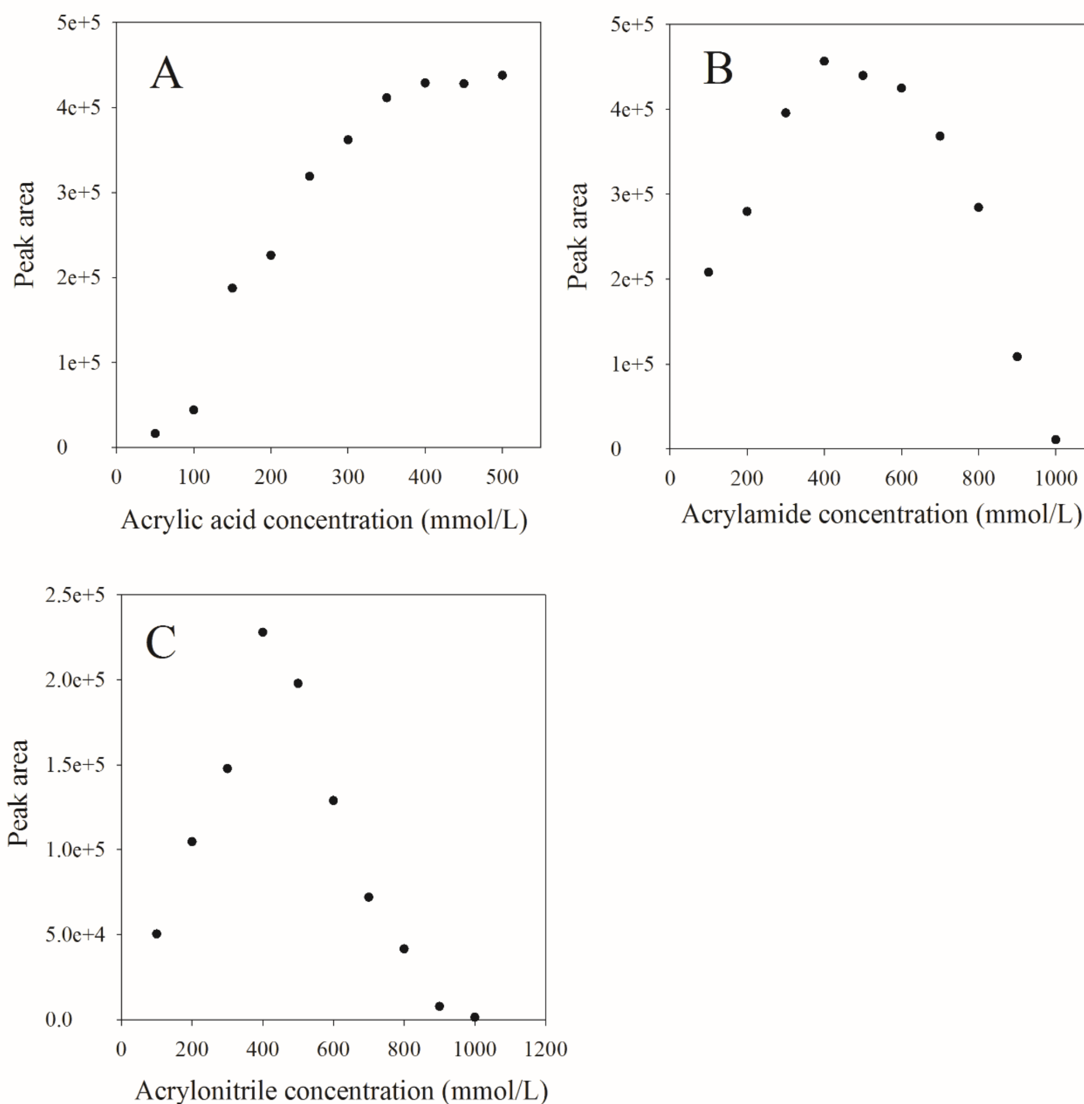


Fig. 2.21. Effect of concentration of acrylic acid on the TMA peak area measured as the acrylic acid derivative (A). Effect of concentration of acrylamide on the TMA peak area measured as the acrylamide derivative (B). Effect of acrylonitrile concentration on the TMA peak area measured as the acrylonitrile derivative (C).

The optimum concentration of acrylic acid, acrylamide, or acrylonitrile was approximately 400 mmol/L in the reaction solvent (Fig. 2.21). The response decreased at greater concentrations of derivatizing reagent, suggesting that an undesirable reaction may be occurring which destroys the derivative. The interference in the mass spectrometer from the excess alkenes may be problematic at higher concentrations. While, these alkenes have fairly low boiling points, some polymerization is likely to be occurring inside the mass spectrometer. Adding disodium hydrogen phosphate

produced the largest peak area for the TMA derivatives. Addition of sodium carbonate only produced a small TMA derivative peak with acrylamide. In the absence of drying agent no product peaks were observed (data not shown). The reaction of trimethylamine with alkenes is acid catalyzed. However, the addition of the more acidic reagent sodium dihydrogen phosphate produced a lower yield of derivative than disodium hydrogen phosphate, possibly by protonating trimethylamine. The sodium dihydrogen phosphate used was not anhydrous, so would not be an effective drying agent. While the reaction requires the presence of hydrogen ions to proceed, it is likely that adding too much acid protonates TMA, whereas the reaction requires the uncharged form of trimethylamine. This would explain why the reaction does not proceed as well under strong acid conditions.

There was no TMA peak observed when injecting a pure TMAO standard derivatized with acrylic acid or acrylamide, showing that TMAO does not react with the alkenes to produce the same derivative as TMA.

The use of acrylamide is preferable to acrylic acid. While both reagents derivatize TMA efficiently, acrylic acid reacts with TMA to produce propiobetaine (sometimes referred to as β-alanine betaine),[111] which is used as an osmolyte in plants, and may therefore be present in the diet. Any endogenous propiobetaine in the samples will give the appearance that more TMA is present than there actually is, potentially leading to a false diagnosis. The lesser known product of acrylamide and TMA, trimethylammonium propylamide, has not reported to be found in the diet. Acrylamide has been reported as a cancer risk in foods where it is found as a by-product of cooking.[112] Acrylamide is not likely to be present in human samples in concentrations that would significantly interfere with the TMA reaction, whereas a concentration of 400 mmol/L of acrylamide is required for complete reaction with TMA.

After injecting multiple samples containing acrylamide or the other alkenes, the signal of the trimethylamine derivative dropped, and the mass spectrometer was found to be visibly dirty with contamination across a wide mass range after the injection of multiple samples containing acrylamide reagent. It is likely that excess reagent is polymerizing and accumulating inside the mass spectrometer, which required the source and capillary to be thoroughly cleaned. For this reason, acrylamide and related alkenes may not be appropriate for use as derivatizing reagents for mass spectrometric detection.

Improved measurement of trimethylamine and trimethylamine-N-oxide in urine by nuclear magnetic resonance spectroscopy

Introduction

Nuclear Magnetic Resonance (NMR) spectroscopy has been traditionally used to measure TMA and TMAO for the diagnosis of trimethylaminuria.[56, 105b] NMR has advantages over other techniques because of simple sample preparation and the ability to measure TMA and TMAO simultaneously in a single spectrum. However, the measurement of TMA and TMAO in urine has previously been problematic. Acidification of the sample is required to separate the methyl resonances of TMAO and betaine in the spectrum.[40, 56] However, acidification of the samples has the side effect of protonating TMA which splits the peak into a doublet, therefore increasing the detection limit and reducing the sensitivity. Ways to prevent the TMA from splitting were investigated. Furthermore, the TMA peak is present on the side of a peak from the citric acid quadruplet, which reduces the sensitivity of the TMA assay even more.

Improvements to the analysis of TMA and TMAO by NMR were made, and this method was compared with the mass spectrometry methods that have been developed.

Methods

Reagents and chemicals

Trimethylacetamide and deuterium oxide (D_2O) were obtained from Sigma. Aluminium chloride was purchased from BDH (Poole, England). Hydrochloric acid was obtained from Merck.

Sample preparation

An NMR reagent was made up by adding 500 mmol/l of HCl, 760 mmol/L of $AlCl_3$, and 10 mmol/L trimethylacetamide to D_2O. This solution was centrifuged and stored in the fridge for up to three months. Two hundred µL of the NMR solution was added to 600 µL of urine sample or aqueous standard. Samples were then centrifuged

(11,000 *g*) and 700 µL was transferred to 5 mm NMR tubes and capped. Samples were measured within four hours of being prepared.

NMR analysis

^{1}H NMR spectra were obtained using a Varian INOVA 500 MHz NMR spectrometer. Spectra were obtained using the method described in Lee *et al*. [7]. A water suppression technique (PRESAT) was used to remove the large water signal in the spectra. Eight transients were collected with a 5 second delay. The trimethylacetamide methyl resonance singlet peak was set to 1.0 ppm in each spectra. Analyte peaks were integrated and peak area ratios to the internal standard were used for quantification. To standardize the peaks, the internal standard was set to 100 arbitrary units in each spectrum. Aqueous standards containing known concentrations of TMA and TMAO were run with each batch to construct calibration curves and calibrate the data.

Validation

Eight replicates of a urine sample with a high and a low added level of TMA and TMAO were run and a CV was calculated to show the precision of the method. The low urine sample contained an added level of 50 µmol/L TMA and 250 µmol/L TMAO. The high urine sample contained an added level of 500 µmol/L TMA and 2.000 mmol/L TMAO. The linearity of the method was shown by running three replicates of four different levels of TMA and TMAO by NMR. Accuracy of the method was indicated by the recovery of TMA and TMAO added to urine.

Results

Reagents

Only aluminium chloride was effective in removing the citrate peaks from the spectrum and stopping the TMA methyl resonance from splitting. The use of aluminium chloride hexahydrate did not achieve this. The citrate peaks were still present and TMA methyl resonance was still split when aluminium chloride hexahydrate was used. The reagents, aluminium potassium sulfate, and aluminium ammonium sulfate were also tried in the method, however they were not sufficiently soluble to make up the desired concentration.

NMR spectroscopy

Adding aluminium chloride to the urine samples before NMR analysis stopped the TMA peak from splitting, and a singlet peak was observed for TMA rather than a doublet (Fig. 2.22). The citric acid peaks were removed from the spectrum after aluminium was added to the sample (Fig. 2.22B). TMA was observed at 2.89 ppm, and TMAO was observed at 3.54 ppm when the trimethylacetamide internal standard was set to 1.18 ppm and TSP (trimethylsilyl-2,2,3,3-tetradeuteropropionic acid) was set to 0 ppm.

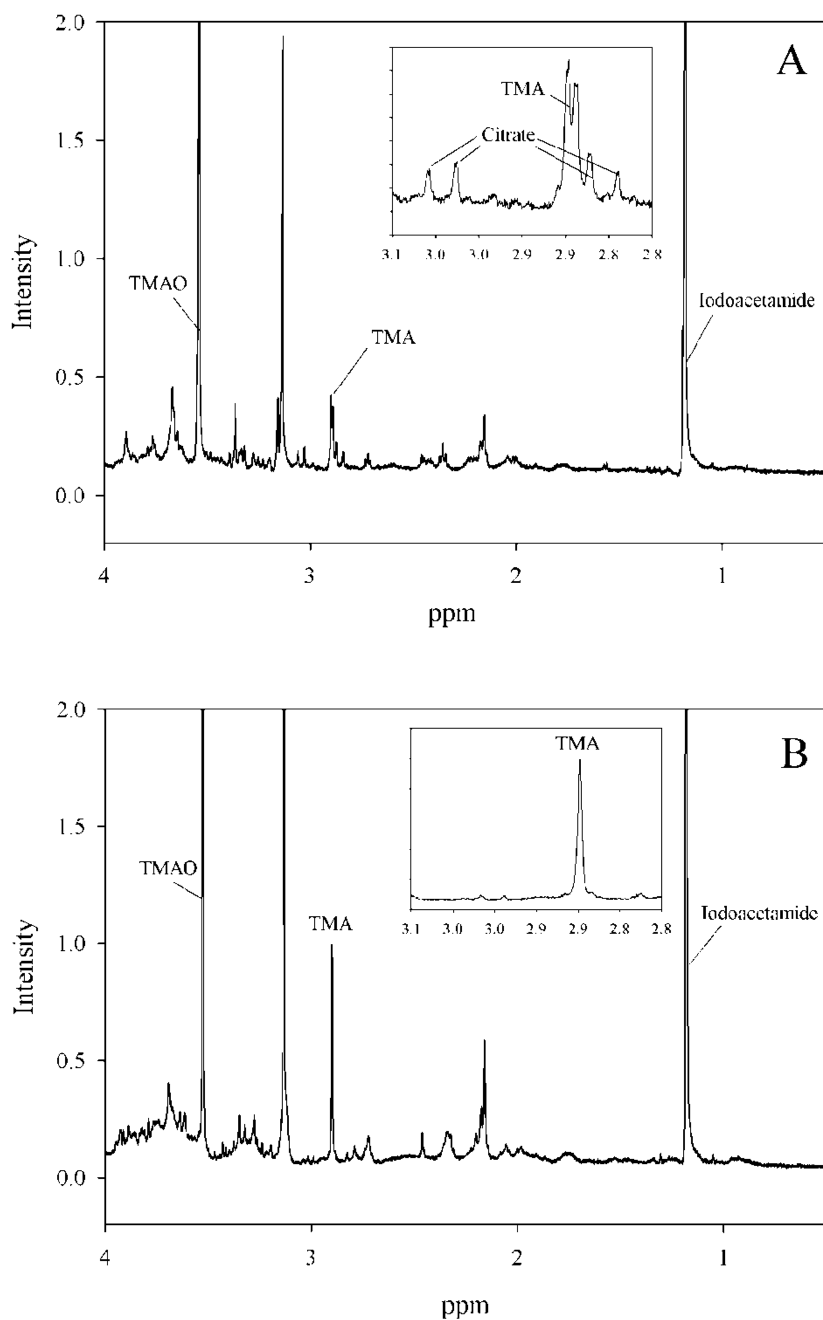


Fig. 2.22. NMR spectra of a urine sample without $AlCl_3$ added (A), and with $AlCl_3$ added (B). The urine sample shown had 500 μmol/L TMA and 2.0 mmol/L TMAO added.

Method validation

Precision

The coefficient of variation (CV) for TMAO was 4.7% for a urine concentration of 0.760 mmol/L, and the CV was 1.7% at a higher concentration of 2.50 mmol/L in urine. The coefficient of variation for TMA in urine was 8.1% at a concentration of 85 µmol/L. The coefficient of variation for TMA in urine was 4.0% at a concentration of 532 µmol/L in urine.

The limit of detection for TMA was 16 µmol/L using aluminium chloride, and 36 µmol/L without aluminium chloride. The detection limit for TMAO was 14 µmol/L.

Accuracy

The recovery of TMA added to urine was 97%. The recovery of TMAO added to urine was 96%.

Linearity

The linearity of the method was indicated by an r^2 value of 0.994 for TMA and an r^2 value of 0.996 for TMAO (Fig. 2.23).

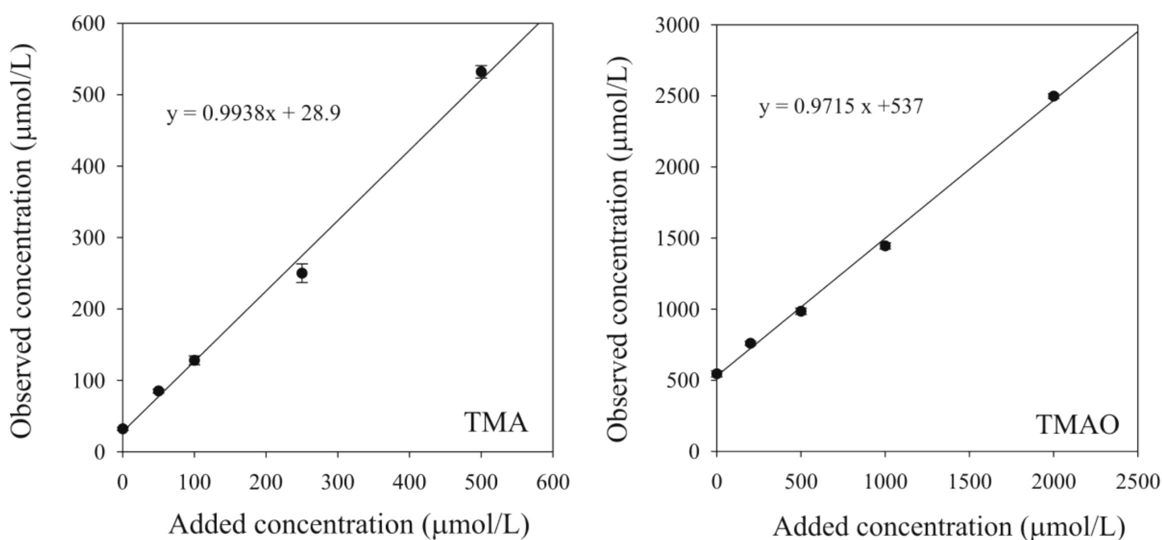


Fig. 2.23. Linearity of TMA and TMAO added to urine and measured by NMR spectroscopy. The error bars represent standard errors.

Discussion

An improved method for the measurement of TMA and TMAO by NMR spectroscopy is described here. NMR spectroscopy is useful for measuring a range of metabolites simultaneously. However, it has the disadvantage that the sensitivity is intrinsically low compared to other techniques such as mass spectrometry. Improved sensitivity may be achieved by increasing the number of transients collected when obtaining the NMR spectrum. However, this makes the acquisition times very long, and the gain is only small. In order to be able to quantify TMA in a urine sample, it is important that the sample is collected after a TMAO load. Usually the patient is asked to eat a meal of marine fish, which is high in TMAO, before sample collection.[56]

It has been documented that there is a need to acidify the samples to separate the methyl resonances of TMAO and betaine.[40] However, acidifying the sample has the side effect of protonating TMA and splitting the TMA peak into a doublet. By adding aluminium ions which coordinate with TMA and make TMA appear as a singlet rather than a doublet in the spectra of acidified samples, the peak height is increased and the sensitivity is improved.

Citric acid is known to complex with metal ions. For example, Sambano *et al*.[113] used lithium ions to complex citric acid and showed that citric acid can be removed from an NMR spectrum by adding lithium chloride. The addition of aluminium ions to the sample has two benefits. Firstly, it prevents TMA from protonating at low pH and causing peak splitting, and secondly, it complexes with citrate and removes this interference from the samples. The citrate ^{1}H NMR signal interferes with the TMA resonance in the spectrum. When the citrate complexes with aluminium ions, it precipitates out of solution and is removed by centrifugation of the sample prior to analysis by NMR spectroscopy. The two methylene groups in citric acid are not equivalent and produce an AB quartet system. These peaks may vary in position depending on the field strength of the NMR spectrometer. However, they were found interfere significantly when measuring TMA on a 500 MHz NMR system.

The use of an appropriate internal standard is also important for achieving reproducible results. Trimethylacetamide (pivalamide) is a non-volatile solid that has nine identical protons, as do the analytes TMA and TMAO. Trimethylsilyl-2,2,3,3-tetradeuteropropionic acid (TSP) has traditionally been used to as an internal standard to measure water soluble compounds by NMR spectroscopy.[114] However, more

inconsistent results can be obtained when using TSP as the internal standard, which is probably due to a lower solubility of TSP in acidified samples, and the TSP free acid may tend to come out of solution when the ionic strength is high. Lee *et al.*[40] used acetonitrile as the internal standard which is volatile, difficult to pipette accurately, and has only one methyl group. Acetonitrile also has a resonance at 2.0 ppm which is in a region where there is more matrix interference than trimethylacetamide which resonates at 1.0 ppm.

In summary, improvements to the NMR assay for TMA and TMAO have been made by adding aluminium chloride to the sample to overcome the problem of the TMA peak splitting when samples are acidified. This improved NMR method was used in the following sections to compare results obtained using LC-MS and LC-MS/MS. However, the lower detection limits mean that NMR spectroscopy may be less useful compared to mass spectrometry methods for the measurement of TMA and TMAO in urine and the diagnosis of trimethylaminuria.

Comparison of liquid chromatography – mass spectrometry methods and NMR for the measurement of TMA and TMAO

Methods

Urine samples (n = 26) were analyzed by ^{1}H NMR for TMA and TMAO using a Varian INOVA 500 MHz instrument. The samples were re-analyzed using the current LC-MS method, and by LC-MS/MS (using an ABSciex API4000) and the results were compared using Passing-Bablok regression analysis (Acomed Statistik V3).[100]

Results

Method comparison – NMR versus LC-MS/MS

Results obtained by tandem mass spectrometry TMA results were in agreement with the NMR spectroscopy method (r^2 = 0.997) (Fig. 2.24). Passing-Bablok analysis showed that the 95% confidence interval for the slope for TMA results was 0.859 to 1.016.

The results for TMAO were significantly higher by LC-MS/MS than NMR (the 95% confidence interval for the slope was 0.700 to 0.996). The LC-MS/MS results were particularly under estimated at concentrations over 10 mmol/L, high-lighting the need to further dilute these samples by at least ten fold. This is likely to be due to the limited linear range of the tandem mass spectrometer.

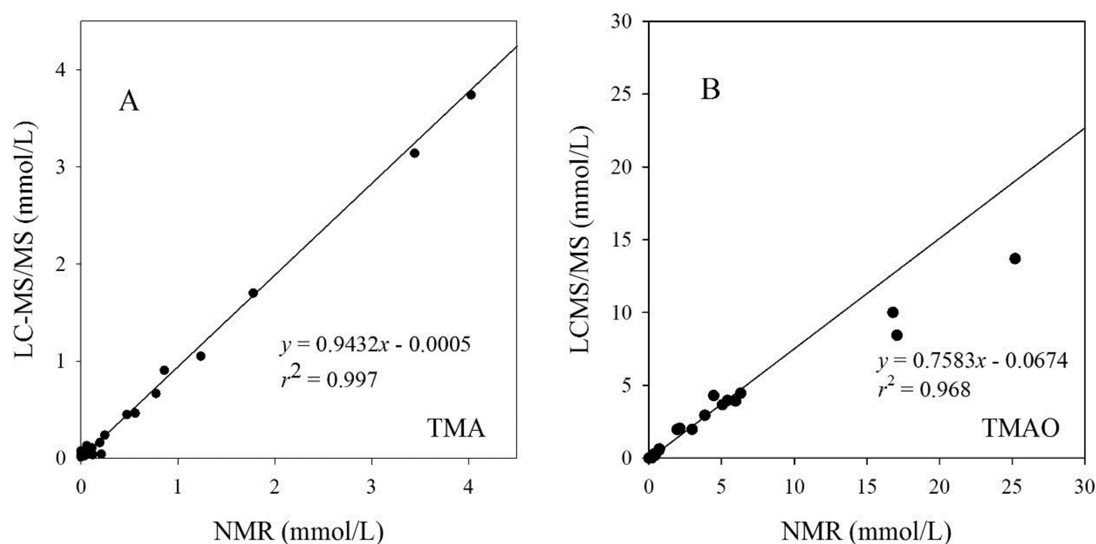


Fig. 2.24. NMR vs LC-MS/MS data for TMA and TMAO. Passing-Bablok regression lines are shown.

Method comparison – NMR versus LCMS

The single quadrupole mass spectrometry TMA results were in agreement with NMR results (r^2 = 0.988). The slope of the Passing-Bablok regression for TMA of 1.146 indicates that the LCMS results are significantly higher than the NMR results as the 95% confidence interval for the slope (1.011 to 1.28) did not include 1 (Fig. 2.25).

TMAO showed agreement between the methods with a slope of 0.953 and an r^2 of 0.980. Bablok passing analysis showed that TMAO results obtained by the NMR and mass spectrometric methods are not significantly different, as the 95% confidence intervals for the slope (0.853 to 1.009) included 1, and the 95% confidence intervals for the intercept included 0.

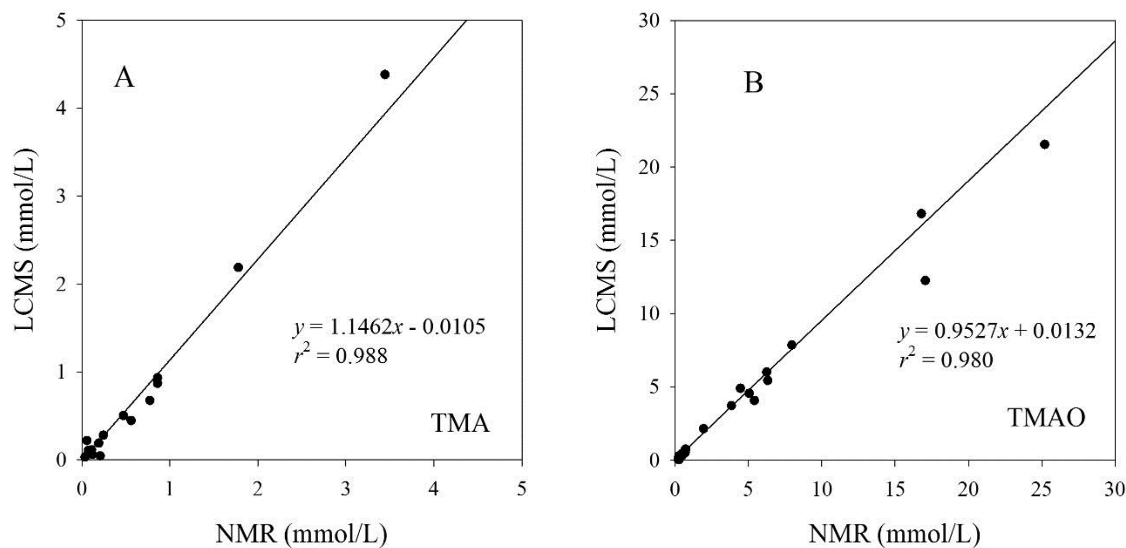


Fig. 2.25. TMA and TMAO concentrations calculated by LC-MS compared to NMR. Passing-Bablok regression lines are shown.

LC-MS and LC-MS/MS have both been shown to be useful for the measurement of TMA and TMAO for the diagnosis of trimethylaminuria. There was generally good agreement between the mass spectrometric methods and NMR spectroscopy. Mass spectrometry methods provide increased sensitivity compared to NMR. However, care needs to be taken to ensure samples are within the linear range of the spectrometer. The higher agreement of TMAO concentrations between LC-MS and NMR, compared to LC-MS/MS and NMR, probably reflects the greater linear range to the single quadrupole instrument.

2.4. The measurement of taurine in plasma and urine

Introduction

Because the sulfonic acid group of taurine is strongly acidic, taurine cannot easily be protonated to make it cationic. Therefore, taurine cannot be measured at the same time as the methylamines and requires the development of a separate LC-MS/MS method using negative ion mode.

Methods

Reagents and chemicals

Taurine was purchased from Sigma. 2-Aminoethane-D_4-sulfonic acid (deuterated taurine) was obtained from CDN isotopes. Acetonitrile was purchased from Merck.

Sample preparation

Fifty µL of aqueous standard, plasma, or urine sample was extracted into 1.0 mL of extraction solvent containing 80% acetonitrile, 20% methanol, and 10 µmol/L D_4-taurine (internal standard). Samples were vortexed and centrifuged at $13,000 \times g$ for 5 minutes.

LC-MS/MS

A Waters (Ireland) XBridge 2.1 × 100 mm, 3.5 µm amide column was used to separate taurine with an isocratic mobile phase containing 15% water and 85% acetonitrile. A flow rate of 0.3 mL/min was used, with an injection volume of 10 µL, an oven temperature of 40ºC, and a run time of 6 minutes. Samples were analyzed using an ABSciex API4000 tandem mass spectrometer with an electrospray ion (ESI) source connected to a Shimadzu Prominence HPLC system. Taurine was detected in negative ion mode using the mass transition $124 \rightarrow 80$. The internal standard for the deuterated internal standard was $128 \rightarrow 80$. The decoupling potential was -55 V, the collision energy was -30 V, and the collision cell exit potential was -13 V.

Taurine can be also measured using single quadrupole mass spectrometry by monitoring the 124 ion for taurine, and the 128 ion for the D_4-taurine in negative ion mode with an ESI source.

Method validation

Six batches of four replicates were analyzed to investigate the precision of the assay for taurine plasma and urine samples containing a high and normal level. The within batch and between batch CVs were calculated for each compound. The linear range of the aqueous standards was investigated by measuring aqueous taurine standards up to 2 mmol/L. Linearity was also investigated by adding taurine concentrations up to 400 µmol/L to plasma, and 1000 µmol/L to urine. Each concentration level was analyzed in triplicate for taurine.

Results and discussion

The Waters XBridge amide column provided sufficient retention of taurine (Fig. 2.26) separating it from interfering peaks in the MRM chromatograms in the urine and plasma samples. Taurine eluted much earlier on an unmodified silica column.

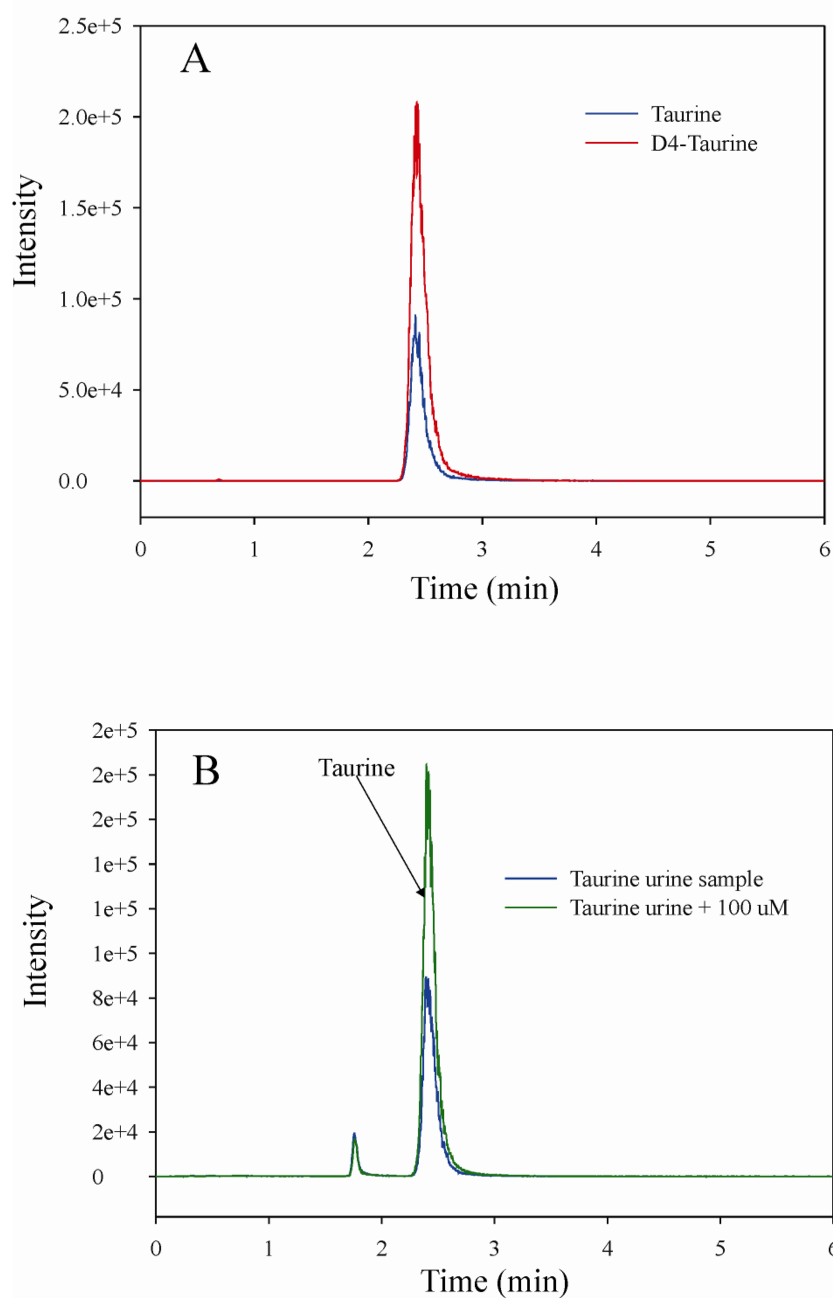


Fig. 2.26. MRM chromatograms showing: A). Taurine standard (100 µmol/L) shown with the internal standard. B). A urine sample and the same sample with 100 µmol/L taurine added.

The recovery of taurine was 81% when 100 µmol/L was added to plasma. However, other concentrations added to plasma had >89% recoveries (see Fig 2.28A). The within batch CV was below 2%, and the between batch CV was below 3% (see Table 2.11).

The recovery of 200 µmol/L taurine added to urine was 83%. The within batch CV was below 2.0%, and the between batch CV was below 1.2%. The limit of detection for taurine was 0.06 µmol/L (*S/N* =3).

Table 2.11. Method performance data for the measurement of taurine in plasma and urine.

Taurine	Mean (µmol/L)	Within batch CV%	Between batch CV%	Recovery%
Low plasma	29.6	1.7	2.9	
High plasma	110.9	1.2	2.7	81%

Taurine	Mean (µmol/L)	Within batch CV%	Between batch CV%	Recovery%
Low urine	147	1.12	1.97	
High urine	313	1.32	1.11	83%

Linear range

The method was not linear for aqueous standards above 250 µmol/L (Fig. 2.27). The linear range was sufficient in plasma up to 400 µmol/L with an r^2 of 0.999 (Fig. 2.28A). The linearity of taurine in urine appeared to be sufficient up to 1000 µmol/L with an r^2 of 0.998 (Fig. 2.28B). However, the lower urine concentrations (0 to 250 µmol/L) had a different slope, suggesting that concentrations above 250 µmol/L should be diluted.

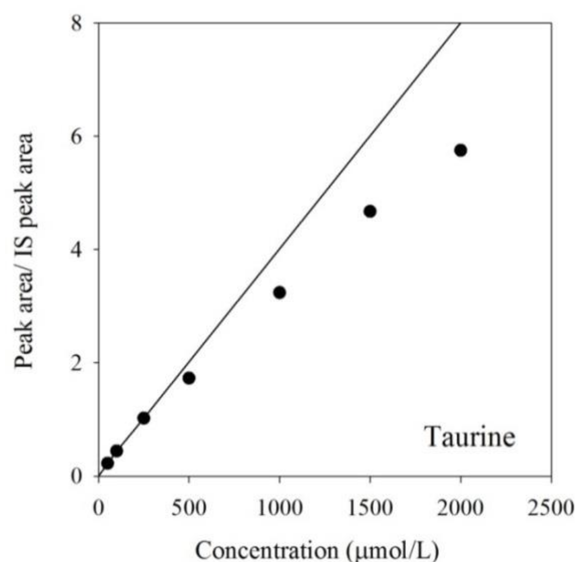


Fig. 2.27. Calibration curve of aqueous taurine standards showing data with peak area ratios to the internal standard peak area. The regression line was calculated using concentrations up to 250 µmol/L only.

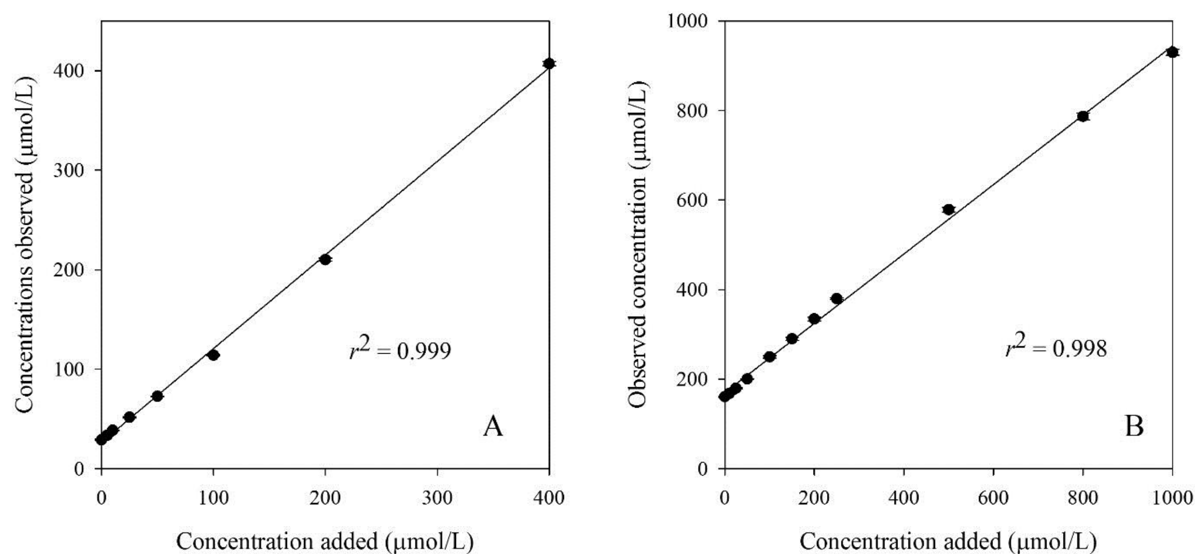


Fig. 2.28. Linearity of taurine added to plasma (A), and urine (B). Means ± standard errors are shown for each concentration.

Summary

Taurine can be measured by LC-MS/MS in negative ion mode in plasma and urine, although care needs to be taken to make sure the assay is operating in linear range of the instrument. The linear range was particularly limited in the aqueous calibration standards. An amide column provided sufficient retention of taurine to help ensure that the peak is away from potential interference from the sample matrix.

2.5. Chapter summary

Methods for the analysis of betaine, DMG, choline, TMAO, carnitine, acetylcarnitine, and taurine have been developed and validated so that they can be reliably measured in plasma and urine. The concentrations of these analytes in clinical samples were measured in Chapter 4 in order to investigate their metabolism in health and disease.

Similar results can be obtained using tandem mass spectrometry (LC-MS/MS) or single quadrupole mass spectrometry (LC-MS) for most methylamines. However, HPLC techniques that require derivatization and UV detection can produce greatly different results, which is likely to be a consequence of interfering compounds present in the samples. Mass spectrometry offers superior specificity compared to HPLC with UV (or fluorescence detection) following derivatization. The improved efficiency of mass spectrometry based methods compared to HPLC-UV methods allows for a greater number of samples to be analyzed in a shorter time, making mass spectrometry more suitable for epidemiological studies.

Separation on an amide column followed by LC-MS/MS in negative ion mode has been shown to be a useful technique analysis of taurine in plasma and urine.

Lee *et al.*[40] validated an NMR method for the analysis of methylamines using a 500 MHz NMR spectrometer, and reported that the limit of detection for these analytes in urine ranged from 15 to 25 µmol/L. While there may be enough sensitivity to measure some methylamines in urine, it is unlikely that this instrument would have sufficient sensitivity to detect most methylamines in plasma, even if the problem of interference from the blood proteins was resolved. While more sensitive NMR systems exist, they require dedicated facilities, and they are cost prohibitive for use as a quantitative instrument in New Zealand clinical laboratories.

3. Chapter Three - The Measurement of Polyols in Biological Samples

3.1. Introduction

The polyol osmolytes, sorbitol and *myo*-inositol, are particularly difficult to measure in complex biological samples. In this Chapter, the problems with measuring polyols are discussed, and potential ways to overcome these problems are described.

Polyols are neutral compounds with no chromophores or reactive functional groups (other than multiple hydroxyls) that can easily be derivatized. Polyols do not possess an aldehyde group, unlike closely related sugars. Sorbitol and *myo*-inositol have multiple isomers, many of which only differ in the orientation of the hydroxyl groups. They have complex splitting patterns on proton NMR that are difficult to distinguish from sugars (such as glucose) that are often present in much higher concentrations in biological samples.

Interference from isomers of sorbitol and *myo*-inositol are likely to be problematic. For example, mannitol (Fig. 3.1, **17**) and galactitol (**18**) are structurally similar isomers of sorbitol (**5**) that produce ions of the same mass and are expected to also have similar fragmentation patterns. They only differ in the arrangement of the hydroxyl groups on the molecule. Mannitol is not produced by mammals, but is used as an osmolyte by plants and fungi, and can be added to foods as a sweetener, so may be present in humans depending on their diet.[115]

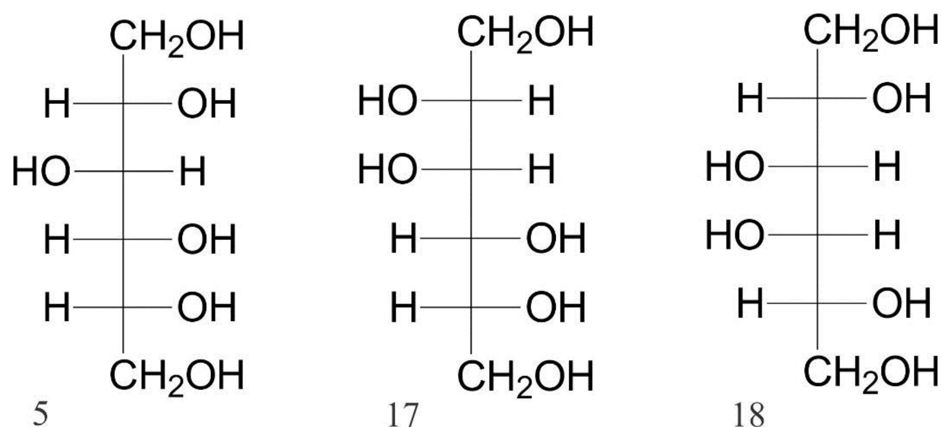


Fig. 3.1. Structure of isomers, sorbitol (**5**), mannitol (**17**), and galactitol (**18**).

There are nine different stereo-isomers of inositol of which *myo*-inositol is just one. The inositols have multiple roles in nature. *myo*-Inositol is an important mammalian osmolyte.[71] Concentrations of *myo*-inositol in the body are affected by

diabetes.[116] The only inositol isomers that are likely to be present in human samples in detectable concentrations are *myo*-inositol (Fig. 3.2, **4**), *scyllo*-inositol (**19**), and *chiro*-inositol (**20**). *chiro*-Inositol may play a metabolic role in mammals and has been associated with insulin resistance.[75b, 116b] *chiro*-Inositol is found in the diet, but can also be synthesized *in vivo* from *myo*-inositol.[116b] *scyllo*-Inositol is present in humans from dietary sources such as seafood[117] and citrus fruits.[8] *myo*-Inositol is present in many foods and can also be synthesized in the body from glucose.[116b] *myo*-Inositol is also found in mammals as phosphates (such as phytic acid), and phosphatidylinositol, which is used in cell membranes.[116b] Glucose, fructose, and mannose all have a molecular mass of 180.2 g/mol, and are also isomers of the inositols. Therefore, care needs to be taken to avoid interference from these sugars when using mass spectrometry. Glucose is present in mammalian blood in much higher concentrations than *myo*-inositol, so it is likely to be important to separate them chromatographically.

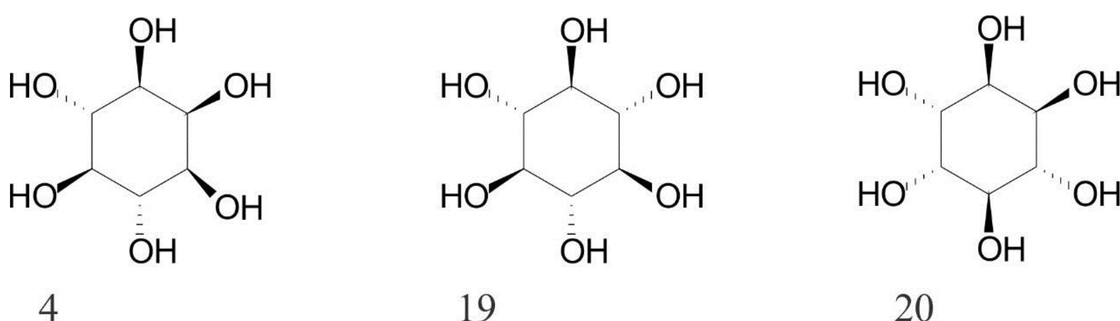


Fig. 3.2. Structure of *myo*-inositol (**4**), *scyllo*-inositol (**19**), and *chiro*-inositol (**20**).

There are different strategies which can be employed to detect polyols. They can be measured directly using refractive index detection, or by using mass spectrometry with an APCI ion source. They can also be measured by mass spectrometry using adducts of ions such as halides.[118] Alternatively, the hydroxyl groups of polyols can be derivatized using highly reactive reagents that contain a chromophore to aid detection.[44, 119] Polyols can also form complexes with transition metals,[120] or boronic acids.[121] Previous methods for measuring sorbitol and inositol have used GC-MS following reaction with a reagent such as butylboronic acid,[52a, 52b, 122] enzymatic assay[52c] and HPLC with refractive index detection[6] or pre-column derivatization with phenylisocyanate,[44, 123] benzoyl chloride,[124] or *p*-nitrobenzoate.[119] Some of the GC methods described for polyols have been able to separate sorbitol

from its isomers. However, they involve intensive sample preparation that includes drying down the samples and pre-column derivatization, and they typically have long run times of around 40 minutes per sample.[52b] Enzymatic assays used to measure sorbitol using sorbitol dehydrogenase are non-specific, polyols other than sorbitol are substrates for this enzyme, and these methods have been shown to significantly over-estimate the results.[52c] HPLC with refractive index detection is not sensitive or selective enough to measure polyols in complex biological samples such as plasma or urine. HPLC methods using derivatizing reagents such as phenylisocyanate and *p*-nitrobenzoate are not selective for *cis*-diol polyols, and multiple peaks are likely to be present in the chromatograms for each of the many compounds in biological samples which contain hydroxyl groups. Polyols and sugars have been measured directly by LC-MS/MS[125] or as chloride adducts in negative mode[118b] and cesium adducts in positive mode.[126] A method has been described using the direct measurement of plasma and urine *myo*-inositol in negative ion mode by LC-MS/MS.[127] However, a large lead column with a long run time of 55 minutes was required to separate *myo*-inositol from other isomers and the peaks were very broad.[127] The sulfonated resin columns in the lead or calcium form, that have traditionally been used to separate polyols, are not very compatible with mass spectrometry. They are only available in wide-bore format and require long run times and a solvent splitter after the column to reduce the flow into the mass spectrometer. This in turn reduces the sensitivity of the assay and reduces sample throughput. These columns are also restricted to using mainly water in the mobile phase and will only tolerate a small organic solvent content.

Polyols such as sorbitol and *myo*-inositol form complexes with boronates to produce anionic species which can be detected by mass spectrometry in the negative ion mode.[122, 128] Boronates are known to complex with *cis*-diols. These interactions are potentially useful to improve both the separation and detection of polyols. The hydroxyl groups on the polyol isomers have different orientations. Complexation with boronates may enhance these structural differences, and improve the ability to separate them chromatographically. Boronate *cis*-diol complexes are volatile cyclic species that should be easily ionizable.

Boric acid can also complex with polyols. Ackloo *et al.*[129] showed that adding ethanediol in the mobile phase avoids leaving boric acid residue in the mass spectrometer. The ethanediol boric acid complex is volatile, whereas ammonium

borate by itself leaves a white residue on drying. The polyol osmolytes, sorbitol and *myo*-inositol, may compete with the ethanediol on the column. And an ion containing both sorbitol and ethanediol bound to a boric acid has been reported.[129]

The interaction of polyols with various chemicals was investigated with the aim of improving the extraction, chromatography, and detection of polyol osmolytes. Mass spectrometry techniques, as well as interactions of polyols with boronic acids, transition metals, and derivatizing reagents were investigated. The objective was to develop and validate methods so that they can be used to reliably measure sorbitol and *myo*-inositol in plasma and urine.

3.2. Extraction of polyols

The samples were initially extracted into acetonitrile (90%) and methanol (10%). However, polyols have a low solubility in organic solvents such as methanol and acetonitrile, and the extraction efficiency was low.

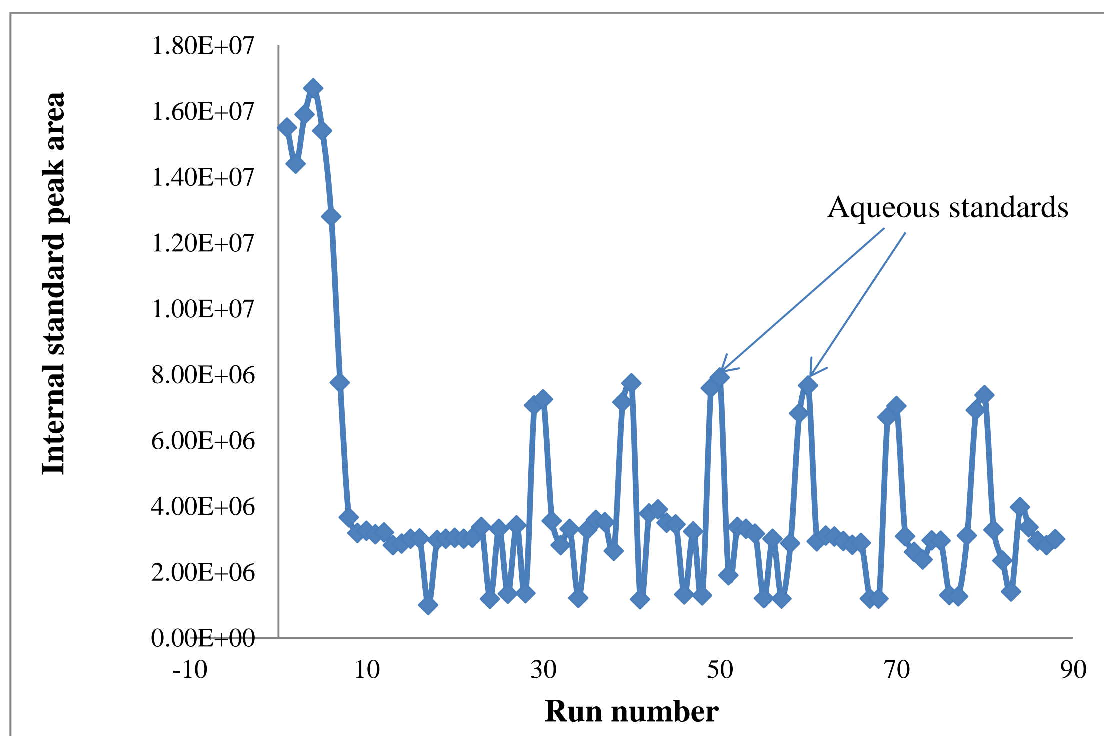


Fig. 3.3. Run number versus peak area for the D_6-*myo*-inositol internal standard measured as the iodide adduct by LC-MS/MS.

The inositol signal was variable throughout the run, as seen by a plot of the D_6-*myo*-inositol peak area (Fig. 3.3). Here, the signal started off high, then dropped off

after the fifth injection. There were regular spikes where the signal increased for a couple of injections, then dropped off again. The samples where the signal for the internal standard was higher correspond to the aqueous standards. This suggested that ion suppression in the urine samples may be a problem. While the internal standard adjusted calibration corrects for this variation in signal, the sensitivity of the method was affected. The main issue with the variation in signal is the difference in detection limit between the aqueous standards and the urine samples. Adding urea to the standards did not change the peak area significantly, which suggests that urea was not an interfering substance in the urine. However, adding sodium chloride to a standard reduced the signal significantly. Removing the cations from urine (Fig. 3.4) and plasma (Fig. 3.5) by adding Dowex 50 cation exchange resin before extraction appeared to improve the sensitivity. However, it was noted that there was high pressure in the column after injecting the samples, particularly the plasma. It was also noted that the protein was not precipitated in the extraction solvent after Dowex 50 was added to the sample, possibly due to acidifying it. It was then questioned whether the difference in response between the standards and the samples is actually a solubility problem instead of an ion suppression problem. Inositols are highly hydrophilic compounds which are not very soluble in organic solvents. It is therefore possible that the inositols were going out of solution with the precipitate during sample extraction and centrifugation. To attempt to solve this problem, the protein in the samples was precipitated with trichloroacetic acid before adding the sample to the acetonitrile / methanol extraction solvent.

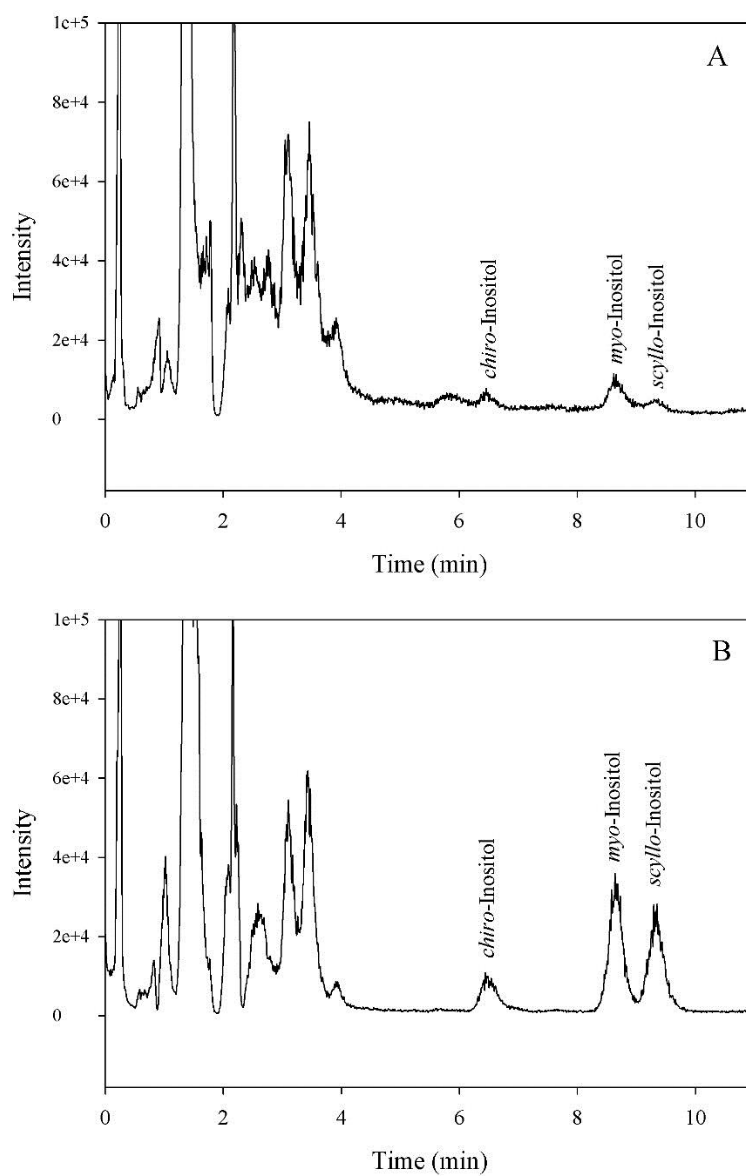


Fig. 3.4. A urine sample (A), the same urine sample with Dowex 50 resin added before extraction (B). The XBridge column was used here. The mobile phase for this separation contained 15% water, 85% acetonitrile, and 10 mmol/L diiodomethane.

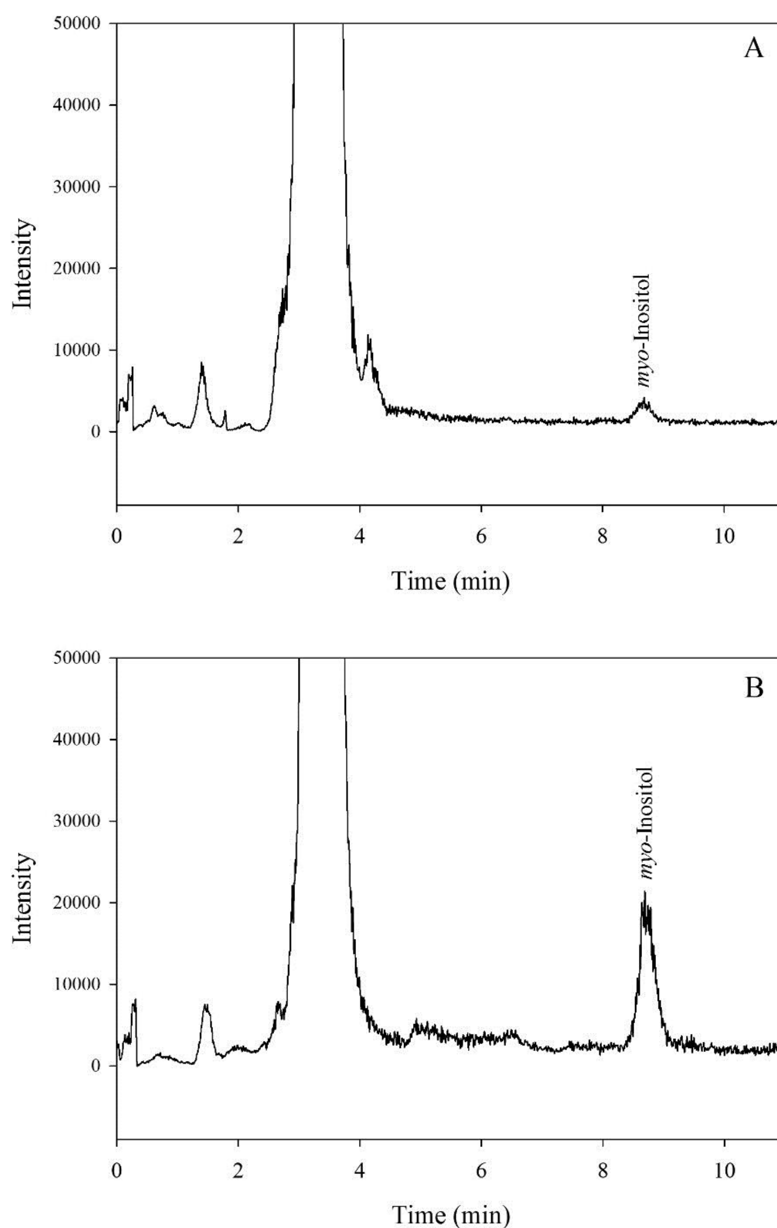


Fig. 3.5. A plasma sample (A), and the same plasma sample with Dowex 50 resin added before extraction (B).

Another approach to improve the extraction efficiency was to pipette the samples into an extraction solution where the inositols were more soluble. To investigate the solubility of inositol in different solvents and solvent mixtures, three replicates of a plasma or urine sample (50 µL) were extracted into 1.0 mL of each of the following solvents: acetonitrile (A); 10% methanol / 90% acetonitrile (B); acetone (C); or 25% dimethylsulfoxide (DMSO) / 75% acetone (D). Samples were measured

directly in negative ion mode by LC-MS with an APCI source after separation on an amide column (see Section 3.3). Inositols were found to have a high solubility in dimethylsulfoxide (DMSO). Figure 3.6 shows the highest extraction efficiency was achieved in a mixture of 25% DMSO and 75% acetone. Extraction of samples into 25% DMSO / 75% acetone gave approximately 70% recovery of *myo*-inositol in plasma samples compared to the aqueous standards, as determined by the D_6-*myo*-inositol internal standard peak area. The extraction efficiency of inositols in acetonitrile and acetone was low. However, addition of 10% methanol to acetonitrile improved the extraction of inositols, particularly in the urine. Addition of DMSO to acetone greatly improved the extraction efficiency of inositols in both plasma and urine.

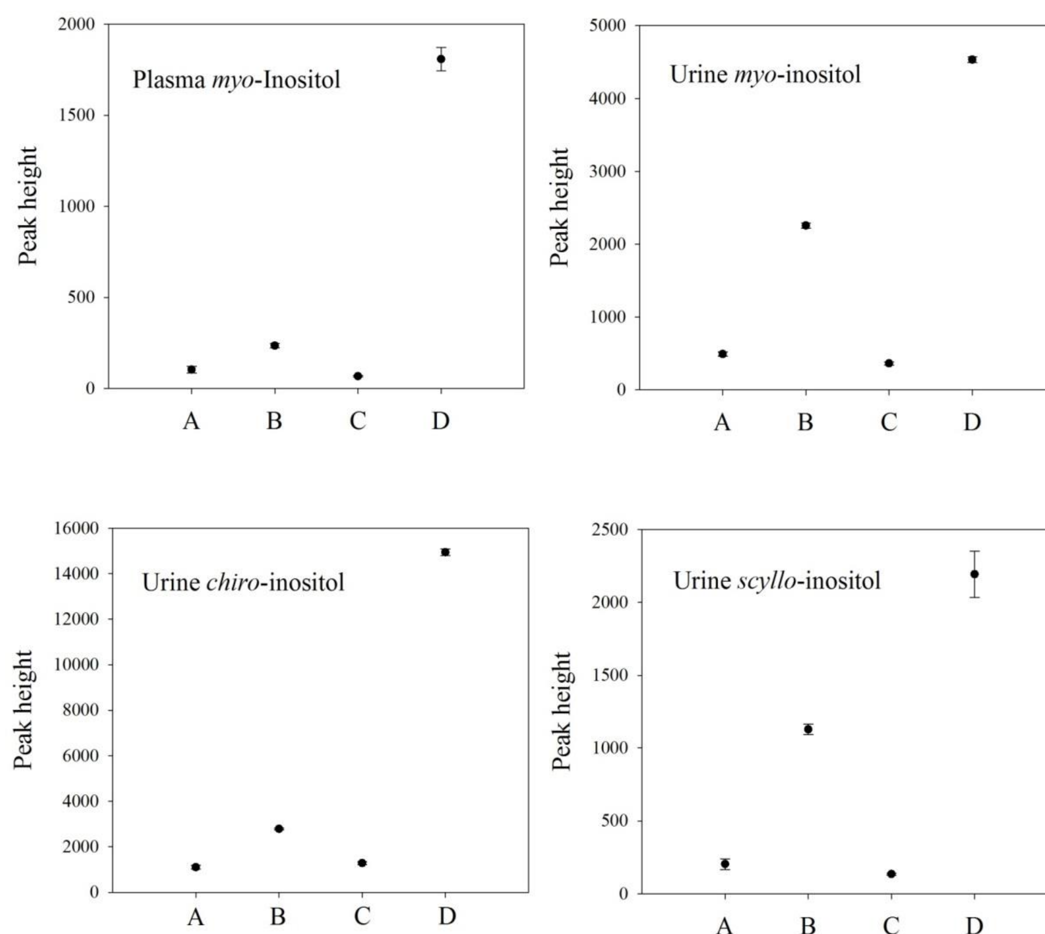


Fig. 3.6. Extraction efficiencies of inositols in plasma and urine using different extraction solvents: acetonitrile (A); 10% methanol / 90% acetonitrile (B); acetone (C); and 25% DMSO / 75% acetone (D). Means and standard errors of peak heights are shown.

3.3. Chromatography of polyols

Introduction

In order to reliably quantify sorbitol and *myo*-inositol, it is important to chromatographically separate the structural isomers. Columns that have previously been used to separate polyols are mainly sugar columns with large volumes that are not ideal for mass spectrometry. They are typically wide bore columns that require high flow rates, and a high water content in the mobile phase. They require long run times and produce wide peaks. These columns contain beads of sulfonated resin which requires regular regeneration with ions such as lead or calcium in order to work. Normal phase columns such as silica, amino, diol, or amide have also been reported for the separation of some polyols and sugars.[130] How some of these columns separate sorbitol and *myo*-inositol was investigated here.

Methods

A range of different columns were investigated for the separation of the polyols of interest, including: silica (Kinetex HILIC core-shell silica 2.6 µm, 2.1 × 100 mm, Phenomenex), amide (Waters XBridge 3.5 µm, 2.1 × 100 mm or Waters Acquity UPLC 1.7 µm, 2.1 × 100 mm), titania (Sachtopore, 3 µm, 2.1 × 150 mm), amino (Phenosphere 3 µm, 150 × 4.6 mm, Phenomenex; Shodex Asahipak 5µm, 250 × 4.6 mm, Phenomenex), and an ion exclusion column (100 × 7.8 mm, Fast Carbohydrate, Pb column, Biorad). In order to optimize the chromatography, a Jasco refractive index detector was used to detect polyol standards eluted through the various columns. During these investigations, the refractive index detector flow cell cracked and the instrument was uneconomical to repair. Further work developing the chromatography of polyols was carried out using mass spectrometric detection with either an AB Sciex API4000 tandem mass spectrometer, or an Agilent 6120 single quadrupole mass spectrometer.

Results and discussion

Examples of chromatographic separations of polyols are shown and discussed here.

Lead column

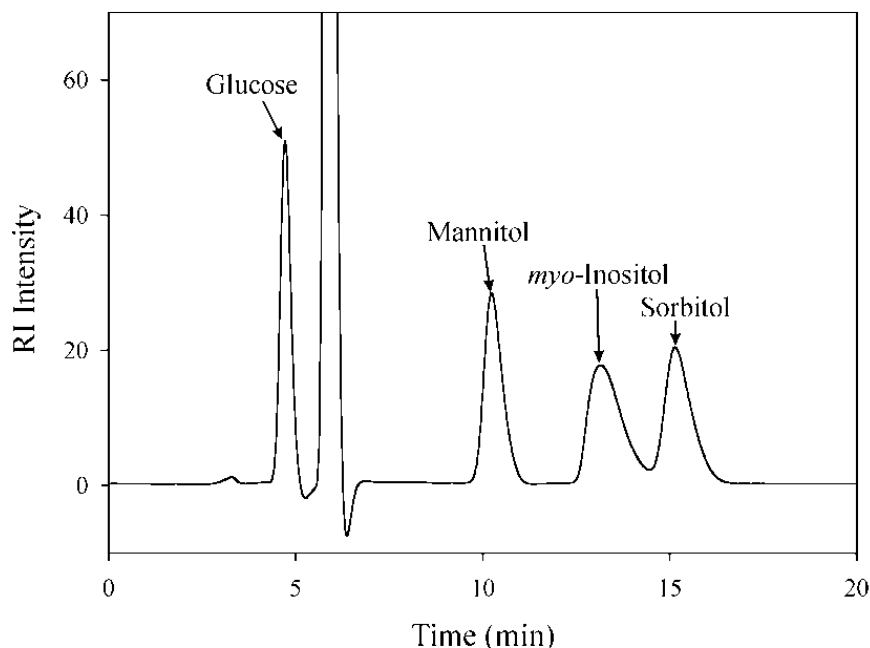


Fig. 3.7. Chromatogram showing separation of polyols on a BioRad fast carbohydrate lead ion exclusion column. This separation was carried out by injecting a 2.5 mmol/L mixed standard of glucose, mannitol, *myo*-inositol, and sorbitol with a flow rate of 0.6 mL/min. The column was operated at 80ºC, and polyols were detected using refractive index detection.

The BioRad lead ion exclusion 150 × 7.8 mm, 10 µm column produced a separation of mannitol and sorbitol within 20 minutes (Fig. 3.7), although the peaks were broader than on other columns. Galactitol and sorbitol were co-eluting on the lead column (Fig. 3.8). Ion exclusion columns (containing sulfonated resin in metal ion forms) have been used extensively for the measurement of carbohydrates because they are known to be effective at separating polyol isomers. A lead column has been shown to effectively separate isomers of inositol.[127] Lead columns give a better separation of sorbitol and mannitol than calcium columns. How these columns separate these isomers is not well understood. However, it is likely to be a result of the hydroxyl groups on the polyols interacting with the metal ions on the column. As sorbitol is retained longer, it is likely to form a stronger chelate with lead ions than mannitol. There are limitations with the use of ion exclusion columns, as well as the fact that they produce broad peaks. They are generally large with an internal diameter

of 7.8 mm, and do not come in smaller sizes for mass spectrometry. However, a flow splitter can be used after the column to reduce the flow into the mass spectrometer. According to the manufacturer, these columns must also be operated with water as the mobile phase with no more than 10% organic solvent added. Aqueous samples should be injected when using the ion exclusion columns, meaning that a simple solvent extraction is not appropriate. Alternative sample clean-ups are required, such as protein precipitation using trichloroacetic acid (TCA), or drying down the sample after a solvent extraction and reconstituting it in water. The manufacturers also recommend that they are operated at high temperatures, typically 60-80ºC.

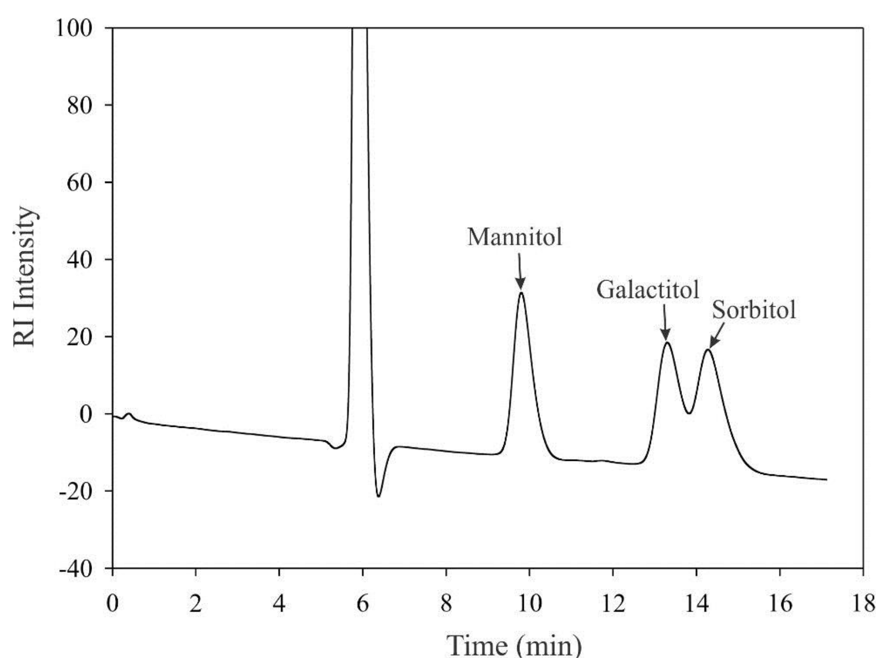


Fig. 3.8. Chromatogram showing separation of isomers mannitol, galactitol, and sorbitol on a BioRad fast carbohydrate lead ion exclusion column. This separation was carried out by injecting a 3.3 mmol/L mixed standard of mannitol, galactitol, and sorbitol with a flow rate of 0.6 mL/min. The column was operated at 80ºC, and polyols were detected using refractive index detection.

As well as investigating the chromatography using refractive index detection, the lead ion exclusion column was also used with mass spectrometric detection with both Q1 and MS/MS monitoring in negative ion mode (Fig. 3.9). The mobile phase was 100% distilled water. The flow rate was 0.6 mL/min and a flow splitter was used after the column to reduce the flow into the mass spectrometer to 0.3 mL/min. The

injection volume was 50 µL and the oven temperature was 60ºC. A high background noise was observed which is likely to be caused by the bleeding of lead ions from the column into the mass spectrometer leading to ion interference in the chromatograms. The 100 µmol/L polyol standards were barely detectable using both monitoring of the parent ion (Q1) and tandem mass spectrometry (MS/MS) when using the BioRad lead column. Using this column with mass spectrometry was therefore lacking the necessary sensitivity to measured polyols in plasma and urine samples.

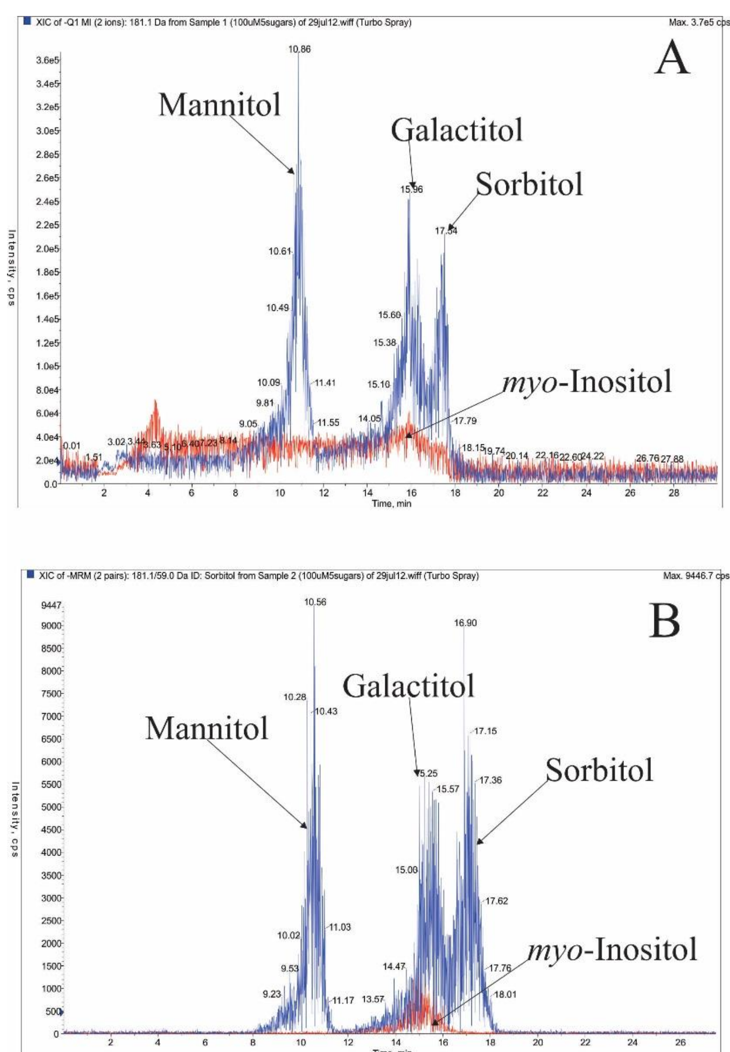


Fig. 3.9. Ion chromatograms of a mixture of 100 µmol/L aqueous standard of sorbitol, *myo*-inositol, glucose, mannitol, and galactitol separated on a BioRad 100 × 7.8 mm lead ion exclusion column. A = Q1 at 181 Da (blue), and 179 (red) in negative ion mode. B = MS/MS mass transitions 181 → 59 (blue), and 179 → 89 (red). Detection was carried out on an Applied Biosystems API4000 mass spectrometer.

Silica, amino, and metal oxide columns

Silica was effective at separating sorbitol, *myo*-inositol, and glucose. However, sorbitol, galactitol, and mannitol were not separated (Fig. 3.10). The silica based amino columns also gave no significant separation of sorbitol and mannitol.

The amino columns produced a noisy baseline signal in the mass spectrometer, suggesting that the bleeding of amino groups was causing ion interference in the baseline. The Asahipak amino column provided a slight separation of sorbitol and mannitol, and produced less ion interference in the mass spectrometer than the Phenosphere amino column.

Metal oxide stationary phases were unsuccessful at separating polyols. No peaks were observed using titania, zirconia, and alumina stationary phases, suggesting that the polyols were retained for a long time on these columns. Elution with high water content in the mobile phase (>50%) did not appear to elute the polyols off these columns.

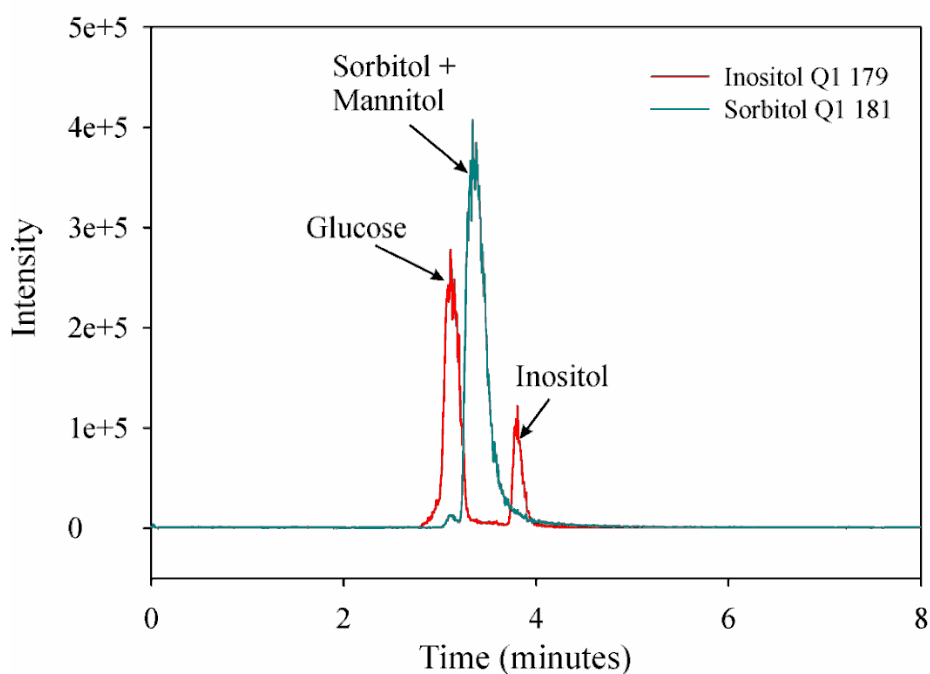


Fig. 3.10. Q1 Selected ion monitoring (SIM) chromatograms collected in negative ion mode at 179 Da and 181 Da. The sample contained 100 µmol/L sorbitol, 100 µmol/L *myo*-inositol, 500 µmol/L mannitol, and 1 mM glucose. Separation was performed on a Kinetix HILIC (silica) 2.1 × 100, 2.6 µm column. Detection was on an API4000 mass spectrometer.

Amide columns

The amide columns provided the best separation of *myo*-inositol, *chiro*-inositol, and *scyllo*-inositol (see Figs. 3.11 and 3.12). The Waters X-bridge amide column separated *myo*-inositol from its isomers (Fig. 3.11) with only *myo*-inositol and *scyllo*-inositol co-eluting. However, it did not separate sorbitol, mannitol, or galactitol from each other. These hexitols all eluted in the same region as glucose.

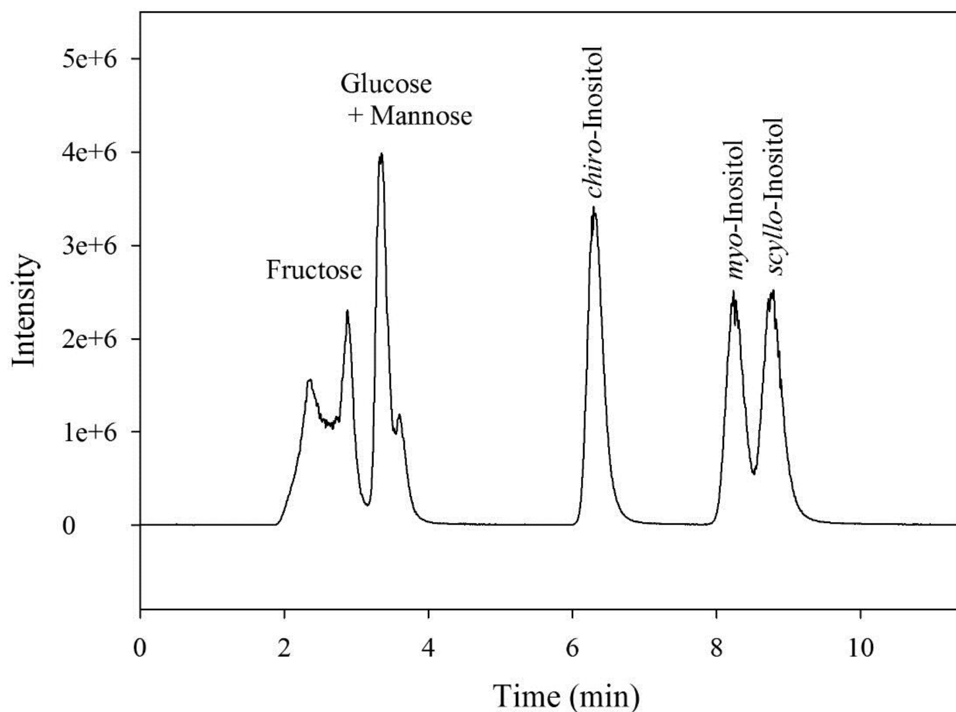


Fig. 3.11. LC-MS/MS chromatograms of iodide adducts collected in negative ion mode on a Waters XBridge 3.5 µm, 2.1 × 100 mm amide column. The mass transition 307 → 127 was used for monitoring *myo*-inositol and isomers. The sample contained 500 µmol/L of: *myo*-inositol, *scyllo*-inositol, *chiro*-inositol, glucose, mannose, and fructose. Detection was carried out on the API4000 mass spectrometer.

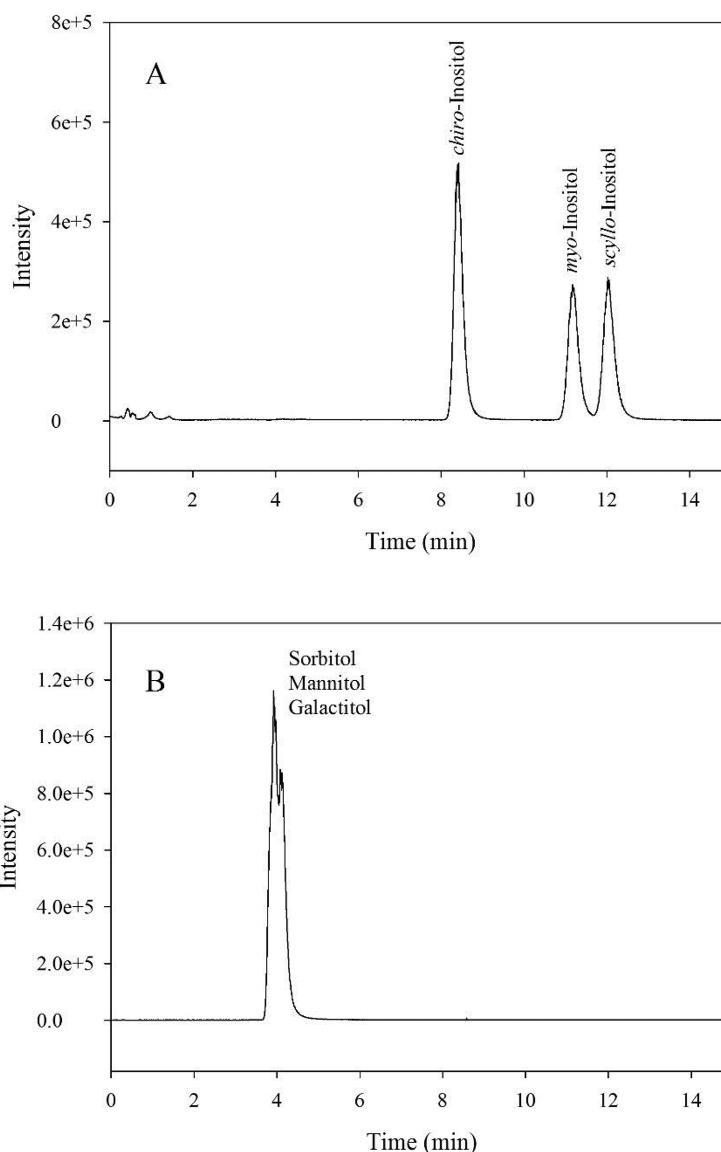


Fig. 3.12. Separation of a 100 µmol/L mixed standard of polyols on a 1.7 µm, 2.1 × 100 mm Waters Acquity UPLC amide column. Chromatograms of inositols (A), and hexitols (B) are shown. The mobile phase contained 15% water, 85% acetonitrile, and 10 mmol/L diiodomethane. Detection was achieved using tandem mass spectrometry of the iodide adducts.

Baseline separation of *myo*-inositol and *scyllo*-inositol was achieved on a waters 1.7 µm particle size (UPLC) amide column (Fig. 3.12A). The Acquity UPLC amide column with 1.7 µm particle size improved the separation of *myo*-inositol and *scyllo*-inositol compared to the 3.5 µm column, with nearly complete separation observed between the peaks. The UPLC column also produced a slight separation of the sorbitol isomers, although not sufficient for reliable quantitation of sorbitol (Fig.

3.12B). While there are nine possible inositol isomers, only *myo*-inositol, *chiro*-inositol and *scyllo*-inositol are likely to be present in humans in significant concentrations.[127] The sugars with the same mass as *myo*-inositol, glucose, mannose, and fructose all eluted earlier than the inositols on the amide column (before 5 minutes). The double peaks that are observed for the sugars: fructose, glucose, and mannose are caused by the separation of the two anomeric forms of the sugar molecules (*i.e.* the ring and open chain form) on the amide column. A single peak can be obtained on amide stationary phases for sugars such as glucose by operating at higher column temperatures (up to 90ºC),[131] which speeds up the equilibrium between the two forms. However, this is not necessary when just measuring the inositols which do not form anomers.

Summary of the chromatography of polyols

The lead ion exclusion column provided the best separation of the hexitol isomers, sorbitol, mannitol, and galactitol. However these columns are not suitable for mass spectrometry. They have a large volume, give broad peaks, and bleed ions into the mass spectrometer, increasing the background noise and causing poor sensitivity. Silica provided some separation of polyols, but amide columns provided greater separation with inositols being well separated on a small particle size (UPLC) amide column. The good separation of inositols observed on the amide columns is likely to be due to hydrogen bonding between the amide and the hydroxyl groups on the polyols. It is not clear why amide groups separated the inositols better than the hexitols. Unfortunately, no commercially available (mass spectrometry compatible) column was found that produced a sufficient separation of sorbitol, mannitol, and galactitol.

3.4. Detection of polyols

Direct mass spectrometric detection of sorbitol and myo-*inositol*

Introduction

Polyols such as sorbitol and *myo*-inositol can shed a proton to form $[M-H]^-$ ions which can be detected in negative ion mode. This provides a simple way of measuring these polyols, either directly as the parent ions, or by fragmenting them and monitoring the mass transitions (MRMs). However, because most polyols tend to fragment most strongly by losing a water molecule, the monitoring of MRM transitions is unlikely to help separate *myo*-inositol from glucose, or other sugar isomers. The separation of polyols from interfering substances such as glucose is therefore likely to rely on the chromatography.

Methods

Reagents and chemicals

Sorbitol, *myo*-inositol, galactitol (dulcitol), and mannitol were purchased from Sigma (MO, USA). D-Glucose was obtained from BDH (Pool, England). *scyllo*-Inositol was purchased from TCI (Tokyo, Japan), and *chiro*-inositol was purchased from Acros Organics (NJ, USA). Acetonitrile (LiChrosolv) was from Merck (Darmstadt, Germany).

LCMS

Detection was investigated operating the mass spectrometer in both Q1 mode (selected ion monitoring of the molecular ion), and in MS/MS mode (tandem mass spectrometry).

An aqueous standard containing 100 µmol/L sorbitol and 100 µmol/L *myo*-inositol, 1 mM glucose 500 µmol/L mannitol was made up. The aqueous standard was diluted 1:5 with acetonitrile, then injected on to a Kinetix HILIC 100 mm × 2.1 mm, 3 µm column (Phenomenex). The mobile phase contained 80% acetonitrile and 20% water, and was delivered in isocratic mode. The injection volume was 10 µL, the flow rate was 0.3 mL/min, and the oven temperature was 40ºC. To measure sorbitol and

myo-inositol using the parent ion mass, the mass spectrometer was programmed to monitor the signal at 179 Da and 181 Da in negative ion mode. To optimize the compound-specific parameters for polyols by MS/MS, 5 µmol/L standards of sorbitol and *myo*-inositol were infused into the API4000 (Applied Biosystems) mass spectrometer using a syringe pump at 10 µL/min, and compound optimizations were performed for MS/MS in negative ion mode.

Results and discussion

Q1 ions

There was good separation on the Kinetex HILIC column of *myo*-inositol and glucose (Fig. 3.13). However, sorbitol and mannitol were not separated using this chromatography system. The sensitivity was likely to be sufficient for some applications by monitoring in Q1 ion mode. The background noise was high when detecting ions in Q1 mode on the API4000 tandem mass spectrometer. However, the Agilent 6120 single quadrupole mass spectrometer operated with an atmospheric pressure chemical ionization (APCI) source was later found to produce increased sensitivity compared to the tandem mass spectrometer operated in single quadrupole (Q1) mode.

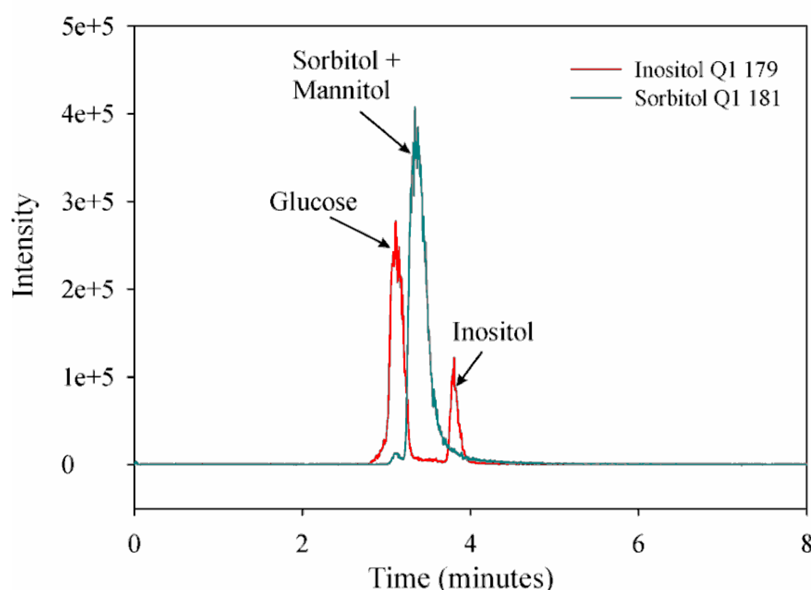


Fig. 3.13. Q1 Selected ion monitoring (SIM) chromatograms collected in negative ion mode at 179 Da and 181 Da. The sample contained 100 µmol/L sorbitol, 100 µmol/L *myo*-inositol, 500 µmol/L mannitol, and 1 mM glucose.

MS/MS

Using the mass transition 179 → 86.8 for *myo*-inositol, there was no significant glucose peak observed in the chromatogram (Fig. 3.14). The limit of detection for the inositol using MRM was 25 µmol/L. The 181 → 59 transition appeared to give better sensitivity for sorbitol. However, it was not possible to calculate a limit of detection for sorbitol as it was co-eluting with mannitol on the Kinetex HILIC column. It is likely that the ionization efficiency of the uncharged polyols would be improved by operating the mass spectrometer with an APCI source. The voltages from the compound optimizations are shown in Table 3.1. The source parameters were, ion source temperature = 350ºC, source gas 1 = 40 L/min, source gas 2 = 45 L/min.

Table 3.1. Compound optimization data for fragmentation of polyols.

Sorbitol MS/MS	DP (V)	CE (V)	CXP (V)	Intensity
181.1 → 59.0	-65.0	-36.0	-3.0	32478
181.1 → 70.8	-65.0	-32.0	-3.0	28058
181.1 → 89.0	-65.0	-20.0	-5.0	14387
181.1 → 101.0	-65.0	-20.0	-25.0	9993
181.1 → 55.0	-65.0	-30.0	-7.0	6327

Inositol MS/MS	DP (V)	CE (V)	CXP (V)	Intensity
179.0 → 86.8	-65.0	-20.0	-13.0	25845
179.0 → 59.0	-65.0	-38.0	-7.0	10152
179.0 → 70.8	-65.0	-30.0	-1.0	6352
179.0 → 85.1	-65.0	-20.0	-13.0	5202
179.0 → 54.9	-65.0	-44.0	-3.0	4570

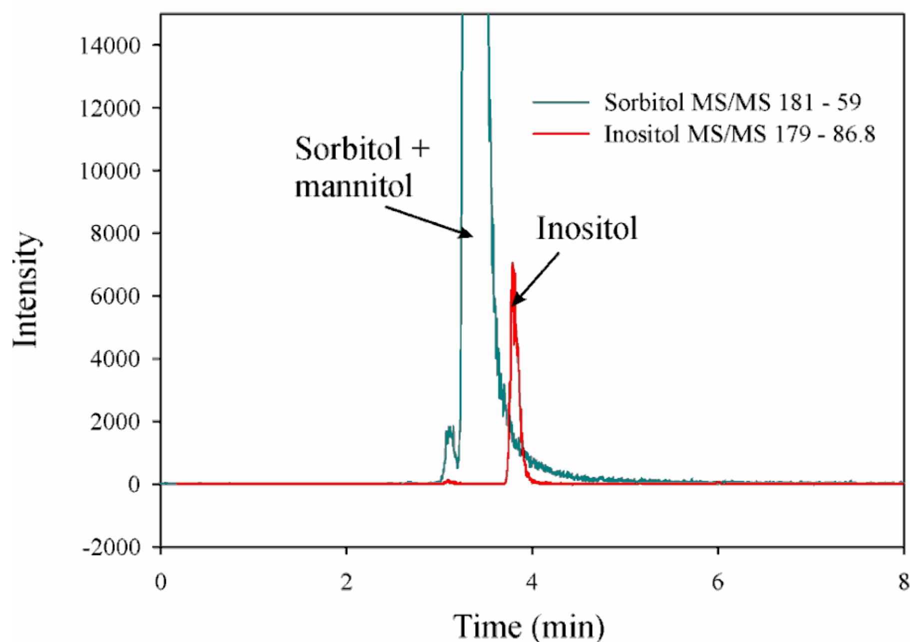


Fig. 3.14. LC-MS/MS multiple reaction monitoring (MRM) chromatograms collected in negative ion mode. Mass transitions were 181 → 59 for sorbitol monitoring, and 179 → 86.8 for *myo*-inositol monitoring. The sample contained 100 µmol/L sorbitol, 100 µmol/L *myo*-inositol, 500 µmol/L mannitol, and 1 mM glucose.

Mass spectrometric detection of polyols as halide adducts

Introduction

Sorbitol is known to form adducts with halides such as chloride, iodide, or fluoride.[118] These adducts can be fragmented in the collision cell of a tandem mass spectrometer to remove the halide. The choice of halide is important. Fluoride is known to cause strong ion interference, and chlorine and bromine have the disadvantage that there is more than one main isotope, which complicates the method, and reduces the sensitivity. Chorine has an abundance of 76% ^{35}Cl and 24% ^{37}Cl, and bromine has 50% ^{79}Br and 50% ^{81}Br. The iodide adduct was chosen for quantitation because it has only one abundant isotope at 126.9 Da. A high abundance of different isotopes splits the mass signal and reduces the sensitivity at any particular mass.

Sorbitol and glucose have previously been measured as iodide adducts by adding iodoform to the mobile phase.[131]

Methods

LC-MS/MS

The mobile phase contained 85% acetonitrile and 15% water, and the stationary phase was a Kinetex HILIC (Phenomenex, 2.1 × 10 mm, 2.6 µm) column. Tandem mass spectrometry was carried out on an API4000 instrument (AB Sciex). The MS/MS conditions were determined by infusing a 5 µmol/L standard of sorbitol and *myo*-inositol in the mobile phase containing iodine into the mass spectrometer and performing a compound optimization.

Iodine (BDH) and diiodomethane (Sigma) were investigated as mobile phase additives for forming iodide adducts. Different concentrations (1, 5, 10, 20 mmol/L) of diiodomethane were tested in the mobile phase to investigate the optimum sensitivity.

Results and Discussion

The sensitivity for *myo*-inositol was improved by measuring the adduct mass transition 307 → 127 compared to MS/MS of the free polyol (Fig. 3.15). Iodide readily dissociates from the polyol with low collision energy, allowing for an intense ion transition signal for quantitation. The optimum concentration of diiodomethane in the mobile phase was 10 mmol/L. Increasing the diiodomethane concentration in the mobile phase did not increase the peak sizes. While iodine (I_2) readily formed iodide adducts with polyols, it appeared to build up in the mass spectrometer, gradually reducing the sensitivity. For both the sorbitol and inositol iodide adducts, the transition from the adduct to the iodide ion was the most intense mass transition (Table 3.2). Sorbitol may form a stronger adduct with iodide than inositol, since it is not cyclic and can potentially attach to an iodide ion using both ends of the molecule.

Table 3.2 Compound optimization data for fragmentation of iodide adducts of polyols.

Sorbitol MS/MS	DP (V)	CE (V)	CXP (V)	Intensity
309.1 → 126.80	-30.0	-22.0	-5.0	7792000
309.1 → 125.07	-30.0	-130.0	-35.0	44000
309.1 → 97.16	-30.0	-42.0	-3.0	22000
309.1 → 79.65	-30.0	-106.0	-1.0	21000
309.1 → 96.57	-30.0	-40.0	-5.0	19000

myo-Inositol MS/MS	DP (V)	CE (V)	CXP (V)	Intensity
307.1 → 126.70	-30.0	-14.0	-7.0	143017
307.1 → 199.40	-30.0	-28.0	-3.0	992
307.1 → 125.48	-30.0	-130.0	-35.0	725
307.1 → 96.47	-30.0	-38.0	-7.0	417
307.1 → 79.95	-30.0	-114.0	-5.0	167

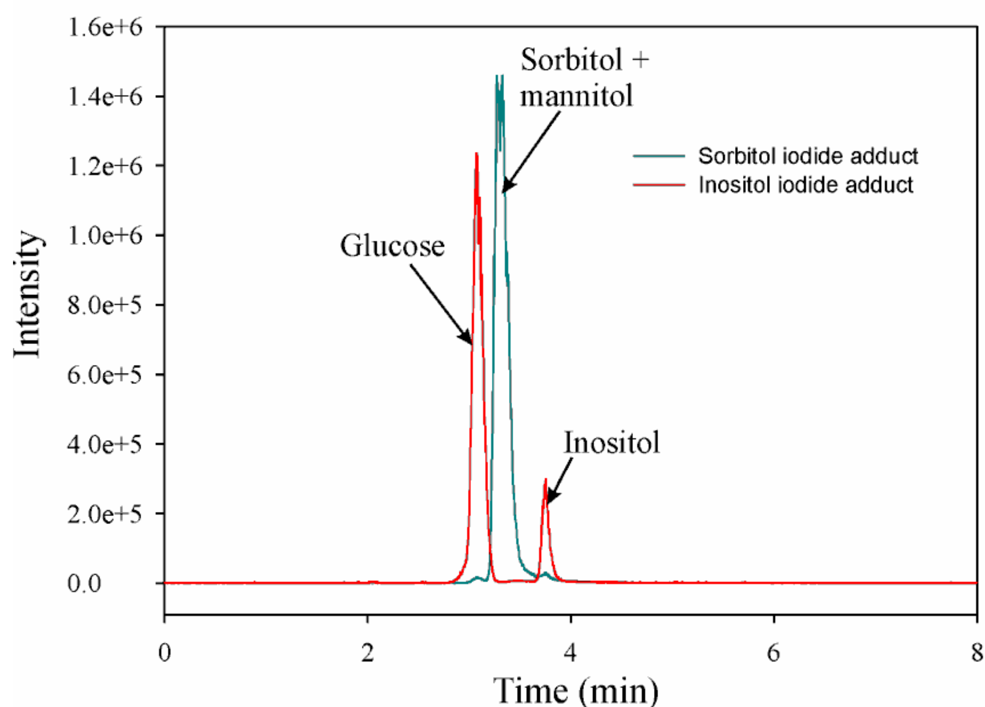


Fig. 3.15. LC-MS/MS chromatograms of iodide adducts separated on a Kinetix silica column. Mass transitions were monitored in negative ion mode at 309 → 127 for sorbitol monitoring, and 307 → 127 for *myo*-inositol. The sample contained 100 µmol/L sorbitol, 100 µmol/L *myo*-inositol, 500 µmol/L mannitol, and 1 mM glucose.

Detection of polyols as iodide adducts using a single quadrupole mass spectrometer

Introduction

Because a mass transition consisting of a halide ion disassociating from a polyol molecule is trivial, tandem mass spectrometry offers little advantage in the way of specificity. Therefore single quadrupole mass spectrometry can be expected to as effective as tandem mass spectrometry in this situation.

Methods

Samples and standards (50 µL) were extracted into an extraction solvent containing 75% acetone and 25% dimethylsulfoxide (DMSO). Flow injection analyses (FIA) were performed to optimize the source parameters for the iodide adduct of sorbitol and *myo*-inositol in the Agilent 6120 single quadrupole mass spectrometer using negative ion mode with an electrospray ion source. Two µL of a 50 µmol/L standard of sorbitol was injected repeatedly while altering the fragmentor voltage, capillary voltage, source gas temperature and source gas flow rate. No column was attached, and the mobile phase contained 15% water, 10 mmol/L diiodomethane, and 85% acetonitrile. While altering the other parameters had little effect on the intensity, the fragmentor voltage was found to have a strong effect on the yield of the iodide adduct (Fig. 3.16). At fragmentor values over 50 V, there was a lower yield of the iodide adduct, and at fragmentor values over 100 V, there was more free sorbitol present in the mass spectrometer than the iodide adduct. The sensitivity was approximately four times lower for *myo*-inositol than for sorbitol. A relatively low fragmentor value is required to avoid the dissociation of the iodide from the polyols in the source. The optimal capillary voltage was 4000 V, the optimal drying gas flow rate was 6 L/min, and the optimal source gas temperature was 275°C.

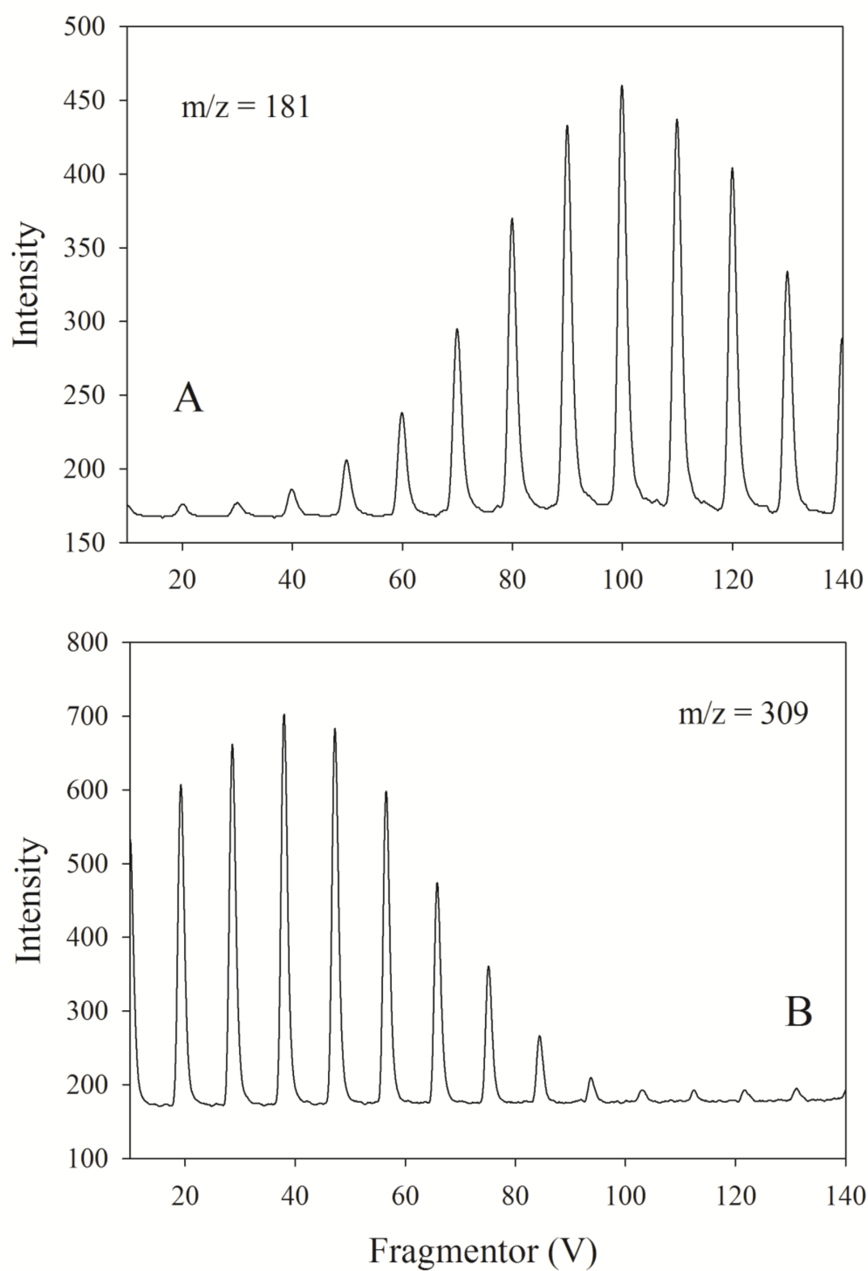


Fig. 3.16. Flow injection analysis (FIA) experiment for sorbitol in the single quadrupole mass spectrometer (Agilent 6120), varying the fragmentor voltage. A is the FIA for free sorbitol in negative ion mode, and B is the sorbitol iodide adduct in negative ion mode.

Results and discussion

Sample extraction and recoveries

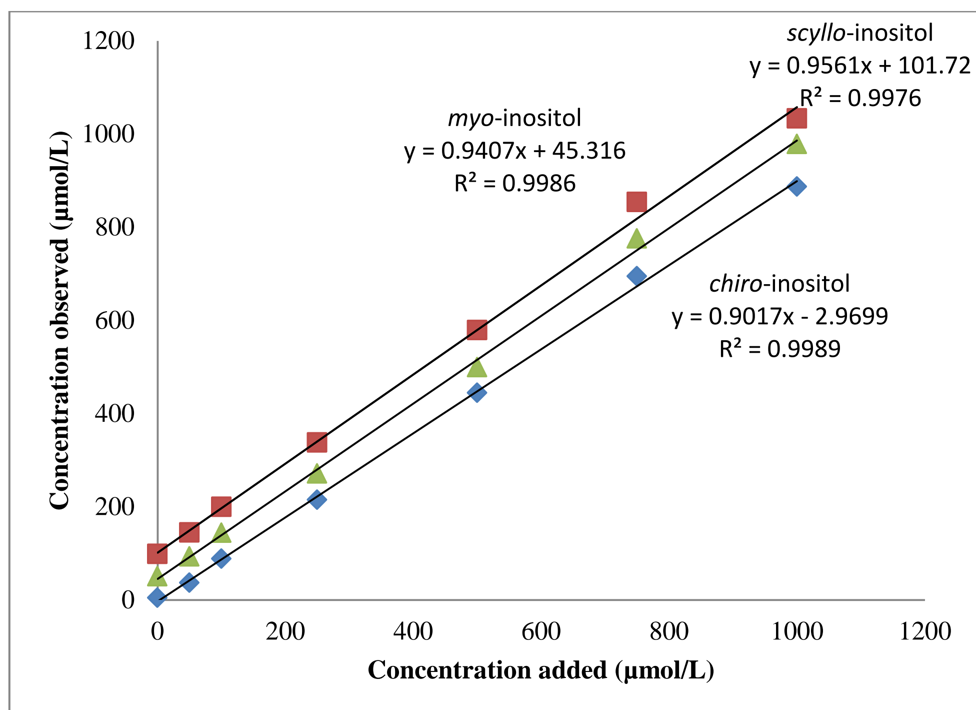


Fig. 3.17. Urine with added levels of *myo*-inositol (green), and *scyllo*-inositol (brown). Samples were extracted in 75% acetone and 25% DMSO, and measured by single quadrupole mass spectrometry after separation on an amide column.

Recoveries of *myo*-inositol and *scyllo*-inositol added to urine were 100% and 95% respectively (Fig. 3.17). *chiro*-Inositol was most problematic with smaller than expected peaks and low recoveries in urine. This is likely to be caused by the presence of ion interference in the urine chromatograms where the *chiro*-inositol elutes, as the other inositols were not affected in this way.

To test for ion interference, 50 µmol/L of *myo*-inositol was added to the mobile phase and a reagent blank, plasma and urine samples were injected. A negative peak was observed in the chromatograms. This interfering substance was found to come from the DMSO in the extraction solvent. Therefore, a cleaner grade of DMSO (ACS, 99.9%) was purchased. Recoveries of *chiro*-inositol were found to be over 90% after this grade of DMSO was further purified by elution through silica (50-200 mesh).

The recoveries depend on the efficiency of the extraction, even though the use of internal standards can help to account for this. Polyols have a low solubility in most organic solvents. However, they are quite soluble in DMSO, especially the inositols. By using DMSO in the extraction solvent, there was no need for a two-step extraction by precipitating the proteins with TCA before extracting the samples into organic solvent. The samples need to be in an injection solvent containing high organic solvent content for normal phase chromatography.

Comparison of APCI and ESI with and without formation of iodide adducts

Introduction

Methods for the detection of polyols are compared here. The aim was to determine whether detecting polyols as iodide adducts provides better sensitivity than direct detection. Two ion sources, atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI), are compared.

Methods

Sample preparation

Fifty microlitres of sample was added to an extraction solvent containing 75% acetone, 25% DMSO. The samples were vortexed for 20 seconds, then centrifuged (13,000 × g, 3 min), and the supernatant was transferred to a HPLC vials and capped for analysis.

LC-MS

Detection of polyols was compared using ESI and APCI with and without diiodomethane in the mobile phase.

Inositols were separated using an Agilent 1200 Series HPLC system and detected using an Agilent 6120 single quadrupole mass spectrometer with either an electrospray ion source for detecting inositols as the iodide adducts, or an APCI source for direct detection. An isocratic system was used with a mobile phase containing 85% acetonitrile, 15% water. When measuring the iodide adducts, 10 mmol/L of diiodomethane was added to the mobile phase. The flow rate was 0.3 mL/min, the injection volume was 10 µL, and the oven temperature was 40ºC. The run time was 20 min, although the samples were diverted to waste post-column for 2 minutes after injection. The chromatographic separation was performed on a Waters Acquity BEH amide 2.1 × 100 mm, 1.7 µm UPLC column (Ireland). Mass spectrometer parameters were optimized by flow injection analysis with no column.

Inositols were monitored directly using selected ion monitoring (SIM) in negative ion mode at m/z = 179 for inositols, sorbitol was monitored at m/z = 181. When measuring the iodide adducts with electrospray ionization, inositols were

detected in negative ion mode at m/z = 307, sorbitol was monitored at *m/z* = 309, and glycerol was monitored at m/z = 219. The fragmentor was set to 50 V, the capillary voltage was 4000 V, the drying gas flow rate was 6 L/min, and the source gas temperature was 275°C.

For direct detection with APCI, the fragmentor voltage was set to 90 V, the capillary voltage was set to 3000 V, the corona current was set to 6 µA, nebulizing pressure was 60 psi, the drying gas was 9 L/min, the drying gas temperature was 175ºC, and the vaporizing temperature was 300ºC.

Results and discussion

The best sensitivity was observed when detecting polyols directly using APCI without diidomethane in the mobile phase (Table 3.3, Fig. 3.18). If no APCI source is available, then it is preferable to measure polyols as iodide adducts using ESI. The peak heights were greater using direct detection with ESI, however, the noise was also greater than was observed when detecting them as the iodide adducts with diiodomethane in the mobile phase.

Table 3.3. Comparison of sorbitol and *myo*-inositol measured in negative ion mode by APCI and ESI with direct detection, and detection as the iodide adducts.

		chiro-Inositol	*myo*-Inositol	*scyllo*-Inositol	Sorbitol
APCI	Peak height	371	441	280	1401
	LOD µmol/L	2.4	1.4	3.2	0.4
APCI (iodide adduct)	Peak height	nd	nd	nd	nd
	LOD µmol/L				
ESI	Peak height	312	320	215	1078
	LOD µmol/L	7.8	7.5	11	1.7
ESI (iodide adduct)	Peak height	296	199	186	486
	LOD µmol/L	2.0	3.0	3.2	1.2

Note: Data was collected by injecting a 100 µmol/L aqueous standard. LOD was calculated as the estimated concentration when the signal to noise ratio equals three. nd = not detected.

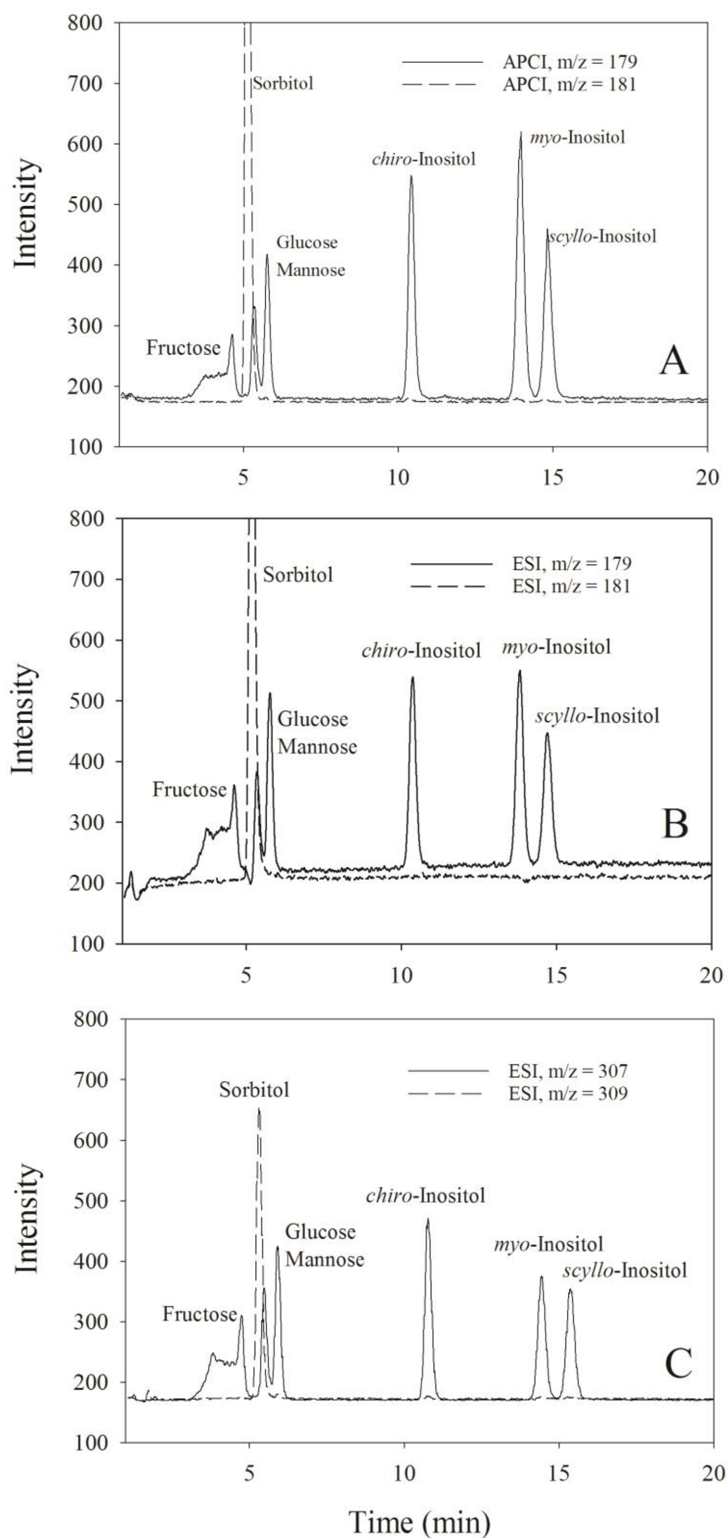


Fig. 3.18. Polyols detected directly using an APCI source (A). Polyols detected directly with an electrospray source (B). Polyols detected as iodide adducts with diiodomethane in the mobile phase using an ESI source (C). All data was collected by injecting a 100 μmol/L aqueous mixed standard.

Complexes using polyols as ligands

Complexes of polyols with boric acid

Introduction

Polyol *cis*-diols bind to boric acid under alkaline conditions to form cyclic borate esters. It was expected that the predominant ion present when there is an excess of ethanediol and boric acid in solution will be the polyol-boric acid-ethanediol complex (Figs. 3.19B and 3.20B). The ethanediol complexes are formed when the polyols are injected into the LC-MS/MS spectrometer and interact with boric acid and ethanediol in the mobile phase. As ethanediol binds relatively weakly with boric acid, the polyol *cis*-diols should compete for binding with boric acid. The reason for adding ethanediol to the mobile phase is that the complex it forms with boric acid is volatile, whereas boric acid is not volatile and will deposit inside the mass spectrometer. Ackloo *et al*.[129] showed that the predominant ion was boric acid bound to two sorbitol molecules in the presence of excess boric acid (Figs. 3.19C and 3.20C). Sorbitol and *myo*-inositol form complexes with boronic acids or transition metal ions which can potentially be used to separate the polyols from chemically similar isomers.

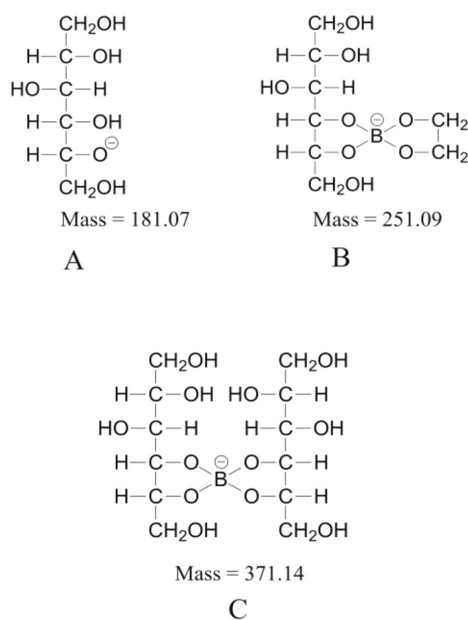


Fig. 3.19. Structures of sorbitol and complexes with boric acid and ethanediol in negative ion mode. A.) Sorbitol ion, B.) sorbitol-boric acid-ethanediol complex, C.) boric acid complexed with two sorbitol molecules.

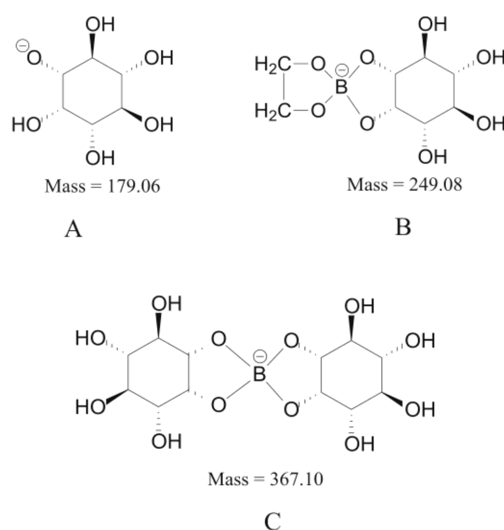


Fig. 3.20. Structures of *myo*-inositol and complexes with boric acid and ethanediol in negative ion mode. A.) *myo*-inositol negative ion, B.) inositol-boronate-ethanediol complex, C.) boronate complexed with two inositol molecules.

Methods

Reagents and chemicals

Ammonium tetraborate, sorbitol and *myo*-inositol were obtained from Sigma (St Louis, MO, USA). Ethanediol was obtained from BDH (Poole, England). Ammonium tetraborate was found to leave a residue on drying. However, when ethanediol was added to the solution it was volatile and no residue was left on drying, making the solution suitable for mass spectrometry.

Chromatography

To separate the polyol complexes, a reversed phase alumina column (Alusphere, RP Select, 150 × 4 mm, 4 µm, Merck, Germany), and a reversed phase titania (Sachtopore, 150 × 4 mm, 3 µm) column were investigated. A gradient system was used going from A to B over 8 minutes. Mobile phase A contained 10 mmol/L ammonium tetraborate, 100 mmol/L ethanediol, and 80% water and 20% methanol. Mobile phase B was 100% methanol. Standards were injected with no column attached and good sensitivity was observed for the inositol complex ion, but not as good for the sorbitol complex ion.

Mass spectrometry

One hundred micromolar standards of *myo*-inositol and sorbitol were made up in 10 mmol/L ammonium tetraborate and 100 mmol/L ethanediol in 50% water and 50% methanol. These solutions were infused into the mass spectrometer and a Q1 scan was performed on the standard solutions in negative ion mode. To determine the fragmentation pattern, MS/MS was performed on the parent ions corresponding to the complex with one mole of boric acid and one mole of ethanediol. Compound optimizations were performed on the parent ions of each polyol, and the mass spectrometer MS/MS parameters are shown in Table 3.4.

Table 3.4. Compound optimization. Negative ion mode.

Sorbitol ion with boric acid and ethanediol mass of 251.1				
MS/MS	DP (V)	CE (V)	CXP (V)	Intensity
251.1 → 86.9	-40	-36	-13	257000
251.1 → 162.9	-40	-22	-1	128000
251.1 → 100.9	-40	-30	-15	119000
251.1 → 84.92	-40	-30	-13	88000
Inositol ion with boric acid and ethanediol mass of 249.1				
MS/MS	DP (V)	CE (V)	CXP (V)	Intensity
249.1 → 186.8	-75	-26	-9	103000
249.1 → 86.9	-75	-42	-13	52000
249.1 → 124.9	-75	-30	-9	41000
249.1 → 160.9	-75	-26	-1	37000

DP = decoupling potential, CE = collision energy, CXP = collision cell exit potential. Data collected using API4000 LC-MS/MS.

Results and discussion

Chromatography

The boric acid complexes did not bind well to the amide column and eluted considerably earlier than the uncomplexed polyols. In the single quad mass spectrometer, the polyol-borate to polyol-borate-ethanediol complex ratio was 2:1, which reduces the sensitivity. The uncomplexed sorbitol ion was approximately 1% of the intensity of the polyol-boronate ion. The high pH of the mobile phase also restricts the use of silica based columns. Chromatographic columns containing particles of

titania, alumina, or zirconia are stable at high pH (> pH 7) and therefore potentially more useful for separating polyol-borate complexes. However, no peaks were observed when using these columns.

Mass spectrometry

A Q1 ion scan of infused solutions showed that the expected ions were present. With an excess of ethanediol, the main ion formed was boric acid bound to one polyol molecule and one ethanediol molecule as seen in Fig. 3.19B and Fig. 3.20B. A complex containing boric acid bound to two molecules of sorbitol or inositol was not observed. There was however some of the free polyols observed in negative ion mode in the Q1 scan with ions at 181.2 Da for sorbitol and 179.0 for inositol which corresponds to the mass of these polyols minus a hydrogen ion. There was also a large signal at 131 Da which corresponds to boric acid bound to two molecules of ethanediol.

The MS/MS fragmentation of sorbitol and inositol formed an ion with a mass of 87 Da (Fig. 3.21) which was reported by Ackloo *et al.*[129] This gives confidence that the mass spectrometer is fragmenting the correct ions for the sorbitol and inositol complexes. The ion fragment at 101 was observed for sorbitol but not inositol. Ackloo *et al*[129] showed that mannitol forms a stronger complex with borate than sorbitol with a significant peak observed for the uncomplexed polyol 181 Da in the mass spectrum which was approximately the same size as the [2M] complex ion 371 Da.

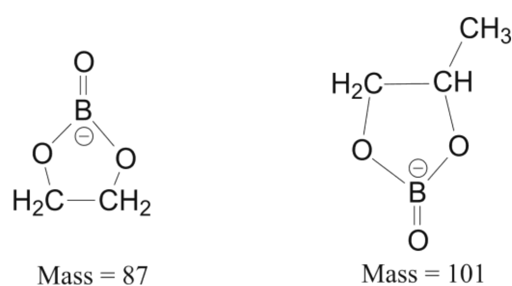


Fig. 3.21. MS/MS fragment ions formed from polyol-borate-ethanediol complexes.

Complexes of polyols with boronic acids

Boronic acids, such as methylboronic acid and phenylboronic acid, are also known to form complexes with *cis*-diols (Fig. 3.22) which may also be useful for measuring polyols by mass spectrometry. The reaction of boronic acids with *cis*-diols is reversible and dependent on the pH and the solvent. Boronate esters are more stable at higher pH (>10) where the boronate ions are present in higher concentrations.[121, 132]

There are various ways in which boronic acids can potentially aid the separation and detection of polyols. For example, some boronic acids fluoresce more when bound to carbohydrates.[133] The functional groups attached to boronic acids may also interact with stationary phases to help with separations. Some ways in which boronic acids can be used to detect polyols are investigated in this section.

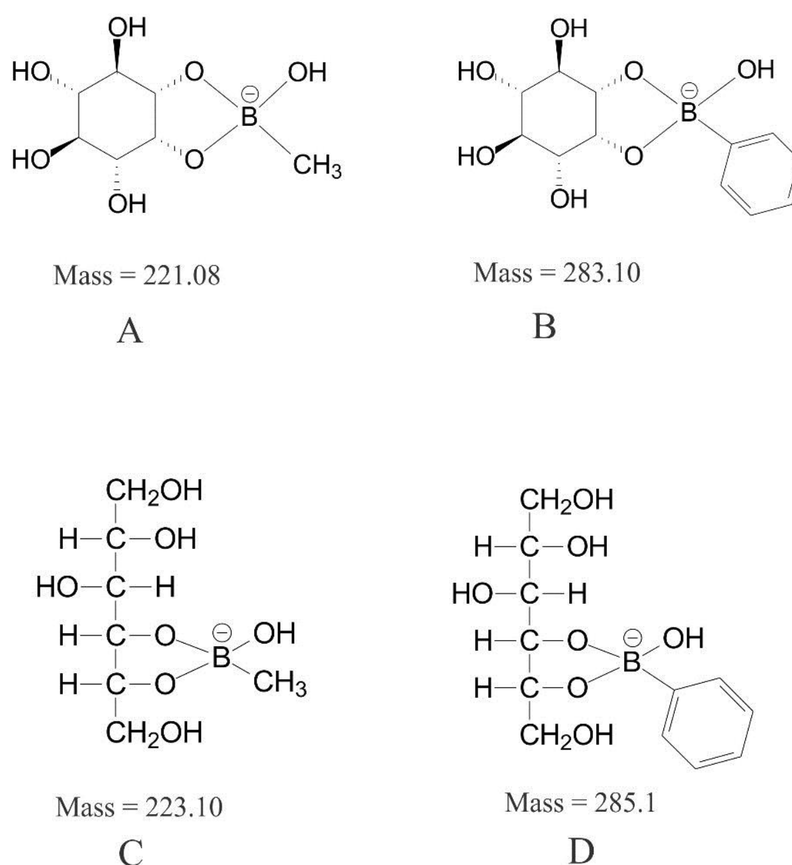


Fig. 3.22. Boronate complexes with sorbitol and inositol. A.) Inositol with methylboronic acid, B.) inositol with phenylboronic acid, C.) sorbitol with methylboronic acid, D.) sorbitol with phenylboronic acid.

Polyol complexes with phenylboronic acid

Introduction

The phenyl group on phenylboronic acid may be useful for the separation of polyols. The theory is that the aromatic group may bind (by π interactions) with the graphite particles of a column such as Hypercarb (Thermo), and therefore provide a separation of polyols that are complexed to the boronic acid.

Methods

LC-MS/MS

A Thermo Hypercarb 100 × 3 mm, 5 µm column (Phenomenex) was used with 5 mmol/L ammonia and 10 mmol/L phenylboronic acid in the mobile phase. Various gradients were tried, including: 50% H_2O and 50% acetonitrile going to 10% water and 90% acetonitrile over 8 minutes; and 90% water and 10% acetonitrile going to 10% water and 90% acetonitrile over 8 minutes. Other columns that were tried (that are stable at high pH) include a Merck 150 × 4 mm, 4 µm Aluspher RP Select, and an Asahipak ODS 250 × 4 mm, 5µm column. These columns were used with a flow splitter post column as the required flow rate was too great for the mass spectrometer. The mass transitions with the greatest intensities were used for the sorbitol and inositol phenylboronic acid complexes. These were 285 → 267 for sorbitol and 283 → 265 for inositol (Table 3.5). These mass transitions both have a mass difference of 18 corresponding to a loss of a water molecule from the polyol complexes.

Table 3.5. Compound optimization data for phenylboronic acid complexes of sorbitol and inositol.

Sorbitol - phenylboronic acid complex, mass = 285.07				
MS/MS	DP	CE	CXP	Intensity
285.1 → 267.1	-30	-12	-1	11753
285.1 → 225.1	-30	-18	-13	6273
285.1 → 121.1	-30	-28	-9	6055
285.1 → 118.9	-30	-26	-21	1298
285.1 → 43.0	-30	-56	-5	1727
Inositol - phenylboronic acid complex, mass = 283.24				
MS/MS	DP	CE	CXP	Intensity
283.2 → 264.9	-70	-14	-7	10000
283.2 → 42.7	-70	-82	-1	6000
283.2 → 43.4	-70	-58	-5	6000
283.2 → 120.7	-70	-24	-5	6000
283.2 → 85.0	-70	-40	-3	5000

DP = decoupling potential, CE = collision energy, CXP = collision cell exit potential.
Data collected using API4000 LC-MS/MS.

Results and discussion

The peaks were not well separated on the Hypercarb column. Changing the gradient only made a small difference, and when higher water contents were used in the initial gradient step the sorbitol peak was split. The Aluspher and Asahipak reversed phase columns did not separate the phenylboronic acid complexes either. Sorbitol, mannitol, and galactitol all eluted early and were co-eluting. The baseline noise was high with phenylboronic acid in the mobile phase.

The mass transitions corresponding to the loss of a water molecule in the collision cell were the most abundant fragment ions, *i.e.* sorbitol 285 → 267 and inositol 283 → 265. Other mass transitions correspond to the loss of the fragment ion corresponding to phenylboronic acid (121 Da) for both polyol complexes. The mass transition of 285 → 225 corresponds to the loss of an ethanediol group from sorbitol.

A comparison of Figs. 3.23 and 3.24 shows that the selectivity changes. Sorbitol elutes last when the starting conditions include a higher water content. Two peaks were observed for the mass transition 285 → 267 for sorbitol after increasing the gradient. Sorbitol can possibly form a complex with phenylboronic acid using one of the end chain hydroxyl groups. This alternative sorbitol complex would have the same mass and may produce the same fragments in the collision cell. This may explain the existence of two peaks in the chromatogram (Fig. 3.24).

The Hypercarb column was investigated for separating polyol-phenylboronic acid complexes because it contains graphite particles which are stable at all pH. The graphite stationary phase was also expected to help the separation by way of π interactions with the benzene ring on the phenylboronate-polyol complex. However, this was not found to be the case. With the high background noise, this approach would be unlikely to produce the necessary sensitivity to measure polyols, even if a useful separation could be achieved for the phenylboronate esters.

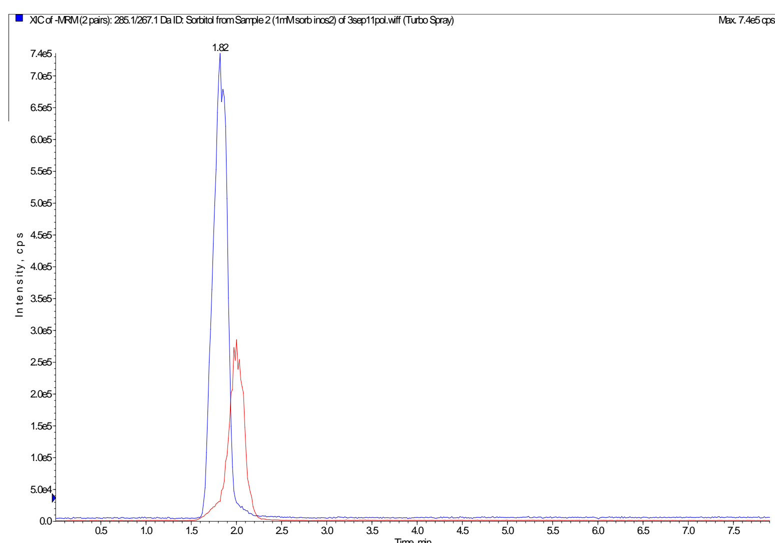


Fig. 3.23. LC-MS/MS chromatograms showing: 1 mmol/L aqueous standards of sorbitol (blue) and inositol (red) complexed with phenylboronic acid. Sorbitol complex mass transition = 285 → 267, inositol complex mass transition = 283 → 265. A Hypercarb column was used, with a mobile phase gradient: starting with 50% water going to 10% water over 8 minutes. Detection was carried out on an Applied Biosystems API4000 tandem mass spectrometer.

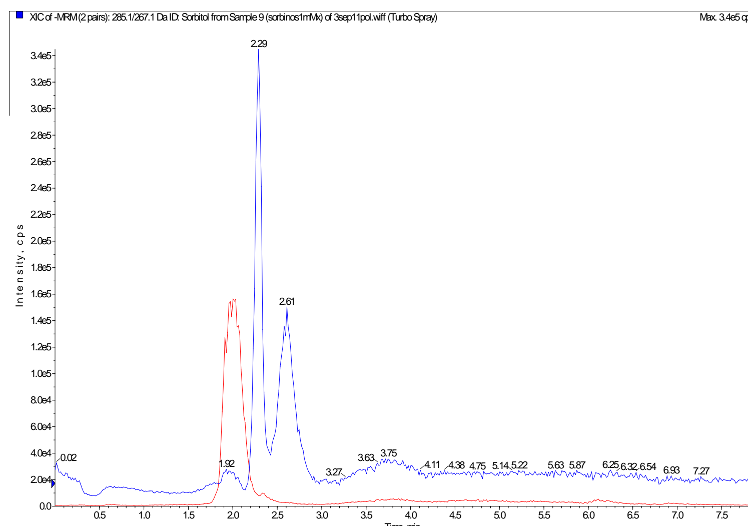


Fig. 3.24. LC-MS/MS chromatograms showing: 1 mmol/L aqueous standards of sorbitol (blue) and inositol (red) complexed with phenylboronic acid. Sorbitol complex mass transition = 285 → 267, inositol complex mass transition = 283 → 265. A Hypercarb column was used here with a gradient starting with 90% water and acetonitrile going to 10% water over 8 minutes. Detection was carried out on an Applied Biosystems API4000 tandem mass spectrometer.

Modification of a stationary phase to enhance the separation of polyols

Introduction

As *cis*-diols interact with boronates, a column with a boronate coated surface may be useful for the separation of polyols. Boronate affinity columns which are useful for separating small molecules such as these polyols are not available commercially. The only boronate columns available commercially are designed to separate glycoproteins and have a high pore size and particle size. Boronate affinity columns can be made by derivatizing the surface of silica with boronic acid groups[134] which should provide a useful interaction with *cis*-diols on the column, therefore providing a mode of separation for polyols. It may be possible to modify a silica column with boronic acids by pumping a reaction mixture through an unmodified silica column to create a column with a boronate functional group attached to the silica particles. Boronic acids can be bound to silica using triethoxysilane.[134c] However, triethoxysilane is pyrophoric and could not be shipped to New Zealand because it is too hazardous, so an alternative synthetic route was investigated.

The aim was to produce a stable HPLC column containing silica particles derivatized with boronates so that it can be used to separate polyol isomers such as sorbitol, mannitol, and galactitol.

Methods

An HPLC column containing unmodified silica (150 × 3 mm, 3 μm, Luna, Phenomenex) was modified with *m*-aminobenzeneboronic acid using an adaptation of a synthesis described by Li *et al.*[135] (Fig. 3.25). To activate the silica for attachment of the boronic acid, 15 mL of γ-chloropropyltrimethoxysilane (Sigma) was added to 150 mL of toluene. This solution was recycled through the silica column overnight at room temperature at a flow rate of 0.3 mL/min. The γ-chloropropylsilane substituted silica column was washed with dichloromethane then methanol. A solution was made up containing 1 g of *m*-aminobenzeneboronic acid (Sigma) and 1 mL of 1 M NaOH in 100 mL of distilled water. This solution was eluted through the column at 80ºC overnight at a flow rate of 0.3 mL/min. The column was washed with water and then methanol.

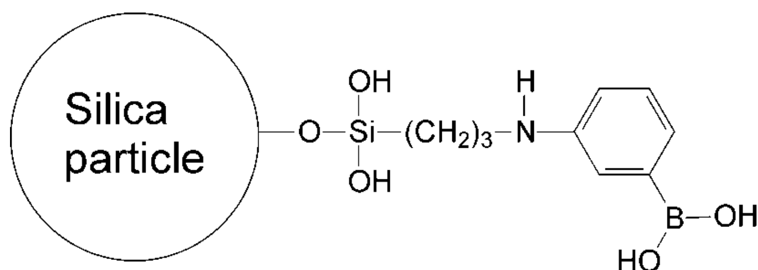


Fig. 3.25. A silica column particle derivatized with a boronic acid functional group.

Results and discussion

The boronate substituted silica column was not successful at separating the polyol isomers. With a mobile phase containing 20% water and 80% acetonitrile, no peaks were observed (with LC-MS-APCI). When some ethanediol (1 mL/L) was added to the mobile phase, a single broad tailing peak was observed for a mixture of sorbitol, mannitol, and galactitol. While binding of the polyols to the boronic acid would most likely be increased at higher pH, the silica based column would not be stable under those conditions.

NMR detection of polyol-methylboronic acid complexes

Introduction

The hypothesis is that different polyols bound to methylboronic acid would lead to differences in the chemical shift of the methyl group singlet in the proton NMR spectrum.

Methods

600 µL of 10 mmol/L methylboronic acid in water was added to a 1.5 mL microcentrifuge tube. 200 µL of a standard containing: 10 mmol/L sorbitol, mannitol, or galactitol, and a water blank were added. 50 µL of 1 mol/L sodium hydroxide was added along with 20 µL of acetonitrile and 200 µL of D_2O. The mixtures were vortexed and transferred to 5 mm NMR tubes. Samples were measured using a Varian 500 MHz NMR using the PRESAT water suppression technique. 8 scans were collected.

Results and discussion

The methylboronate ion was observed at -0.524 ppm as a singlet (using acetonitrile as a reference standard at 1.9 ppm). The methyl group for the polyol complexes of methylboronic acid were observed on the side of the methylboronate singlet as a major and a minor peak for each ion. The methylboronic acid methyl resonances were slightly different when bound to the different hexitol isomers. Sorbitol (-0.483 ppm), mannitol (-0.500 ppm), and galactitol (-0.495 ppm). This result shows that the methyl group was in a different chemical environment when complexed to the different polyols. This could be the basis for a novel detection method that can separate these polyols. However, it is unlikely to provide the resolution or the sensitivity required for the detection of hexitols and other polyols in biological samples. For future work, better resolution may be obtained by collecting more scans. The potential interference from other *cis*-diols such as glucose and the effect of pH should also be investigated. Further investigations involving methylboronic acid - polyol complexes with NMR detection were not carried out due to a major mechanical failure of the 500 MHz NMR at the University of Canterbury.

Complexes of polyols with transition metals

Introduction

Polyols can form stable complexes with metal ions in the presence of base.[136] The hydroxyl groups on sugars have been shown to interact with the metal oxides, alumina[137] and zirconia.[138] Columns that have traditionally been used to separate carbohydrates are likely to rely on the complexation of hydroxyl groups with metals such as lead or calcium to separate polyols. Those columns contain beads of ion exchange resin which are in the lead or calcium form. The transition metals, manganese and cobalt, may be useful for measuring polyols in combination with mass spectrometry, considering that Mn and Co both have just one abundant isotope.

Methods

Samples were prepared by mixing 1 mL of a 10 mmol/L aqueous standard containing sorbitol, or galactitol, or mannitol with approximately 3 mL of a 10 mmol/L aqueous solution of cobalt acetate and 3 mL of an aqueous solution of 20 mmol/L lithium hydroxide in a test tube. The test tubes were vortexed and any colour change was noted.

Results and discussion

Sorbitol and its isomers galactitol and mannitol all formed an olive green colour when mixed with cobalt acetate and lithium hydroxide. However, a sample blank containing just water and no polyol remained a pale pink colour. The addition of sorbitol and other polyols forms a complex which could potentially be separated on an HPLC column and detected using electrochemical detection or mass spectrometry. Mannitol binds to cobalt (II) and cobalt (III) in a 1:1 ratio and may be present either as a bidentate or tridentate ligand.[136b]

The polyol metal complexes were not stable enough to measure without having metal ions and base present in the mobile phase. Addition of metal ions to the mobile phase was problematic when using electrochemical detection. The pressure increased gradually in the electrochemical cells when cobalt acetate was pumped through it. This approach was not attempted with mass spectrometric detection due to the high likelihood of background interference and the risk of contaminating the mass

spectrometers with metal ions. Detecting the colour change in the visible spectrum when polyols bind to transition metals is unlikely to provide the necessary sensitivity.

Polyols also appear to bind strongly to metal oxides. Sorbitol and inositols were found to bind very strongly to zirconia, titania, and alumina stationary phases with no peaks observed within 45 minutes. Attempts to elute the polyols off the metal oxide columns by increasing the water content of the mobile phase were not successful.

Detection of polyols using derivatization

Derivatization with isocyanates

Introduction

The hydroxyl groups of carbohydrates can be derivatized with isocyanates to form UV absorbing or fluorescent urethane derivatives (Fig. 3.26).[139] Since isocyanates are readily hydrolyzed by water, the reaction of isocyanates with alcohols requires anhydrous conditions.[139] The reaction also requires the presence of base to catalyse the reaction. Previous isocyanate methods have shown that by adding a large excess of derivatizing reagent during sample preparation, and heating the samples to 55ºC, the maximum number of hydroxyl groups are derivatized and only a single peak is seen in the chromatograms for each polyol.[44, 123] Measuring polyols as their urethane derivatives is likely to enhance the separation by providing more structural differences to the molecules, allowing for better chromatography. Sorbitol and other polyols have been detected by UV using pre-column derivatization with phenylisocyanate.[44, 123] There have been no reports of isocyanates used to measure polyols in blood plasma or urine. If derivatization with isocyanates is useful in these samples, then sensitivity could be improved by the choice of reagent. For example, 1-naphthyl isocyanate derivatives can be expected to have a higher molar absorbtivity than phenylisocyanate, and are also likely to be fluorescent.[140]

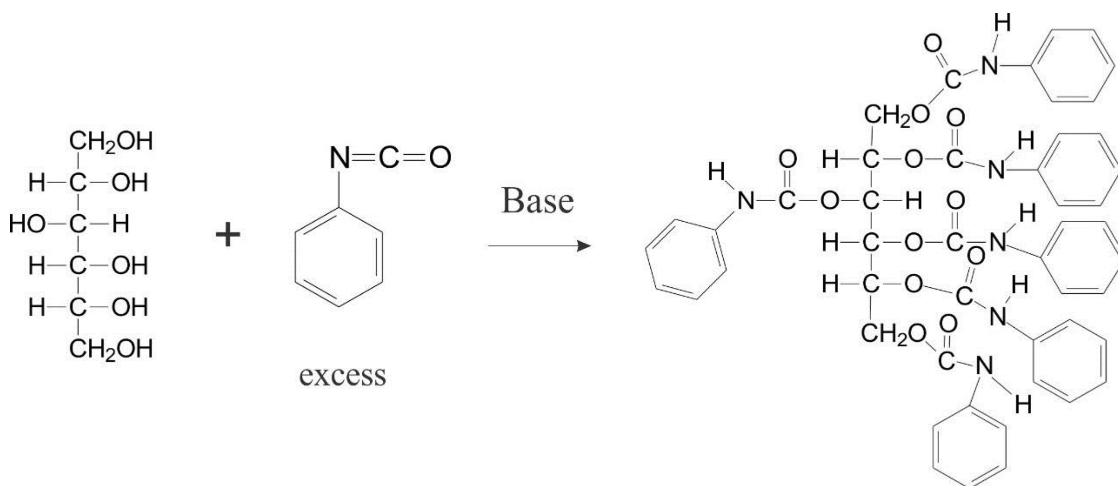


Fig. 3.26. Reaction of sorbitol with phenyl isocyanate showing derivatization of the maximum possible number of hydroxyl groups.

Methods

The method of Miwa *et al*.[44] was adapted to investigate aqueous standards. Fifty µL of 2 mmol/L aqueous polyol standards were dried down under nitrogen. 70 µL of pyridine and 20 µL of phenylisocyanate were added to the residue, and the mixture was reacted in sealed tubes at 55°C for 1 hour. 20 µL of methanol was then added to react with the excess isocyanate, and 300 µL of 50% water and 50% acetonitrile were added. The excess isocyanate precipitated as a white solid, which was centrifuged out at 13,000 × *g* for 5 minutes. The supernatant was transferred to HPLC tubes and capped.

Attempts to add 20 µL of phenylisocyanate to the sample in pyridine, and remove the water (using sodium sulfate) failed to produce any derivative peaks. The samples had to be dried down under nitrogen and dry pyridine added for the method to work.

Fifty µL of derivatized sample was injected onto a Supelco reversed phase LC18 (250 × 4.6 mm, 5 µm, Sigma) column using a gradient starting with 30% acetonitrile and 70% water, going to 20% water, 40% acetonitrile, and 40% methanol over 45 minutes. Detection was carried out using a Shimadzu SPD-20A UV detector set to 240 nm.

Results and discussion

The method produced polyol urethane derivatives with a high concentration of isocyanate present (2:7 isocyanate to pyridine ratio) added to a sample after drying

down under nitrogen. Fig. 3.27 shows the separation of phenylisocyanate polyol derivatives on the LC18 column. To make the reaction of polyols and isocyanates more specific, the *cis*-diols can be protected using boric acid. Only the hydroxyl groups not bound to the boric acid could then react with the isocyanates. This could potentially lead to less derivatives being formed for each individual polyol, therefore reducing the number of peaks in the chromatogram from each polyol.

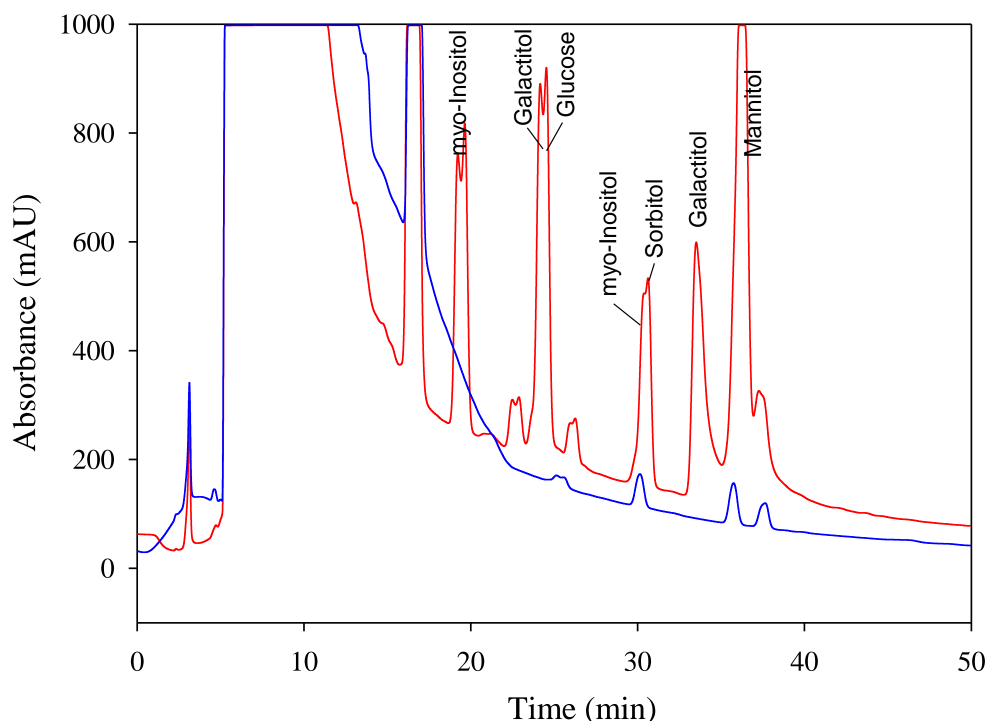


Fig. 3.27. Derivatization of polyols with phenylisocyanate showing a mixed standard containing 2 mmol/L sorbitol, mannitol, galactitol, *myo*-inositol, and glucose (red), and a blank containing no polyols (blue).

Derivatization with isocyanates generally produces too many poorly resolved peaks, often with multiple peaks for one polyol analyte. This approach was therefore considered unlikely to be suitable for measuring sorbitol and inositols in biological samples such as plasma and urine and was not pursued further.

Reaction of polyols with methylisatoic anhydride

Introduction

Hydroxyls can react with methylisatoic anhydride (MIA) to form fluorescent esters and release carbon dioxide (Fig. 3.28).[141] When the reaction takes place in the presence of boric acid, the *cis*-diols may be protected, and the number of reactive sites that the polyols can be derivatized by MIA may be reduced. By removing the *cis*-diols from derivatization by MIA, the structural differences between the polyols and their isomers may be exaggerated, making chromatographic separation more achievable. Using methylisatoic anhydride as the derivatizing reagent has advantages over isocyanates in that the reaction takes place under mild conditions at room temperature, and tolerates the presence of water. Methylisatoic anhydride has been used to measure inositol by capillary electrophoresis.[142] However, there are no reports of its use to measure polyols by HPLC. MIA derivatives are reported to fluoresce at excitation wavelengths in the range of 350-360 nm and an emission wavelength around 430-450 nm.[141]

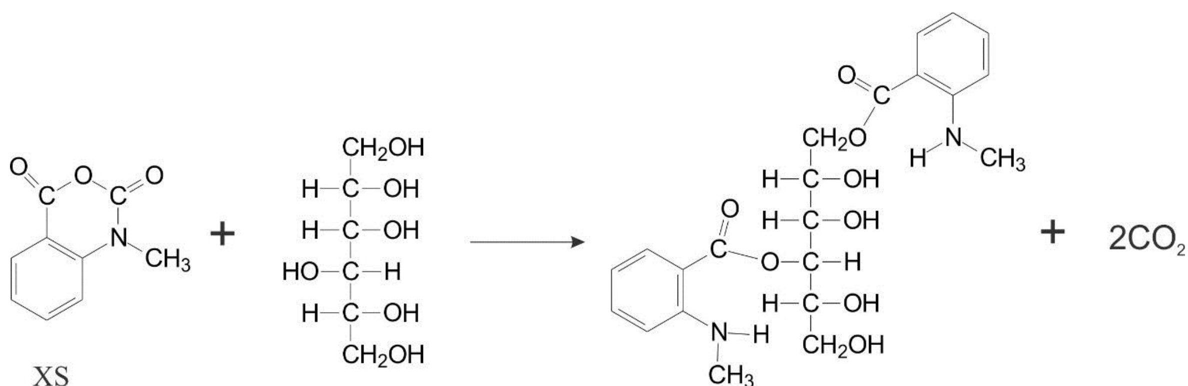


Fig. 3.28. Reaction of MIA with sorbitol.

Methods

Sample preparation

Fifty microlitres of 10 mmol/L aqueous polyol standard was added to a 1.5 mL microcentrifuge tube. To this was added 50 µL of 50 mmol/L triethylamine borate in dimethylsulfoxide (DMSO) and the mixture was vortexed briefly. One hundred µL of 30 mg/mL MIA in acetonitrile was added and the samples were vortexed at room

temperature for 10 minutes. 200 µL of distilled water was added to the samples, and they were vortex mixed, then centrifuged and transferred to HPLC vials.

HPLC

Attempts were made to separate the MIA derivatives using a reversed phase column, and on a strong cation exchange SCX column. The SCX column was a Phenosphere SCX (Phenomenex, 250 × 4.6 mm, 5 µm). The mobile phase contained 5 mmol/L triethylamine, 10 mmol/L succinic acid, 5% water and 95% acetonitrile. The flow rate was 1 mL/min and the oven temperature was set to 40ºC.

A reversed phase C30 Develosil (Phenomenex, 250 × 4.6 mm, 5 µm) column was used with a gradient going from 20% acetonitrile and 80% water to 30% acetonitrile and 70% water over 20 minutes, then to 100% acetonitrile at 35 minutes, and back to starting conditions at 40 minutes. The run time was 45 minutes, the flow rate was 1 mL/min, and the oven temperature was 40 ºC. The Shimadzu (RF-10AXL) fluorescence detector was set to 355 nm for excitation and 440 nm for emission.

Results and discussion

The polyol MIA derivatives did not separate well on a strong cation exchange (SCX) column (Fig. 3.29). A broad peak was observed for sorbitol when using the SCX column which eluted at around 7 minutes. Mannitol and galactitol had slightly different retention times but were not well separated. A broad peak corresponding to *myo*-inositol was observed at 10 minutes. Diluting the buffer in the mobile phase did not change the retention time of the derivatives, suggesting that cation exchange is not the primary mode of separation. The nitrogen bound to the benzene ring on the derivative is likely to be only weakly cationic at low pH, therefore resulting in low retention of the derivatives using SCX chromatography.

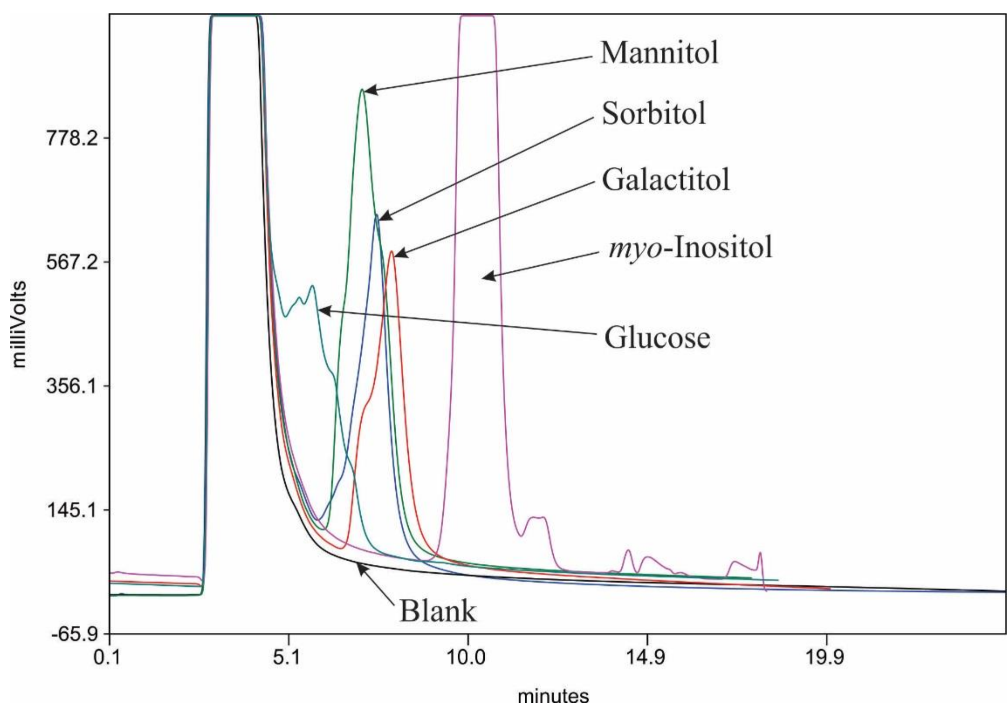


Fig. 3.29. Separation of polyol-MIA derivatives on an SCX column.

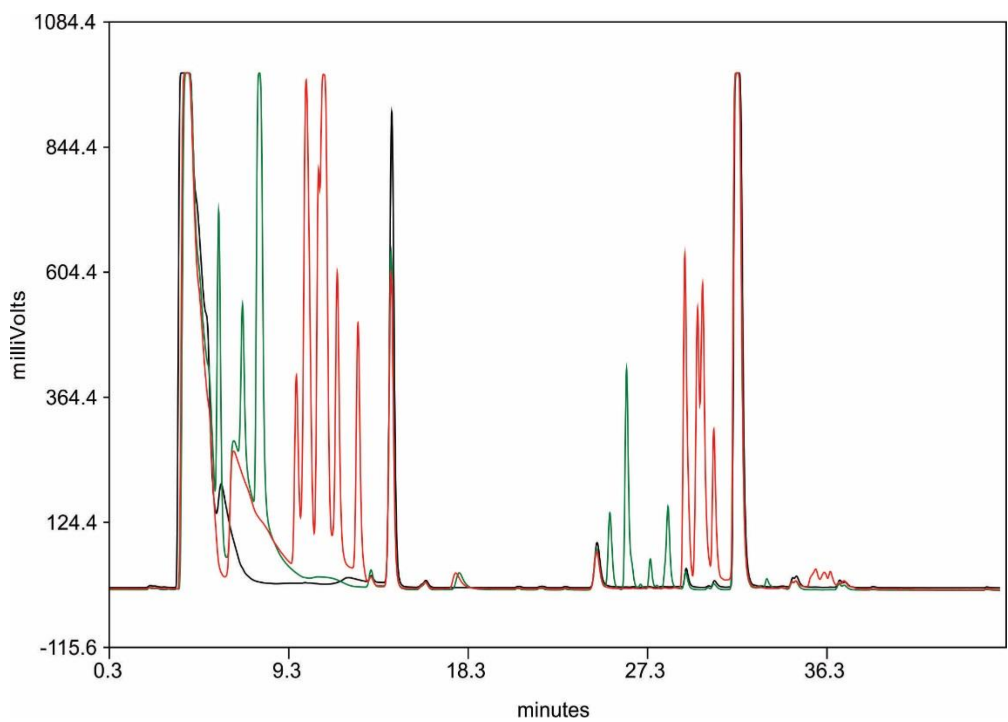


Fig. 3.30. Separation of polyol MIA derivatives on a C30 reversed phase column. Chromatograms shown are: a water blank (black); a 10 mM *myo*-inositol standard (green), 10 mM glucose standard (red).

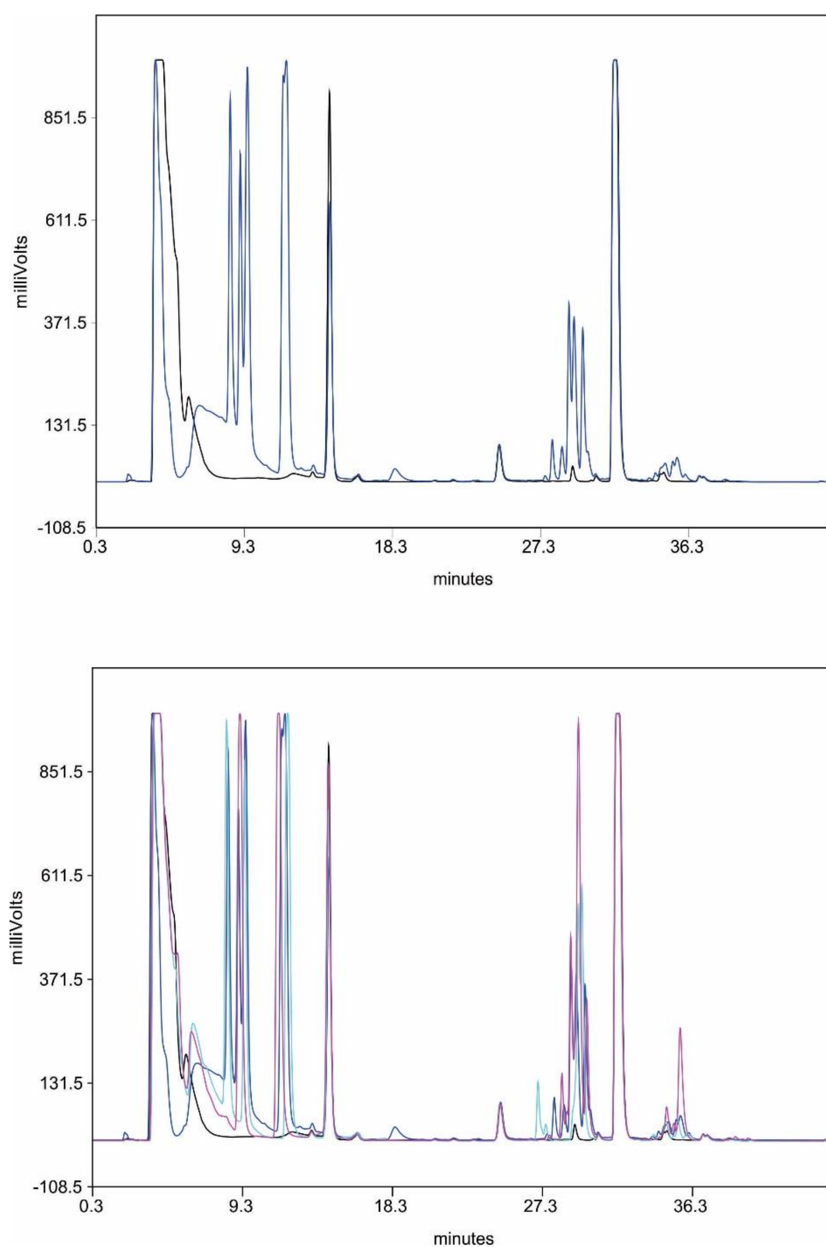


Fig. 3.31. Separation of polyol MIA derivatives on a C30 reversed phase column. Black = blank, dark blue = 10 mM sorbitol, pink = 10 mM mannitol, and light blue = galactitol.

Multiple MIA derivatives were observed on the C30 reversed phase column for each polyol: sorbitol, mannitol, galactitol, *myo*-inositol, and glucose (Figs. 3.30 and 3.31). Most of the sorbitol peaks were overlapping with mannitol and galactitol or only separating slightly. The *myo*-inositol peaks were better separated from the glucose, sorbitol, mannitol, and galactitol peaks. The multiple peaks observed for each polyol make separation and quantification difficult, especially when considering the

large number of hydroxylated compounds present in biological samples. These multiple peaks are likely to be due to a mixture of compounds formed when MIA reacts with different hydroxyl groups on the polyols. It was hypothesized that the borate added to the sample would complex with the *cis*-diols before derivatization, and therefore reduce the number of hydroxyl sites where derivatization can occur on the polyols.

Heating the samples during the reaction with MIA to 60ºC did not reduce the number of peaks present for each polyol. However, heating the samples did increase the size of the peaks in the 27 – 37 minute region corresponding to an increase in the formation of the more non-polar derivatives, *i.e.* products with a higher number of hydroxyls on the polyols derivatized by MIA. When no triethylamine borate was added to the sample, there were no peaks observed for the early eluting (more polar) derivatives.

3.5. Validation of assays for the measurement of inositols

Validation of a method for the measurement of myo-inositol in urine by LC-MS/MS using iodide adduct formation

Introduction

It has been shown that a mass spectrometry compatible amide column provides sufficient separation of the three common inositol isomers, *myo*-inositol, *scyllo*-inositol, and *chiro*-inositol. All three isomers are found in human urine samples. On an amide column, the inositol peaks also separate well from sugars such as glucose which have an identical mass. Using an amide column, a useful chromatographic separation can be achieved, with *myo*-inositol sufficiently separated from *chiro*-inositol and *scyllo*-inositol, as well as from sugars with the same mass such as glucose. All three isomers are found in human urine samples. The use of diiodomethane in the mobile phase allows for the detection of iodide adducts of the inositols with good sensitivity. A method is developed and validated here to measure *myo*-inositol by LC-MS/MS as the iodide adduct following separation on an amide column. This approach is a good option for measuring inositols because of the good separation of inositol isomers and the low baseline noise. Halide adducts have been described for the measurement of polyols by mass spectrometry.[44, 118b, 131] Iodide adducts are preferable to other halides because iodine has only one abundant stable isotope and readily forms adducts with polyols. Chloride and bromide adducts are problematic because natural chlorine and bromine consist of two abundant isotopes, which split the mass signal and reduce sensitivity.

Materials and methods

Reagents and chemicals

myo-Inositol, diiodomethane, and Dowex 50 WX8-200 resin were obtained from Sigma, *chiro*-inositol was obtained from Acros Organics (NJ, USA), and *scyllo*-inositol was obtained from TCI (Tokyo, Japan). D_6-*myo*-inositol was purchased from

CDN Isotopes. Dowex 50 was obtained from Sigma. Acetonitrile (LiChroSolv) was obtained from Merck.

Sample preparation

An extraction solvent was made up containing 25% dimethylsulfoxide (DMSO) and 75% acetone, and 10 µmol/L of both D_6-*myo*-inositol. Fifty microlitres of sample or standard was pipetted into 1000 µL of extraction solvent, and vortexed. The samples were centrifuged at 13,000 × *g* for 5 minutes and transferred to 96 well microtitre plates and sealed with aluminium foil.

LC-MS/MS

The mobile phase contained 85% acetonitrile, 15% distilled water, and 10.0 mmol/L of diiodomethane. A Waters XBridge (Ireland) amide 100 × 2.1 mm, 3.5 µm column was used for the chromatographic separation. The flow rate was 0.3 mL/min, the injection volume was 10 µL, and the oven temperature was 50ºC. Samples were analyzed using a Shimadzu Prominence HPLC system connected to an API4000 (ABSciex) triple quad mass spectrometer with an electrospray ionization source. The polyols were monitored as the iodide adducts. The mass transition used to monitor *myo*-inositol was 307 → 127 (D_6-*myo*-inositol 313 → 127). The decoupling potential was -30 V, the collision energy was -14 V, and the collision cell exit potential was -7 V. The ion source temperature was 350ºC, the ion spray voltage was 5500 V, the source gas 1 was set to 10, source gas 2 was set to 15, the collision gas (nitrogen) was delivered at 6 L/min, and the curtain gas was 17 L/min. Polyols were quantified using the ratio of peak area to the corresponding internal standard. The data was calibrated using aqueous standards of known concentration.

Method validation

Linearity

The linear range of the assay was investigated by adding different concentrations (ranging from 10 µmol/L to 1000 µmol/L) of *myo*-inositol to urine.

Precision and accuracy

Six batches of four urine samples with a low concentration of *myo*-inositol and a high *myo*-inositol concentration level (urine with 500 µmol/L *myo*-inositol added) samples were analyzed for *myo*-inositol. Accuracy was evaluated as the concentration of *myo*-inositol added to urine that was recovered.

Results

The maximum peak area for *myo*-inositol was observed when 10 mmol/L of diiodomethane was added to the mobile phase.

Linearity

The method was linear for *myo*-inositol up to 1000 µmol/L, as shown in Fig. 3.32, with an r^2 of 0.997 for *myo*-inositol added to urine.

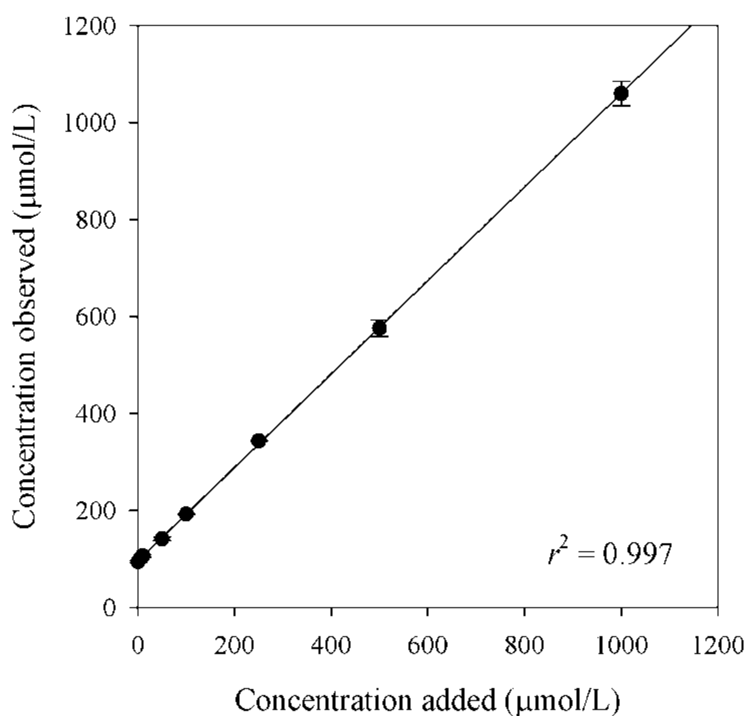


Fig. 3.32. *myo*-Inositol added to urine, error bars represent standard errors.

Precision and accuracy

Within batch and between batch CVs were below 5.1%. The recovery of *myo*-inositol added to urine was 95% (Table 3.6). The limit of detection ($S/N = 3$) for *myo*-inositol was 2.6 µmol/L.

Table 3.6. Precision and accuracy of *myo*-inositol added to urine

myo-Inositol	Mean	Within batch CV%	Between batch CV%	Recovery
Low urine	92.4	5.03	3.18	
High urine	566	3.66	2.08	95%

Discussion

Diiodomethane was the preferred reagent for forming iodide adducts in the mass spectrometer. Diiodomethane produced low background noise in the mass spectrometer, and had minimal effects on the chromatography at a concentration of 10 mmol/L in the mobile phase. Iodine could be added to the mobile phase as it also had no noticeable effects on the chromatography and formed iodide adducts with the polyols. However iodine in the mobile phase appeared to build up in the mass spectrometer, lowering the sensitivity after a while. The use of an amide column provided a useful separation of inositol isomers without the need to use traditional sugar columns which are less compatible with mass spectrometry. Amide columns are reported by the manufacturers to be more stable than amino columns, which can form Schiff bases with sugars. There have been no previous reports of amide stationary phases being used to separate inositol isomers. Amide columns are often avoided for the separation of sugars because they are known to separate anomers at lower temperatures, *e.g.* two peaks have been observed for glucose. However, polyols such as sorbitol and inositol do not form anomers as they are fixed as either open chain (sorbitol) or cyclic (inositol) hexitols. The XBridge amide column used here provided a separation of *myo*-inositol from its isomers, *chiro*-inositol and *scyllo*-inositol. Adding the diiodomethane to the mobile phase means that no post-column mixing is required, allowing for a much simpler and efficient method than those previously described.

Inositols were found to be more soluble in DMSO than methanol. Therefore, an extraction solvent made up of 25% DMSO and 75% acetone proved to be more efficient at extracting inositols from plasma. This procedure gives a one-step sample preparation which is compatible with either APCI detection or detection of inositols as the iodide adducts with electrospray ionization. By having an extraction solvent which the inositol analytes are more soluble in gives a better recovery by reducing the partition of the analytes into the aqueous layer associated with the protein precipitate.

Another intermittent problem causing decreased sensitivity was high background noise. This appeared to be present in the mobile phase and was particularly bad when using water from the distilled water from the Canterbury Health Laboratories reversed osmosis system. Using higher quality purified water (from a Barnstead, Easypure II ultra-pure water system) in the mobile phase improved the sensitivity. However, the mobile phase needed to be prepared fresh for each sample run.

Unfortunately, sorbitol does not easily separate from mannitol and galactitol on an amide column. Furthermore, sorbitol could not be quantified as a total hexitol (sorbitol + mannitol + galactitol) concentration because of interference from glucose in the ion chromatograms.

Validation of a method for the measurement of inositols in urine and plasma by liquid chromatography atmospheric pressure chemical ionization mass spectrometry (LC-APCI-MS)

Introduction

Atmospheric pressure chemical ionization (APCI) is useful for ionizing neutral compounds such as inositols.[47] It is shown here that APCI mass spectrometry gives enough sensitivity to measure inositols in human plasma and urine samples without the need to form halide adducts. A method is developed and validated using a single quadrupole mass spectrometer with APCI detection for the measurement of *myo*-inositol in plasma, and *myo*-inositol, *chiro*-inositol, and *scyllo*-inositol in urine.

Materials and methods

Reagents and chemicals

myo-Inositol, was obtained from Sigma, *chiro*-inositol was obtained from Acros Organics, and *scyllo*-inositol was obtained from TCI Chemicals. D_6-*myo*-inositol was purchased from CDN Isotopes. Acetonitrile (LiChrosolv) was obtained from Merck.

Sample preparation

One hundred microlitres of sample (or standard) and 25 µL of internal standard (500 µmol/L D_6-*myo*-inositol) were added to a 1.5 mL microcentrifuge tube. Twenty five µL of 50% (w/v in water) trichloroacetic acid (TCA) was then added and the mixture was vortexed for 20 seconds. One hundred microlitres of dichloromethane was then added and the mixture vortexed for a further 20 seconds. The samples were then centrifuged (13,000 × *g*, 3 min) and 50 µL of the top layer was transferred to a separate tube containing 500 µL of 10% methanol, 90% acetonitrile. The samples were then centrifuged and transferred to HPLC vials and capped for analysis.

LC-MS

A Waters Acquity BEH amide 2.1 × 100 mm, 1.7 µm ultra-performance liquid chromatography (UPLC) column (Ireland) was used for the chromatographic separation. An isocratic system was used because a gradient system did not improve the separation of the inositols. The mobile phase contained 85% acetonitrile, 15% water. The flow rate was 0.3 mL/min, the injection volume was 10 µL, and the oven temperature was 50ºC. The run time was 15 min, although the samples were diverted to waste post-column for 2 minutes after injection. Samples were analyzed using an Agilent 1200 series HPLC system connected to a 6120 mass spectrometer with an APCI ion source. The inositols were monitored using selected ion monitoring (SIM) in negative ion mode at *m/z* 179.2 for inositols, and 185.2 for D_6-*myo*-inositol. The capillary voltage was set to 2500 V, the corona current was set to 6 µA, the drying gas was 12 L/min, the drying gas temperature was 250ºC, the vaporizing temperature was 400ºC, and the fragmentor was set to 115 V. Inositols were quantified using the ratio of peak area to the peak area of the internal standard (D_6-*myo*-inositol). The data was calibrated using aqueous standards of known concentration.

Method validation

Precision and accuracy

To determine the within batch and between batch variation, four batches of six replicates of a urine sample with a low concentration, a urine sample with a medium level (urine with 100 µmol/L *myo*-inositol, *chiro*-inositol, and *scyllo*-inositol added), and urine with a high inositol level (urine with 500 µmol/L *myo*-inositol, *chiro*-inositol, and *scyllo*-inositol added) were analyzed for *myo*-inositol, *chiro*-inositol, and *scyllo*-inositol. Four batches of six replicates of plasma with a low and a high level (100 µmol/L added) of *myo*-inositol were measured to determine the precision and accuracy in plasma.

Linearity

The linear range of plasma was investigated by adding 25 µmol/L to 125 µmol/L of *myo*-inositol to normal pooled plasma. The linear range of the urine assay was investigated by adding concentrations (ranging from 10 µmol/L to 1000 µmol/L) of *myo*-inositol, *chiro*-inositol, and *scyllo*-inositol to normal pooled urine.

Results

Chromatography

myo-Inositol was the only inositol detected in the plasma samples (Fig. 3.33). The inositols separated on the amide column. Inositols eluted in the order: *myo*-Inositol, *scyllo*-inositol, *chiro*-inositol with baseline separation (Fig. 3.34). A gradient system did not improve the separation of the inositols. The inositols were retained on the column longer than the sugars: glucose, mannose, and fructose, which all eluted before 6 minutes. The sugars gave a much lower signal than the inositols, with millimolar concentrations of glucose in the plasma giving a relatively small peak. There was a small interfering peak co-eluting with *chiro*-inositol in the urine sample. *chiro*-Inositol and *scyllo*-inositol were not detected in the plasma where they are present in nanomolar concentrations.[28]

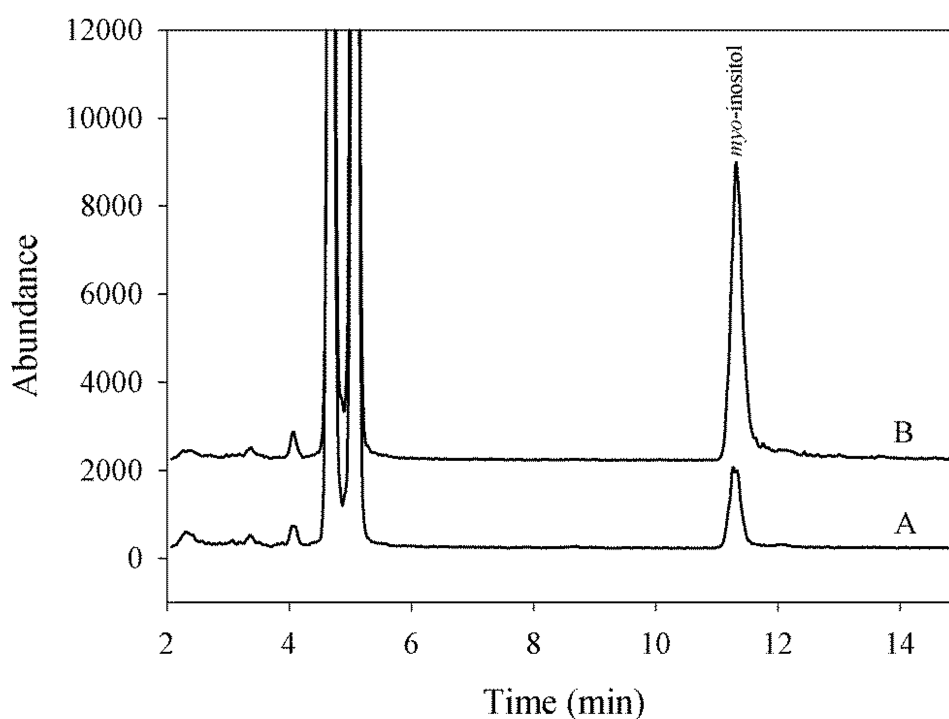


Fig. 3.33. Extracted ion chromatograms (EIC) at 179 Da, showing: A, a plasma sample, and B, plasma with 100 µmol/L *myo*-inositol added.

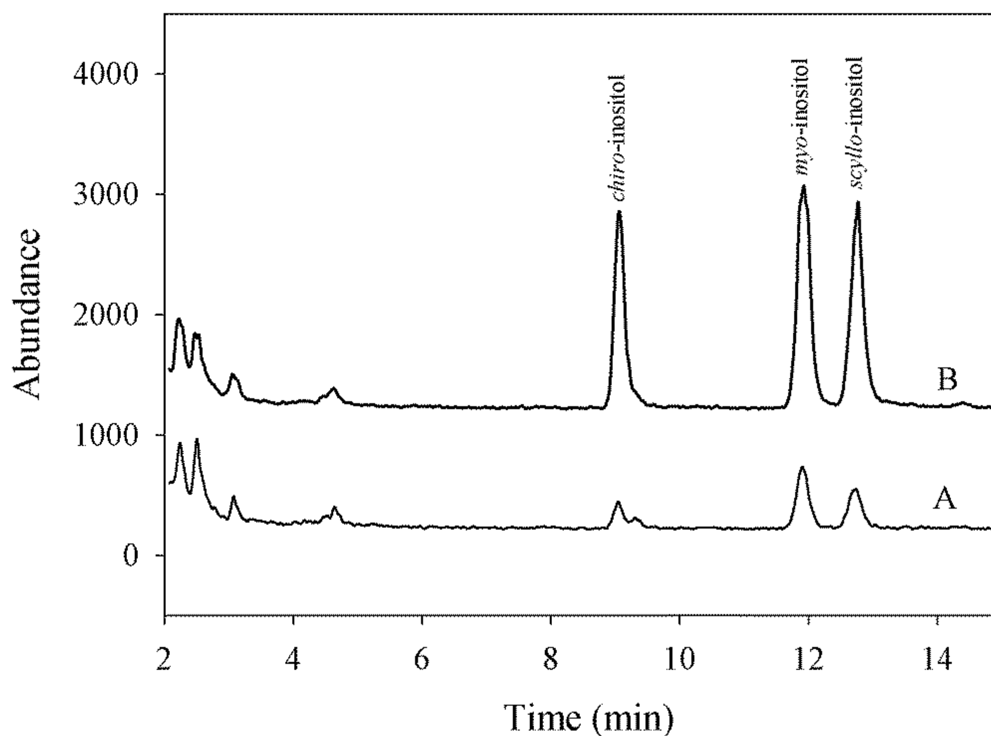


Fig. 3.34. EIC chromatograms at 179 Da, showing: A, a urine sample, and B, urine with 100 µmol/L *chiro*-inositol, *myo*-inositol, and *scyllo*-inositol added.

Precision and accuracy

Within batch and between batch CVs were below 8.3% for the measurement of *myo*-inositol in plasma. The recovery of *myo*-inositol added to plasma was 106% (Table 3.7). The imprecision for the inositols was higher at the lower levels where the concentrations were closer to the detection limits. The limit of detection ($S/N = 3$) for the inositols was calculated to be 1.4 µmol/L.

Table 3.7. Precision and accuracy of inositols in plasma and urine using LCMS with APCI detection

	Mean	Within batch CV%	Between batch CV%	Recovery
Plasma *myo*-inositol				
Plasma	28.0	7.4	8.2	
Plasma + 100 µmol/L added	134	4.7	6.7	106%
Urine *myo*-inositol				
Urine	35.5	12.6	21.5	
Urine + 100 µmol/L added	124.1	8.5	4.9	89%
Urine + 500 µmol/L added	511.3	6.1	3.6	95%
Urine *chiro*-inositol				
Urine	9.6	9.3	49.6	
Urine + 100 µmol/L added	101	7.3	3.7	91%
Urine + 500 µmol/L added	489	6.0	6.1	96%
Urine *scyllo*-inositol				
Urine	20.9	14.0	37.8	
Urine + 100 µmol/L added	123	6.9	3.6	102%
Urine + 500 µmol/L added	495	5.5	2.9	95%

Linearity

The regression equation for *myo*-inositol concentrations added to plasma was $y = 1.018x + 24.5$. Added concentrations of *myo*-Inositol were linear in plasma with an r^2 of 0.994 (Fig. 3.35). The method was linear in urine up to 1000 µmol/L (as shown in Fig. 3.36) with an r^2 of 0.988 for *chiro*-inositol, an r^2 0.992 for *myo*-inositol, and an r^2 of 0.994 for *scyllo*-inositol added to urine. The linear regression equation for *myo*-inositol added to urine was $y = 0.929x + 36.5$, the regression equation for *scyllo*-inositol added to urine was $y = 0.952x + 37.0$, and the regression equation for *chiro*-inositol added to urine was $y = 0.852x + 28.3$. The slopes of the regression equations suggested that the recoveries were greater than 90% for *myo*-inositol and *scyllo*-inositol, and the slope suggested the recoveries were around 85% for added concentrations of *chiro*-inositol in urine. The regression lines intercept the y axis at the endogenous concentration in the sample.

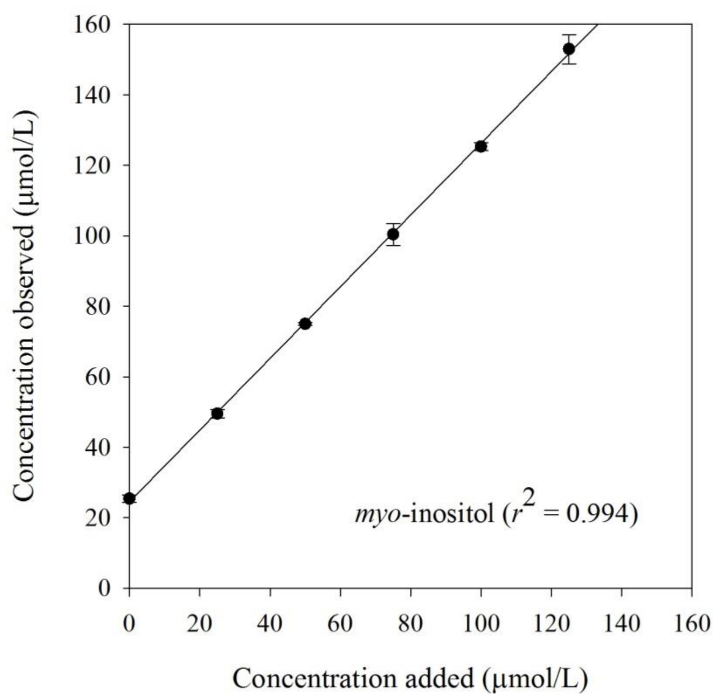


Fig. 3.35. *myo*-Inositol added to plasma, error bars represent standard errors.

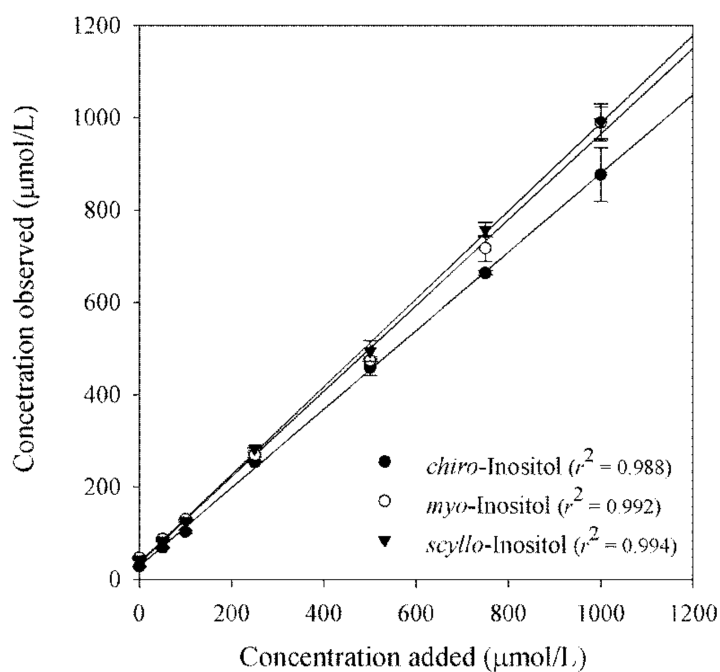


Fig. 3.36. *chiro*-Inositol, *myo*-inositol, and *scyllo*-inositol added to urine. Error bars represent standard errors

Discussion

Inositols have successfully been measured in plasma and urine samples using single quadrupole mass spectrometry with an APCI source with high accuracy and precision. The problem caused by the low solubility of inositols in organic solvents was successfully avoided by precipitating the proteins before extraction, which produced similar peak areas for the samples compared to the aqueous standards. However, extraction of the samples into a solution of DMSO and methanol has also shown to be effective, and this has the benefit of reducing the number of steps involved in the sample preparation. The method was linear ($r^2 = 0.994$) for *myo*-inositol concentrations added to plasma, and $r^2 = 0.992$ for *myo*-inositol concentrations added to urine. However, the between batch imprecision for inositols was high for the low urine concentration (CVs > 20%), which is likely to be a consequence of the relatively high limits of detection.

An efficient separation of *myo*-inositol, *scyllo*-inositol, and *chiro*-inositol was achieved on a mass spectrometry-compatible amide column. Using a UPLC amide column with 1.7 µm particle size improves the resolution so that *myo*-inositol and *scyllo*-inositol are completely separated. The inositols were also well separated from the sugar isomers such as glucose, mannose, and fructose. The sugars appeared to ionize less efficiently using APCI compared to the inositols, as the sugar peaks were smaller than expected in the samples (see Fig. 3.18).

Most published methods describing the measurement of polyols and sugars by LC-MS use the formation of halide adducts to enhance the sensitivity.[44, 143] However, direct detection of inositols using atmospheric pressure chemical ionization (APCI) ionization with selected ion monitoring (SIM) using a single quadrupole mass spectrometer was found to give similar detection limits, and similar recoveries for *myo*-inositol and *scyllo*-inositol.

There are few published methods for the measurement of inositols that provide information about the performance of the assays. However, it is shown here that *myo*-inositol can be reliably measured in human plasma and urine with sufficient precision and accuracy to investigate them in conditions such as diabetes. The analysis of *chiro*-inositol and *scyllo*-inositol in urine is also possible by this method.

3.6. Chapter Summary

The separation of polyols using traditional sugar columns, such as those containing sulfonated resin in the lead form, is not ideal for mass spectrometry. However, a mass spectrometry-compatible amide column proved to be effective at separating inositol isomers, but did not separate sorbitol from its isomers, mannitol and galactitol. The use of iodide adducts to monitor the polyols provides better sensitivity than what is observed when directly monitoring the polyols when using electrospray ionization. The combination of an amide column and diiodomethane in the mobile phase is a useful approach for measuring inositols in biological samples by LC-MS. Alternatively, APCI can be used to detect the inositols directly without the need to add diiodomethane as a source of halide ions.

It was difficult to find appropriate conditions for the use of boronic acids to separate and detect polyols using LC-MS/MS. The boronate esters are only stable at high pH or in non-aqueous conditions. A suitable chromatography system was not found to separate and detect the derivatives. Some boronic acids, such as 8-quinolineboronic acid, fluoresce only when bound to hydroxyl groups, or fluoresce at different wavelengths when bound to hydroxyl groups. However, adding boronic acids to the mobile phase creates a high background noise signal in the mass spectrometer and was found to be unsuitable for the detection of polyols in biological samples.

Derivatization of polyols with isocyanates is a possible option, but the sample preparation is labour intensive and not suitable for analyzing large numbers of samples. Isocyanates only derivatize polyols in the complete absence of water, *i.e.* the samples require drying down under nitrogen before they are derivatized. Attempts to dry the extraction solvent with sodium sulfate were not effective, and no polyol peaks were observed. The reaction would only work with pyridine as the reaction solvent which acts as a weak base and nucleophilic catalyst. Isocyanates also generated multiple peaks for some polyols, and the many peaks were not well chromatographically separated on a reversed phase column. Methylisatoic anhydride has the advantage that it reacts with polyols with a high water content (50%) present in the reaction mixture, so drying down the samples is not necessary. However, there were many derivatives formed for each polyol and they were difficult to separate by HPLC, especially sorbitol and its isomers mannitol and galactitol. These hexitol

derivatives also chromatograph in the same region as the glucose derivatives. This method is therefore unlikely to be useful for the measurement of sorbitol, especially in diabetes samples where high blood glucose is often present.

Attempts to use electrochemical detection to measure polyols were unsuccessful. Adding electrochemically active reagents such as transition metals or ferroceneboronic acid to the mobile phase clogged up the electrochemical cells, causing a constant increase in the pressure of the HPLC system.

The problems with measuring polyols include the low solubility in organic solvents, the lack of easily ionizable functional groups and the presence of isomers in biological samples which only differ by the orientation of the hydroxyl groups. The solubility problem can be overcome by extracting samples into a mixture of DMSO and acetone, which gave a greater extraction efficiency than a mixture of methanol and acetonitrile. Another option is to precipitate the proteins with TCA prior to solvent extraction.

All attempts to derivatize or form complexes with boronic acids or transition metals to aid resolution and enhance detection were unsuccessful due to the formation of too many derivatives, poor chromatography, or ion interference. Mass spectrometric methods with or without iodide adduct formation were the most useful for measuring polyols. Adding diiodomethane to the mobile phase had no significant effect on the chromatography. While sorbitol and glucose have a mass difference of two, there were still significant interfering glucose peaks co-eluting with the sorbitol peak. This may be from the low mass resolution of the spectrometer, and may also be explained by the presence of naturally occurring isotopes such as ^{13}C, ^{17}O, and ^{18}O, considering glucose is present in much higher concentrations in plasma than sorbitol. Direct detection of inositols with an APCI source produced similar sensitivity to electrospray ionization when detecting inositols as iodide adducts. If APCI is not available, then electrospray ionization with diiodomethane in the mobile phase and detection as iodide adducts provides better signal to noise than direct detection with electrospray ionization.

4. Chapter Four - Betaine, Other Osmolytes, and Other One-Carbon Metabolites in Health and Disease

4.1. Introduction

The work described in previous chapters has shown that liquid chromatography – mass spectrometry techniques have improved the analysis of a wide range of osmolytes and related metabolites, some of which were particularly difficult to measure by older HPLC-UV methods. Osmolyte concentrations can be expected to differ in disease due to changes in dietary intake, changes in metabolic processes, and changes in urinary excretion. Assays that have been developed for the measurement of betaine, other osmolytes, and other one-carbon metabolites were used here to analyze clinical study samples in order to improve knowledge about their metabolism, and to help show the potential value of these analytes as risk markers in disease. How these metabolites correlate with each other, as well as with markers of glycemic control and renal function, helps to show what information can be obtained by measuring them. The biological variation of osmolytes (and related metabolites) was also investigated in order to help evaluate the usefulness of single measurements as markers of disease. Other factors that were investigated include the effect of different diet treatment, and medications on the concentrations of betaine, other osmolytes, and other one-carbon metabolites. While people with type 2 diabetes have been shown to have high urinary betaine excretion,[14] the lipid lowering drug bezafibrate has also been associated with increased urinary betaine excretion.[15] The effect of bezafibrate on urine betaine concentrations in people with diabetes was investigated to test if these factors are cumulative for elevated urinary betaine excretion. A different fibrate drug, fenofibrate, was investigated in healthy subjects before and after treatment to determine if it increases urine betaine excretion. This is the first study to test whether there may be a causal relationship between fibrates and high urinary betaine excretion.

4.2. Osmolytes and related metabolites in overweight subjects with type 2 diabetes

Background

Diabetes is the most common cause of chronic kidney disease and renal failure.[144] Many people with diabetes also go on to develop eye damage, and problems with the cardiovascular system.[145] This is often attributed to the glycation of proteins, which is the result of elevated glucose concentrations in the body. A less understood pathological process in diabetes is the disruption of osmolytes in the body. The loss of osmolytes from the body makes it more difficult to maintain cell volume. This may lead to tissue and organ damage, particularly in the kidneys where there is a high osmotic gradient. The urinary excretion of the osmolytes, betaine and sorbitol, is higher and more variable in people with diabetes.[91] However, the biological variation of these osmolytes in people with diabetes has not previously been reported. It is possible that the concentrations of osmolytes in people with type 2 diabetes may be useful as risk markers and predictors of secondary complications such as cardiovascular disease and renal disease.

The major osmolytes: betaine, GPC, taurine, and *myo*-inositol were investigated in overweight people with type 2 diabetes. These osmolytes were chosen because they have been shown to be the major osmolytes present in tissues such as the kidneys and liver[32] and their concentrations are known to be affected by type 2 diabetes.[91] The one-carbon metabolites, choline and DMG, as well as TMAO, carnitine, and acetylcarnitine were also measured in the DEWL samples. Sorbitol and sarcosine were not measured in these samples because of analytical problems that have been previously discussed.

The DEWL study

The samples that were analyzed in this study were collected as part of the Diabetes and Excess Weight Loss (DEWL) study, which was conducted as a collaboration between the University of Otago, and the University of Auckland.[146] The DEWL study recruited 419 people with type 2 diabetes who were overweight (BMI > 27 Kg/m^2). They were aged between aged from 30 to 76 years, and were recruited from Christchurch, Wellington, and Auckland. Of 419 subjects who were recruited, only 294 completed the study which was conducted over a two year period. Four samples from each of these 294 patients were collected (after fasting) over the 2 year period at: baseline (time 0); 6 months; 12 months; and 24 months. This study was initially carried out to compare the effects of different dietary advice (high protein or high carbohydrate diet) on weight loss as described in Krebs *et al*.[146] In the DEWL study, no significant differences in outcomes, such as: body fat, blood pressure, lipids, renal function, or HbA_{1c}, were observed between the treatments.[146] There were no significant differences in health outcomes for the DEWL study subjects by giving dietary advice for a low fat diet containing either a high carbohydrate or a high protein intake. However, both treatments showed a small overall weight loss. Fasting glucose, HbA_{1c}, renal function parameters (plasma creatinine and estimated glomerular filtration rate (eGFR)), body mass index (BMI), cholesterol, triacylglycerides, and saturated fatty acids were measured during the DEWL study at each time point. These measurements were made by the Department of Nutrition, University of Otago.[146] A spread-sheet recording what diet the DEWL study subjects were on, what medications they were taking, age, gender, and biochemistry data was provided by the DEWL study researchers. Some health characteristics of the DEWL subjects are shown in Table 4.1. The average age in the DEWL study was 59. Many of the participants in the DEWL study had poor glycemic control throughout the study with plasma glucose levels over 7 mmol/L, and haemoglobin A_{1C} (HbA_{1c}) values over 7% of total haemoglobin. While most of the study participants had normal renal function, there were some that showed impaired renal function indicated by plasma creatinine levels over the normal range (>120 μmol/L), and a low estimated glomerular filtration rate (eGFR < 80 mL/min for males and < 62 mL/min for females). Renal disease was an exclusion criteria in the DEWL study.[146]

Table 4.1. Some characteristics of the study population

	Median	Interquartile range 25%	75%
Plasma Glucose (mmol/L)	7.84	6.54	9.70
HbA_{1c} (%)	7.73	7.04	8.80
BMI (kg/m^2)	34.6	31.3	39.4
Plasma Creatinine (μmol/L)	74.2	64.3	88.2
eGFR (mL/min/1.73 m^2)	82.2	69.8	95.8
Age (years)	59.8	53.2	66.9

Note: Data is for males and females. Data provided by the DEWL study researchers.[146]

The present study on the DEWL samples

After the DEWL study was complete, the samples were sent to Canterbury Health Laboratories for the purpose of investigating betaine and other osmolyte metabolism. Ethical approval was applied for and granted to Dr Krebs (Department of Medicine, University of Otago, Wellington) by the Multi-region Ethics Committee to investigate betaine metabolism in the DEWL study samples (MEC/06/08/081) without the need to obtain further informed consent from the patients. Patient names were not known in the present study. Individual subjects were only identified using numerical codes which were assigned during the DEWL study. Not all urine samples from the DEWL study were available for the analysis of osmolytes as some had been previously used up for other measurements. Plasma and urine samples from the DEWL study were analyzed using the methods developed in this project for the osmolytes: betaine, GPC, taurine and *myo*-inositol. The methylamines, DMG, choline, TMAO, carnitine, and acetylcarnitine were also measured in the samples.

Concentrations of osmolytes and related methylamines in 64 baseline DEWL subjects were compared with subjects from the betaine and body composition (BBC) study (n = 62) who had features of metabolic syndrome (they were overweight, had high blood lipids, high blood pressure, and no history of diabetes).

Correlations between osmolytes and related metabolites in the baseline DEWL study samples (n = 385 for plasma, and n = 312 for urine) were investigated using the Spearman's rank order test along with other results, including: HbA_{1c}; fasting glucose; plasma creatinine; eGFR; and body mass index (BMI) that were collected during the

original DEWL study sample analyses.[146] The changes in osmolyte levels were investigated over time, and related to markers of glycemic control and renal function.

The DEWL study participants were on a range of prescribed medications which may affect the concentrations of osmolytes and related metabolites in the body. Drugs which the subjects were taking include: diabetic control drugs such as metformin and insulin, fibrates, statins, antidepressants, proton pump inhibitors, as well as multivitamins. For example, it has been shown that betaine excretion is often higher in people who are taking fibrates to lower triglycerides.[15] Because plasma homocysteine was not measured in the original DEWL study, plasma (n = 32) from the subjects taking fibrates were analyzed for homocysteine. Plasma from 64 control subjects who were not on fibrates, were matched for age ± 3 years and sex, and also analyzed for homocysteine. Samples with plasma creatinine > 120 µmol/L were not used for controls to exclude renal failure, which is also known to affect betaine metabolism.[91] The concentrations of betaine and other analytes in the fibrate subjects were compared with the control subjects using Mann-Whitney rank sum tests carried out in SigmPlot (v13, Systat Software Inc, San Jose, CA).

Osmolyte and other metabolite concentrations measured in the present study could not be related to patient outcomes, such as cardiovascular events or mortality, due to ethical restrictions.

Sample analysis

Samples were stored at -80ºC prior to analysis. Fifty microlitres of the samples were extracted into 800 µL of extraction solvent containing 10% methanol, 90% acetonitrile and 10 µmol/L of each of the deuterated internal standards: D_9-betaine, D_3-dimethylglycine, D_9-choline, D_3-carnitine, D_4-taurine, and D_9-trimethylamine-*N*-oxide. The samples were vortexed, centrifuged (13,000 × *g*, 3 minutes) and 200 µL was transferred into two separate 96 well microtitre plates and sealed with aluminium foil. One plate had external standards (50, 100, 250 µmol/L) and controls containing: betaine, DMG, TMAO, choline, carnitine, and acetylcarnitine, and the other plate had internal standards containing taurine added for sample calibration. A separate method was required to measure taurine in the samples because it was measured in negative ion mode. All of the methylamines could be measured on the same injection. For the plasma runs, pooled plasma was spiked with 50 µmol/L of each of the analytes, and

for the urine sample runs, urine was spiked with 100 µmol/L of each of the analytes. These samples with and without added concentrations of analytes were used as controls and were analyzed in duplicate in every sample run.

Samples were analyzed by LC-MS/MS using an Applied Biosystems API4000 tandem mass spectrometer. For separation of the methylamines, a Cogent diamond hydride (100 × 2.1 mm, 4. µm) column was used with a gradient going from 40% water in acetonitrile to 90% water and 10 mmol/L ammonium formate buffer over 8 minutes. For the taurine separation, a Waters X-Bridge Amide (100 × 2.1 mm, 3.5 µm) column was used with an isocratic mobile phase containing 20% distilled water and 80% acetonitrile. For both methods the flow rate was 0.3 mL/min, the sample injection volume was 10 µL, and the oven temperature was set to 40ºC. The methylamines were measured in positive ion mode using multiple reaction monitoring (MRM). The MRM mass transitions and mass spectrometer parameters used for these methods are given in Section 2.2. Urine samples in which values were over the linear range of the assay (250 µmol/L) were diluted ten-fold and re-analyzed. Inositols were measured as the iodide adducts using LC-MS/MS as described in Section 3.5. Due to time restrictions related to ethics approval, inositols were only measured in a subset of the DEWL study samples consisting of 32 subjects who were taking bezafibrate, and 64 age and gender matched controls.

Method performance

To monitor the performance of the method during the sample runs, urine was spiked with 100 µmol/L of each analyte, and plasma was spiked with 50 µmol/L of each analyte. The high and low controls were prepared with each batch and injected throughout each run. Table 4.2 shows the data for the controls that were run in each batch of samples. The recoveries in urine ranged from 65-117%. The recoveries in plasma ranged from 75-101%. The precision was lowest (with CVs at around 20%) for GPC compared to the other analytes because no isotopic standard was available for this compound, so D_9-betaine was used as the internal standard. The recoveries for GPC were also more variable, with 117% recovered in plasma and 75% recovered in urine. The precision for GPC was poorer for GPC in this study compared to the method validation in Section 2.2, where the data was collected over a shorter time. The lower precision and accuracy for GPC observed in the controls during the

measurement of the diabetic samples is likely to be a result of instrument drift, and changes to the chromatographic column over time leading to a decrease in the effectiveness of D_9-betaine as the internal standard. The CVs for the urine controls were generally higher than expected when compared the method validation data. This is likely to be due to instrument drift over the duration of measuring the study samples. There were on-going problems with the API4000 mass spectrometer system (which were, in part, due to damage from the Christchurch earthquakes). The limit of detection was also variable for the analytes in this project due to these instrument problems, and the multi-user environment leading to regular contamination of the mass spectrometer, and constant changing between methods with different instrument parameters. There were also intermittent increases in the baseline noise, especially with the betaine MRM signal, that may have been caused by contamination in the mobile phase by a compound with a similar mass. The ion source could be cleaned with a solution of methanol and water (50/50 v/v) containing 0.1% acetic acid. However, a service engineer was required to clean the quadrupoles.

Table 4.2. Method performance data for quality controls measured throughout the present study by LC-MS/MS.

Plasma	**Betaine**	**DMG**	**Choline**	**GPC**	**TMAO**	**Carnitine**	**Acetyl carnitine**	**Taurine**
Low control								
Mean (μmol/L)	19.6	1.5	102.4	11.0	8.4	43.0	0.3	155.4
SD	1.0	0.2	6.2	2.5	0.5	1.5	0.1	12.8
CV%	5.2	13.2	6.0	22.8	6.2	3.5	39.0	8.2
High control								
Mean (μmol/L)	66.3	47.4	149.2	48.7	56.3	87.3	50.7	196.5
SD	3.0	2.4	7.8	10.2	3.2	3.4	4.9	15.0
CV%	4.6	5.1	5.3	20.9	5.7	3.9	9.6	7.7
Recovery%	93	92	94	75	96	89	101	82
Urine	**Betaine**	**DMG**	**Choline**	**GPC**	**TMAO**	**Carnitine**	**Acetyl carnitine**	**Taurine**
Low control								
Mean (μmol/L)	163.6	79.5	29.6	3.44	341.7	166.4	44.8	126.8
SD	11.1	9.2	7.7	0.71	17.7	12.2	8.1	7.8
CV%	6.8	11.5	26.2	20.7	5.2	7.3	18.1	6.1
High control								
Mean (μmol/L)	245.9	168.1	122.3	120.2	406.8	243.4	143.2	212.7
SD	16.1	11.4	14.7	21.2	21.5	14.2	15.7	12.3
CV%	6.6	6.8	12.0	17.6	5.3	5.8	11.0	5.8
Recovery%	82	89	93	117	65	77	98	86

Abbreviations: *N*,*N*-dimethylglycine (DMG); glycerophosphorylcholine (GPC), trimethylamine-*N*-oxide (TMAO).

Concentrations of betaine,* N,N*-dimethylglycine, choline, glycerophosphorylcholine, trimethylamine-*N*-oxide, carnitine, acetylcarnitine, and taurine in overweight subjects with type 2 diabetes

The median values and interquartile ranges for methylamines in the DEWL study subjects are shown in Table 4.3. This data was calculated on the baseline samples excluding the 32 subjects who were on fibrates.

Table 4.3. Median and interquartile range of the baseline data in the DEWL samples

	Male	Interquartile range			Female	Interquartile range		
Plasma	Median (μmol/L)	25%	75%	n	Median (μmol/L)	25%	75%	n
Betaine	32.1	26.2	39.6	158	28.8	23.1	35.0	227
DMG	2.51	2.09	3.28	158	2.33	1.83	2.88	227
Choline	10.7	9.3	12.4	158	9.95	8.54	11.5	227
GPC	1.95	1.34	2.75	158	1.77	1.28	2.34	227
TMAO	5.9	3.99	10.85	158	5.67	3.92	9.21	227
Carnitine	46.6	40	53.2	158	43.7	38.2	49.0	227
Acetylcarnitine	7.19	6.05	9.29	158	7.57	6.5	10.05	227
Taurine	55.0	45.4	75.2	158	54.0	42.9	72.2	227
Urine	mmol/mol CRN							
Betaine	34.8	18.0	69.1	132	22.9	12.9	57.1	180
DMG	7.85	5.43	12.39	132	7.42	4.9	11.8	180
Choline	4.17	2.99	6.2	132	4.18	3.03	7.35	180
GPC	0.50	0.36	0.72	132	0.61	0.45	0.87	180
TMAO	66.9	47.3	99.3	132	85.2	46.6	138	180
Carnitine	14.1	8.06	22.7	132	12.2	7.0	19.5	180
Acetylcarnitine	4.11	2.32	6.25	132	3.15	1.59	5.55	180
Taurine	46.1	23.6	82.3	132	20.1	6.8	51.2	180

Abbreviations: *N*,*N*-dimethylglycine (DMG); glycerophosphorylcholine (GPC); trimethylamine-*N*-oxide (TMAO); creatinine (CRN).

Mann-Whitney rank sum tests showed that there are significant differences between the genders. Males had significantly higher ($p < 0.01$) plasma betaine, DMG, choline, and carnitine. Males also had significantly higher ($p < 0.01$) urine betaine and urine taurine, and also had significantly lower ($p < 0.01$) urine GPC. Plasma betaine has previously been reported to be higher in males than females.[4b]

The concentrations of osmolytes and other metabolites in plasma (Table 4.3A) are similar to previously reported data on normal populations.[4b,65b,9, 84] Lever *et al*.[91] reported that the median plasma betaine in people with diabetes is not significantly different to the normal population, which is consistent with the findings of the present study. However, the overall median urine betaine level of 25.9 mmol/mol crn in this group is considerably higher than that reported in the normal population (median = 5.9 mmol/mol creatinine (CRN)[65b]). A few individuals in the DEWL study were likely to be excreting more than the estimated New Zealand median dietary betaine intake (227 mg/day[147]). These individuals may be betaine depleted, and therefore dependent on choline oxidation for osmo-regulation and one-carbon metabolism. Elevated urine betaine excretion in diabetes has been previously reported.[91,148,14] No statistically significant trends were detected for betaine excretion to increase with time. Possibly with a small sample size (116 subjects) a longer time than two years may be required to show the pattern of change in betaine concentrations with diabetes. Plasma DMG concentrations are consistent with those reported in other populations.[4b]

Plasma choline concentrations (overall median 10.4 µmol/L) in the overweight subjects with diabetes were not elevated compared to other reports.[83] Cardiovascular events such as myocardial infarction have been associated with high plasma choline concentrations, though the event itself probably causes an elevation. Plasma choline concentrations ≥ 25 µmol/L have been reported to be predictive of major cardiac events.[83] The mean plasma taurine concentration of 53.5 µmol/L observed for the DEWL study samples is in good agreement with Laidlaw *et al*.[9], who reported a mean value of 58 ± 16 µmol/L in normal subjects. There is little data on the osmolyte GPC, especially in diabetes, so it is difficult to compare the results with other studies. However, the present study shows that the concentrations of GPC in plasma and urine of overweight people with diabetes are considerably lower than the other osmolytes, betaine and taurine. This may be because GPC is synthesized intra-cellularly in response to osmotic stress, rather than accumulated by active transport from the circulation.[3]

The overall mean plasma taurine concentration for the DEWL study samples of 61.1 µmol/L is in good agreement with Laidlaw *et al*.,[9] who published a mean value of 58 ± 16 µmol/L in normal subjects. This suggests that plasma taurine is not significantly raised in overweight people with diabetes. There has been very little data published on GPC, especially in diabetes, so it is difficult to compare the results with

other studies. Pomefret *et al*.[149] reported plasma concentrations of GPC (11 ± 1 μmol/L) to be similar to plasma choline (12 ± 1 μmol/L). However, they did not measure GPC by a direct method. The present study shows that the concentrations of GPC in plasma (median = 1.95 μmol/L) and urine (median = 0.50 mmol/mol creatinine) of overweight people with diabetes are considerably lower than the other osmolytes, betaine and taurine.

The plasma TMAO concentrations were highly variable, and most likely affected by the dietary intake of marine foods. The plasma TMAO results were slightly higher than what was reported by Tang *et al*.,[85] where median plasma TMAO was 3.7 μmol/L (interquartile range 2.4 - 6.2). However, the higher and more variable TMAO results observed in the DEWL samples may be related to diabetes or simply reflect the diet. For example, consuming marine fish will lead to a temporary increase in TMAO levels.[45b] The DEWL study samples were collected while fasting. However it is possible that TMAO may still be elevated due to dietary intake. While Tang *et al*.[85] reported that TMAO is a sensitive marker of cardiovascular disease, any short term dietary variations in TMAO could limit its usefulness.

Plasma choline concentrations in the overweight subjects with diabetes were not generally elevated compared to other studies.[83] This suggests that these subjects may not be at immediate risk of cardiovascular events such as heart attacks which have been associated with high plasma choline concentrations.[83] Plasma choline concentrations ≥ 25 μmol/L have been reported to be predictive of major cardiac events.[83] The highest plasma choline value observed in the present study was 20.8 μmol/L.

Osmolytes and related methylamines in the plasma of overweight subjects who have type 2 diabetes compared with overweight subjects who have the metabolic syndrome

The concentrations of plasma metabolites in baseline samples from overweight subjects with type 2 diabetes (n = 64) were compared to baseline samples of overweight male controls with the metabolic syndrome from the betaine and body composition (BBC) study (n = 62) using Mann-Whitney rank sum tests. The DEWL

samples used here were the baseline samples from the matched control subjects who were not on fibrate therapy (32 males and 32 females).

The results are shown in Fig. 4.1 as boxplots. The Mann-Whitney rank sum tests showed that *myo*-inositol ($p < 0.001$) and GPC ($p < 0.001$) were significantly lower in people with type 2 diabetes. Betaine ($p = 0.002$) and DMG ($p = 0.012$) were also lower in people with type 2 diabetes. However, taurine ($p < 0.001$) was significantly higher and more variable in the plasma of people with diabetes. Free choline, TMAO, carnitine, and acetylcarnitine were not significantly different ($p < 0.05$) in the two groups. Betaine and related metabolites are important risk markers for diabetes. Svingen *et al*.[61b] reported that low plasma betaine is a strong predictor of developing type 2 diabetes in people with no previous history of diabetes. Elevated betaine, DMG, and sarcosine in the urine have also been reported to be strong predictors of developing type 2 diabetes.[61b] The diagnostic value of measuring the osmolytes, taurine, *myo*-inositol and GPC as risk markers in diabetes is yet to be determined.

The result that *myo*-inositol is lower in the plasma of people with diabetes compared to people without diabetes contradicts Hasegawa *et al.,*[128] who reported elevated *myo*-inositol in the plasma of people with diabetes compared to normal controls. Ostlund[28] reported that plasma *myo*-inositol concentrations were not different in people with diabetes (median 24.1 µmol/L) compared to non-diabetics (median 24.5 µmol/L). The displacement of other osmolytes from the body by sorbitol can occur in diabetes, where sorbitol is found in particularly high concentrations due to the reduction of glucose. While plasma betaine has previously been reported to be not significantly different in diabetes,[91] it was found here to be significantly lower in the plasma of the DEWL study subjects (overweight people with type 2 diabetes) compared to overweight people without diabetes. This may be in part due to the presence of 50% females in the group with diabetes, whereas the control subjects with the metabolic syndrome were all males. The higher taurine observed in people with type 2 diabetes was unexpected. However, higher plasma taurine in diabetes may be explained by it being displaced from cells and body tissues by increased circulating sorbitol generated by the reduction of glucose. In that case, blood plasma may be a poor indicator of what is actually happening in the tissues. Plasma taurine was much more variable in people with diabetes than in the control group, showing that concentrations of this osmolyte are disrupted.

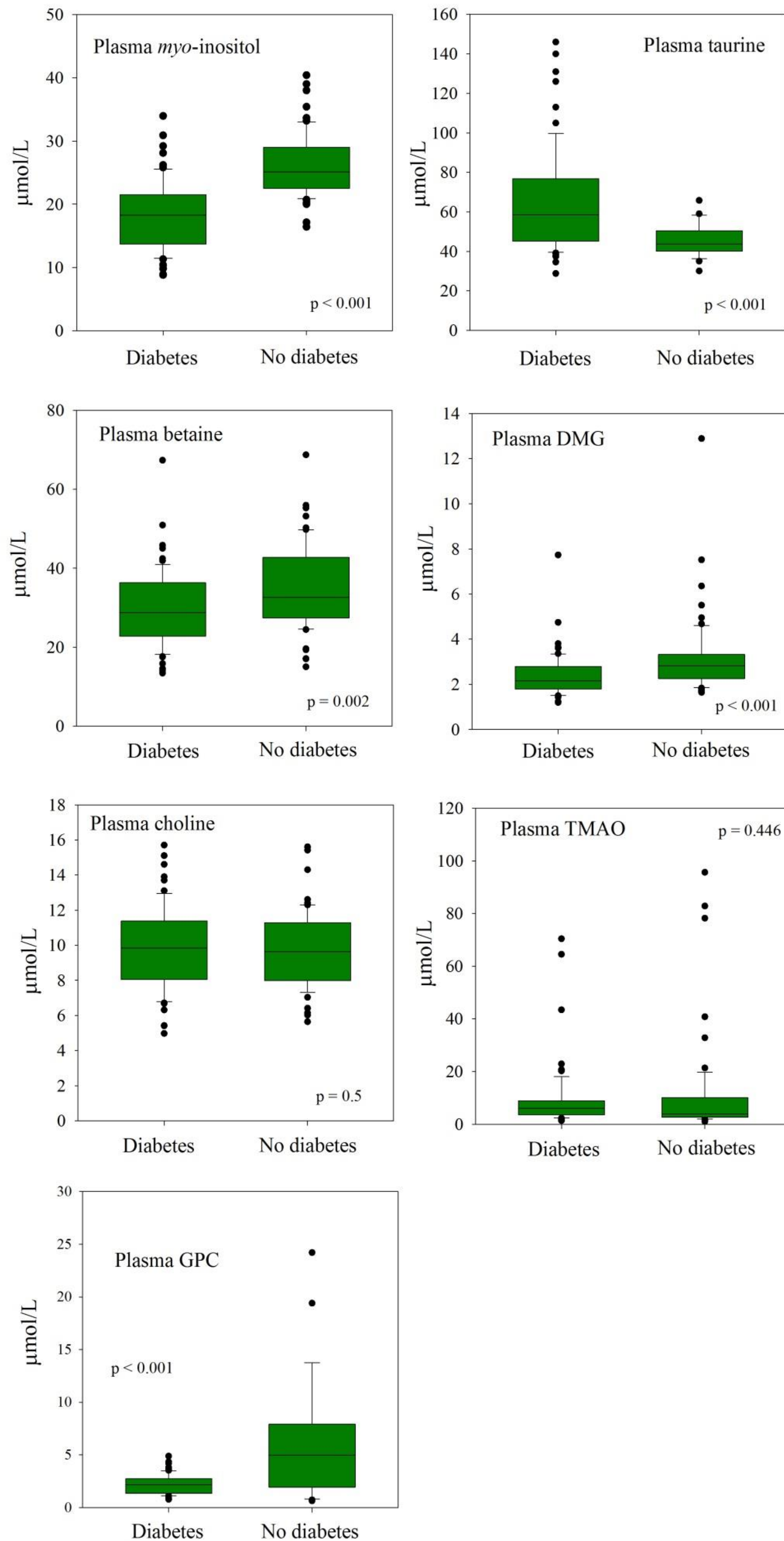


Fig. 4.1. Boxplots showing plasma osmolyte and methylamine concentrations in the DEWL study subjects with diabetes and overweight subjects without diabetes.

Osmolytes and related methylamines in the urine of overweight subjects who have type 2 diabetes compared with overweight subjects who have the metabolic syndrome

The concentrations of urine metabolites from the DEWL study subjects (n = 64) were compared to a group of overweight male non-diabetic controls with the metabolic syndrome from the betaine and body composition (BBC) study (n = 62) (Fig. 4.2). The DEWL samples used here were the baseline samples from the matched control subjects who were not on fibrate therapy (32 males and 32 females). The groups were compared using Mann-Whitney rank sum tests on unmodified data.

Betaine, GPC, *myo*-inositol, *scyllo*-inositol, DMG, choline, and TMAO were all significantly higher ($p < 0.05$) in the urine of overweight people with type 2 diabetes compared to the non-diabetic controls. TMAO was significantly higher in the urine of the subjects with type 2 diabetes ($p = 0.009$). Carnitine and acetylcarnitine were not significantly different ($p > 0.05$) in the urine of people with diabetes compared to the controls. Taurine was also not significantly different in the diabetic urine compared to the control group ($p = 0.065$), but was more variable in people with type 2 diabetes. *chiro*-Inositol was only detected in 12 non-diabetic urine samples. However, the concentrations were generally higher in the group with diabetes (median = 2.99 mmol/mol creatinine compared to median = 1.03 mmol/mol creatinine in the 12 control group samples).

Elevated concentrations of betaine, DMG, *myo*-inositol, *chiro*-inositol, and sorbitol have been previously reported in the urine of people with type 2 diabetes.[71, 28, 91, 150] *scyllo*-Inositol and *chiro*-inositol are likely to be filtered out by the kidneys along with *myo*-inositol as they are chemically similar compounds, only differing by the orientation of the hydroxyl groups.

With the exception of taurine, the concentrations of most osmolytes (betaine, GPC, and *myo*-inositol) were significantly lower in the plasma and higher in the urine of people with diabetes, suggesting that they could be at risk of being depleted of these important nutrients. The effect this has on health outcomes in diabetes should be further investigated.

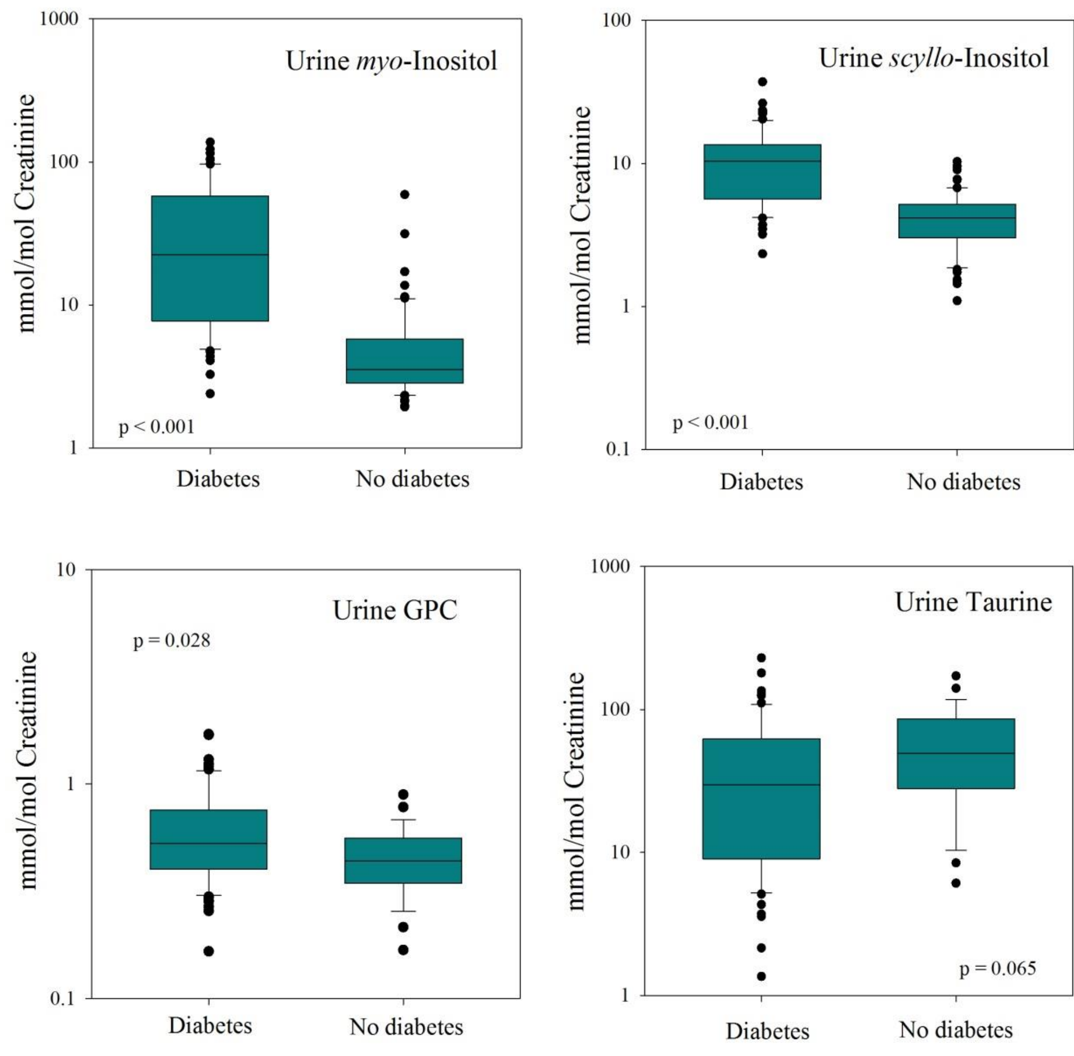


Fig. 4.2A. Inositols, taurine, and GPC in the urine of people with type 2 diabetes versus controls with no diabetes.

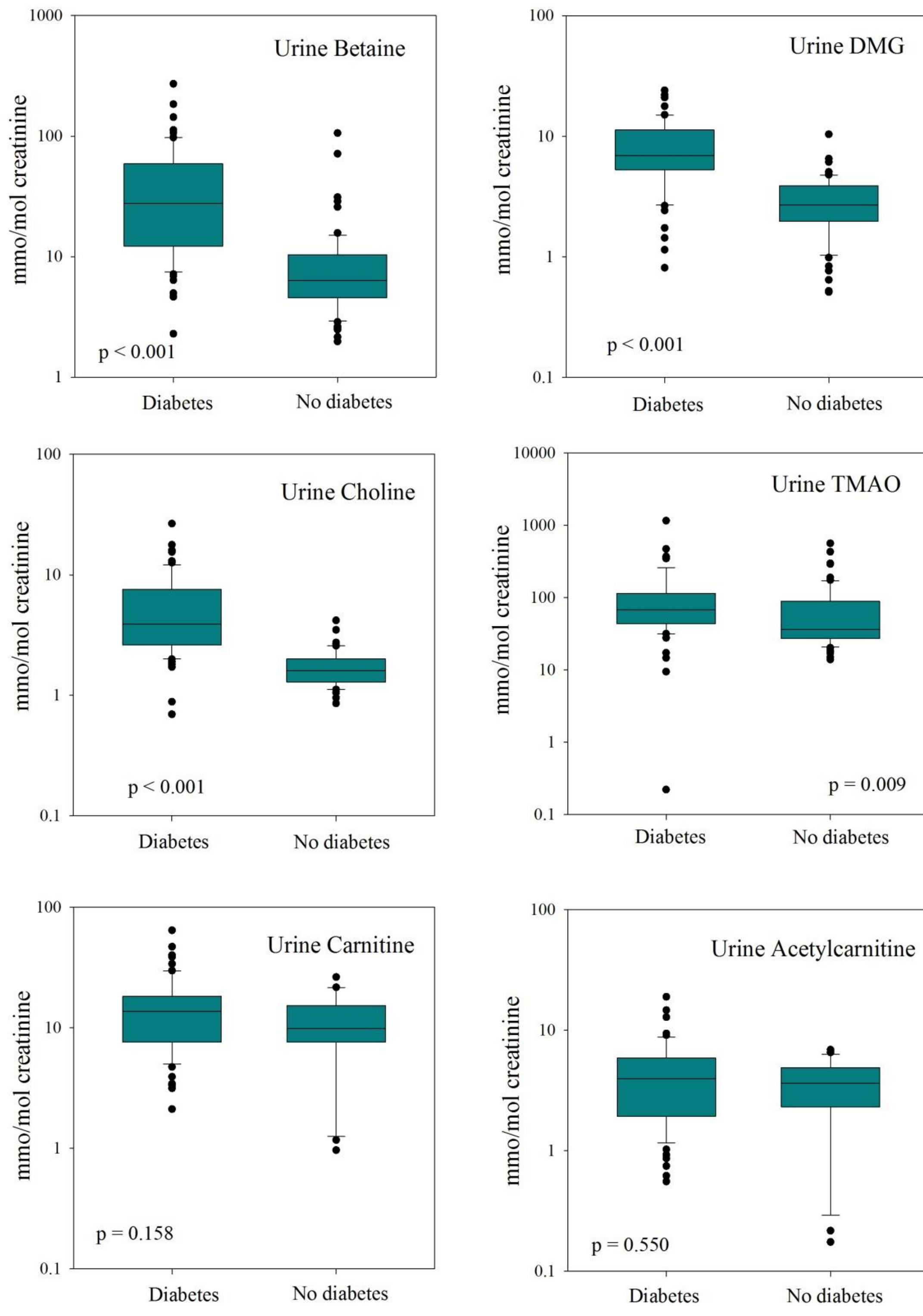


Fig. 4.2B. Boxplots showing urine methylamine concentrations in people with type 2 diabetes and non-diabetic controls.

The effect of diet on the concentrations of betaine other osmolytes, and other one-carbon metabolites

The DEWL study participants were given dietary advice prior to the six month visit, so an increase in plasma betaine concentrations in the group on high carbohydrates was expected at the 6 month time point. However, at 6 months, Mann-Whitney rank sum tests showed no significant differences in any of the osmolytes and related metabolites measured in the present study between the high protein diet and the high carbohydrate control diet prescribed in the DEWL study. These results support the findings of the DEWL study, where few differences were observed in the health outcomes for the subjects on different diets. This could be due to the low compliance of the subjects with the dietary advice that they were given over the duration of the DEWL study.[146] It remains inconclusive whether a high protein or high carbohydrate diet affects the osmolyte distribution in diabetes. However, considering that high carbohydrate diets are likely to contain wheat based products such as bread and pasta, which are very high in betaine,[147] the subjects on high carbohydrate diets were expected to show increased betaine levels. High protein diets are likely to contain more meat, which is high in taurine.[9] Because no significant differences were observed between the DEWL study diet treatments, study subjects on the different prescribed diets were treated as one group for the purposes of investigating the other aspects of osmolytes in diabetes.

Variation of betaine, glycerophosphorylcholine, taurine,* N,N*-dimethylglycine, choline, and trimethylamine-*N*-oxide in the plasma and urine of overweight people with type 2 diabetes over a two-year period

Introduction

The biological variation of: betaine; GPC; taurine; DMG; choline; TMAO; carnitine; and acetylcarnitine was investigated in the DEWL study samples collected over a two year period. The variability of these osmolytes, one-carbon metabolites, and other methylamines in people with type 2 diabetes was previously unknown. In order to compare with other studies, both the coefficient of reliability and the index of individuality were calculated for each metabolite, even though they provide similar information. Log-normal reference change values (RCVs) are also calculated to determine 95% confidence intervals for the percentage change that can be considered significant for repeat measurements. The hypothesis was that osmolyte concentrations in plasma and urine change over a two year period as the diabetes progresses and secondary complications begin to develop. This study has been published in *Annals of Clinical Biochemistry*.[151]

Methods

Statistical analysis

Statistical analyses were performed using SigmaPlot (v13.0). A p-value < 0.05 was considered to be statistically significant. Log-normal RCVs were calculated as described by Fraser.[152] Log-normal RCVs for a positive and negative change were calculated using the equation: $RCV_{pos} = [\exp(1.96 \times 2^{½} \times \sigma) - 1] \times 100$, and $RCV_{neg} = [\exp(-1.96 \times 2^{½} \times \sigma) - 1] \times 100$ where σ is the log-normal distribution defined as $\sigma = [\ln(CV_t^2 + 1)]^{½}$, and CV_t is the total imprecision. A *Z*-score of 1.96 was used to calculate RCVs, corresponding to p = 0.05, or a 95% chance that a change in subsequent measurements is significant, and due to something other than normal biological variation and analytical imprecision.

The intra-individual variation over the four time points was determined by calculating the coefficient of reliability, which is also known as Cronbach's α. The coefficient of reliability was calculated on urine using log-transformed data. A

reliability coefficient close to 1 represents a high degree of intra-individual reliability.[153] The coefficient of reliability was calculated for each compound using only subjects where there was a complete set of four time points (baseline, 6 months, 12 months, and 24 months). There was a complete set of data for 131 subjects for urine, and a complete set of 262 subjects for plasma. To enable comparisons with previous studies, the index of individuality was also calculated for each analyte, whereas a lower number represents higher individuality of the data. The index of individuality was calculated as the ratio of within-individual coefficient of variation (CVi) to between-individual coefficient of variation (CVg).[65b]

Results

There were no significant differences in urine betaine concentrations observed when the four time points were compared by Kruskal-Wallis analysis of variance on ranks. However, there was a tendency for urine betaine concentrations to increase during the study for approximately 10% of the subjects.

The coefficients of reliability and indices of individuality are shown in Table 4.4 for plasma, and Table 4.5 for urine. Because differences were observed between males and females in betaine concentration, data are displayed for each gender.

The results show that there is a high degree of reliability for betaine (coefficient of reliability = 0.75) and DMG (coefficient of reliability = 0.79) in the plasma. Of all the analytes investigated, betaine (coefficient of reliability = 0.83) and DMG (coefficient of reliability = 0.81) concentrations showed the highest degree of reliability in the urine. Concentrations for plasma and urine betaine at all time points are plotted for the first 50 subjects in Fig. 4.3.

While betaine showed a high level of individuality in the plasma and urine, the other osmolytes were more variable. GPC had a moderate degree of reliability in the plasma (coefficient of reliability = 0.42), and urine (coefficient of reliability = 0.59). Taurine was much more variable in the plasma (coefficient of reliability = 0.09) than in the urine (coefficient of reliability = 0.73).

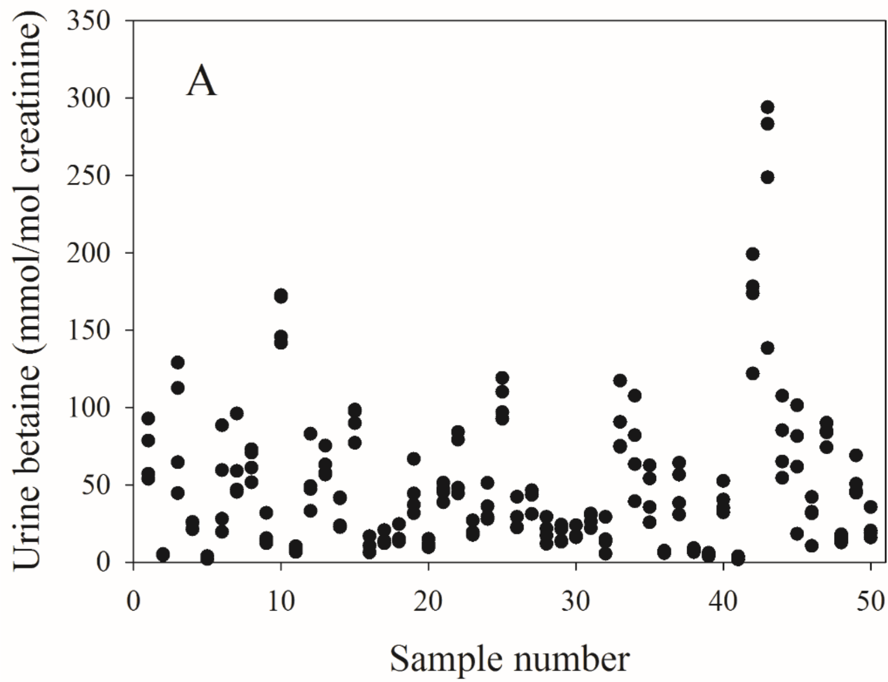
Choline concentrations showed a moderate reliability in the plasma (coefficient of reliability = 0.67) and in the urine (coefficient of reliability = 0.75). TMAO had a low reliability in both the plasma (coefficient of reliability = 0.17), and the urine (coefficient of reliability = 0.31).

The indices of individuality show similar trends for the metabolites. A smaller number for the index of individuality indicates low intra-individual variability (high individuality). Plasma taurine had a particularly high index, representing a low individuality. GPC and TMAO in the plasma also had high indices of individuality of 0.84.

The reference change values (RCVs) were much lower in the plasma than the urine for all analytes. A change in plasma betaine concentration of +51% (or -34%) in males and +56% (or -36%) in females can be considered significant ($p < 0.05$). A change in plasma DMG concentrations of +64% (-39%) is significant ($p < 0.05$) for both males and females. However, a change in plasma TMAO of +403% (or -80%), and a change of plasma taurine of +124% (or -55%) is required to be significant ($p = 0.05$) over both genders. Larger log-normal RCV ranges were observed for urine metabolite excretions compared to the plasma. For example, urine betaine had a positive log-normal RCV of 186 and a negative log-normal RCV value of -65 over both genders. TMAO had the highest RCVs in the urine of +480% (or -83%) over both genders.

Carnitine and acetylcarnitine had high test-retest reliability in both the plasma and urine. The coefficient of reliability for carnitine in plasma was 0.80 and urine was 0.46. Carnitine had a log-normal RCV range of 31.5% to -24% in the plasma, and 272% to -73% in the urine. The reliability coefficient for acetylcarnitine was 0.72 in plasma and 0.40 in urine. Acetylcarnitine had a log-normal RCV range of 65.4% to -39.5% in the plasma, and 332 to -77% in the urine.

Mann-Whitney rank sum tests showed that there are significant differences between the sexes. Males had significantly higher ($p < 0.01$) plasma betaine, DMG, choline, and carnitine. Males also had significantly higher ($p < 0.01$) urine GB. Males had a higher coefficient of reliability for betaine, DMG, and GPC in the plasma (Table 4.4) and urine (Table 4.5) than females. The significantly higher plasma betaine results observed for males compared to females has also been reported in normal populations.[4b]



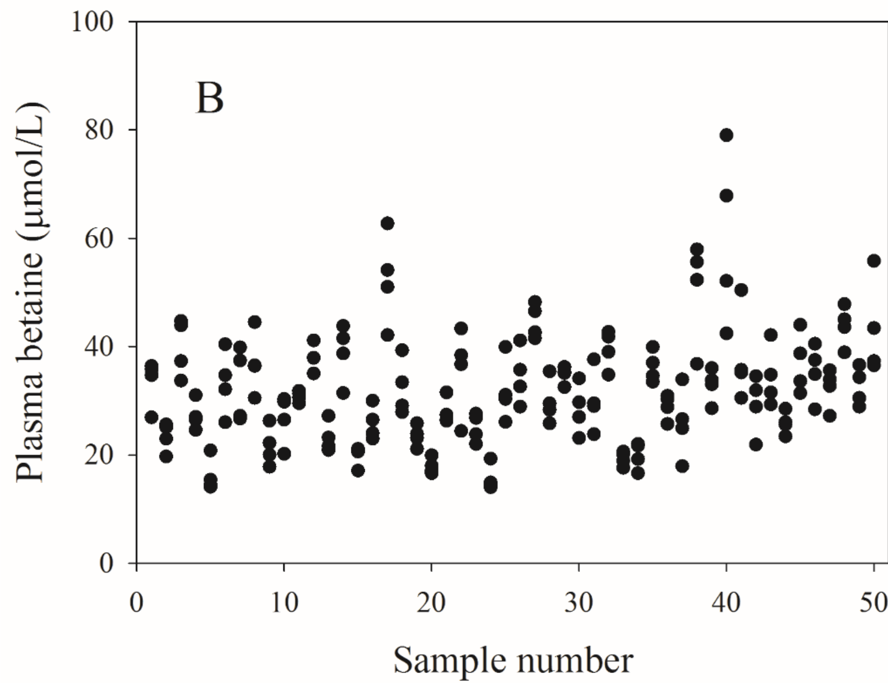


Fig. 4.3. Betaine in the urine of the first 50 subjects (A). Betaine in the plasma of the first 50 subjects (B).

Table 4.4. Individuality and test-retest reliability in plasma for the DEWL study.

	Median (μmol/L)	Interquartile range 25%	75%	Reliability coefficient	CVa %	CVi %	Index of Individuality	RCV % Lognormal Positive	Negative
All subjects (N = 243)									
Betaine	30.3	24.9	36.9	0.75	4.6	11.0	0.38	119.3	-54.4
DMG	2.34	1.89	2.96	0.79	5.1	12.8	0.22	63.6	-38.9
Choline	10.4	8.7	12.3	0.67	5.3	10.0	0.48	52.4	-34.4
GPC	1.54	1.17	2.12	0.42	20.9	9.1	0.25	125.6	-55.7
Taurine	49.3	41.4	60.5	0.09	7.7	22.0	1.12	123.9	-55.3
TMAO	6.31	4.09	10.4	0.17	5.7	57.9	0.76	403.0	-80.1
Carnitine	45.6	39.3	51.5	0.80	3.9	9.9	0.52	31.5	-23.9
Acetylcarnitine	7.72	6.30	9.91	0.72	9.6	18.3	0.58	65.4	-39.5
Males (n = 99)									
Betaine	32.1	26.3	40.0	0.77	4.6	10.2	0.42	50.7	-33.6
DMG	2.50	2.10	3.12	0.83	5.1	12.6	0.22	62.6	-38.5
Choline	10.9	9.27	12.8	0.67	5.3	9.3	0.49	49.6	-33.1
GPC	1.52	1.18	2.15	0.48	20.9	6.8	0.28	112.6	-53.0
Taurine	50.3	42.3	60.9	0.13	7.7	20.5	1.15	115.0	-53.5
TMAO	6.64	4.04	11.0	0.18	5.7	61.5	0.76	442.5	-81.6
Carnitine	47.1	40.3	54.0	0.82	3.9	17.0	0.48	30.2	-23.2
Acetylcarnitine	7.69	6.34	9.57	0.74	9.6	25.7	0.56	63.9	-39.0
Females (n = 144)									
Betaine	28.9	23.4	35.1	0.72	4.6	11.6	0.34	56.2	-36.0
DMG	2.20	1.78	2.77	0.76	5.1	12.9	0.21	64.0	-39.0
Choline	9.92	8.46	11.7	0.67	5.3	10.4	0.42	54.1	-35.1
GPC	1.55	1.14	2.09	0.30	20.9	10.3	0.23	132.8	-57.0
Taurine	48.8	40.8	60.4	0.08	7.7	22.9	1.22	129.2	-56.4
TMAO	6.08	4.14	10.2	0.17	5.7	55.1	0.73	373.3	-78.9
Carnitine	44.9	38.4	49.8	0.79	3.9	7.5	0.40	23.1	-18.7
Acetylcarnitine	7.79	6.29	9.99	0.72	9.6	13.8	0.43	46.3	-31.7

Median values were calculated from all four time points. Abbreviations: analytical imprecision (CVa); within subject coefficient of variation (CVi); reference change value (RCV); *N*,*N*-dimethylglycine (DMG); glycerophosphorylcholine (GPC); trimethylamine-*N*-oxide (TMAO).

Table 4.5. Individuality and test-retest reliability in urine for the DEWL study.

	Median (mmol/mol CRN)	Interquartile range 25%	75%	Reliability coefficient	CVa %	CVi %	Index of Individuality	RCV % Lognormal Positive	Negative
All subjects (N = 116)									
Betaine	25.9	13.0	52.8	0.83	6.6	32.7	0.38	186.2	-65.1
DMG	6.75	4.10	9.76	0.81	6.8	25.9	0.29	141.9	-58.7
Choline	3.63	2.64	5.25	0.75	12.0	23.5	0.26	159.9	-61.5
GPC	0.56	0.41	0.77	0.59	17.6	15.9	0.22	146.9	-59.5
Taurine	29.0	10.3	54.9	0.73	5.8	50.6	0.20	328.8	-76.7
TMAO	70.7	46.3	115	0.31	5.3	65.1	0.33	480.2	-82.8
Carnitine	12.6	7.2	19.4	0.46	5.8	50.2	0.38	272.0	-73.1
Acetylcarnitine	3.38	1.88	5.70	0.40	11.0	56.7	0.49	332.0	-76.9
Males (n = 48)									
Betaine	32.4	13.1	66.5	0.91	6.6	32.7	0.40	186.2	-65.1
DMG	6.92	4.27	9.46	0.89	6.8	19.4	0.38	104.3	-51.0
Choline	3.51	2.46	4.90	0.72	12.0	21.9	0.30	149.3	-59.9
GPC	0.49	0.37	0.72	0.83	17.6	2.9	0.06	75.3	-43.0
Taurine	36.9	19.0	58.1	0.68	5.8	44.3	0.71	271.0	-73.0
TMAO	65.7	45.8	94.7	0.30	5.3	48.2	0.72	301.6	-75.1
Carnitine	13.2	7.6	18.6	0.43	5.8	45.9	0.34	236.0	-70.3
Acetylcarnitine	3.75	2.30	5.77	0.49	11.0	43.3	0.45	257.7	-72.0
Females (n = 68)									
Betaine	25.4	13.8	53.0	0.78	6.6	24.7	0.28	133.5	-57.2
DMG	6.99	4.39	10.8	0.79	6.8	19.3	0.19	103.4	-50.8
Choline	3.94	2.86	5.79	0.78	12.0	14.1	0.15	103.6	-50.9
GPC	0.62	0.46	0.87	0.52	17.6	11.1	0.14	118.2	-54.2
Taurine	19.6	6.44	51.5	0.75	5.8	36.2	0.12	205.9	-67.3
TMAO	86.8	49.4	130.0	0.32	5.3	52.6	0.31	343.8	-77.5
Carnitine	12.5	7.5	19.8	0.48	5.8	37.8	0.26	175.1	-63.7
Acetylcarnitine	3.02	1.67	5.39	0.35	11.0	44.3	0.33	223.0	-69.0

Median values were calculated from all four time points. The coefficients of reliability for the urine results were calculated on log-transformed data. Abbreviations: analytical imprecision (CVa); within subject coefficient of variation (CVi); reference change value (RCV); *N*,*N*-dimethylglycine (DMG); glycerophosphorylcholine (GPC); trimethylamine-*N*-oxide (TMAO).

Discussion

Two measures are used as guides to test-retest reliability, or individuality. The coefficient of reliability ranges from 0 (no consistency on retesting) to 1 (perfect concordance on retesting). The index of individuality has an inverse scale, and perfect concordance of results within each individual would give an index of individuality of 0. The upper range is open-ended but in practice a value approaching 1 or greater indicates low individuality. When the index of individuality is low, and the coefficient of reliability high, reference ranges may be of limited use. More significance is attached to changes than to single values, and reference change intervals are used to estimate the changes that are likely to be clinically significant. However, it has been shown that single values of plasma and urine betaine,[154] and especially plasma DMG,[154-155] are predictive of secondary cardiovascular events in populations with established vascular disease. It is possible that monitoring these markers could show trends with time that have additional clinical value. Both statistics, the index of individuality and the coefficient of reliability, were calculated in order to compare the results with previous studies and other populations. Lever *et al.*[156] reported that abnormal betaine excretions in a small number of patients (n = 10) attending a lipid clinic were highly individual and may persist for many years. The results of the present study are consistent with the high coefficient of reliability of 0.73 which was reported for urine betaine concentrations by Schartum-Hansen *et al.*[148] in a population with coronary artery disease; the coefficient in the subgroup with diabetes was similar (0.70) despite a much higher median excretion in this group.[148] The present study found an even higher coefficient of reliability of 0.83 for urine betaine (0.91 in the males). The main difference in these populations is that the present study investigated people with diabetes who were over-weight, whereas Schartum-Hansen *et al.* investigated a larger group of cardiovascular patients that included a significant minority with diabetes.[148] A high reliability of betaine in the plasma and urine was also reported by Lever *et al.*[65b, 157] in healthy subjects. The coefficients observed for people with type 2 diabetes in the present study are generally consistent with those of Lever *et al.*[65b] who reported coefficients of reliability of 0.43 in the plasma, and 0.78 in the urine for betaine over an 8 week period. Urinary DMG excretion may have slightly lower individuality than betaine, and free choline exhibits lower still.

Plasma DMG is much more individual in this population than in a small group of healthy young males,[65b] and these values can be expected to be population dependent. Svingen *et al*.[155] also reported a high coefficient of reliability (0.93) for plasma DMG in people with stable angina. The predictive value of single measurements of plasma DMG is not just a reflection of its correlation with homocysteine,[155] and as noted before, it could be expected that changes in plasma DMG with time may be a useful additional tool in patient management, and this should be investigated.

The other osmolytes, GPC and taurine were considerably more variable in the plasma than betaine. This is possibly related to variations in osmotic control caused by fluctuations in their blood sugar levels.

The results from the present study show that plasma choline has moderately high individuality (coefficient of reliability = 0.67), and a change of 52% (or -34%) is required to be significant with 95% confidence. This suggests that plasma choline may be useful as an indicator of cardiovascular risk in this population. The high intra-individual variability in plasma taurine (coefficient of reliability = 0.09, positive and negative RCVs = 124% and -55% respectively) observed here suggests that a large change is required to be regarded as significant beyond the biological and analytical variation in this population. However, the urine taurine was much more individual in this population (coefficient of reliability = 0.73; index of individuality = 0.23). Plasma GPC concentrations had low individuality (coefficient of reliability = 0.42).

The plasma TMAO concentrations were highly variable in this study group. Plasma TMAO has been promoted as a predictive marker of cardiovascular disease.[87b] However in the DEWL study group, both plasma concentrations and urinary excretion of TMAO are highly variable. TMAO concentrations are especially affected by the dietary intake of marine foods. TMAO is an osmolyte in many marine animals, but not a mammalian osmolyte, and is often a marker of eating fish. TMAO concentrations are also raised in people on high choline diets or taking lecithin, betaine, or carnitine supplements.[56] Elevated plasma concentration TMAO has been previously proposed as a marker of renal disease.[158] The high intra-individual variability of TMAO concentrations observed in this population would make it difficult to make clinical decisions, at least on the basis of a single measurement. The fact that a large change in plasma TMAO (RCV) of 403% (or -80%) is required to be 95% confident that it is significant (beyond analytical imprecision and biological

variability) implies that there may be a low value of a single measurement as a diagnostic tool for cardiovascular risk in this population with diabetes.

A limitation is that most subjects lost weight during the DEWL study,[146] and the dietary changes may be expected to affect the handling of some metabolites. This makes the high level of consistencies reported here more remarkable, and we may have underestimated the individuality of some of those we studied. However, there were no trends for changes with treatment and time found that approach statistical significance.

There are marked differences in the individualities of the osmolytes and one-carbon metabolites we investigated. The high reliability of betaine plasma and urine betaine in this population suggests that betaine concentrations measured at any given time provide information about the betaine status of each individual. Although this has been shown to have prognostic value, monitoring changes with time may provide significantly more information. The high reliability of betaine in plasma and urine appears to be more related to its role in one-carbon metabolism, than to its role as an osmolyte. Like betaine, the other one-carbon metabolites, choline and DMG, had high reliability in the urine and plasma. The other osmolytes, GPC and taurine, were considerably more variable than betaine. The high intra-individual variation observed for plasma and urine TMAO in this study throws doubt on its value as a disease marker (at least in this population) despite evidence for its association with vascular disease.[87b]

Correlations between betaine, other osmolytes, and other one-carbon metabolites in subjects with type 2 diabetes

Methylamines and taurine

Correlations were identified in the baseline samples from the DEWL study using Spearman's rank order analysis, and the results are summarised in Fig. 4.4. Plasma betaine correlated significantly with the following metabolites in the plasma: DMG ($p < 0.001$), choline ($p < 0.001$), GPC ($p = 0.0096$), and carnitine ($p < 0.001$) and acetylcarnitine ($p < 0.001$). While plasma betaine did not correlate significantly with taurine in the plasma ($p = 0.078$), it did correlate with taurine ($p = 0.002$) in the

urine. There was a negative correlation between plasma betaine and total cholesterol ($p < 0.001$). There was a weaker correlation between plasma betaine and creatinine ($p = 0.035$), but not eGFR (p = 0.51). There were no significant correlations observed between plasma betaine and plasma glucose or HbA_{1c}. There were strong correlations between betaine, DMG, and choline and carnitine and acetylcarnitine in the plasma ($p < 0.001$). Urine betaine correlated strongly with many of the other metabolites including DMG, choline, GPC, taurine, carnitine and acetylcarnitine ($p < 0.001$). Urine betaine correlated strongly with HbA_{1c} ($r = 0.475$, $p < 0.001$) and plasma glucose ($r = 0.456$, $p < 0.001$). Betaine fractional clearance (FC) also correlated with HbA_{1c} ($p = 0.01$), and plasma glucose ($p < 0.001$). Urine DMG and choline also showed a significant correlation with HbA_{1c} ($p < 0.001$) and plasma glucose ($p < 0.001$).

Plasma DMG correlated significantly with markers of renal function (plasma creatinine p < 0.001, eGFR $p < 0.001$). There was also a weak correlation between plasma DMG and plasma glucose ($p = 0.043$).

Plasma choline also correlated significantly with markers of renal function (plasma creatinine $p < 0.001$, eGFR $p < 0.001$). Plasma choline also correlated with HbA_{1c} ($p = 0.007$).

GPC correlated strongly with taurine in the plasma ($r = 0.5$, $p < 0.001$) (Fig. 4.5). GPC did not correlate with betaine, but did correlate with carnitine ($p < 0.001$) in the plasma. Plasma GPC correlated with cholesterol ($p = 0.001$) and BMI ($p = 0.01$). Urine GPC correlated with HbA_{1c} ($p = 0.015$) and glucose ($p = 0.0017$). Urine GPC also had a strong negative correlation with plasma creatinine ($r = -0.279$, $p < 0.001$) and positive correlation with eGFR ($r = 0.201$, $p = < 0.001$). These correlations suggest that higher GPC in the urine is associated with better kidney function.

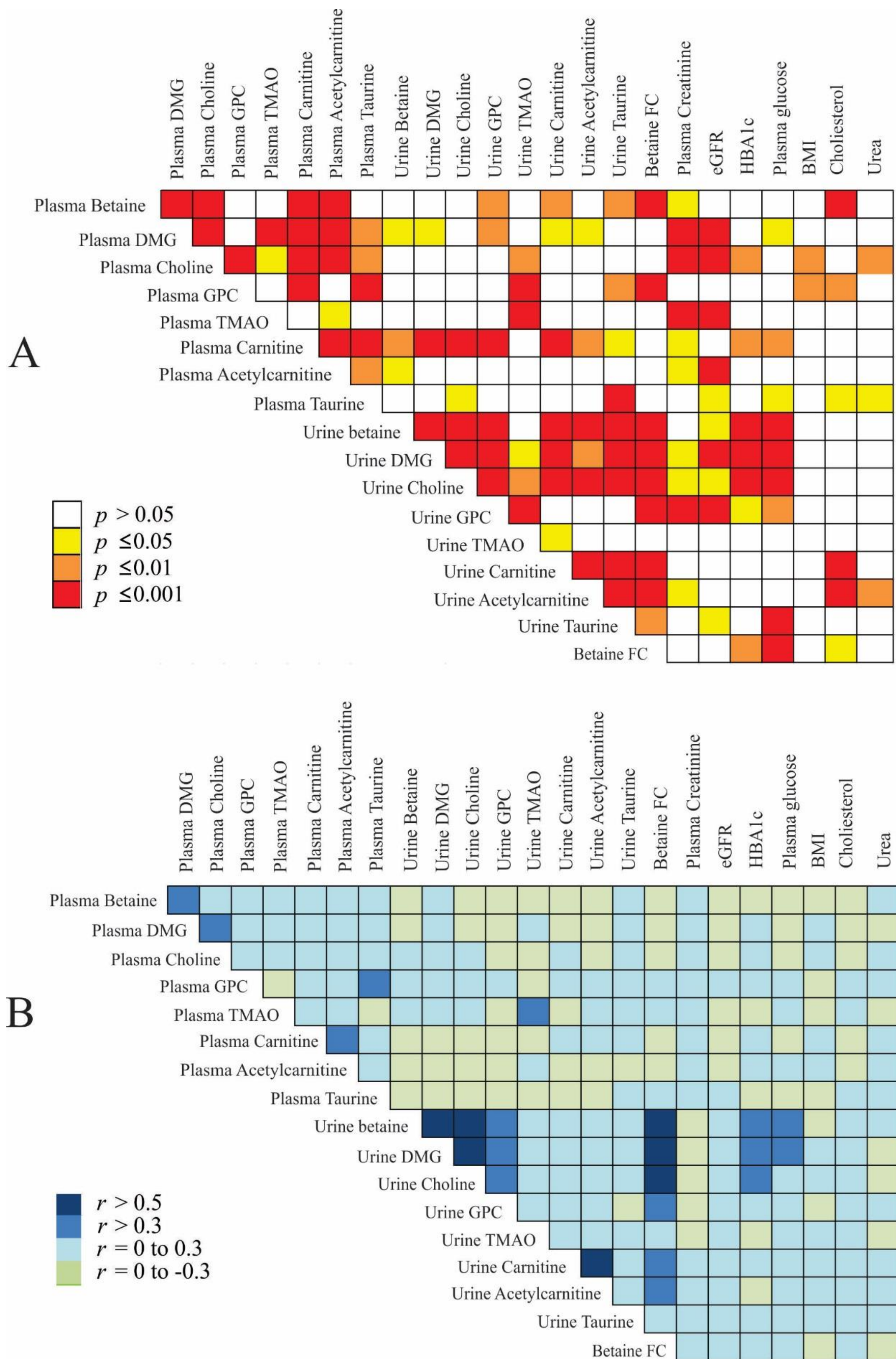


Fig. 4.4. Spearman’s rank order correlations for the baseline samples from the DEWL study, showing *p*-values (A), and *r*-values (B). Note: betaine FC is betaine fractional clearance.

Plasma TMAO correlated with acetylcarnitine ($p = 0.027$), and also correlated with plasma creatinine and eGFR ($p < 0.001$).

Plasma taurine did not correlate significantly with plasma betaine, but there was a weak correlation between taurine and DMG ($p = 0.002$) and choline ($p = 0.005$) in the plasma. Urine taurine correlated significantly with plasma glucose ($p < 0.001$). However, urine taurine did not correlate significantly with HbA_{1c} ($p = 0.054$).

Plasma carnitine correlated with many compounds in the plasma and the urine including: acetylcarnitine, taurine, betaine, DMG, choline, and GPC ($p < 0.001$). Plasma carnitine also correlated with plasma creatinine ($p = 0.035$), and plasma glucose ($p = 0.003$) and HbA_{1c} ($p = 0.007$). Plasma acetylcarnitine also correlated with betaine, DMG, and choline ($p < 0.001$), and correlated with GFR ($p < 0.001$). Urine carnitine and acetylcarnitine correlated with total cholesterol ($p < 0.001$).

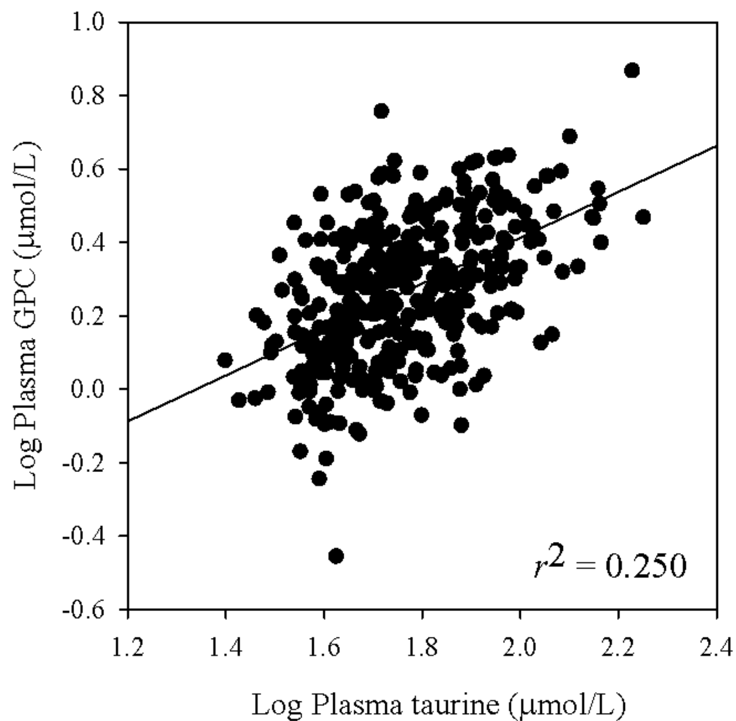


Fig. 4.5. Correlation between plasma taurine and plasma GPC.

Inositols

To investigate the concentrations of inositols in diabetes, a subset of the DEWL study sample was analyzed for inositols. This subset consisted of the 32 subjects who were taking bezafibrate, and 64 matched controls. *myo*-Inositol was measured in plasma, and the inositol isomers *myo*-inositol, *chiro*-inositol, and *scyllo*-inositol were measured in the urine. The correlations discussed here are from Spearman's rank order correlation analysis on the control data (Table 4.6). Urine *myo*-inositol correlated strongly with urine betaine ($r = 0.70$, $p < 0.001$) (Fig. 4.6), and its metabolites DMG ($r = 0.48$, $p < 0.001$), and choline ($r = 0.57$, $p < 0.001$). Urine *myo*-inositol also correlated with the osmolyte GPC in the urine ($r = 0.545$, $p < 0.001$), but not with urine taurine ($r = 0.23$, $p = 0.71$). Urine *myo*-inositol also correlated significantly with markers of glycemic control, plasma glucose ($r = 0.45$, $p < 0.001$), and HbA_{1c} ($r = 0.27$, $p = 0.03$). Urine *myo*-inositol did not correlate significantly with eGFR. Urine *myo*-inositol correlated with other inositols in the urine, *i.e.*, *chiro*-inositol ($r = 0.66$, $p < 0.001$) and urine *scyllo*-inositol ($r = 0.60$, $p < 0.001$). There were significant correlations between urine *chiro*-inositol, *scyllo*-inositol, and other metabolites in the urine (including: carnitine, acetylcarnitine, TMAO, GPC, choline, betaine, and DMG). The strong correlation observed between *scyllo*-inositol and carnitine ($r = 0.48$, $p < 0.001$) and acetylcarnitine ($r = 0.51$, $p < 0.001$) may suggest that *scyllo*-inositol plays some role in fatty acid metabolism. The three inositol isomers in the urine did not correlate significantly with plasma methylamines or plasma taurine. *chiro*-Inositol and *scyllo*-inositol did not correlate significantly with HbA_{1c}, and only *chiro*-inositol weakly correlated with plasma glucose ($p = 0.049$).

myo-Inositol was the only inositol detected in the plasma, and had a mean value of 18.4 µmol/L and a standard deviation of 5.6 µmol/L. Plasma *myo*-inositol correlated strongly with markers of renal function, plasma creatinine and eGFR, but not with markers of glycemic control (Table 4.7). There was also a strong correlation between plasma *myo*-inositol and plasma homocysteine, which is likely to be because homocysteine correlates strongly with eGFR, and may be an artefact of renal function. Plasma *myo*-inositol correlated significantly with the plasma osmolytes, betaine and taurine, but not with GPC. There were also significant

correlations between *myo*-inositol and DMG, carnitine, and acetylcarnitine ($p <$ 0.05).

While *myo*-inositol correlated strongly with betaine in the diabetic urine, it did not correlate significantly with betaine in the small number of healthy subjects from the Solvay study (n = 26, $p = 0.302$). *myo*-Inositol, which is the only inositol isomer known to be a mammalian osmolyte, was the only one to show a strong correlation with plasma glucose, showing that people with poor glycemic control excrete more *myo*-inositol in the urine. However, *chiro*-inositol has been shown to be a marker of insulin resistance,[75b] which may explain its correlation with the osmolytes and its weak correlation with plasma glucose.

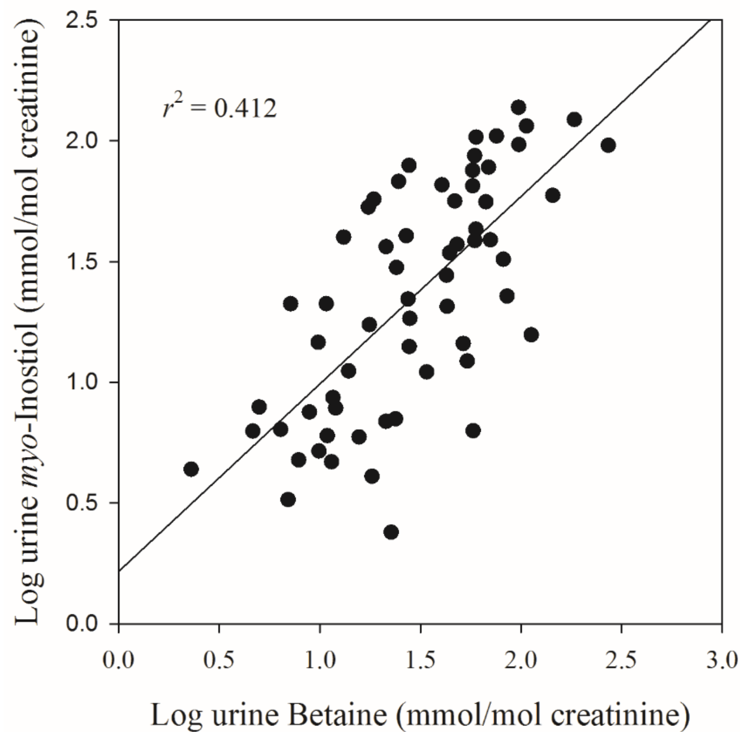


Fig. 4.6. Plot showing the correlation between betaine and *myo*-inositol in urine.

Table 4.6. Spearman's rank order correlations of inositols with other metabolites.

	Urine *myo*-Inositol	Urine *chiro*-Inositol	Urine *scyllo*-Inositol
HbA_{1c}	**r = 0.270**	r = 0.166	r = -0.008
	p = 0.034	p = 0.196	p = 0.952
Plasma Glucose	**r = 0.451**	**r = 0.252**	r = 0.110
	p < 0.001	**p = 0.049**	p = 0.393
Plasma Creatinine	r = 0.186	r = -0.022	r = 0.044
	p = 0.148	p = 0.865	p = 0.732
eGFR	r = -0.052	r = 0.073	r = -0.066
	p = 0.690	p = 0.57	p = 0.609
BMI	r = -0.079	r = 0.0328	r = -0.199
	p = 0.561	p = 0.809	p = 0.141
Cholesterol	r = 0.009	r = -0.009	r = 0.175
	p = 0.944	p = 0.944	p = 0.173
Urine Betaine	**r = 0.705**	**r = 0.476**	**r = 0.354**
	p < 0.001	**p < 0.001**	**p = 0.005**
Urine DMG	**r = 0.477**	**r = 0.382**	**r = 0.306**
	p < 0.001	**p < 0.001**	**p = 0.016**
Urine Choline	**r = 0.567**	**r = 0.517**	**r = 0.389**
	p < 0.001	**p < 0.001**	**r = 0.002**
Urine GPC	**r = 0.545**	**r = 0.389**	**r = 0.410**
	p < 0.001	**p = 0.002**	**p = 0.001**
Urine TMAO	r = 0.192	**r = 0.452**	**r = 0.304**
	p = 0.135	**p < 0.001**	**p = 0.016**
Urine Carnitine	**r = 0.315**	r = 0.222	**r = 0.475**
	p = 0.013	p = 0.082	**p < 0.001**
Urine Ac Carn	**r = 0.355**	r = 0.178	**r = 0.507**
	p = 0.005	p = 0.165	**p < 0.001**
Urine Taurine	r = 0.231	**r = 0.177**	**r = 0.303**
	p = 0.071	**p = 0.167**	**p = 0.017**
Urine *scyllo*-Inositol	**r = 0.600**	**r = 0.614**	
	p < 0.001	**p < 0.001**	
Urine *chiro*-Inositol	**r = 0.657**		
	p < 0.001		

Note: Data from DEWL study subjects, controls used in fibrate study (n = 62). Statistically significant (p < 0.05) correlations are highlighted in bold.

Table 4.7. Spearman's rank order correlations of plasma *myo*-inositol with other metabolites.

	Plasma *myo*-Inositol
Plasma Glucose	$r = 0.042$
	$p = 0.741$
HbA_{1c}	$r = 0.0196$
	$p = 0.877$
Plasma Creatinine	**$r = 0.374$**
	$p = 0.002$
eGFR	**$r = -0.506$**
	$p < 0.001$
Plasma Betaine	**$r = 0.248$**
	$p = 0.048$
Plasma Hcy	**$r = 0.505$**
	$p < 0.001$
Plasma DMG	**$r = 0.301$**
	$p = 0.016$
Plasma Choline	$r = 0.220$
	$p = 0.081$
Plasma GPC	$r = 0.119$
	$p = 0.350$
Plasma Taurine	**$r = 0.336$**
	$p = 0.007$
Plasma Carnitine	**$r = 0.340$**
	$p = 0.006$
Plasma Acetylcarnitine	**$r = 0.339$**
	$p = 0.007$

Note: Data from DEWL study subjects, controls used in fibrate study (n = 62). Statistically significant ($p < 0.05$) correlations are highlighted in bold. Abbreviations: Haemoglobin A_{1c} (HbA_{1c}); estimated glomerular filtration rate (eGFR); homocysteine (Hcy); *N*,*N*-dimethylglycine (DMG); glycerophosphorylcholine (GPC).

Correlations between betaine, other osmolytes and other one-carbon metabolites in the urine of healthy individuals

Correlations between osmolytes and other metabolites were investigated in the urine of healthy subjects and compared to the correlations observed in type 2 diabetes. Fig. 4.7 shows correlations of some osmolytes and other metabolites in the urine of 26 healthy individuals aged 48-60 years from the fenofibrate study (see Section 4.3) at baseline (before treatment).

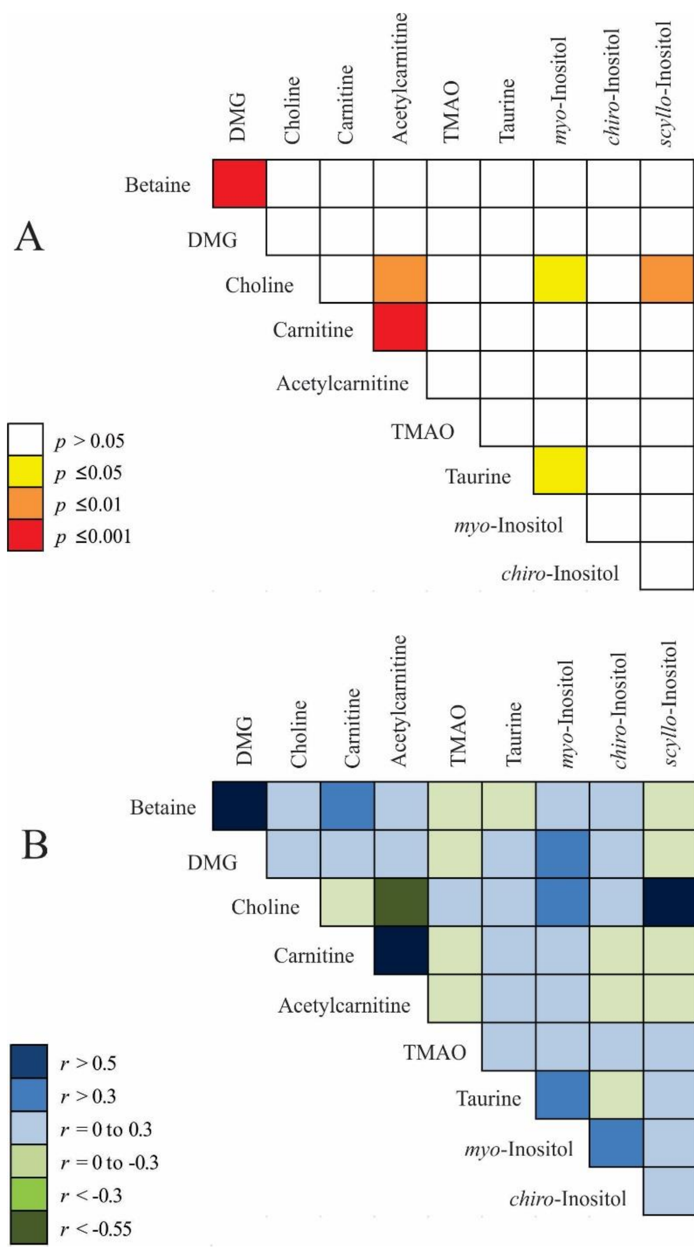


Fig. 4.7. Spearman's rank order correlations for metabolites in the urine of healthy subjects showing *p*-values (A), and *r*-values (B).

Less significant correlations were observed in the urine of healthy subjects, than for people with type 2 diabetes. *myo*-Inositol did not correlate with *scyllo*-inositol and *chiro*-inositol in the urine of healthy subjects, unlike in subjects with diabetes where the inositols all correlated strongly in the urine. The inositols did not correlate with betaine in healthy individuals. This may be partly due to the low sample number of 26 healthy controls. However, *myo*-inositol correlated with the other osmolyte, taurine, in the healthy individuals. There was a significant correlation between *scyllo*-inositol and choline in the urine of healthy subjects. Plasma was not available for this set of healthy study subjects, so correlations in plasma were not investigated.

Osmolytes and glycemic control in type 2 diabetes

The increased in urinary betaine excretion observed in diabetes has been shown not to be a direct result of high glucose levels.[159] However, the results from the DEWL study samples show that the osmolytes GPC, and betaine, and its metabolites choline and DMG in the urine, are all somehow related to markers of glycemic control (see Fig. 4.4). People with diabetes who have higher HbA_{1c} and plasma glucose excrete more of the osmolytes: betaine, taurine, GPC, and *myo*-inositol in the urine. Of the osmolytes investigated, urine betaine correlated the strongest with plasma glucose ($r = 0.456$, $p < 0.001$) and HbA_{1c} ($r = 0.475$, $p < 0.001$) (Fig. 4.8). Urine *myo*-inositol correlated strongly with plasma glucose ($p < 0.001$), and less strongly with HbA_{1c} ($p = 0.034$). Urine taurine also correlated significantly with plasma glucose ($p < 0.001$), but not with HbA_{1c} ($p = 0.054$). This suggests that the concentrations of *myo*-inositol and taurine in the urine are affected by the short-time sugar concentrations in the body, whereas urine betaine is affected by both the short-term and long-term sugar concentrations. A correlation between plasma glucose (and HbA_{1c}) and betaine excretion has been previously reported in people with diabetes.[14] The reason that more betaine and GPC are excreted by the kidneys by people with poorer glycemic control may be related to increased sorbitol levels displacing the other osmolytes in the kidneys. Alternatively, the betaine transporter proteins may become glycated in diabetes which may alter their function leading to a loss of betaine from cells and from the kidneys.[91] It is not clear why the betaine metabolites, DMG and choline, in the urine also correlate with plasma glucose and HbA_{1c}. However, it appears that the disruption in osmolyte concentrations (or the increase in circulating

glucose) affects the betaine / homocysteine pathway because DMG in the urine also correlates quite strongly with plasma glucose and HbA_{1c}. DMG is only formed in the body from metabolism of betaine by BHMT activity. Betaine is normally preferentially retained by the kidneys, so the increased loss which can occur in people with diabetes may lead to other health problems. As well as its role in osmo-regulation, betaine is also involved in reducing homocysteine which is associated with cardiovascular disease, and as well as GPC is also used as a counteracting solute to maintain protein structure, and therefore betaine (and GPC) depletion may play a significant role in tissue and organ degeneration.

The negative correlation between plasma carnitine and plasma glucose (and HbA_{1c}) ($p < 0.01$) suggests that people with poor glycemic control may tend to have less efficient fatty acid metabolism.

The correlation between plasma choline and HbA_{1c} ($p < 0.01$) is consistent with an increased risk of cardiovascular disease that is associated with diabetes. Increased plasma choline is known to be associated with an increased risk of cardiovascular events such as heart attack or stroke.[83]

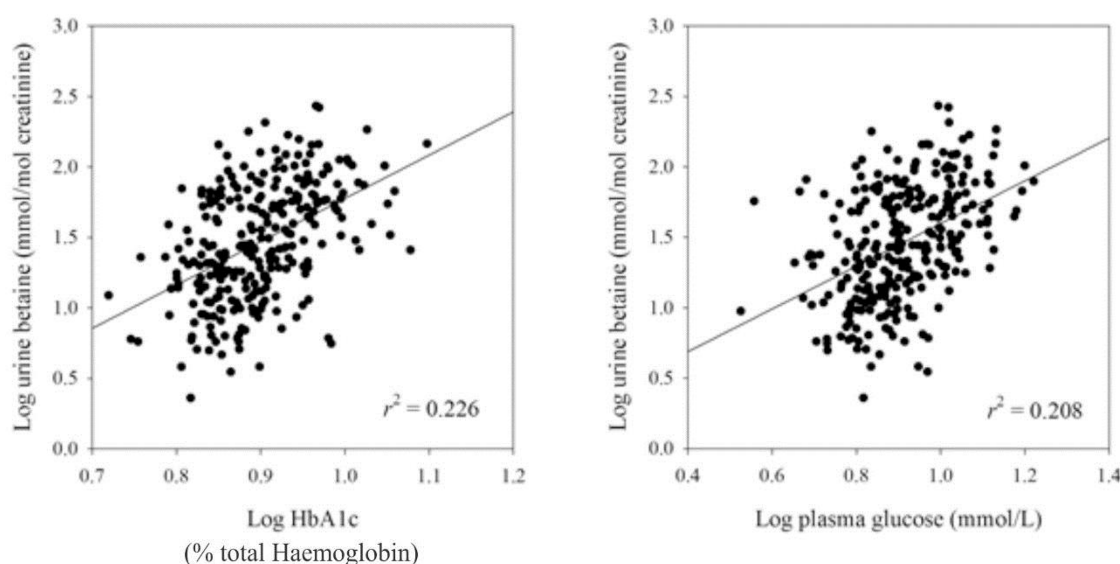


Fig. 4.8. Linear regression correlations between markers of glycemic control and urine betaine.

Osmolytes and renal function

The correlations that have been observed between osmolytes, other osmolytes, and other low molecular weight metabolites (such as carnitine and TMAO) in the urine are likely to be a consequence of the function of the kidney and its ability to filter out low molecular weight metabolites. The filtration of these metabolites is likely to vary between person depending on their renal function and glycemic control. It is therefore not surprising that so many of these compounds correlate in the urine samples.

There were significant negative correlations between GPC and DMG in the urine with plasma creatinine, and there were also significant positive correlations between eGFR and urine DMG, GPC, and taurine (see Fig. 4.4). This suggests that DMG, GPC, and taurine are less efficiently excreted (or more poorly resorbed) in people with poor renal function. However, urine betaine did not significantly correlate with plasma creatinine or eGFR. There was a positive correlation between urine inositol and plasma creatinine, showing that inositol excretion is increased in people with diabetes who have poorer renal function. These results show that people with diabetes who have poor renal function tend to excrete less GPC, DMG, and taurine and excrete more inositol into the urine. There was no significant correlation between urine betaine and markers of renal function (plasma creatinine and eGRF) or renal damage (microalbumin). However, plasma DMG correlated with microalbumin ($p < 0.001$). Plasma betaine, DMG, choline, TMAO all correlated with plasma creatinine to some degree. Plasma DMG, choline, TMAO, and acetylcarnitine all correlated negatively with estimated glomerular filtration rate (eGFR). TMAO has previously been reported to correlate strongly with plasma creatinine in people with chronic renal failure.[158] eGFR is generally considered to be a better marker of renal function than plasma creatinine as it takes into account factors such as the patient's weight and gender. While urine taurine did not correlate with markers of renal function or glycemic control, it did correlate with: urine betaine, DMG, choline, and acetylcarnitine. The kidneys accumulate GPC in response to high urea as well as during osmotic stress.[3] However, in the present study there was no observed correlation with urine GPC and urea.

Plasma DMG, TMAO, and choline correlated with plasma creatinine and negatively correlated with eGFR, showing that these betaine metabolites are

accumulated in bodies of people with poorer renal function. The increase in plasma choline concentrations observed in people with poor renal function is also suggestive that these people may be at greater risk of developing cardiovascular disease.[83]

Osmolytes, lipid metabolism, and body weight

People with raised low density lipoprotein (LDL) cholesterol are reported be at greater risk of having a heart attack.[160] The results of this study show that people with high LDL cholesterol tend to have lower betaine in the plasma ($p < 0.001$), and have slightly raised levels of the other osmolytes, GPC ($p < 0.01$) and taurine ($p < 0.05$) in the plasma. Carnitine ($p < 0.001$) and acetylcarnitine ($p < 0.01$) in the urine tends to be raised in patients with high LDL cholesterol. Plasma GPC was also negatively correlated with total body fat ($p < 0.001$), showing that the more fat people have, the lower the plasma GPC. Plasma triacylglycerides (TAG) was associated with low plasma betaine and raised plasma choline and GPC. Saturated fatty acids significantly correlated with plasma carnitine, choline, GPC, and TMAO ($p < 0.05$).

Betaine supplementation has been associated with weight loss in animals,[4b] so the loss of betaine may also be a contributing factor in obesity in diabetes. However, no significant correlation was observed between betaine and weight (or BMI) in the present study, which does not indicate that the more obese people are losing more betaine in the urine. However the negative correlation between betaine and LDL cholesterol is suggestive that low plasma betaine is an important marker of cardiovascular risk. The positive correlation between LDL cholesterol and plasma GPC and urine carnitine and acetylcarnitine may is likely to reflect the role of these metabolites in lipid metabolism.

One carbon metabolism in diabetes

The fact that many metabolites correlated in the urine is likely to be an artefact of filtration by the kidneys and renal function. However, urine DMG and choline both correlated particularly strongly with urine betaine (Fig. 4.9). These metabolites are all involved in one-carbon metabolism and the betaine-homocysteine methyl transferase (BHMT) pathway. An increase in BHMT activity will cause increased DMG in the urine as it can only be formed by this biochemical pathway in the body. The fact that

the urine betaine correlates more strongly with the BHMT metabolites than with the other osmolytes (taurine and GPC) suggests that the high betaine loss observed in diabetes is more complex than a simple displacement of osmolytes caused by increased circulating sorbitol concentrations. The betaine metabolites, DMG and choline correlated with HbA_{1c} and plasma glucose stronger than the other osmolytes, taurine and GPC. It seems possible from these results that a disruption of the BHMT pathway may be an important factor in the observed increased urinary loss of betaine, which is associated with poor glycemic control in diabetes.

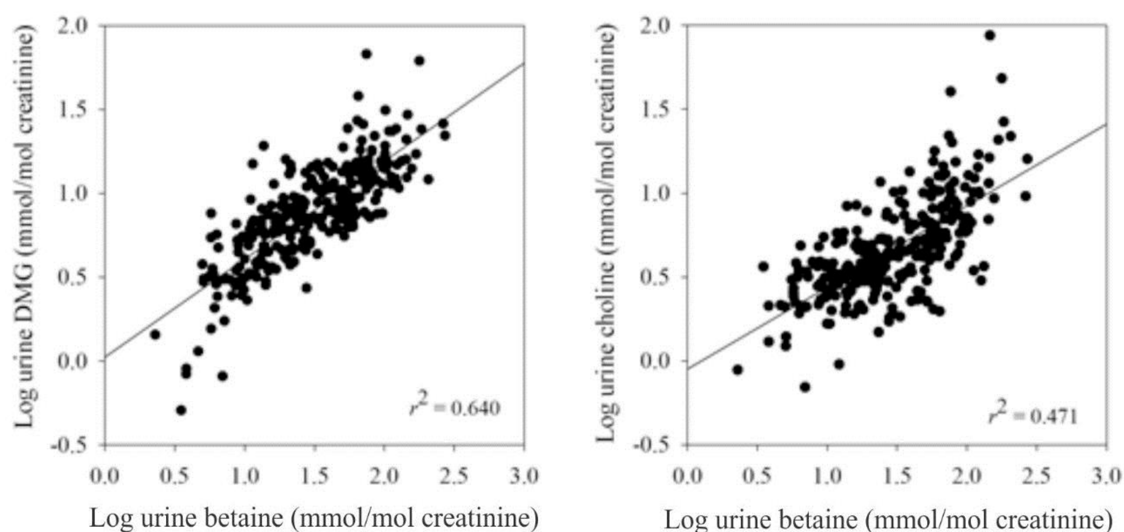


Fig. 4.9. Plots showing relationships of betaine with *N*,*N*-dimethylglycine (DMG) and choline in urine of people with type 2 diabetes.

Plasma homocysteine was not measured during the DEWL study. However, it was measured in the fibrate and control samples to compare with the betaine results. Correlations of one-carbon (and some other) metabolites with plasma homocysteine in the 64 control samples are shown in Table 4.8.

Table 4.8. Spearman's rank order correlations with plasma homocysteine

	Plasma homocysteine
Plasma betaine	$r = 0.140$
	$p = 0.270$
Plasma DMG	**$r = 0.310$**
	$p = 0.013$
Plasma choline	$r = 0.128$
	$p = 0.314$
Plasma carnitine	**$r = 0.346$**
	$p = 0.005$
Plasma acetylcarnitine	**$r = 0.263$**
	$p = 0.036$
Plasma *myo*-inositol	**$r = 0.505$**
	$p < 0.001$
Plasma creatinine	**$r = 0.300$**
	$p = 0.016$
Urine betaine	$r = -0.120$
	$p = 0.344$
Urine DMG	$r = -0.109$
	$p = 0.389$
Urine choline	$r = -0.233$
	$p = 0.064$
urine GPC	**$r = -0.263$**
	$p = 0.036$
Urine *chiro*-inositol	**$r = -0.302$**
	$p = 0.017$

Note: Significant correlations ($p < 0.05$) are shown in bold. Abbreviations: *N*,*N*-dimethylglycine (DMG), glycerophosphorylcholine (GPC).

Plasma DMG was expected to correlate with plasma homocysteine, because DMG is only formed in the body by BHMT activity. The significant correlations of homocysteine with carnitine and acetylcarnitine may be related to lipid metabolism. Homocysteine also correlated negatively with urine GPC and *chiro*-inositol, but did not significantly correlate with the other inositols in the urine. As has been mentioned, the strong correlation between homocysteine and plasma *myo*-inositol may be partly related to kidney function, as they both correlate significantly with plasma creatinine (see Table 4.7).

The effect of medications on plasma and urine concentrations of betaine, other osmolytes, and other one-carbon metabolites

Introduction

Elevated betaine concentrations have previously been reported in the urine of people taking the lipid lowering drug bezafibrate.[15] However, it was not known if other osmolytes such as taurine, GPC, and *myo*-inositol are also excreted into the urine at abnormal concentrations by people taking bezafibrate. The study on the effects of bezafibrate has been published in *Cardiovascular Drugs and Therapy*.[161] Differences in the concentrations of betaine, other osmolytes, and other one-carbon metabolites were also investigated for subjects who were taking other medications such as metformin and insulin.

Methods

There were 32 DEWL study subjects (16 males and 16 females) on the lipid lowering drug bezafibrate. A control group was selected for comparison. The control group consisted of a subset of the DEWL study participants (n = 64, 32 males and 32 females) who were matched for age and gender with the bezafibrate group. Samples were analyzed for betaine, DMG, choline, taurine, GPC, and *myo*-inositol using an AB Sciex API4000 tandem mass spectrometer using methods described in Chapters 2 and 3. *myo*-Inositol was measured using an Agilent 6120 single quadrupole mass spectrometer with an APCI source. The subjects on fibrate therapy were compared to the control group using Mann-Whitney rank sum tests.

Results and discussion

Of the 32 DEWL study subjects who were on the drug bezafibrate, only one person had a normal betaine concentration in the urine (18.3 mmol/mol creatinine). The rest of the fibrate subjects were above the normal range (> 32.5 mmol/mol creatinine[156]). Mann-Whitney rank sum tests showed that the DEWL study subjects who were taking bezafibrate (n = 32) had significantly higher betaine ($p < 0.001$) and DMG ($p < 0.001$) in the urine (Fig. 4.10). The plasma betaine was significantly lower

($p < 0.001$) in people on fibrates. Plasma homocysteine was significantly higher ($p < 0.001$) in people on fibrate therapy. There were no differences in the urine choline concentrations between the subjects taking fibrates and the subjects not taking fibrates. There was no significant correlation between urinary betaine excretion or plasma betaine with homocysteine in the subjects on fibrates or the control group. There was a significant correlation ($p = 0.01$) between plasma DMG and homocysteine in the control group.

There were no significant differences in the plasma or urine between subjects taking bezafibrate and not taking bezafibrate for the other osmolytes: taurine, GPC, and *myo*-inositol. *scyllo*-Inositol and *chiro*-inositol were not significantly different in the urine of fibrate subjects compared to the controls either (Table 4.9). *myo*-Inositol correlated with triacylglycerides (TAG, data not shown) in the subjects on fibrate therapy ($r = 0.594$, $p < 0.001$), but not in the control group ($p = 0.57$).

Mann-Whitney rank sum tests confirmed that urine betaine and urine DMG were greater in patients on fibrate therapy ($p < 0.001$) (Fig. 4.10). Mann-Whitney rank sum tests also showed that DEWL subjects taking bezafibrate had significantly lower urine acetylcarnitine ($p < 0.001$), and higher plasma carnitine ($p < 0.001$) and acetylcarnitine ($p < 0.001$) (Fig. 4.11). Mann-Whitney rank sum tests also showed that plasma betaine is significantly lower in people on fibrates ($p < 0.001$) (Fig. 4.10), and that carnitine is significantly higher in the plasma of subjects taking fibrates ($p < 0.001$), see Fig. 4.11. Urine TMAO was significantly elevated in the urine of people taking fibrates ($p = 0.04$).

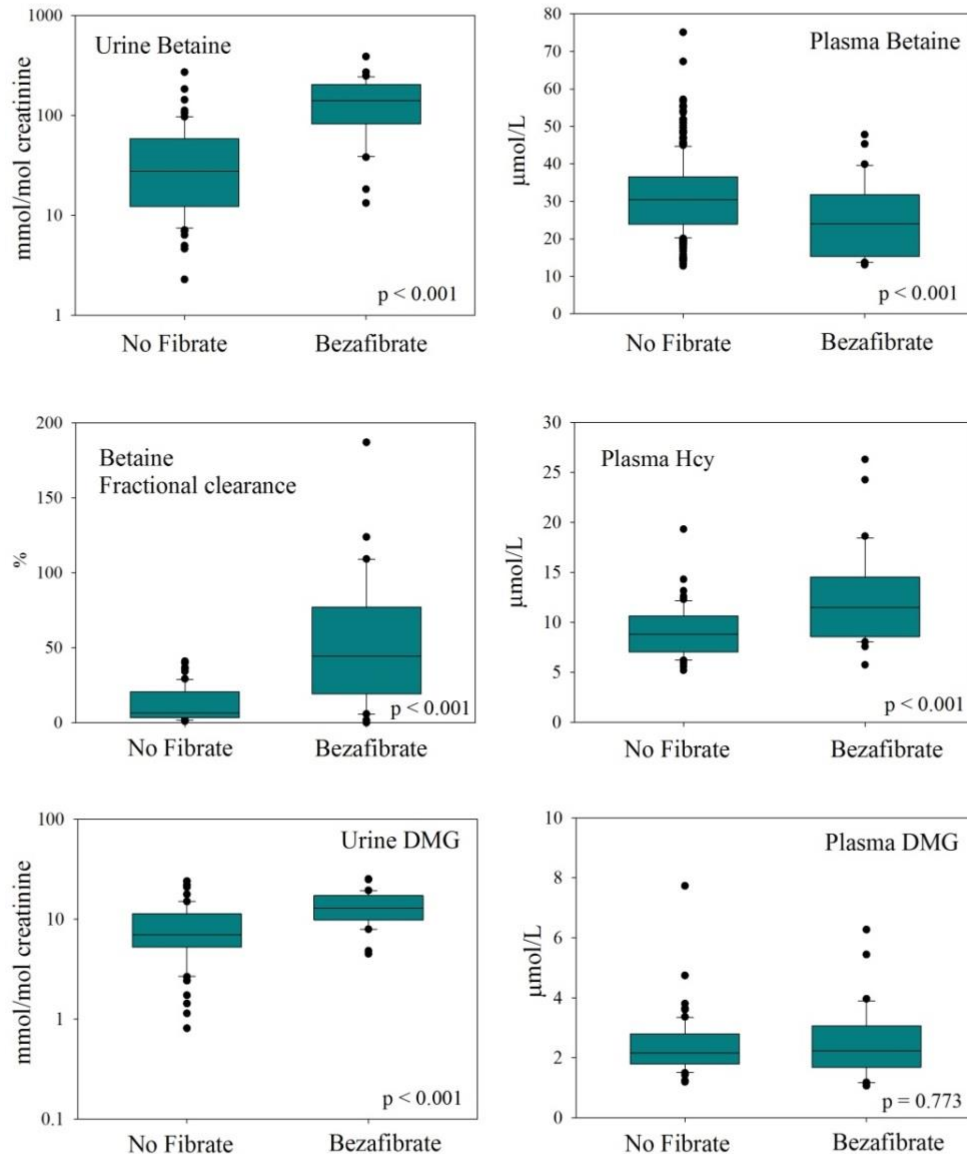


Fig. 4.10. Box plots showing betaine and dimethylglycine (DMG) concentrations in the urine and plasma, the fractional clearance of betaine, and plasma homocysteine (Hcy) in subjects with type 2 diabetes not taking fibrates and taking bezafibrate.

Subjects who were taking metformin showed significantly elevated urine betaine ($p < 0.001$), DMG ($p < 0.001$), choline ($p < 0.001$), and GPC ($p < 0.001$). Subjects on metformin had significantly higher plasma glucose ($p = 0.02$), and HbA_{1c} ($p = 0.002$). People with type 2 diabetes requiring insulin (isophane) also had increased urine betaine ($p = 0.034$), DMG ($p = 0.008$), choline ($p = 0.006$), and GPC ($p = 0.016$). There were no significant differences in the urine concentrations of osmolytes in people with type 2 diabetes taking gliclazide, aspirin, or ACE inhibitors. Subjects requiring insulin and on metformin had higher betaine, DMG, choline, and

GPC concentrations in the urine than subjects who were not on these medications. People on metformin and insulin are likely to have increased urinary betaine, DMG, and choline excretion because their diabetes is more advanced than people not yet on these medications. This is supported by the significantly higher plasma glucose and HbA_{1c} in subjects on metformin and isophane, which shows that these patients did have poorer glycemic control. Metformin works by inhibiting the production of glucose in the liver and is commonly given to people with diabetes.

The benefits of taking the lipid lowering drug bezafibrate in diabetes have been well studied.[162] However, the negative effect on betaine metabolism has been largely over-looked. It has been shown that people taking the fibrate drug bezafibrate have high betaine excretion in a population with acute coronary syndrome.[154] It is not known if other fibrates affect betaine metabolism, as the only fibrate available in New Zealand until recently was bezafibrate. It is shown here that overweight people with type 2 diabetes show the same effect on betaine metabolism when taking bezafibrate. However, even without taking fibrates many people with type 2 diabetes excrete abnormally high concentrations of betaine in the urine. It has been shown in the present study that taking fibrates exacerbates the problem of betaine loss in type 2 diabetes. Considering that betaine is an important nutrient which protects against cardiovascular disease, it may be beneficial for people who have diabetes to be supplemented with betaine, especially those who are on fibrates. Betaine supplementation in these patients would allow for them to better maintain their tissue cell volume and may also improve health outcomes by protecting against a range of diseases such as: cardiovascular disease, kidney disease, and cancer.[4b] The fact that other osmolytes do not show the same pattern as betaine in patients on fibrates suggests that the effect is likely to involve the disruption of a betaine transporter in kidneys, such as the IMINO porter SLC6A20, which is specific for betaine and proline.[36]

The observation that free carnitine and acetylcarnitine were increased in the plasma and decreased in the urine of patients taking fibrate was novel. This change in carnitine metabolism is likely to be a result of the lipid lowering action of fibrates. Because carnitine is responsible for the transport of fatty acids, a decrease in circulating lipids may lead to more free and acetylcarnitine in the blood. This observation is consistent with the findings of Henninger *et al.*,[163] where carnitine was increased in the liver of rats taking the drug fenofibrate.

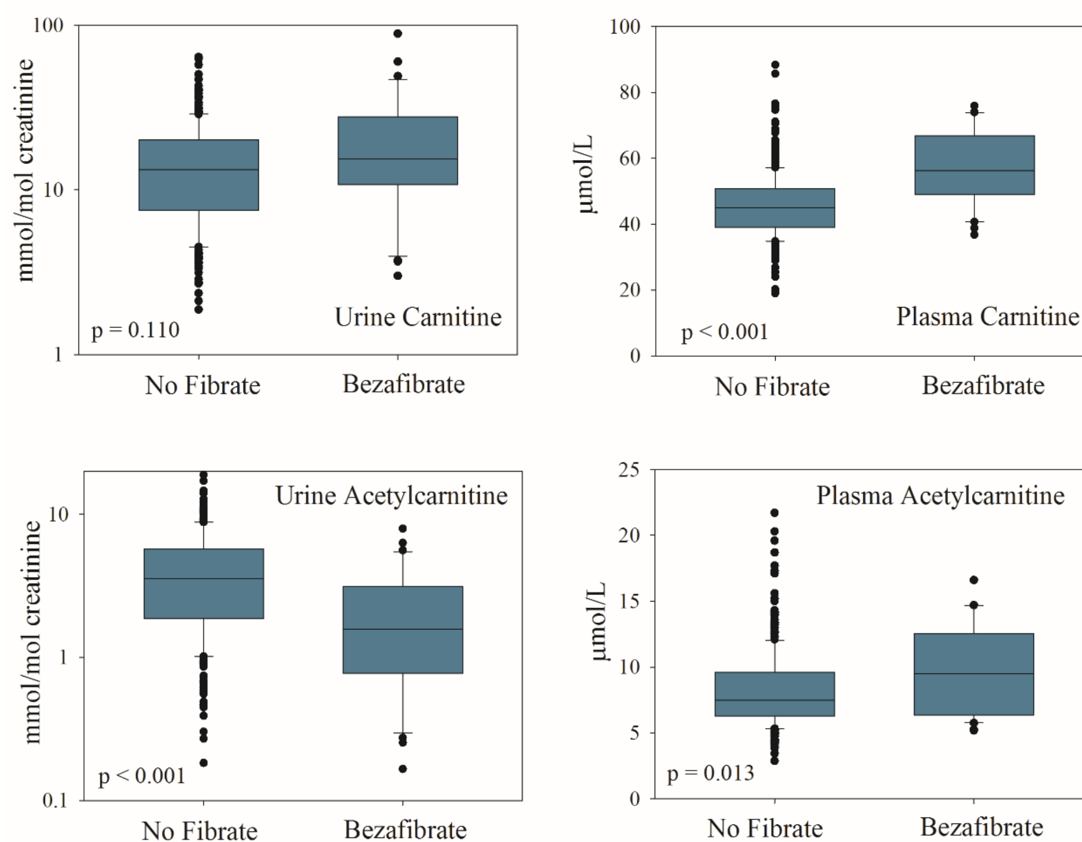


Fig. 4.11. Box plots showing carnitine and acetylcarnitine concentrations in the urine and plasma of people with type 2 diabetes not taking fibrates and taking bezafibrate.

Table 4.9. Urine inositol concentrations in subjects on bezafibrate and controls

	Controls (n = 62)	Interquartile range		Subjects on fibrates (n = 31)	Interquartile range	
	Median (mmol/mol crn)	25%	75%	Median (mmol/mol crn)	25%	75%
myo-Inositol	22.4	7.8	57.3	31.1	18.4	45.5
scyllo-Inositol	10.4	5.7	13.5	8.3	6.8	12.6
chiro-Inositol	3.0	1.8	8.1	2.0	1.6	3.9

Note: The controls were age and gender matched DEWL study subjects (overweight people with type 2 diabetes) who were not on fibrate therapy.

Summary

It was originally hypothesized that subjects who were on a high protein diet or a high carbohydrate diet would have different concentrations of betaine, other osmolytes, and one-carbon metabolites. However, there were no significant differences ($p < 0.05$) in concentrations of these metabolites observed between the two diets the subjects were placed on during the DEWL study. This result may be inconclusive due to the reported low compliance of the study subjects with dietary advice that they were given.[146]

The overweight people with type 2 diabetes who were enrolled in the DEWL study generally had normal metabolite concentrations in their plasma. However, the increased urinary betaine excretion in this group is a concern, especially with over half of the subjects having abnormally high betaine in the urine. The betaine loss was made even worse when they were taking the drug bezafibrate. This high urinary loss of betaine in diabetes could be expected to lead to problems with osmotic control and associated tissue damage. *myo*-Inositol was also elevated in the urine of the DEWL study subjects compared to healthy controls. However, urine taurine was not significantly elevated in the DEWL study subjects.

It was not previously known if osmolytes other than betaine are affected by the drug bezafibrate. However, it has been shown here that the high urinary betaine excretion was not observed for the other osmolytes, and a mechanism other than a general disruption to osmotic control is likely to be responsible. The fact that DMG is also increased in the urine of people on bezafibrate suggests that there is increased BHMT activity occurring, and it is not simply a case of affecting the betaine transporters such as BGT1. The increased plasma homocysteine observed in people on fibrate therapy is likely to be caused by betaine depletion and the decreased ability to convert homocysteine to methionine. There is a need for further research to determine the reason for the observed loss of betaine in people who are taking bezafibrate. The low intra-individual variability of betaine in the urine suggests that the subjects who are losing high levels of betaine continue to do so. It is therefore likely to be beneficial to supplement these people with betaine to help prevent the onset of further complications, as has been suggested by Lever *et al*.[164]

The correlations in the DEWL study samples show that there are a number of other factors that affect the concentrations of betaine, other osmolytes, and other one-

carbon metabolites in people with diabetes, including: lipid metabolism; glycemic control; and renal function. For example, the strong correlations between betaine (and related metabolites DMG, choline) with glucose and HbA_{1c} show that more betaine is excreted by the kidneys in people with poorer glycemic control. The other osmolytes, taurine, and GPC, also correlated with markers of glycemic control, although less strongly. While plasma *myo*-inositol didn't correlate with plasma glucose, urine *myo*-inositol did. Plasma *myo*-Inositol concentrations correlated with markers of renal function, and with homocysteine, showing that it is an important risk marker for a number of different reasons and is likely to be useful as a diagnostic marker of disease.

The accelerated loss of betaine is likely to be detrimental for various reasons, including: a decrease in osmotic control; choline depletion from cells and cell membranes; and elevated homocysteine due to reduced BHMT activity. It would be useful to test the hypothesis that people with diabetes who are losing large amounts of betaine are more likely to go on to develop other complications (such as, heart disease, renal failure, and cataracts). Low plasma betaine is a risk marker for developing diabetes.[73] Unfortunately, ethics approval could not be obtained to relate osmolyte concentrations from this project to health outcomes.

While it is likely that supplementing patients who have diabetes with osmolytes such as betaine, taurine, and *myo*-inositol may be beneficial, it is not known if it improves health parameters such as: glycemic control, weight, and renal function.

4.3. The effect of fenofibrate treatment on betaine, other osmolytes, and other methylamines in the urine of healthy individuals

Introduction

The lipid lowering drug, bezafibrate, has been associated with elevated betaine concentrations in the urine.[15] However, it is not known if fibrates are causative for betaine loss, and it is not known if fibrate drugs other than bezafibrate have the same effect on urinary betaine excretion. There has been no previous study where fibrates have been given to healthy people and the concentrations of betaine and other osmolytes measured before and after treatment. Increased plasma homocysteine has been previously reported in patients taking fenofibrate or bezafibrate.[165] Elevated plasma homocysteine and high urinary betaine excretion has been observed in the DEWL study subjects taking bezafibrate,[161] and has also been reported by Lever *et al*.[15] The fibrate medication, fenofibrate, is investigated here to determine if betaine excretion is elevated after administering it to normal healthy individuals. The hypothesis was that betaine concentrations significantly increase in the urine of healthy subjects after treatment with fenofibrate compared to baseline urine betaine concentrations. This study has been published in the *Journal of Clinical Lipidology*.[166]

Methods

Study design

A clinical study was conducted by Solvay Pharmaceuticals (Abbott) at the Guy's Hospital in London. Ethics approval for this study was obtained by Solvay Pharmaceuticals.

Twenty six healthy individuals (15 males and 11 females) were given a dose of 145 mg of the lipid lowering drug, Lipanthyl (fenofibrate), for six weeks. Individuals were aged between 40 and 65 years, and had normal folate and vitamin B_{12} levels. Subjects fasted for at least 10 hours before sample collection. Urine was collected before fenofibrate was given (baseline), then at the end of the study (6 weeks), giving a total of 52 samples. The urine samples were shipped to Christchurch, New Zealand, as part of a collaboration with Solvay Pharmaceuticals to measure betaine concentrations at Canterbury Health Laboratories. Betaine, choline, DMG, TMAO,

taurine, *myo*-inositol, carnitine, and acetylcarnitine were measured in the urine as part of the present project. Plasma homocysteine, methionine cysteine, *S*-adenosyl-methionine (SAM), and *S*-adenosyl-homocysteine (SAH) data were provided by Solvay Pharmaceuticals. Unfortunately, the plasma samples from the Solvay study were not available for the analysis of betaine and related metabolites.

Sample preparation

Fifty microlitres of sample was extracted into 1.0 mL of extraction solvent containing 90% acetonitrile, 10% methanol, and 10 μmol/L D_9-betaine, D_9-choline, D_3-dimethylglycine, D_9-carnitine, and D_9-trimethylamine-*N*-oxide. For negative ion mode, the extraction solvent contained 10 μmol/L D_4-taurine, and D_6-*myo*-inositol. Samples were vortexed for 20 seconds and centrifuged at 13,000 × g for 3 minutes, then transferred to HPLC vials and capped for analysis.

LC-MS

Urine samples were analyzed for methylamines and taurine using an AB Sciex API4000 after separation on a Cogent diamond hydride silica column (100 × 2.1 mm, 4 μm, Microsolv Technologies) as described in Chapter 2. An Agilent 1260 Infinity HPLC system connected to an Agilent 6120 single quadrupole mass spectrometer with an APCI source was used for inositol analysis as described in Section 3.5.

Statistical analysis

Wilcoxon signed rank tests were carried out on the data to compare subjects before and after treatment for most analytes. However, paired t-tests were performed on DMG, taurine, and *S*-adenosyl-homocysteine (SAH), because they fitted the criteria for normal distribution.

Results

Wilcoxon signed rank tests (or paired t-tests) showed that betaine and DMG were significantly elevated ($p < 0.001$) in the urine after treatment with fenofibrate for 6 weeks compared to baseline (Fig. 4.12). Urine betaine increased with varying degrees in all subjects after treatment. Ten subjects (38.5%) were excreting betaine amounts higher than the normal range (>32.5 mmol/mol creatinine) when on

fenofibrate. Whereas, all subjects had normal betaine concentrations at baseline (range = 1.7 to 17.4 mmol/mol creatinine). A Wilcoxon signed rank test showed a significant increase ($p = 0.011$) in urine choline with fenofibrate treatment. TMAO was not significantly different before and after treatment ($p = 0.648$). Carnitine was not significantly different in the urine with fenofibrate treatment ($p = 0.525$), but acetylcarnitine was significantly lowered in the urine ($p < 0.001$) (Fig. 4.12). Taurine ($p = 0.065$) and *myo*-inositol ($p = 0.213$) concentrations were not significantly different in the urine after treatment with fenofibrate.

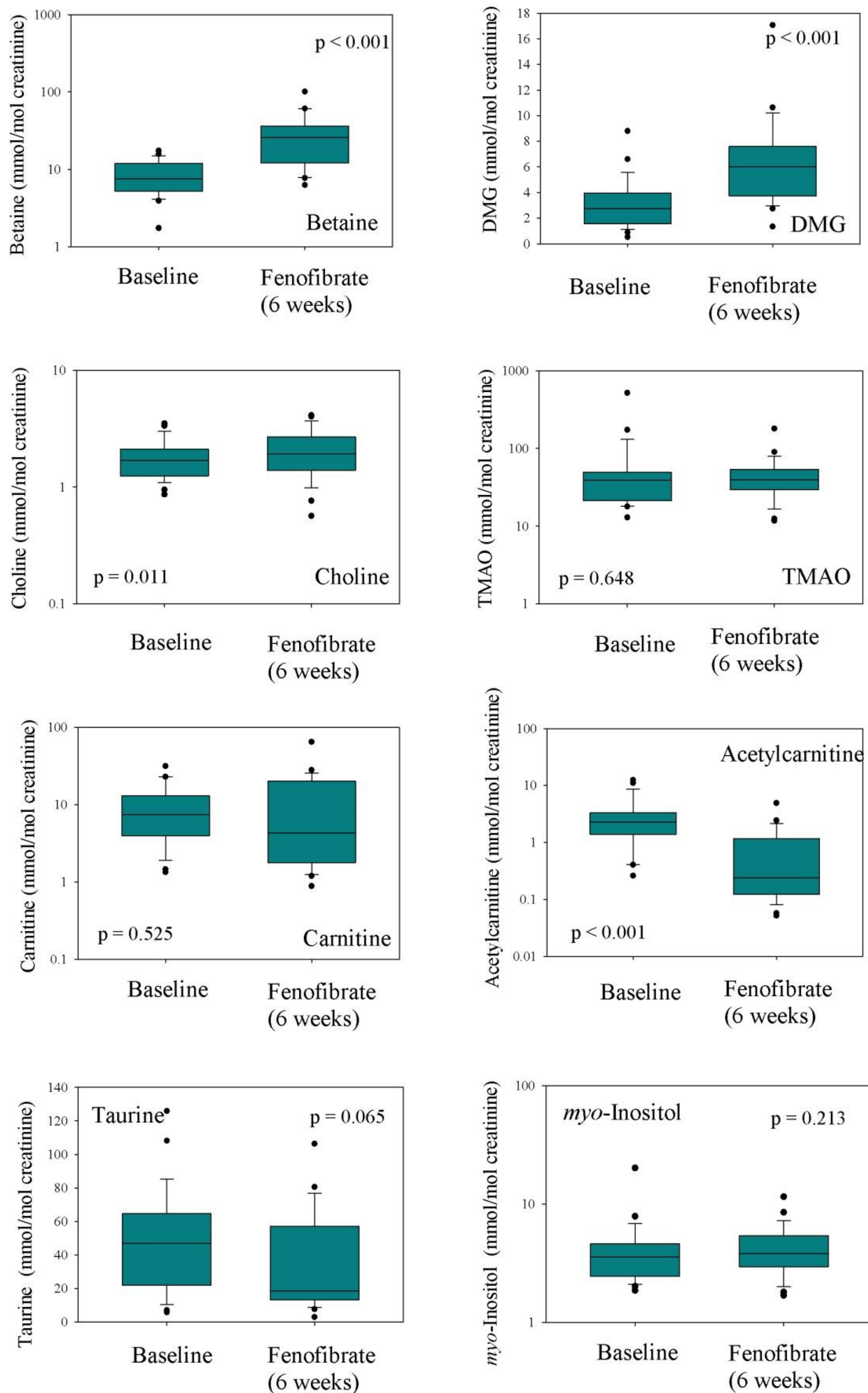


Fig. 4.12. Boxplots showing urine betaine, DMG, choline, TMAO, carnitine, acetylcarnitine, taurine, and *myo*-inositol concentrations in healthy subjects before and after treatment with fenofibrate. p-Values shown are from Wilcoxon signed rank tests, or paired t-tests (for DMG and taurine).

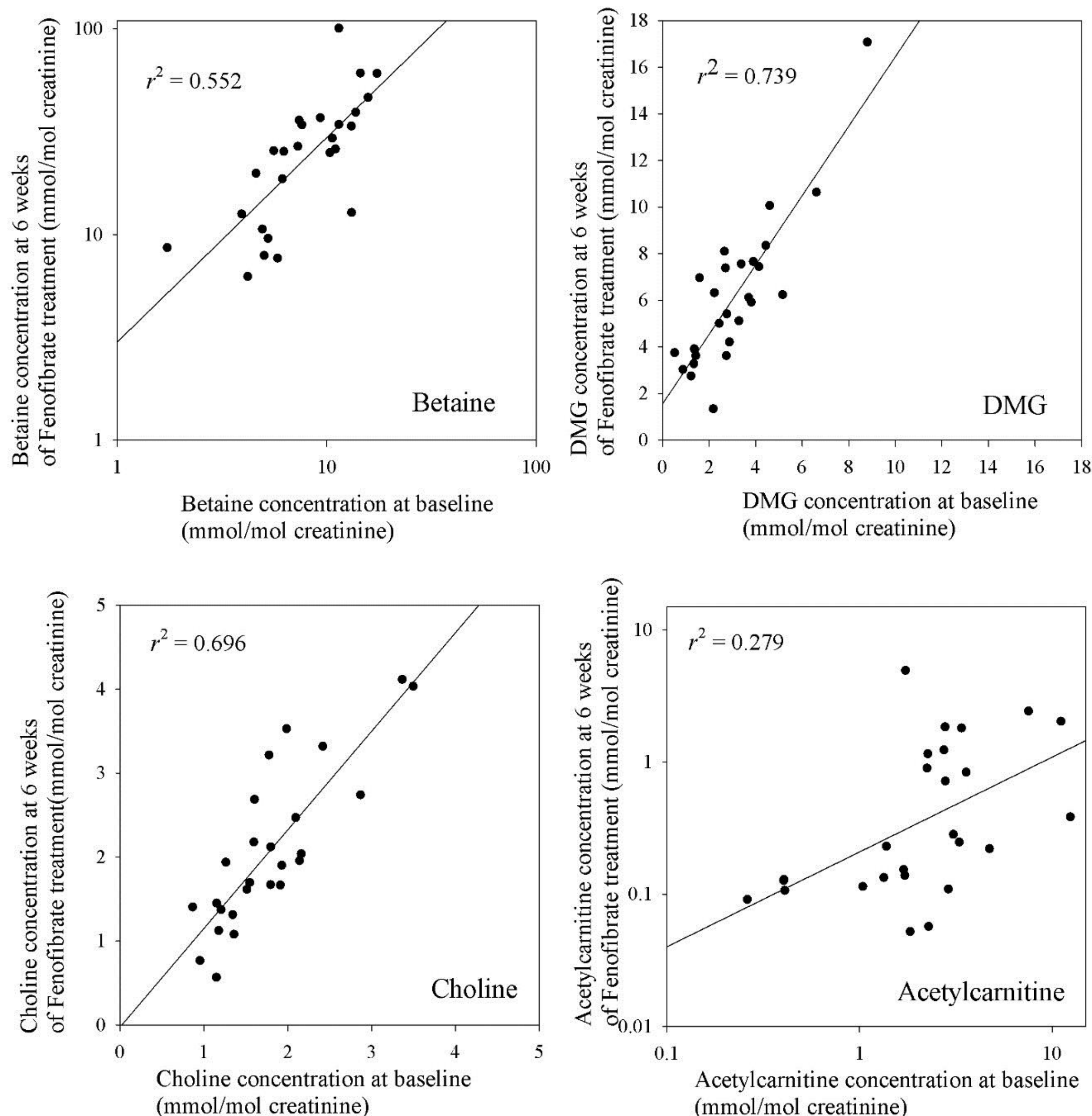


Fig. 4.13. Plots showing baseline versus 6 weeks of treatment with fenofibrate for urine betaine, dimethylglycine (DMG), choline, and acetylcarnitine. Regressions were performed on log-transformed data for betaine and acetylcarnitine to meet the requirement for the data to be normally distributed.

There were strong correlations between the individuals at baseline and at 6 weeks of fenofibrate treatment for betaine, DMG, and choline (Fig. 4.13). Betaine showed a logarithmic relationship between the time points. Acetylcarnitine showed a weaker correlation (r^2 = 0.279) than the one-carbon metabolites.

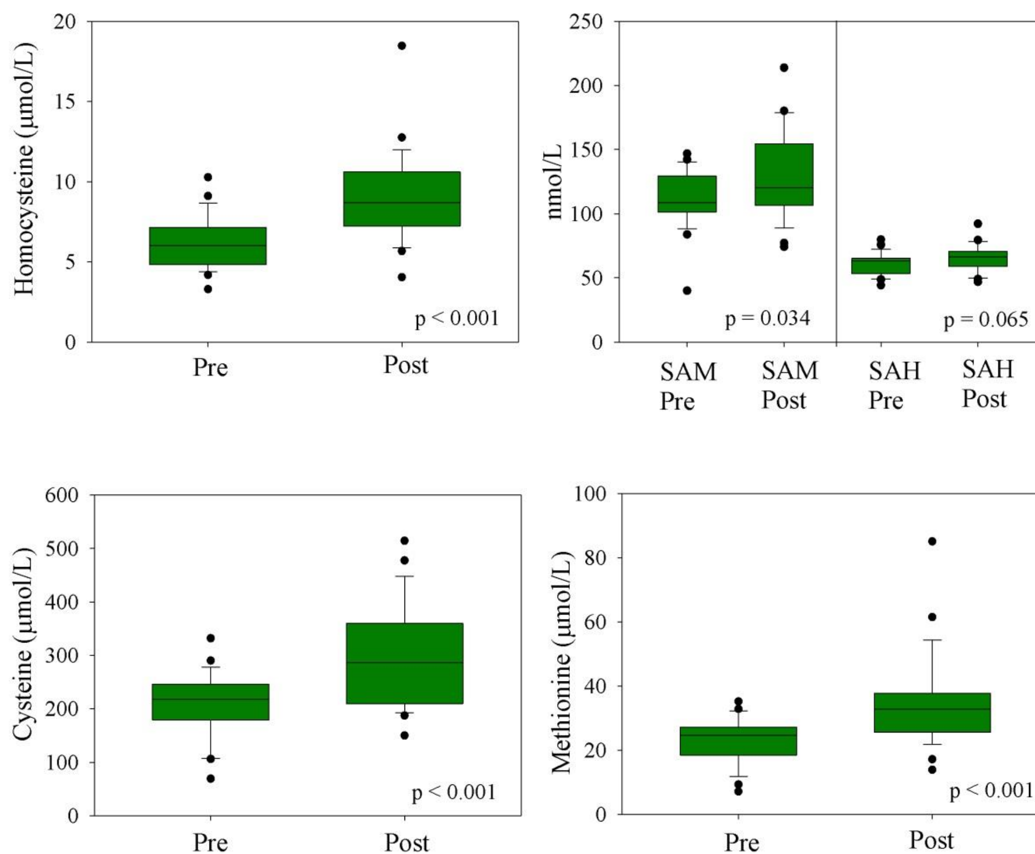


Fig. 4.14. Boxplots showing baseline (pre) and 6 weeks on fenofibrate treatment (post) for homocysteine and metabolites in plasma. p-Values show the results of Wilcoxon signed rank tests, or paired t-test (for SAH). Abbreviations: *S*-adenosyl-methionine (SAM); *S*-adenosyl-homocysteine (SAH). Data provided by Solvay Pharmaceuticals.

Homocysteine and its metabolites *S*-adenosyl-methionine (SAM), cysteine, and methionine went up significantly ($p < 0.05$) on fenofibrate treatment (Fig. 4.14). Spearman's rank correlations showed that urine betaine did not correlate with homocysteine at baseline ($p = 0.738$) or 6 weeks ($p = 0.428$).

Discussion

This study has shown strong evidence that the drug fenofibrate causes an increase in the urinary excretion of betaine and DMG in healthy individuals. This is the first time a fibrate drug other than bezafibrate, namely fenofibrate, has been associated with increased urinary betaine excretion. The only previously published

betaine data for people on fibrates were from cardiovascular patients[15] and people with type 2 diabetes who were placed on fibrate treatment by their doctors due to high blood lipids (see Section 4.2).[161] It was previously unproven whether fibrates caused elevated betaine in the urine, or whether this effect was simply a consequence of the people who are prescribed fibrates having a high blood lipid profile. The strong correlations before and after treatment with fenofibrate for betaine, DMG, and choline confirm the high individuality for these metabolites in urine. There was an exponential relationship between the time points for betaine, showing that the higher the initial concentration of betaine, the larger the percentage increase observed after treatment with fenofibrate. This is particularly concerning when patients with diabetes, who are already likely to have high betaine excretion, are placed on fibrates.

While betaine increased in the urine of healthy subjects on fenofibrate, the other osmolytes, taurine and *myo*-inositol did not significantly change. This suggests that the mechanism for urinary betaine loss with fibrate therapy is unlikely to be related to the role of betaine as an osmolyte, and is not the same mechanism that causes elevated urinary excretion of betaine and other osmolytes in diabetes. Whether or not fibrates affect the function of betaine transporters requires further investigation.

While fibrate therapy may provide significant benefits to patients by lowering their blood lipids, they may also be doing damage by removing betaine from the body and placing it under greater osmotic stress. Low plasma betaine has been shown to be a strong predictor of developing diabetes.[61b] However, the causality of betaine concentrations in developing the disease needs to be established to determine whether it is a risk factor or risk marker. The replacement of betaine by supplementation in patients who are on fibrate patients may help to counteract these effects.

Plasma homocysteine was seen to increase significantly in healthy people given fenofibrate over a six week period. However, there was no correlation observed between plasma homocysteine and urine betaine, and the urinary betaine loss cannot be proven to be the cause of the increased plasma homocysteine with fibrate therapy. It is unclear why the SAM, cysteine, and methionine were increased in the plasma with fenofibrate therapy. Methionine is produced from homocysteine *via* betaine homocysteine methyltransferase (BHMT) activity,[167] and there is likely to be more methionine produced due to the higher levels of homocysteine present, and the body's attempt to metabolize it.

The decrease in urine acetylcarnitine after fenofibrate treatment is consistent with what was observed in the DEWL study data, where people on bezafibrate had lower urine acetylcarnitine. This fenofibrate data shows that fibrates are causal in lowering the acetylcarnitine, and that fibrates affect carnitine metabolism. It is likely that the plasma carnitine was elevated after subjects were placed on fenofibrate, which would also be consistent with the cross-sectional baseline DEWL study data. However, it was not possible to investigate metabolites in the fenofibrate study plasma as these samples were not available for analysis. The effects of fibrates on carnitine metabolism are likely to be related to the role of fibrates as lipid lowering drugs, considering that carnitine transports fatty acids across the mitochondrial membrane. The fact that betaine excretion changed significantly after administration of fenofibrate to healthy people provides strong evidence that fibrates cause the body to shed betaine. The mechanism remains unknown, and does not appear to be related to the function of betaine as an osmolyte.

4.4. Chapter summary

The studies described in this chapter show that important information about the metabolism of osmolytes can be obtained using the LC-MS/MS methods that have been developed in this project. The greater number of osmolytes that can be measured, and the improved sample throughput, allows for much more information to be gained when analyzing study samples.

These studies have shown that the concentrations of many osmolytes are different in diabetes compared to other populations, and that many osmolytes correlate with known risk factors of disease such as LDL cholesterol, creatinine, and homocysteine. However, these studies are mostly cross sectional, and much more research is required to show the true value of osmolytes as risk markers in disease.

5. Chapter Five - The Distribution of Betaine, Other Osmolytes, and Related Metabolites in Different Blood Components

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Introduction

The liquid chromatography – mass spectrometry methods that have been developed were used to investigate the distribution of betaine and related metabolites in the different components of blood. The concentrations of osmolytes in blood cells may better represent the concentrations in tissues than plasma or whole blood. A study on rats has shown that betaine concentrations in organs such as the liver and kidneys are in the millimolar range, whereas plasma betaine is in the micromolar range.[168] Betaine concentrations in tissues are not necessarily reflected in the plasma concentrations.[168] Betaine concentrations in whole blood have been reported to be nearly six times greater than in the plasma.[169] Blood cells actively uptake betaine for osmo-regulation and for methylation. For example, blood platelets have been reported to express the betaine and γ-aminobutyric acid transporter BGT-1.[170] Macrophages have also been shown to use the BGT-1 transporter in the mouse.[171] Red cells, platelets, white cells, and plasma will be analyzed for betaine and related compounds to investigate the distribution. There is little published data on the distribution of betaine and other osmolytes in the different cell types found in blood. Measuring betaine and related metabolites and osmolytes in blood components such as: platelets, red cells, or white cells may give a better representation of the osmolyte status in the tissues than plasma betaine which has been traditionally measured. While there are several other types of white blood cells (including neutrophils, eosinophils, basophils, lymphocytes, and monocytes), they are not differentiated here. It would be difficult to separate and isolate them without using a buffer that would most likely alter the osmotic status of the cells and change the osmolyte concentrations.

Methods

Sample preparation

Thirteen baseline EDTA blood samples from men with the metabolic syndrome were collected during the betaine and body composition (BBC) study. Blood samples were centrifuged at 3000 rpm (1800 × *g*) for 10 minutes. Plasma was collected from the top layer, and red cells were collected from the bottom layer after the white cells were removed. White cells were enriched by collecting the buffy coat from four tubes with a pipette, re-centrifuging, and collecting the buffy coat again. After separation, the samples were stored at -20°C until analysis.

Sample analysis

Fifty µL of each of the sample fractions (including: whole blood, plasma, red blood cells, and white blood cells) were extracted into 1.0 mL of extraction solvent containing 10 µmol/L of each of the deuterated internal standards, and placed on a vortex mixer for 10 minutes before centrifugation (13,000 *g*, 5 minutes). Samples were analyzed by single quadrupole mass spectrometry for the osmolytes: betaine; taurine; and *myo*-inositol, as well as: choline; DMG; carnitine; acetylcarnitine; and TMAO as described in Chapters 2 and 3. *myo*-Inositol was measured directly with an APCI source.

The concentrations of osmolytes and other methylamines were compared in the different blood components by Kruskal-Wallis analysis of variance on ranks using SigmaPlot (v13).

Results and discussion

There were significant differences in osmolyte concentrations in the various blood components (Fig. 5.1). Median values and interquartile ranges of osmolytes and related methylamines are shown in Table 5.1. Kruskal-Wallis analysis of variance on ranks indicated that betaine was significantly higher in the white blood cell fractions compared to the plasma ($p < 0.01$). However red cell betaine was not significantly elevated compared to plasma betaine ($p = 0.29$). Taurine concentrations were considerably higher in the white cell fractions than in the plasma and whole blood ($p < 0.001$), but were not significantly elevated in the red cells. *myo*-Inositol was significantly elevated in the white blood cells compared to all other blood fractions ($p < 0.001$), and was also not significantly different in the red cells compared to the plasma.

The observation that taurine and *myo*-inositol concentrations were higher in the white blood cell enriched fractions compared to the other blood components, indicates that these osmolytes are accumulated by white cells for osmo-regulation, even more so than betaine.

The red cells contained significantly lower carnitine than all of the other blood components ($p < 0.001$), which probably reflects the fact that these cells do not have mitochondria.

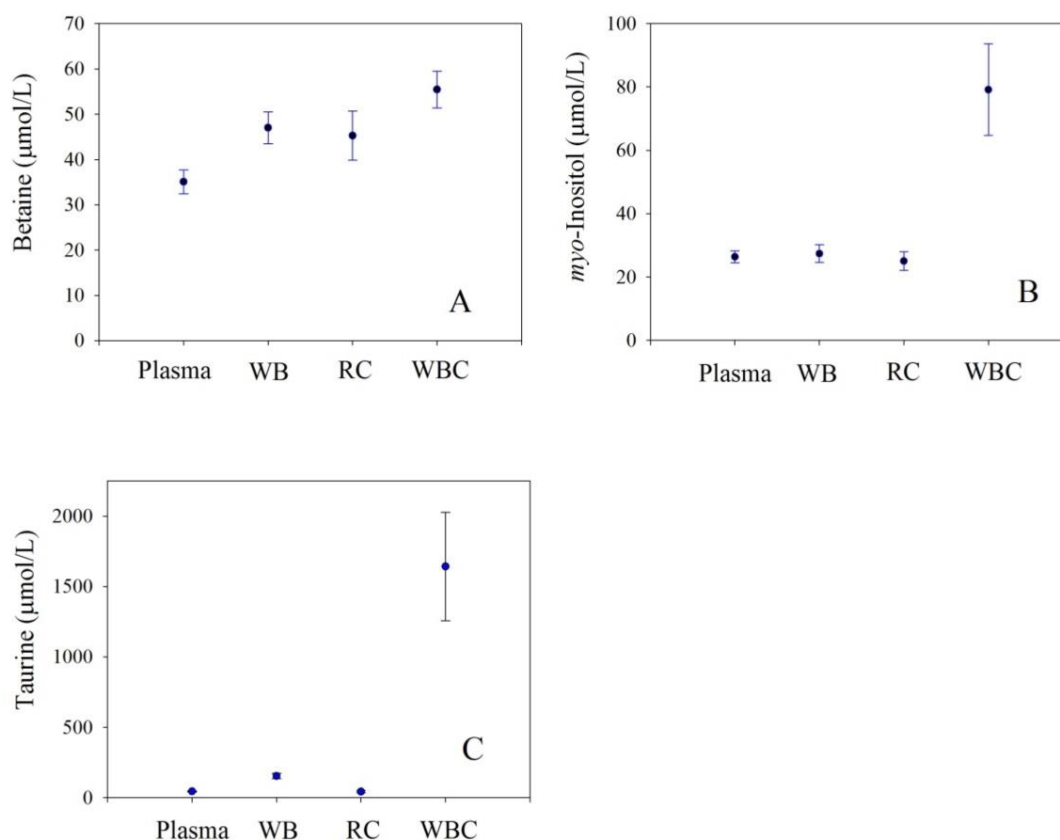


Fig. 5.1. Data (mean ± standard error) showing the distribution of the osmolytes: betaine (A); *myo*-inositol (B), and taurine (C) in blood. Abbreviations: whole blood (WB); red cells (RC); white blood cells (WBC).

DMG, TMAO, and carnitine concentrations were not significantly higher in the cells than in the plasma. The betaine results in whole blood were considerably lower (median = 48.7 µmol/L) in this study compared to those reported by Awward *et al*. (median = 165 µmol/L).[169] Choline was higher in the cell fractions, which may be related to the presence of phosphatidylcholine in cell membranes. Awward *et al*. reported that the median choline concentration in whole blood was 66.6 (10 – 90 percentile 43.2–154.8) µmol/L which was higher than what was observed in the present study (median = 29.7 µmol/L). Awward *et al*.[169] also reported higher DMG concentrations in whole blood compared to plasma, but this was not observed here either. The study by Awward *et al.* was conducted on elderly people without metabolic syndrome.[169] However, this is unlikely to explain the large differences in metabolite concentrations observed here. Awward *et al*. may have over-estimated the metabolite concentrations in whole blood due to an analytical error.

Table 5.1. The distribution of osmolytes and related methylamines in blood.

	Plasma	Whole blood	White blood cells	Red cells
Betaine (µmol/L)	37.2 (28.8 - 41.6)	48.7 (39.0 - 52.4)	59.8 (41.4 - 67.6)	43.6 (26.7 - 66.4)
Taurine (µmol/L)	45.0 (41.1 - 50.6)	143.1 (117.7 - 190.9)	1868 (763 - 2324)	36.9 (30.3 - 49.8)
myo-Inositol (µmol/L)	25.5 (23.3 - 30.1)	27.6 (22.6 - 33.2)	70.5 (52.3 - 99.2)	25.2 (21.2 - 29.9)
Choline (µmol/L)	10.7 (8.8 - 13.3)	29.7 (23.9 - 62.0)	65.2 (30.9 - 123.2)	67.5 (20.0 - 111)
DMG (µmol/L)	2.74 (2.25 - 3.09)	2.18 (1.88 - 2.24)	1.87 (1.33 - 2.27)	0.87 (0.72 - 1.52)
Carnitine (µmol/L)	42.7 (40.6 - 45.4)	33.9 (28.9 - 40.7)	36.9 (29.1 - 46.7)	13.9 (9.68 - 22.0)
Acetylcarnitine (µmol/L)	6.79 (6.26 - 8.75)	9.74 (5.72 - 15.3)	10.4 (2.57 - 37.6)	9.10 (3.15 - 13.4)
TMAO (µmol/L)	5.19 (2.61 - 10.6)	4.91 (2.0 - 8.0)	2.94 (0.48 - 4.57)	2.26 (1.20 - 3.81)

Note: Median values and interquartile ranges are shown.

The accumulation of betaine, taurine, and *myo*-inositol by the white cells suggests that they may be useful as a representation of tissue osmolyte concentrations. A limitation of this approach is that the white cells collected in this study were not pure, and were merely enriched with white cells compared to the whole blood. Therefore the error associated with these measurements is quite high. The types of white cells that accumulate osmolytes are also yet to be identified. Methods used for obtaining pure white cells involve the use of buffers which were be expected to change the osmotic status of the cells, and therefore the osmolyte concentrations would not be accurate either. There were no significant differences in metabolite concentrations in the platelet rich plasma compared to the platelet poor plasma (data not shown). To obtain a useful estimate of metabolite concentrations in platelets would require them to be purified and isolated, and such methods involve the use of buffers that would change the osmotic environment of the cells, and affect the osmolyte concentrations.

6. Chapter Six – *N,N*-Dimethylglycine-*N*-oxide in Human Samples

6.1. The synthesis of *N*,*N*-dimethylglycine-*N*-oxide and identification in plasma and urine using LC-MS/MS

Introduction

The advancement of mass spectrometry in recent years has led to improved sensitivity, which allows for the detection of metabolites that are present in very low concentrations, and may not have been previously discovered. The aim was to investigate if *N*,*N*-dimethylglycine (DMG) may be oxidized in the body to form the expected metabolite, *N*,*N*-dimethylglycine-*N*-oxide (DMGO) (Fig. 6.1). Both the carboxyl group and the *N*-oxygen require protonation to ionize the molecule for measurement by LC-MS/MS in positive ion mode.

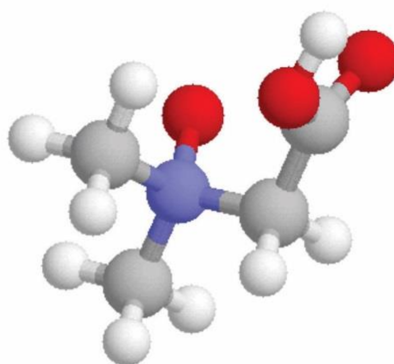


Fig. 6.1. Structure of DMGO.

To investigate if DMGO may be present in human samples, plasma and urine samples were initially analyzed by single quadrupole LC-MS using the methylamine chromatography system described in Section 2.2. The m/z = 120 signal was monitored in positive ion mode and several peaks were observed with this mass to charge ratio that could potentially be DMGO. In order to determine if any of these peaks were from the presence of DMGO, an analytical standard was synthesized and validated, and two different chromatography systems were developed to separate and measure it using tandem mass spectrometry. Mass transitions that correspond to the structure of the molecule were used to measure DMGO.

Methods

Reagents and chemicals

N,*N*-Dimethylglycine free base, peracetic acid (32% wt in dilute acetic acid), formic acid, ammonium formate, and Dowex 50WX8 (200 – 400 mesh) strong cation exchange resin were purchased from Sigma. Acetonitrile and ammonia (25% v/v) were purchased from Merck. D_3-*N*,*N*-Dimethylglycine (HCl) was purchased from CDN Isotopes.

Synthesis of DMGO

N,*N*-Dimethylglycine (4.00 g, Sigma, free base) was dissolved in 25 mL of peracetic acid (32% wt in dilute acetic acid, Sigma). The reaction mixture was refluxed in a round bottom flask over a heating mantle for 2.5 hours. The excess peracetic acid was removed on a rotary evaporator at 40°C, and the reaction mixture was acidified with sulfuric acid and then added to a column containing acidified Dowex 50 strong cation exchange resin. The impurities were removed by washing approximately three column volumes of water. When the eluent no longer tested positive for peroxides using the potassium iodide starch test, the DMGO product was eluted off the column with aqueous ammonia solution (8% v/v). The ammonia and water were removed on a rotary evaporator at 40°C and DMGO crystallized as a white powder.

The purity of the DMGO was tested by LCMS, NMR spectroscopy, and elemental analysis. Elemental analysis was performed by the Campbell Microanalytical Laboratory at the Chemistry Department, University of Otago, New Zealand.

For NMR analysis, approximately 10-20 mg of DMG or DMGO was added to 1 mL of D_2O, and approximately 10 mg of 3-(trimethylsilyl)propionic-2,2,3,3-D_4 acid sodium salt (TSP, Sigma) was added as a reference standard. The TSP ^1H signal was set to 0 ppm. A proton NMR spectrum was obtained in 5 mm NMR tubes using a Varian INOVA 500 MHz NMR spectrometer with 8 scans.

Deuterated *N*,*N*-dimethylglycine-*N*-oxide (D_3-DMGO) was made for use as an internal standard by reacting 0.0142 g of D_3-*N*,*N*-dimethylglycine HCl (CDN Isotopes, Quebec, Canada) with 178 µL of peracetic acid (32 wt%, Sigma) at 60°C for 2.5 hours in a sealed reaction tube. The reaction mixture was added to acidified

Dowex 50 cation exchange resin and the peroxide washed out with distilled water before elution with ammonia (8% v/v). The excess water and ammonia were dried down to a small volume in a rotary evaporator, and the contents made up to 10 mL in a volumetric flask to form a solution containing approximately 10 mmol/L D_3-DMGO.

An initial attempt was made to synthesize DMGO by reaction with hydrogen peroxide in the presence of excess acetic acid as described by Ikutani and Matsumura.[172] However, oxidation of DMG by this method resulted in low reaction yields and around half the DMG starting material left in the reaction mixture. The excess hydrogen peroxide was difficult to remove from the product. It was presumed that the most effective oxidizing agent in the reaction mixture was peracetic acid. Commercially available peracetic acid was found to oxidize much more efficiently than hydrogen peroxide, with complete reaction at 2.5 hours. DMGO was found to be difficult to crystallize in organic solvents such as 2-propanol or acetone. Drying down the reaction mixture produced an impure sticky white solid which gave an extra singlet peak at 2.712 ppm on the NMR. However, by placing the reaction mixture onto acidified strong cation exchange resin, the impurities such as peroxides could be washed off with water, then the purified DMGO was eluted with ammonia and the eluent dried down.

Sample preparation

When measuring DMGO in urine an extraction solvent was used containing 10 µmol/L D_3-*N*,*N*-dimethylglycine-*N*-oxide (internal standard) in 20% methanol and 80% acetonitrile. When measuring plasma DMGO, the internal standard was diluted to 100 nmol/L in the extraction solvent. Fifty µL of plasma, urine, or aqueous standards were pipetted into 1 mL of extraction solvent. Samples were vortexed and centrifuged at 13000 × *g* for 5 minutes, then transferred to HPLC tubes and capped for analysis.

LC-MS/MS

In order to show that DMGO is present in plasma and urine, two different chromatography systems were used. The first system used a Cogent diamond hydride silica column (100 × 2.1 mm, 4 µm, Microsolv Technologies, NJ, USA), and the

second system used an Epic strong cation exchange column (SCX, 125 × 3 mm, 3 µm, ES Industries, NJ, USA).

The chromatography system for the Cogent diamond hydride silica column was as follows: Mobile phase solvent A contained 10 mmol/L formic acid, and 10 mmol/L ammonium formate in 50% distilled water and 50% acetonitrile (v/v). Solvent B contained 7.5 mmol/L trifluoroacetic acid (TFA) and 15 mmol/L formic acid in acetonitrile. A gradient was used starting with 5% A and 95% B, then to 25% A and 75% B at 3 minutes, and 100% A at 7.5 to 8.5 minutes, and then back to starting conditions at 9 minutes. The run time was 12 minutes, the flow rate was 0.3 mL/min, the injection volume was 10 µL, and the column temperature was 40°C.

The chromatography system used for the Epic SCX column was as follows: Solvent A contained 10 mmol/L formic acid and 10 mmol/L ammonium formate in 50% distilled water and 50% acetonitrile. Solvent B contained 7.5 mmol/L TFA and 15 mmol/L acetic acid in acetonitrile. The gradient started at 3% A and 97% B, going to 30% A and 70% B at 4 minutes, then to 100% A from 7.5 to 8.5 minutes, and back to 3% A and 97% B at 9 minutes. The run time was 12 minutes. The injection volume was 10 µL, the flow rate was 0.3 mL/min, and the oven temperature was 40°C.

DMGO was initially detected on an AB Sciex API4000 (Applied Biosystems) triple quadrupole mass spectrometer with an electrospray ion source (ESI) in positive ion mode using the mass transitions m/z = 120.1 → 58.1 and 120.1 → 103.1. A third mass transition for DMGO, 120.1 → 102.1, was also monitored in urine, but was below the detection limits in plasma. The decoupling potential was 41 V, the collision energy was 27 V for the m/z = 120.1 → 58.1 mass transition (and 17 V for 103 and 102 fragments), and the collision cell exit potential was 6 V. Samples were quantified using external aqueous standards of DMGO with D_3-*N*,*N*-dimethylglycine-*N*-oxide (D_3-DMGO) used as an internal standard. The mass transition for D_3-DMGO was 123 → 61, the decoupling potential was 46 V, the collision energy was 29 V, and the collision cell exit potential was 4 V.

As sensitivity was found to be a problem with detecting DMGO in plasma, a more sensitive tandem mass spectrometer, an Agilent 6490 (Mulgrave, VIC, Australia) with an ESI source was also used. A fourth mass transition for DMGO (120 → 42) was identified during compound optimization on the Agilent 6490 instrument. The collision energy was 32 V for the 120 → 58 mass transition, 56 V for the 120 → 42 mass transition, and 10 V for the 120 → 103 and 120 → 102 mass transitions. The

collision energy used for the internal standard mass transition (123 → 61) was 32 V. The gas temperature was 220°C, the gas flow rate was 12 L/min, the sheath gas temperature was 350°C, the sheath gas flow rate was 11 L/min, the capillary voltage was 3000 V, and the cell accelerator voltage was 5 V.

Method performance

To test the linearity of the urine method, 25, 50, and 100 µmol/L of DMGO was added to urine, and 3 replicates of each level of were measured by tandem mass spectrometry. To test the precision and accuracy of the method, six replicates of a urine sample, and the same urine sample with 25 µmol/L DMGO added, were analyzed for DMGO using tandem mass spectrometry.

To estimate the precision and accuracy of the plasma DMGO method, 6 replicates of a plasma sample and the same plasma sample were analyzed for DMGO using tandem mass spectrometry. To investigate the linearity of the method in plasma, three replicates of plasma containing different spike levels (0, 0.1, 0.25, 0.5, 1, 5, and 10 µmol/L) were analyzed for DMGO.

DMGO was measured in 62 plasma and urine samples from males with metabolic syndrome, and 16 plasma and urine samples from healthy males, using the two chromatography systems and various mass transitions.

Results and discussion

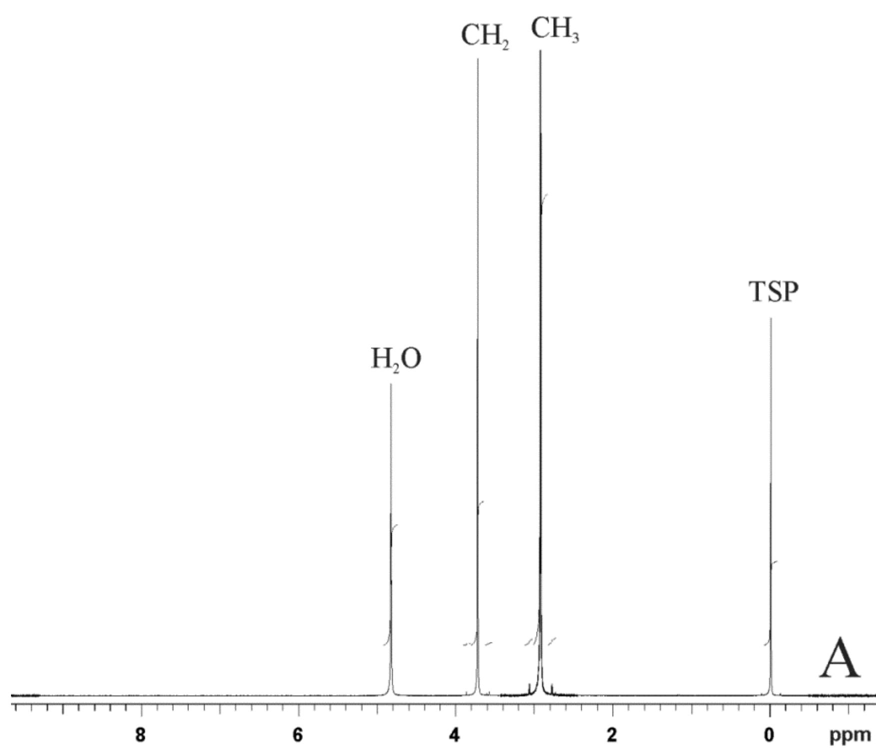
Synthesis of DMGO

The yield of DMGO was 4.13 g (89%). The elemental analysis results for DMGO were: %C 40.31, %H 7.83, %N 11.79. This is consistent with the DMGO crystallizing as the free base with a molecular weight of 119.12 g/mol. The expected composition for this was: %C 40.33, %H 7.62, %N 11.76, %O 40.29. The pH of DMGO dissolved in distilled water was 3.98.

There was no detectable unreacted DMG present in the product by LC-MS, or NMR spectroscopy. The ^{1}H NMR spectrum of DMGO (Fig. 6.2) showed the product to be pure. The methyl group resonance shifted from 2.931 ppm to 3.485 ppm, and the CH_2 resonance shifted from 3.731 ppm to 4.157 ppm with the addition of the oxygen

to the nitrogen. The peak area ratio of the CH_3 peak to the CH_2 peak was 3:1 as expected.

The yield of D_3-DMGO was estimated to be approximately 93%, considering there was still a small D_3-DMG peak present by LC-MS/MS with approximately 7% the peak height of the product. The incomplete reaction of D_3-DMG with peracetic acid may be because D_3-DMG was purchased as the hydrochloride. It may be preferable to convert the D_3-DMG to the free base before reacting it with peracetic acid.



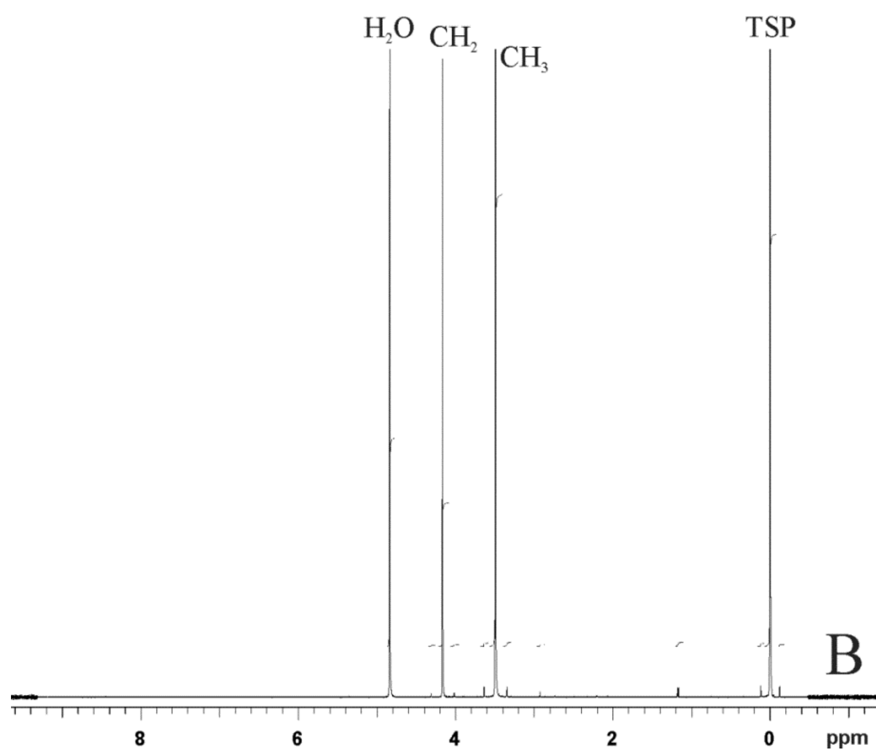


Fig. 6.2. ^{1}H NMR spectrum of DMG (A), and ^{1}H NMR spectrum of DMGO (B).

DMG: ^{1}H NMR (500 MHz, D_2O) δH 2.931 (3 H, s, CH_3), 3.731 (2 H, s, CH_2)
DMGO: ^{1}H NMR (500 MHz, D_2O) δH 3.485 (3 H, s, CH_3), 4.157 (2 H, s, CH_2)

LC-MS/MS of DMGO

The mass transitions 120 → 103 and 120 → 102 required a low collision energy (10 V), because the fragment ions correspond to the removal of a hydroxyl group and a water molecule from the nitrogen of DMGO, which are relatively weak bonds. The *m/z* 120 → 58 corresponds to the formation of a trimethylamine fragment ion, which is also observed when TMAO fragments in the mass spectrometer. The fragment ion that was most likely observed at *m/z* = 42 is $[CH_2NCH_2]^+$ (Fig. 6.3).

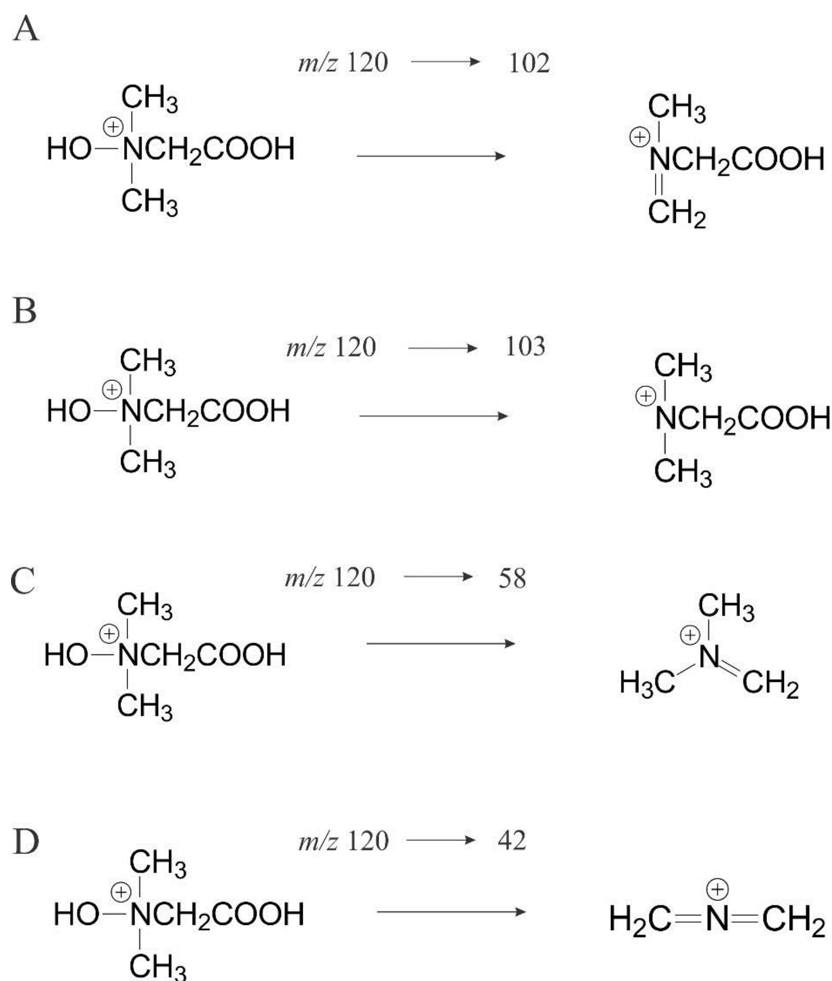


Fig. 6.3. Mass transitions of DMGO in the Agilent 6490 observed in the triple quadrupole mass spectrometer. *m/z* 120 → 102 (A), *m/z* 120 → 103 (B), *m/z* 120 → 58 (C) and *m/z* 120 → 42 (D).

Detection of DMGO in plasma

DMGO eluted before 5 minutes on the Cogent Diamond Hydride silica column (Fig. 6.4). DMGO was retained longer on the strong cation exchange (SCX) column, eluting around 7.5 minutes (Fig. 6.5). The optimal chromatography for DMGO was achieved using the Cogent diamond hydride silica column. The baseline noise was greater when using the SCX column.

DMGO initially gave poor chromatography with a broad tailing peak on the diamond hydride column, and peak splitting on the SCX column, which was likely to be caused by incomplete protonation of DMGO in the mobile phase. However, the addition of TFA to the mobile phase improved the peak shape of DMGO. As both the *N*-oxygen and the carboxyl group need to be protonated in order to make DMGO cationic, a low pH is required in the mobile phase. Sensitivity can be affected by the addition of TFA in the mobile phase, which has been reported to cause ion suppression.[48] With the addition of TFA only to mobile phase B, then eluting with mobile phase A after sample injection, the concentration of TFA is lower when the DMGO peak elutes into the mass spectrometer. Adding a higher concentration of a weaker acid such as acetic acid or formic acid to the mobile phase can also reduce the ion suppression effects of TFA.

DMGO was observed in plasma at all four mass transitions using the more sensitive Agilent 6490 mass spectrometer. However, DMGO was only observed with the *m/z* 120 → 58, and 120 → 103 mass transitions on the AB Sciex API4000. There were peaks other than DMGO present in plasma for all mass transitions. Plasma components with the same mass as DMGO, such as threonine, may be showing up in the chromatograms.

The intensity of the DMGO peak was different using the various mass transitions. Peak heights were in the following order of intensity: (*m/z* 120 → 102) < (*m/z* 120 → 103) < (*m/z* 120 → 42) < (*m/z* 120 → 58). However, there was slightly greater baseline noise in the *m/z* 120 → 58 transition, so overall sensitivity was not greatly different to the *m/z* 120 → 42 and *m/z* 120 → 103 mass transitions. Using the AB Sciex API4000 mass spectrometer, the limit of detection (S/N = 3) for DMGO in plasma and urine was 0.1 µmol/L for the *m/z* 120 → 58 mass transition, and 0.3 µmol/L for the *m/z* 120 → 103 mass transition, and 1 µmol/L for the *m/z* 120 → 102 mass transition on the SCX column. Detection limits were at approximately ten times lower when using the Agilent 6490 tandem mass spectrometer.

Initial attempts to quantify DMGO in plasma and urine using commercially available deuterated *N*,*N*-dimethylglycine (D_3-DMG) produced poor precision and accuracy. To overcome this problem, D_3-DMGO was synthesized on a small scale for use as an internal standard.

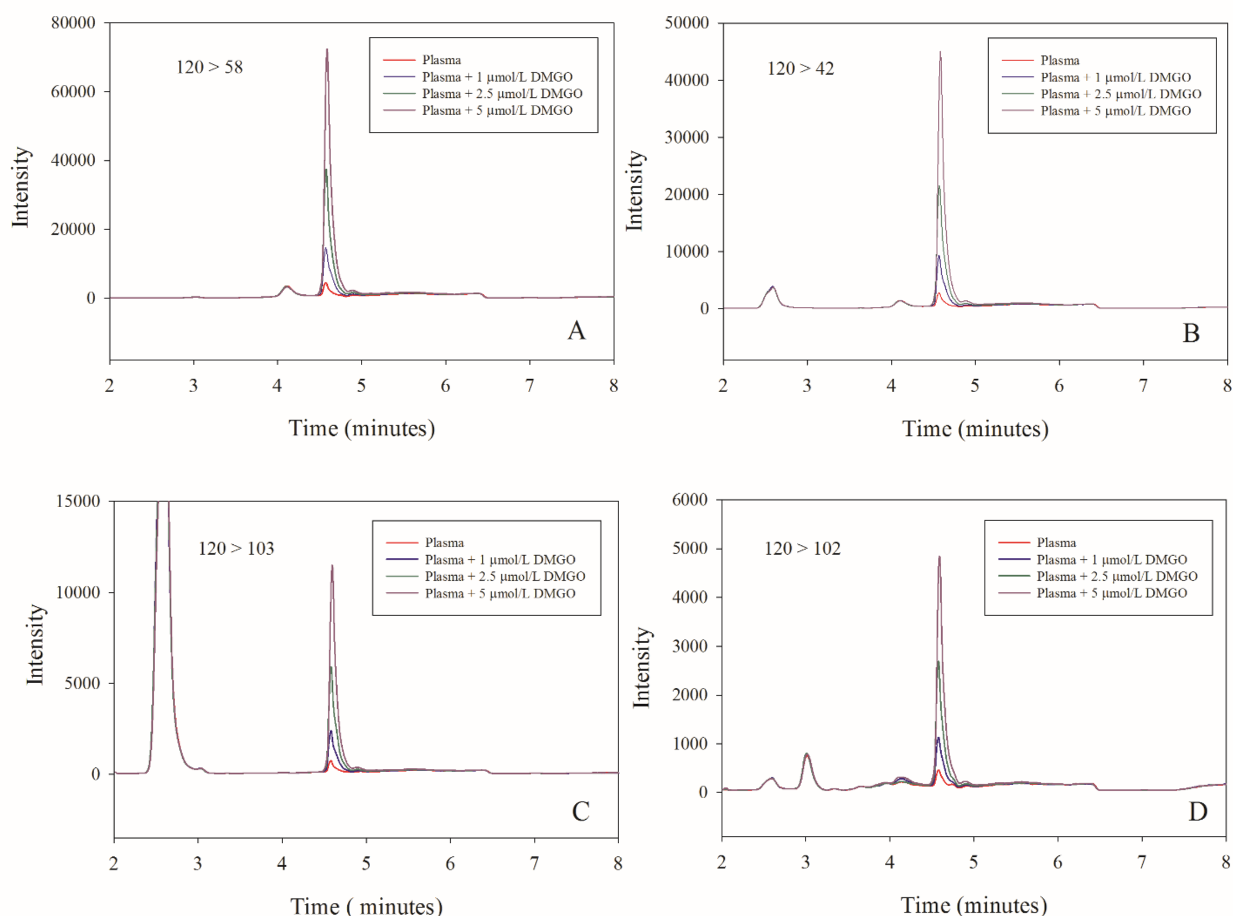


Fig. 6.4. Chromatograms obtained for plasma separated on a Cogent diamond hydride silica column and detected using an Agilent 6490 tandem mass spectrometer. A plasma sample is shown (red) and the same plasma with the added concentrations of DMGO: 1, 2.5, and 5 µmol/L are overlaid. The chromatograms are as follows: *m/z* 120 → 58 (A), *m/z* 120 → 42 (B), *m/z* 120 → 103 (C), and *m/z* 120 → 102 (D).

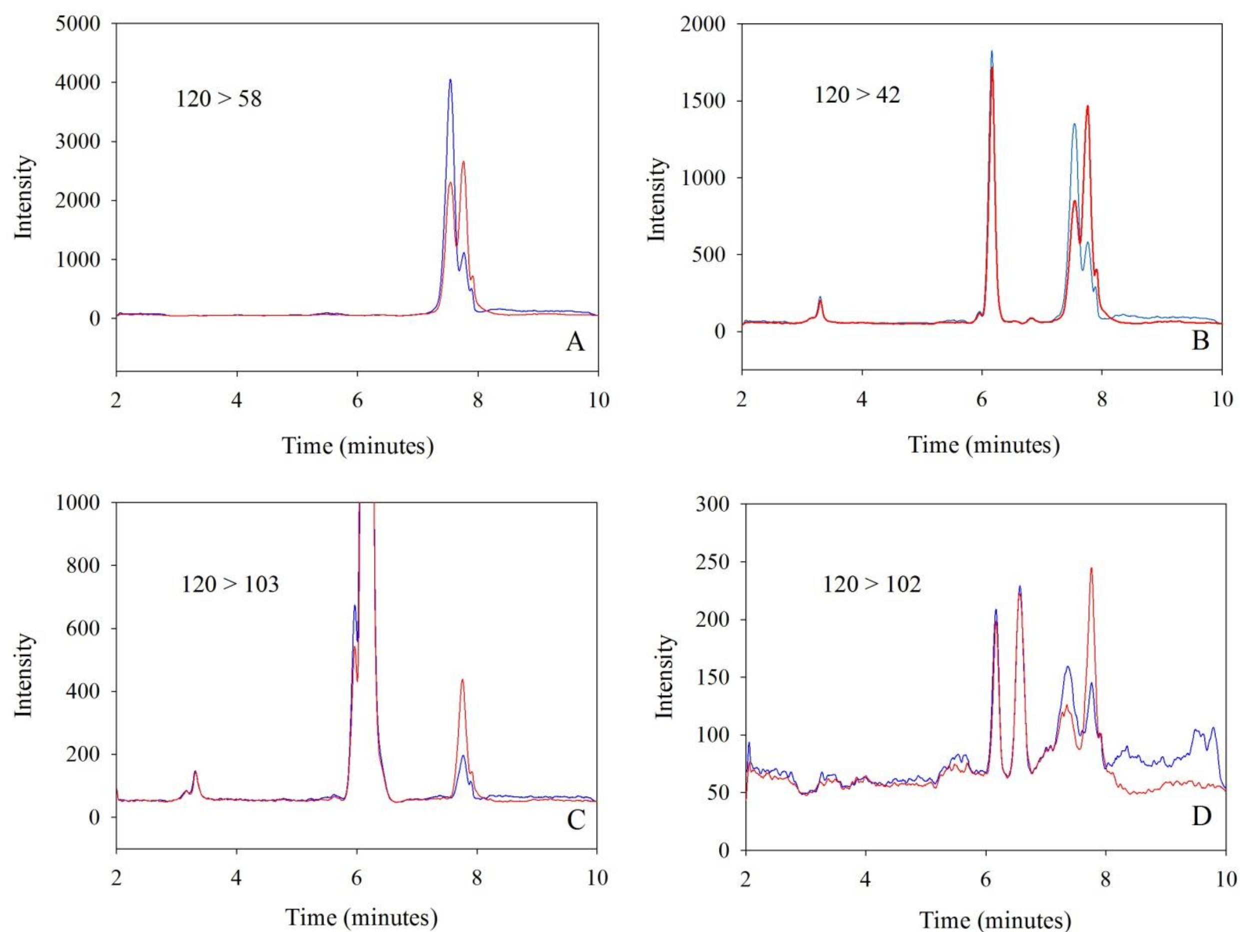


Fig. 6.5. Chromatograms obtained for plasma separated on an ES Industries SCX column. Samples were detected using an Agilent 6490 tandem mass spectrometer. A plasma sample is shown (blue), and the same plasma with an added concentration of 1 µmol/L DMGO is overlaid in red. The chromatograms are as follows: *m/z* 120 → 58 (A), *m/z* 120 → 42 (B), *m/z* 120 → 103 (C), and *m/z* 120 → 102 (D).

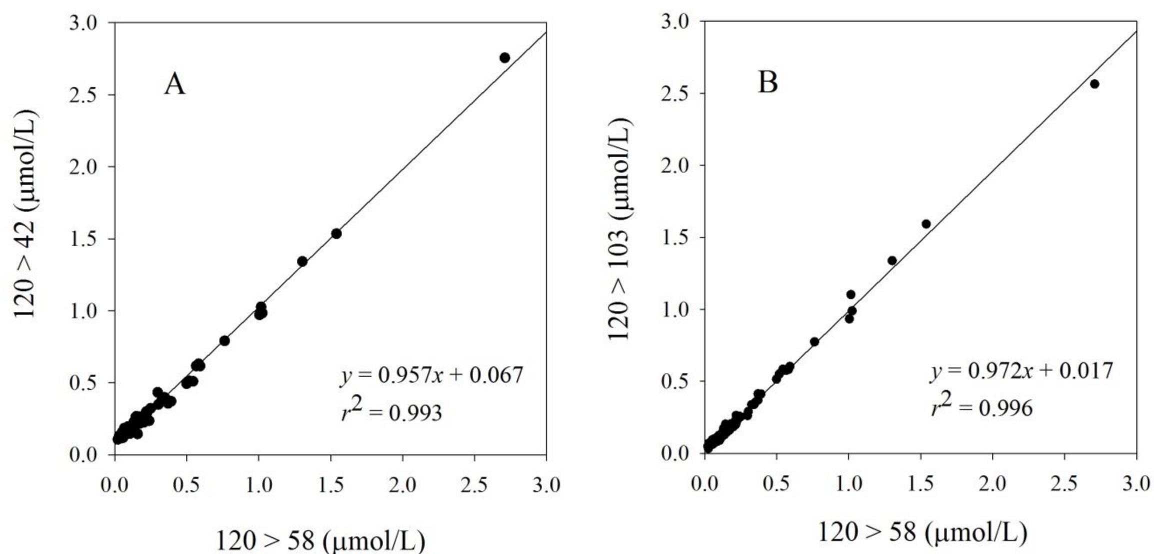


Fig. 6.6. Linear regression comparison of LC-MS/MS results for DMGO in plasma using the 120 → 58 mass transition versus the 120 → 42 mass transition (A), and the *m/z* 120 → 58 mass transition versus the *m/z* 120 → 103 mass transition (B). Results were obtained using the Cogent silica hydride column on the Agilent 6490.

The plasma results obtained using the different mass transitions were in agreement ($r^2 > 0.992$) (Fig. 6.6). This shows that almost identical results can be obtained using the mass transitions 120 → 58, 120 → 42, and 120 → 103. The *m/z* 120 → 102 mass transition is not shown due to insufficient sensitivity.

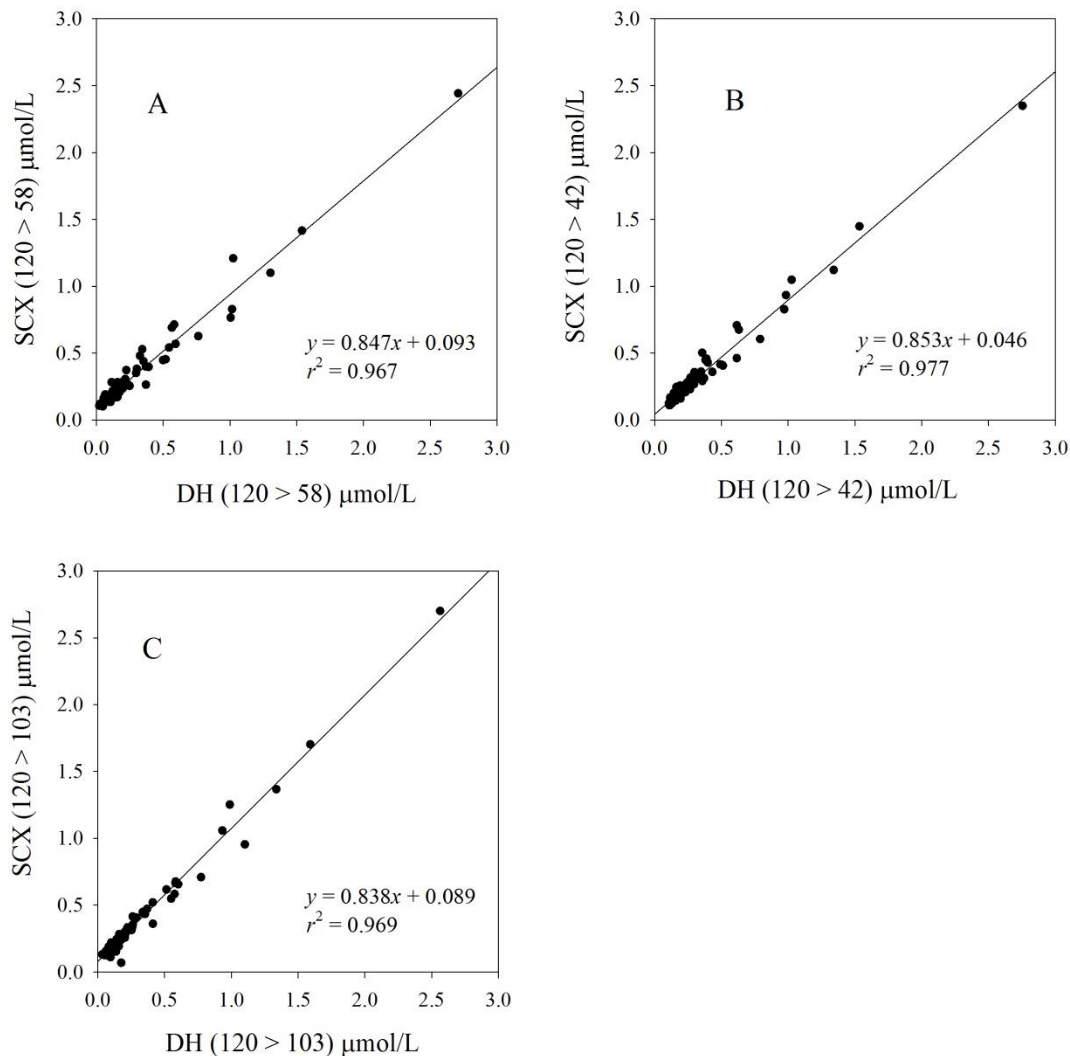


Fig. 6.7. Linear regression results for plasma obtained using the Agilent 6490 for the Cogent diamond hydride silica column (DH) compared to the ES Industries strong cation exchange column (SCX). *m/z* 120 → 58 (A), *m/z* 120 → 42 (B), and *m/z* 120 → 103 (C).

Results for the two chromatography systems were in agreement with $r^2 > 0.966$ for the three most intense mass transitions (Fig. 6.7). The slopes were greater than 0.83. However, there were significant positive intercepts showing some interference was likely to be present when using the SCX chromatography system. The *m/z* 120 → 102 mass transition is not shown due to insufficient sensitivity.

Detection of DMGO in urine

DMGO was observed in urine on both the diamond hydride silica column (Fig. 6.8) and the SCX column (Fig. 6.9). There was more DMGO observed in the urine samples than in the plasma, and interference from other components was less problematic. While there were some peaks other than DMGO observed in the urine chromatograms with both chromatography systems, the DMGO peak was well separated from any interference.

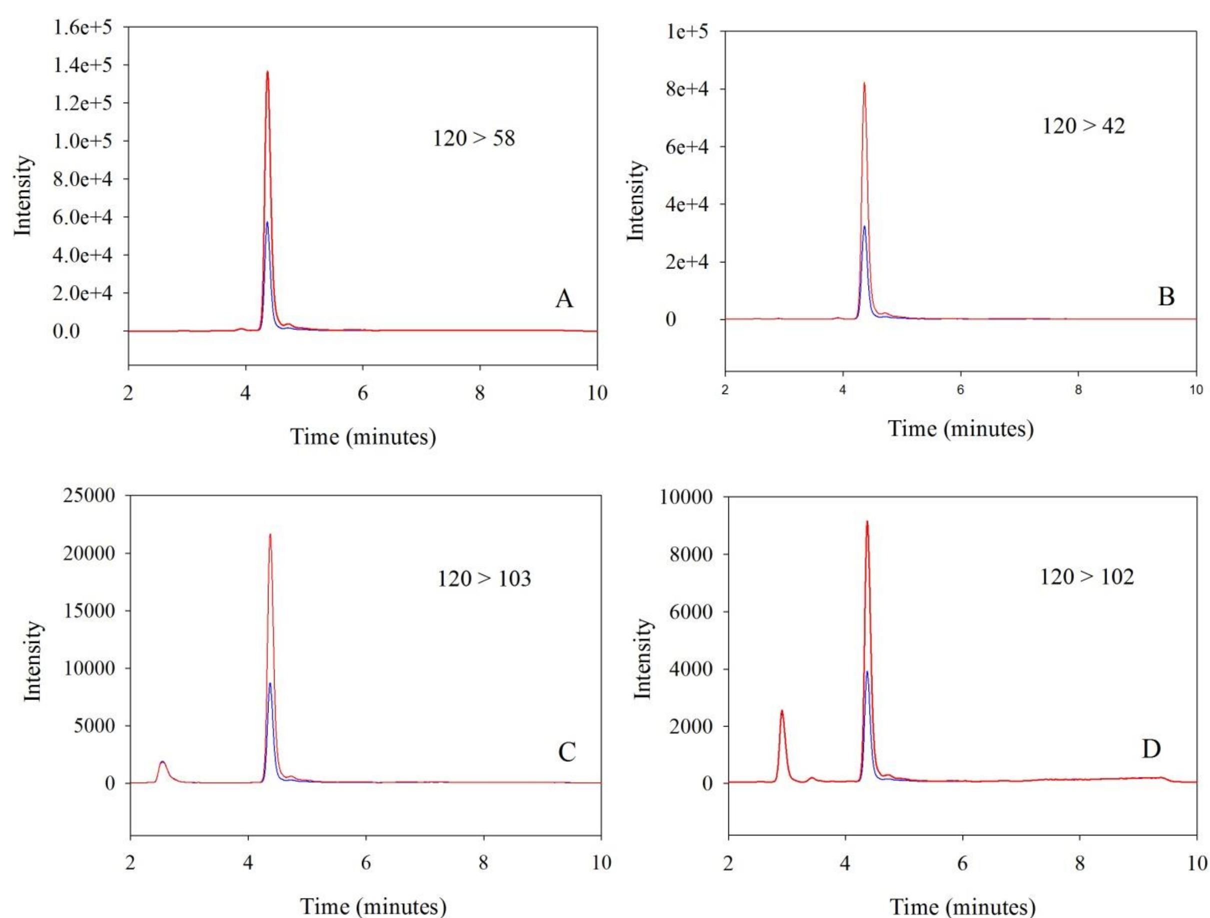


Fig. 6.8. Chromatograms showing the separation of DMGO in urine separated on a Cogent diamond hydride silica column. A urine sample (blue), and the same urine sample with 25 μmol/L DMGO added (red) are shown. The mass transitions shown are: *m/z* 120 → 58 (A), *m/z* 120 → 42 (B), *m/z* 120 → 103 (C), and *m/z* 120 → 102 (D). Samples shown were analyzed using an Agilent 6490 tandem mass spectrometer.

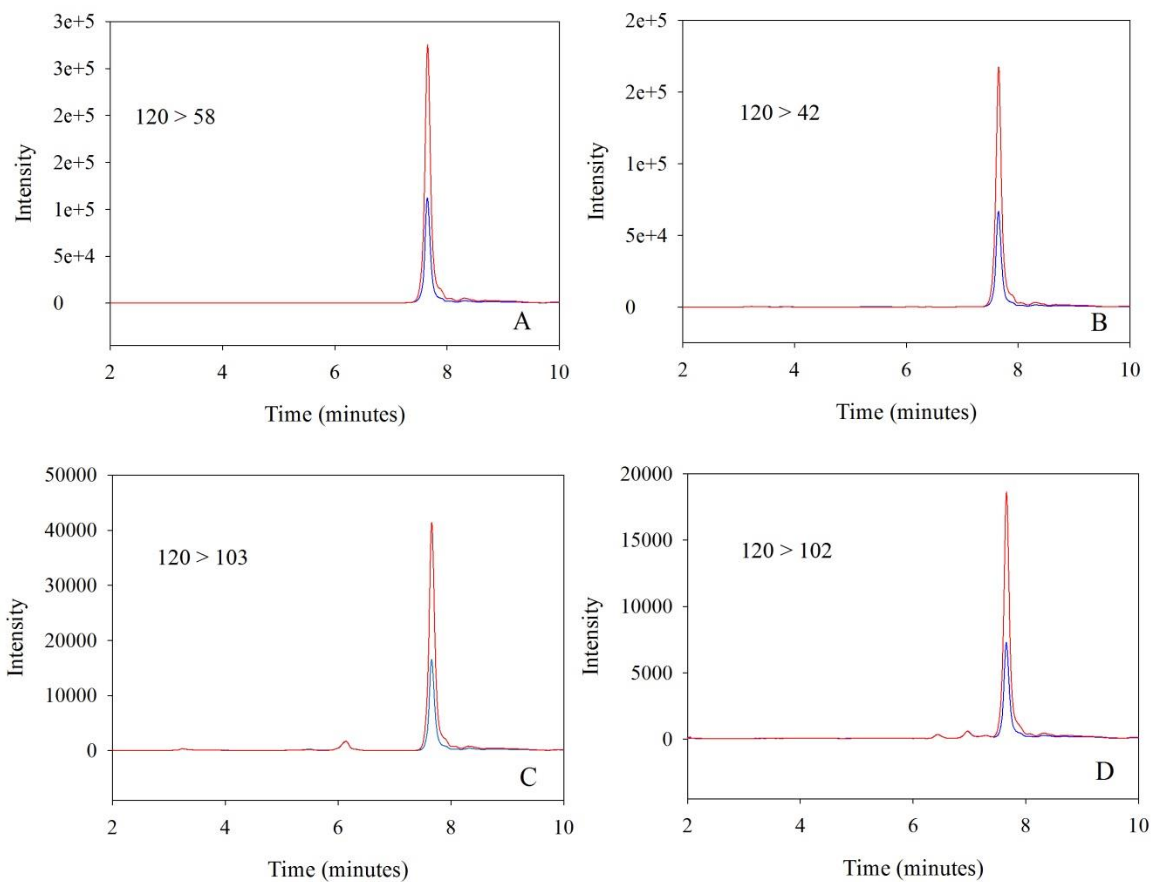


Fig. 6.9. Chromatograms showing the separation of DMGO in urine separated on an ES Industries SCX column. A urine sample (blue), and the same urine sample with 25 μmol/L DMGO added (red) are shown. The mass transitions shown are: *m/z* 120 → 58 (A), *m/z* 120 → 42 (B), *m/z* 120 → 103 (C), and *m/z* 120 → 102 (D). Samples shown were analyzed using an Agilent 6490 tandem mass spectrometer.

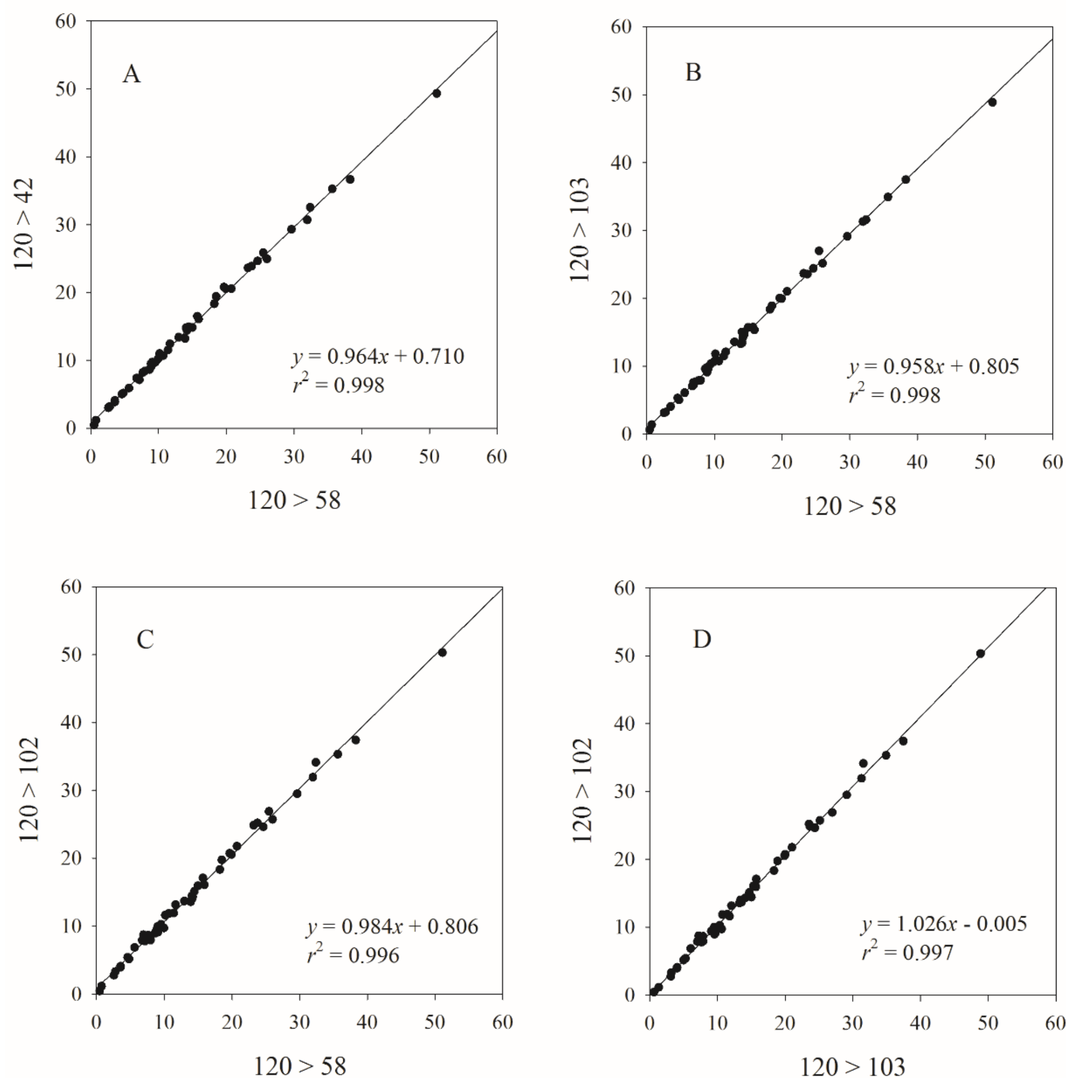


Fig. 6.10. Linear regression analyses showing the comparison of LC-MS/MS results for DMGO in urine using: the *m/z* 120 → 58 mass transition versus the *m/z* 120 → 42 mass transition (A); the *m/z* 120 → 58 mass transition versus the *m/z* 120 → 103 mass transition (B); the *m/z* 120 → 58 mass transition versus the *m/z* 120 → 102 mass transition (C); and the *m/z* 120 → 103 mass transition versus the *m/z* 120 → 102 mass transition (D). Results were obtained using the Cogent silica hydride column on the Agilent 6490 LC-MS/MS system.

Similar results were obtained in urine using all mass transitions ($r^2 > 0.995$) (Fig. 6.10), which indicates that there is no significant interference affecting the quality of the results for data collected using any particular mass transition.

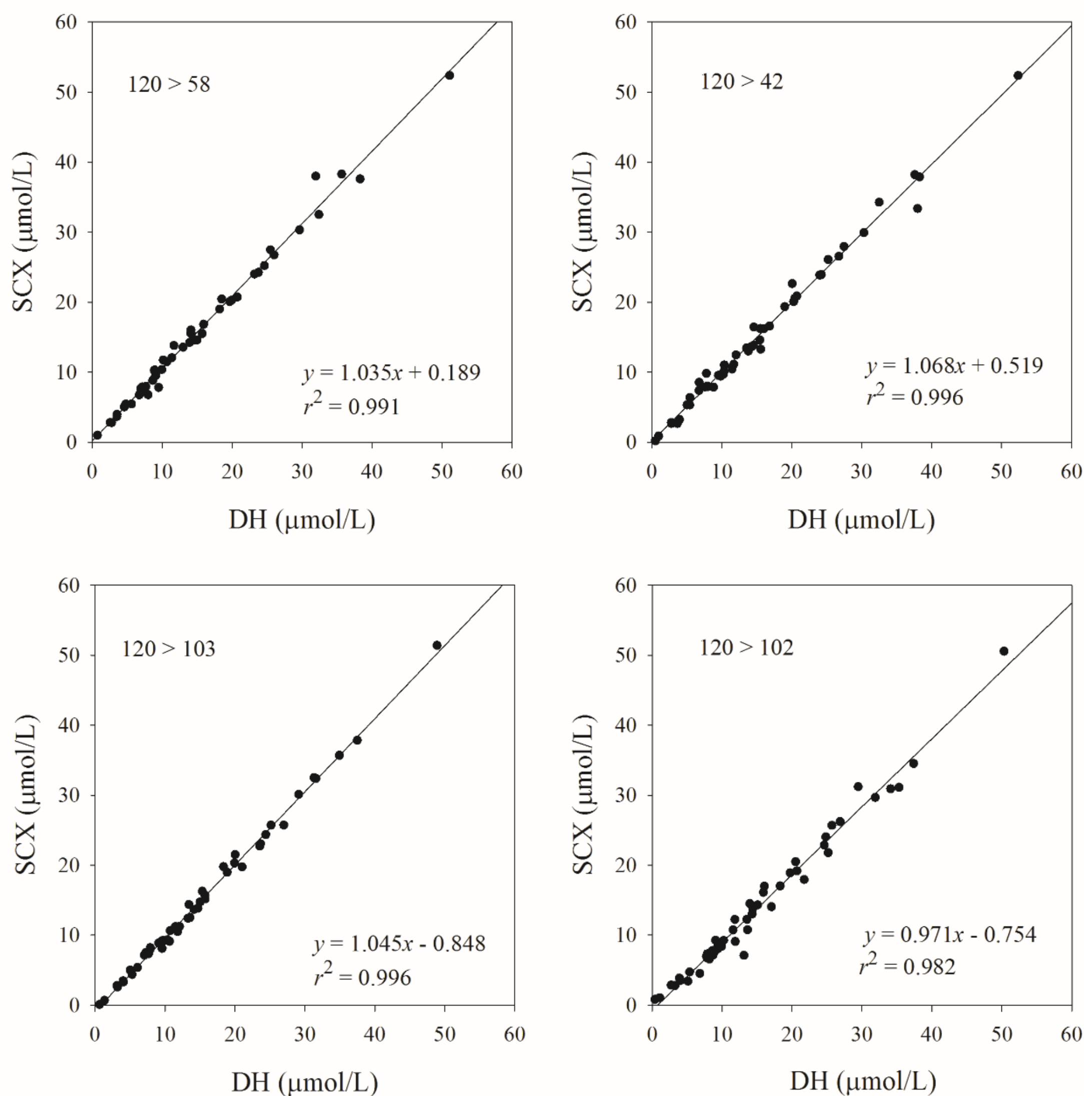


Fig. 6.11. Urine DMGO results obtained using a Cogent silica diamond hydride (DH) column compared with results obtained with an ES Industries SCX column. Samples were measured for DMGO using the Agilent 6490 instrument. Results were obtained using D_3-DMGO as the internal standard.

The urine results obtained using the two different chromatography systems agreed well ($r^2 = 0.982$ to 0.996) for all four mass transitions (Fig. 6.11). This provides a high degree of confidence that DMGO is present in the samples and that the peak is not a product of interference from the sample matrix. It is unlikely that an interfering compound would chromatograph in the same place as DMGO on two different chromatography systems, and have the same fragmentation pattern.

Method performance for DMGO in plasma

The use of deuterated DMGO (D_3-DMGO) and the internal standard was found to be necessary to produce satisfactory precision and accuracy.

The *m/z* 120 → 58 mass transition produced the largest peaks for DMGO. However, the baseline noise was greater, and the sensitivity was similar for the 42 and 103 fragment ions. The imprecision for plasma was indicated by coefficients of variation ranging from 4.9% to 18% for a low concentration of 0.03 µmol/L, and CVs 6% and lower at a concentration of 1 micromolar (Table 6.1). The DMGO concentration in some plasma samples were close to the detection limits, and may be below the theoretical limit of quantitation. Therefore, imprecision is higher at the lower concentrations. The limit of detection can also vary depending on how clean the mass spectrometer is on any particular day. Recoveries were over 83%. However, the slope of the curves in Fig. 6.12 indicates that the recoveries over the different concentration levels were between 100 and 106% for plasma. Greater method performance for plasma DMGO was possible when using the diamond hydride silica column. The SCX chromatography system had more background interference and co-eluting peaks. Therefore the imprecision was greater, and the limits of detection were approximately twice as high.

Table 6.1. Method performance results for DMGO in plasma. The diamond hydride silica column method was used to obtain these results.

	m/z 120 → 58	*m/z* 120 → 42	*m/z* 120 → 103
Plasma, mean	0.030	0.028	0.039
Plasma, SD	0.005	0.003	0.002
Plasma CV%	17.3	8.8	4.9
Plasma + 1 µmol/L, mean	0.895	0.864	0.947
Plasma + 1 µmol/L, SD	0.054	0.038	0.030
Plasma + 1 µmol/L, CV%	6.0	4.4	3.2
Recovery%	87	84	91
LOD µmol/L (S/N = 3)	0.015	0.02	0.02

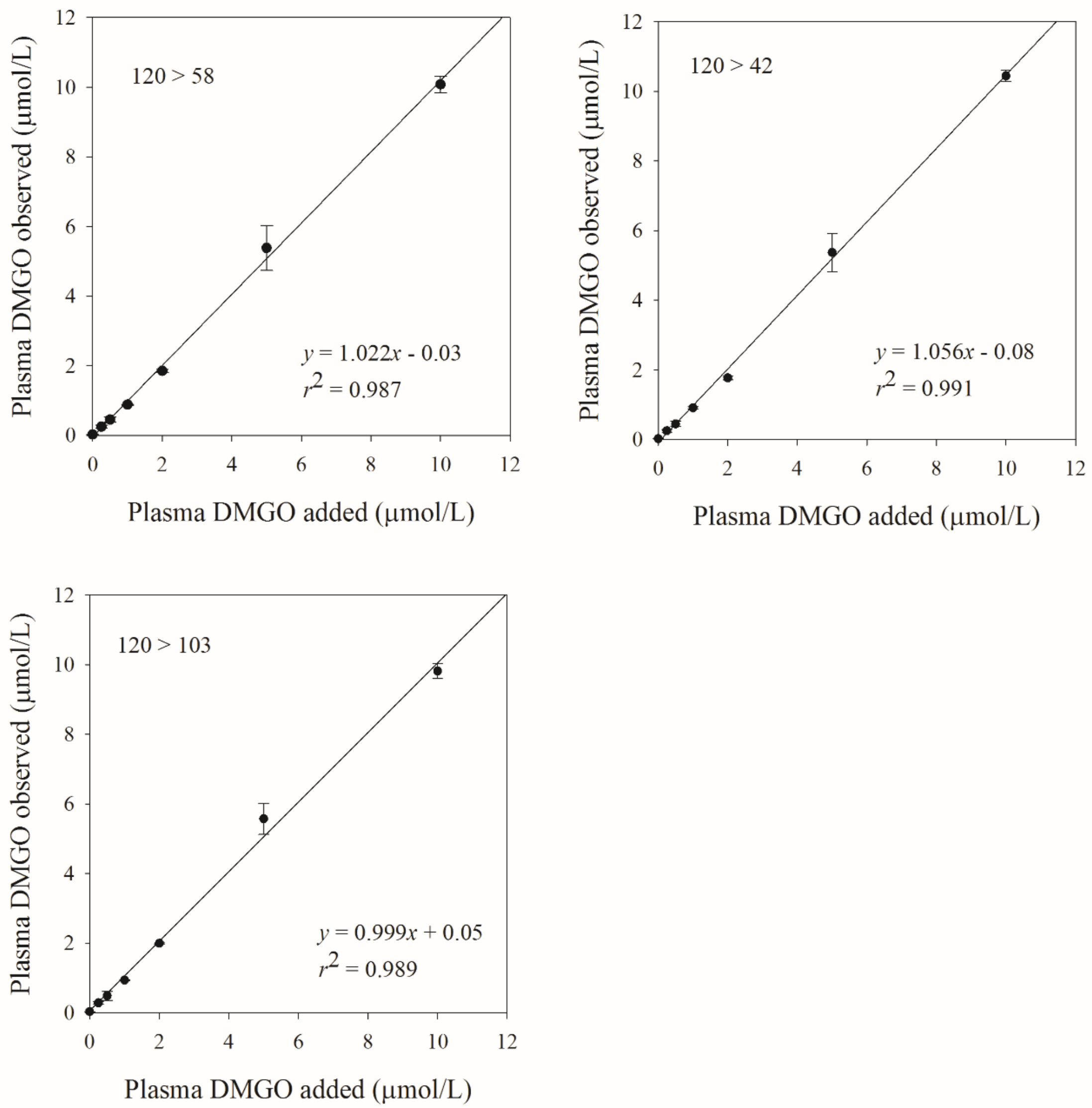


Fig. 6.12. Plasma with added DMGO concentrations detected using different mass transition. Means ± SE are shown. DMGO was separated using a diamond hydride silica column, and detected with and Agilent 6490 tandem mass spectrometer.

Method performance for DMGO in Urine

The imprecision for urine DMGO was indicated by coefficients of variation (CVs) below 4.03% when using the diamond hydride silica column, and below 5.08% using the SCX column. The recovery of DMGO added to urine was over 95% for both columns (Table 6.2). The r^2 value for DMGO added to urine was > 0.997 for the mass transitions: 120 → 103; 120 → 58; and 120 → 42 (Fig. 6.13).

Table 6.2. Method performance results for DMGO in urine.

Diamond hydride silica	*m/z* 120 → 58	*m/z* 120 → 42	*m/z* 120 → 103
Urine, mean	12.26	12.30	12.63
Urine, standard deviation	0.49	0.45	0.46
Urine CV%	4.02	3.64	3.61
Urine + 25 µmol/L, mean	36.56	37.17	37.08
Urine + 25 µmol/L, SD	0.82	0.72	1.37
Urine + 25 µmol/L, CV%	2.26	1.93	3.70
Recovery%	97	99	98

SCX column	*m/z* 120 → 58	*m/z* 120 → 42	*m/z* 120 → 103
Urine, mean	11.98	12.03	11.94
Urine, standard deviation	0.61	0.55	0.19
Urine CV%	5.07	4.55	1.59
Urine + 25 µmol/L, mean	37.39	35.87	37.00
Urine + 25 µmol/L, SD	1.45	0.55	0.93
Urine + 25 µmol/L, CV%	3.88	1.53	2.52
Recovery%	102	95	100

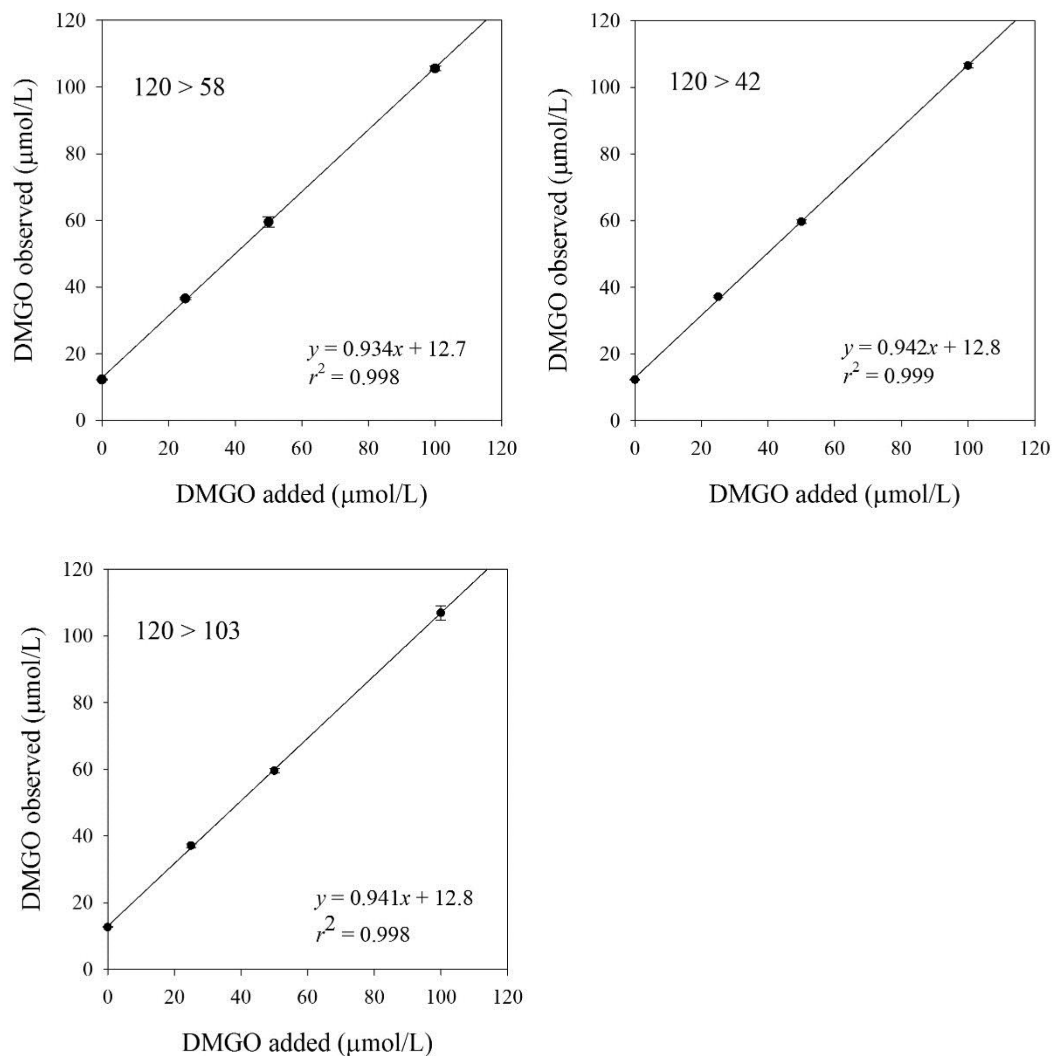


Fig. 6.13. Urine with added DMGO concentrations detected with three mass transitions. Means ± SE are shown. DMGO was separated using a diamond hydride silica column, and detected with and Agilent 6490 tandem mass spectrometer.

Is N-hydroxy-N-methylglycine a metabolite of DMGO?

Introduction

It is not known how DMGO may be further metabolized in the body. It is possible that DMGO may lose a methyl group to form *N*-hydroxy-*N*-methylglycine (HMG). It is hypothesized that if DMGO is demethylated by either dimethylglycine dehydrogenase (DMGDH) or betaine-homocysteine methyltransferase (BHMT), then HMG will also be present in human plasma and urine. The aim was to synthesize HMG and determine if it is present in plasma and urine samples using LC-MS/MS.

Methods

Synthesis of N-hydroxy-N-methylglycine

1.00 g (0.012 mol) of *N*-methylhydroxylamine hydrochloride, 10 g of sodium bicarbonate, and 50 mL of dichloromethane were added to a 100 mL conical flask and stirred for 3 hours. The reaction mixture was filtered, and 1.67 g (0.012 mol) bromoacetic acid dissolved in dichloromethane was added. This mixture was stirred for 3 hours at room temperature and then left to sit for 7 days. An oily substance developed at the bottom of the reaction mixture. Mass spectrometry confirmed that this substance had a mass of 106 corresponding to the expected mass of *N*-methylhydroxyglycine (HMG). 50 mL of distilled water was added and the mixture was well shaken in a separation funnel. The aqueous layer was collected and dried under vacuum. The *N*-methylhydroxyglycine hydrobromide did not crystallize in 2-propanol, and dried down to a yellow syrup.

LC-MS/MS

A compound optimization of HMG produced fragments at *m/z* 42.1, and 60.2. HMG was separated using the SCX chromatography system that was described for separating DMGO. HMG was detected using the 106 → 42, and 106 → 60 mass transitions with a collision energy of 30 V.

Results and discussion

There was no detectable HMG peak in the plasma samples. However, there was a small peak in the urine samples in the region of HMG (Fig. 6.14). It was not possible to reliably quantify this peak in the urine, but it suggests that HMG is likely to be present in sub-micromolar concentrations. The observation that no HMG was detected in plasma and only a small amount of HMG was observed in the urine shows that DMGO is likely to be only a minor substrate for DMGDH or BHMT, if it interacts at all. Another possible fate for DMGO is that it is readily excreted into the urine, and is not required for further metabolism.

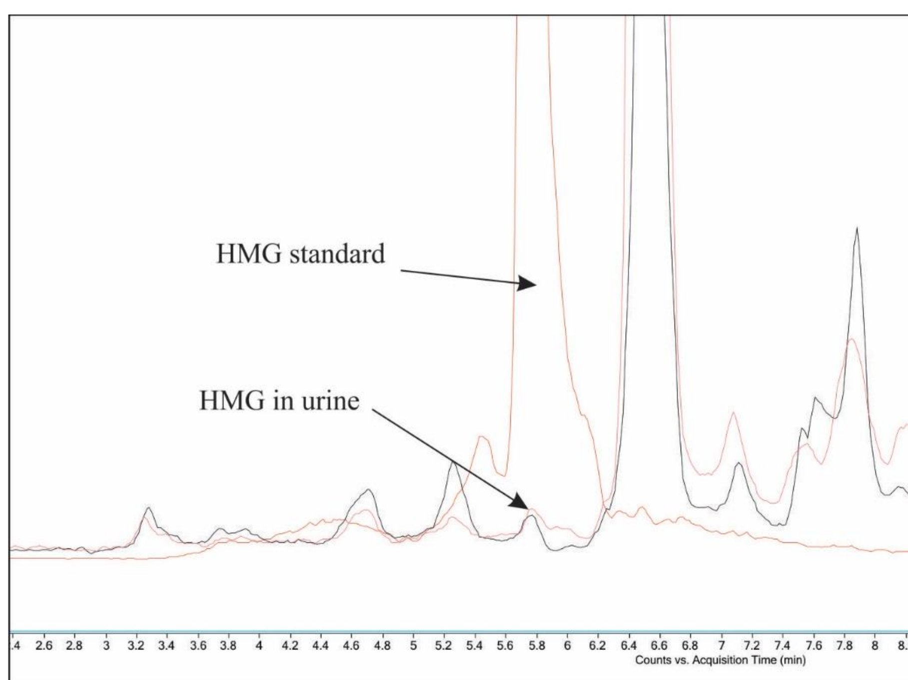


Fig. 6.14. Chromatograms showing the HMG peak in two urine samples and a standard (containing approximately 100 µmol/L HMG) using the 106 → 42 mass transition.

Summary

The results indicate that DMGO is present in plasma and urine samples. While the best chromatography was observed using a diamond hydride silica column, DMGO was also seen on an SCX column, although with more interference in the plasma. The high agreement for DMGO results obtained using different mass transitions corresponding to the structure of DMGO, along with the high agreement between two different chromatography systems, provides strong evidence that DMGO was observed in the samples.

The co-eluting peaks observed in the chromatograms are likely to be compounds that are structurally similar to DMGO, as they are derived from the same mass transitions. Identification of these peaks may be useful for future studies.

D_3-DMG made a poor internal standard for the measurement of DMGO by mass spectrometry. More accurate and precise results were obtained after D_3-DMGO was synthesized and used in the method. Measuring the low concentrations of DMGO that were observed in plasma requires the use of a highly sensitive tandem mass spectrometer.

The fact that there was no HMG peak observed in plasma, and only a small peak (<1 μmol/L) in urine that may be attributable to HMG, shows that demethylation is an unlikely fate for DMGO in the body.

The measurement of the low concentrations of DMGO present in plasma is dependent on using a highly sensitive mass spectrometer. While proton NMR would struggle to detect DMGO in many of the urine samples, NMR was useful for validating the DMGO that was synthesized for use as a quantitative standard.

The presence of DMGO has not previously been reported in human samples, and its identification in plasma and urine paves the way for more research investigating its potential role in one-carbon metabolism.

6.2. *N,N*-Dimethylglycine-*N*-oxide concentrations in subjects with metabolic syndrome, type 2 diabetes, and healthy controls

Introduction

N,N-Dimethylglycine-*N*-oxide (DMGO) has been identified in plasma and urine samples. In this section, DMGO was measured in samples from different populations including people with metabolic syndrome, people with type 2 diabetes, and a healthy population. Differences in DMGO concentrations between these groups, and how DMGO correlates with other metabolites in plasma and urine were investigated. This work has been accepted for publication in Biological Chemistry.[173]

Methods

Plasma and urine from 62 baseline samples from the betaine and body composition (BBC) study were analyzed for DMGO, betaine, DMG, choline, TMAO, creatinine, and *myo*-inositol. These subjects were selected because they had features of the metabolic syndrome, *i.e.* were overweight, had high lipids, and elevated blood pressure.[63] A group with type 2 diabetes (n = 64) from the DEWL study[146] were also analyzed for plasma and urine DMGO. Plasma and urine from a control group were analyzed from a control group consisting of 16 healthy male subjects obtained from a previous diet study[174] which had been stored at -40°C. This control group contained only baseline samples when no treatment had been given. Correlations between the metabolites were observed using Spearman's rank order tests. The groups were compared using Kruskal-Wallis analysis of variance on ranks with SigmaPlot (v13). Fractional clearances were calculated as: ((urine metabolite concentration / plasma metabolite concentration) / (urine creatinine concentration/ plasma creatinine concentration)) x 100.

Fifty microliters of urine was extracted into 1 mL of extraction solvent (80% acetonitrile, 20% methanol containing 10 µmol/L of D_3-*N,N*-dimethylglycine-*N*-oxide (D_3-DMGO) as an internal standard). Fifty microliters of plasma sample was extracted into1 mL of extraction solvent (80% acetonitrile, 20% methanol containing 0.1 µmol/L of D_3-DMGO as an internal standard). Samples were analyzed for DMGO

using an Agilent 6490 LC-MS/MS system with a Cogent diamond hydride silica column (100 × 2.1 mm, 4 µm, Microsolv Technologies, NJ, USA). Mobile phase solvent A contained 10 mmol/L formic acid, and 10 mmol/L ammonium formate in 50% distilled water and 50% acetonitrile (v/v). Solvent B contained 7.5 mmol/L TFA and 15 mmol/L formic acid in acetonitrile. A gradient was used starting with 5% A and 95% B, then to 25% A and 75% B at 3 minutes, and 100% A at 7.5 to 8.5 minutes, and then back to starting conditions at 9 minutes. The run time was 12 minutes, the flow rate was 0.3 mL/min, the injection volume was 10 µL, and the column temperature was 40°C. The results reported in this Section were obtained using the most intense mass transition for DMGO, *m/z* 120 → 58, on an Agilent 6490 tandem mass spectrometer after separation on a Cogent diamond hydride silica column. Further mass spectrometry details were provided in Section 6.1.

Results and discussion

Comparison of DMGO concentrations in the different groups

Descriptive statistics are shown for plasma and urine DMGO, DMG, and betaine concentrations in people with the metabolic syndrome and the control group in Table 6.3. The median DMGO concentration in the plasma for the group with the metabolic syndrome was 0.13 µmol/L, the median plasma DMGO in the subset with diabetes was 0.19 µmol/L (Fig. 6.15), and the median DMGO concentration for the healthy control group was 0.57 µmol/L. The range was much narrower for plasma DMGO in the groups with metabolic syndrome and diabetes than for the controls.

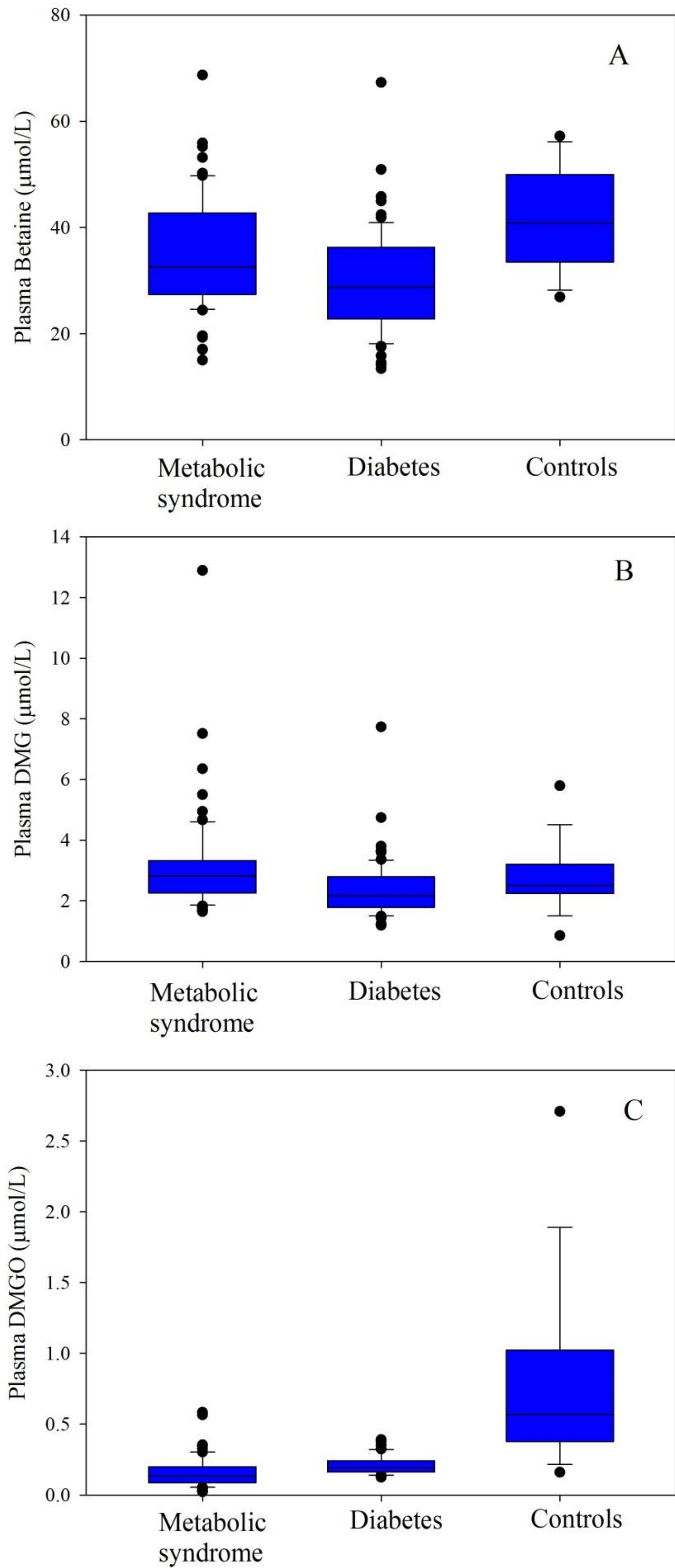


Fig. 6.15. Concentrations of: betaine (A), DMG (B), and DMGO (C) in plasma for people with metabolic syndrome, type 2 diabetes, and healthy controls.

Table 6.3. Descriptive statistics for DMGO, DMG, and betaine in metabolic syndrome subjects, people with type 2 diabetes, and the control group.

Metabolic syndrome	Max	Min	Median	25%	75%
Plasma Betaine (µmol/L)	68.7	15.0	32.6	27.4	42.8
Plasma DMG (µmol/L)	12.9	1.64	2.82	2.25	3.33
Plasma DMGO (µmol/L)	0.583	0.022	0.133	0.087	0.198
Urine Betaine (mmol/mol creatinine)	106.0	1.98	6.32	4.58	10.40
Urine DMG (mmol/mol creatinine)	10.37	0.51	2.69	1.97	3.87
Urine DMGO (mmol/mol creatinine)	3.91	0.40	1.19	0.94	1.63
Fractional clearance Betaine %	51.3	0.39	1.56	1.06	2.40
Fractional clearance DMG %	20.4	1.94	7.23	4.97	9.88
Fractional clearance DMGO%	229	38.5	75.4	60.8	91.7

Type 2 Diabetes	Max	Min	Median	25%	75%
Plasma Betaine (µmol/L)	67.3	13.4	28.8	22.8	36.3
Plasma DMG (µmol/L)	7.73	1.19	2.16	1.79	2.79
Plasma DMGO (µmol/L)	0.390	0.124	0.193	0.161	0.24
Urine Betaine (mmol/mol creatinine)	271.2	2.29	27.8	12.3	58.9
Urine DMG (mmol/mol creatinine)	24.0	0.81	6.95	5.29	11.3
Urine DMGO (mmol/mol creatinine)	13.3	0.44	1.96	1.43	3.17
Fractional clearance Betaine %	40.9	0.91	6.56	3.43	20.4
Fractional clearance DMG %	24.0	0.81	6.95	5.29	11.3
Fractional clearance DMGO%	312	21.6	73.5	59.7	103

Healthy controls	Max	Min	Median	25%	75%
Plasma Betaine (µmol/L)	88.1	26.9	43.1	36.5	53.6
Plasma DMG (µmol/L)	5.79	1.78	2.51	2.24	3.21
Plasma DMGO (µmol/L)	2.708	0.159	0.568	0.377	1.02
Urine Betaine (mmol/mol creatinine)	8.13	1.94	4.56	3.58	7.21
Urine DMG (mmol/mol creatinine)	8.41	0.77	2.11	1.56	3.25
Urine DMGO (mmol/mol creatinine)	21.2	0.40	1.12	0.89	1.65
Fractional clearance Betaine %	1.84	0.38	0.74	0.57	1.31
Fractional clearance DMG %	12.6	2.17	4.22	3.02	5.91
Fractional clearance DMGO%	30.3	4.69	15.0	6.88	22.6

Kruskal-Wallis one way analysis of variance on ranks showed that plasma DMGO was significantly lower in the subjects with diabetes and the subjects with metabolic syndrome compared to the healthy controls ($p < 0.001$). Plasma DMGO concentrations in the subjects with type 2 diabetes were also significantly different to the subjects with the metabolic syndrome ($p < 0.001$). Urine DMGO was significantly higher ($p < 0.001$) in the subjects with type 2 diabetes compared to the metabolic

syndrome samples and the healthy controls. Urine DMGO was not significantly different in the metabolic syndrome subjects compared to the healthy controls.

Kruskal-Wallis tests showed that plasma betaine was significantly lower in the subjects with type 2 diabetes compared to the healthy controls ($p < 0.001$). However, plasma betaine in the metabolic syndrome subjects was not significantly different to the controls or the DEWL subjects ($p > 0.05$). Betaine was significantly higher ($p < 0.001$) in the urine of the subjects with type 2 diabetes compared to the people with the metabolic syndrome and the healthy controls. However, urine betaine was not significantly different in the people with the metabolic syndrome compared to the healthy controls ($p = 0.167$).

Plasma DMG concentrations were significantly higher in the group with the metabolic syndrome compared to the group with type 2 diabetes ($p = 0.008$). Neither group had significantly different plasma DMG than the healthy controls.

Kruskal-Wallis one way analysis of variance on ranks showed that the fractional clearance of DMGO in healthy subjects and people with metabolic syndrome were significantly different from each other ($p < 0.001$) (Fig. 6.16). However, people with diabetes and metabolic syndrome did not have significantly different fractional clearances of DMGO. The fractional clearance was higher in the groups with metabolic syndrome and diabetes because the plasma DMGO was lower in these groups compared to the healthy subjects. The urine concentrations of DMGO were not significantly different between the groups with metabolic syndrome and healthy controls. However, the group with diabetes had significantly higher DMGO in the urine compared to the other two groups ($p < 0.001$). The moderately low fractional clearance of DMGO in the healthy subjects (median = 15%) suggests that it is retained by the kidneys for a metabolic purpose. The subjects with metabolic syndrome and diabetes had median fractional clearances for DMGO of 75% and 73%. This shows that their kidneys are less able to retain it, most likely due to their impaired health and poorer metabolic function. The fractional clearance of DMGO may be a more sensitive marker of disease and possibly a predictor of health outcomes. Being an important osmolyte, and one-carbon metabolite, betaine is strongly retained by the kidneys, as shown by its low fractional clearance (Table 6.3).

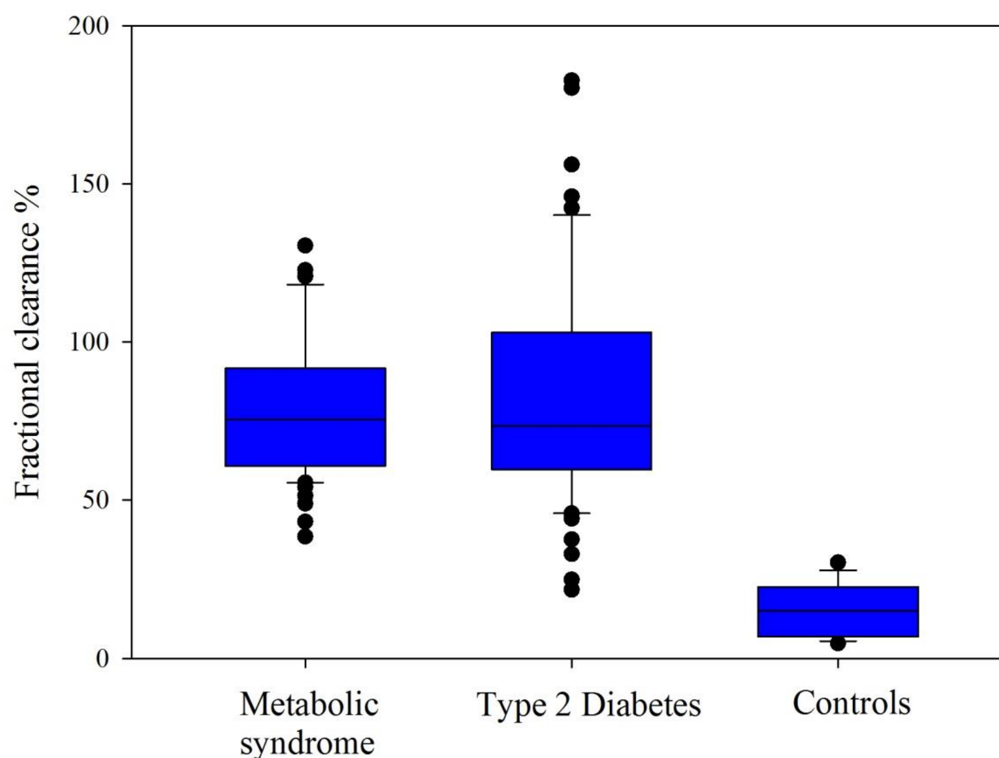


Fig. 6.16. The fractional clearance of *N*,*N*-dimethylglycine-*N*-oxide (DMGO) in metabolic syndrome subjects, people with type 2 diabetes, and the healthy controls.

DMGO in subjects with metabolic syndrome

Spearman's rank order correlation analysis (Table 6.4) showed that in the subjects with metabolic syndrome, plasma DMGO correlated strongly with plasma DMG ($p < 0.001$) and plasma betaine ($p = 0.001$). Plasma DMGO also correlated significantly ($p < 0.05$) with plasma choline and *myo*-inositol ($p = 0.015$). Urine DMGO correlated strongly ($p < 0.001$) with plasma DMGO, plasma DMG, and plasma betaine. Urine DMGO correlated negatively with urine betaine ($p = 0.049$), but did not correlate with urine DMG or urine choline. Some significant correlations with plasma DMGO observed in the metabolic syndrome subjects are shown in Fig. 6.17.

Table 6.4. Spearman's rank order correlations for DMGO in plasma and urine calculated from people with metabolic syndrome.

	Plasma DMGO	Urine DMGO
Plasma DMGO		**r = 0.860** **p < 0.001**
Plasma betaine	r = **0.407** p = **0.001**	**r = 0.421** **p < 0.001**
Plasma DMG	r = **0.673** p < **0.001**	**r = 0.657** **p < 0.001**
Plasma choline	r = **0.263** p = **0.039**	r = 0.149 p = 0.248
Plasma TMAO	r = 0.182 p = 0.064	r = 0.058 p = 0.656
Plasma creatinine	r = 0.620 p = 0.187	r = 0.029 p = 0.821
Plasma *myo*-inositol	r = **0.309** p = **0.015**	r = 0.235 p = 0.067
Urine betaine	r = -0.218 p = 0.088	r = -0.203 p = 0.114
Urine DMG	r = 0.157 p = 0.222	**r = 0.290** **p = 0.022**
Urine choline	r = -0.028 p = 0.826	r = -0.018 p = 0.888
Urine TMAO	r = 0.124 p = 0.336	r = 0.090 p = 0.486

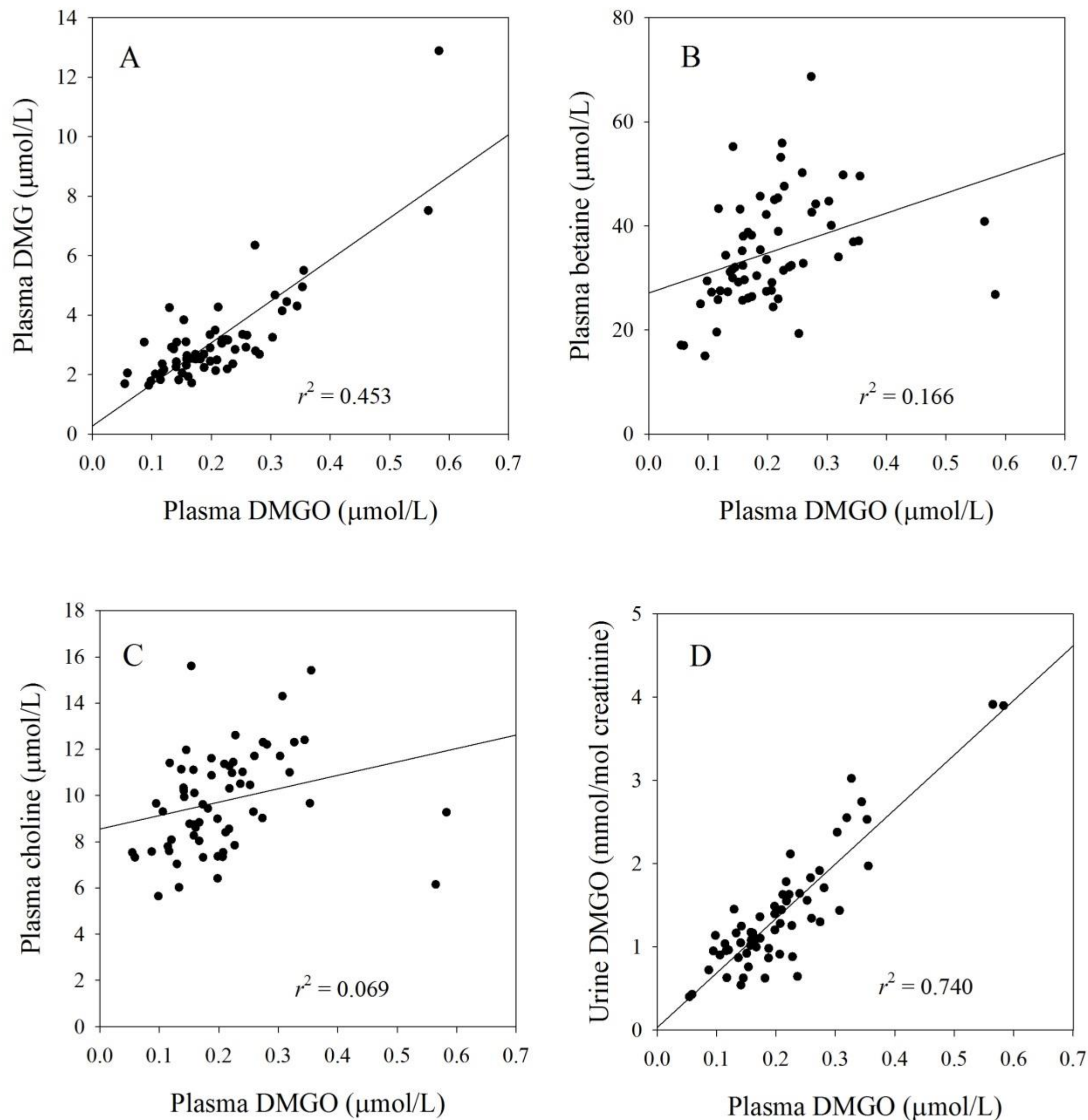


Fig. 6.17. Some significant correlations between plasma DMGO with: plasma DMG (A), plasma betaine (B), plasma choline (C), and urine DMGO (D). Linear regressions lines are shown. The data set is from subjects with metabolic syndrome.

DMGO in subjects with the type 2 diabetes

Spearman's rank order correlations showed that plasma DMGO in the subjects with type 2 diabetes correlated with plasma DMG ($p < 0.001$), but the correlation with plasma betaine did not quite reach significance in this group ($p = 0.051$). Plasma DMGO correlated strongly with urine DMGO ($p < 0.001$). Plasma DMGO correlated negatively with total cholesterol ($p = 0.018$) and low density lipoprotein (LDL) cholesterol ($p = 0.007$), but did not correlate significantly with plasma homocysteine,

glucose, HbA_{1c}, creatinine, or BMI ($p > 0.05$) (data supplied by DEWL study researchers).

Urine DMGO correlated significantly ($p < 0.001$) with plasma DMGO and plasma DMG. Urine DMGO also correlated with urine DMG, urine choline, and urine TMAO (Table 6.5).

Table 6.5. Spearman's rank order correlations for DMGO in plasma and urine calculated from people with type 2 diabetes.

	Plasma DMGO	Urine DMGO
Plasma DMGO		***r* = 0.591** ***p* < 0.001**
Plasma betaine	*r* = 0.120 *p* = 0.349	*r* = 0.120 *p* = 0.349
Plasma DMG	***r* = 0.410** ***p* < 0.001**	***r* = 0.410** ***p* < 0.001**
Plasma *myo*-inositol	*r* = 0.199 *p* = 0.114	*r* = 0.013 *p* = 0.918
Plasma TMAO	*r* = 0.049 *p* = 0.701	*r* = 0.049 *p* = 0.701
Plasma taurine	*r* = -0.005 *p* = 0.968	*r* = 0.019 *p* = 0.880
Plasma creatinine	*r* = 0.137 *p* = 0.280	*r* = -0.202 *p* = 0.112
Urine betaine	*r* = -0.086 *p* = 0.503	*r* = 0.086 *p* = 0.503
Urine DMG	*r* = 0.128 *p* = 0.314	***r* = 0.420** ***p* < 0.001**
Urine choline	*r* = 0.008 *p* = 0.947	***r* = 0.293** ***p* = 0.020**
Urine TMAO	*r* = 0.157 *p* = 0.214	***r* = 0.363** ***p* = 0.004**

DMGO in the healthy control group

In the control group, urine DMGO correlated with plasma betaine ($p = 0.002$), and with plasma DMG ($p < 0.001$), but not with plasma DMGO ($p = 0.153$), nor plasma *myo*-inositol ($p = 0.67$), and plasma creatinine ($p = 0.16$). Urine DMGO did not correlate with urine betaine ($p = 0.41$) or urine DMG ($p = 0.15$) either. Plasma DMGO correlated with plasma betaine ($p = 0.03$), but not with plasma DMG (p =

0.14) in the control group. Further studies are required to investigate the normal range of DMGO using fresh samples collected on a greater number of healthy subjects.

Summary

The presence of DMGO in plasma and urine suggests that DMG is likely to be oxidized by an FMO enzyme in the same way that trimethylamine is converted to trimethylamine-*N*-oxide. FMO enzymes are found in the kidneys as well as the liver.[175] If DMGO is formed by the oxidation of DMG, then it may play an important biological role in one-carbon metabolism. The proposed metabolic pathway for the formation of DMGO is shown in Fig. 6.18. There is still a question mark for the pathway from DMG to DMGO, and a question mark for the suggestion that an FMO enzyme may be responsible for the oxidation of DMG, because further studies will be required to substantiate these theories.

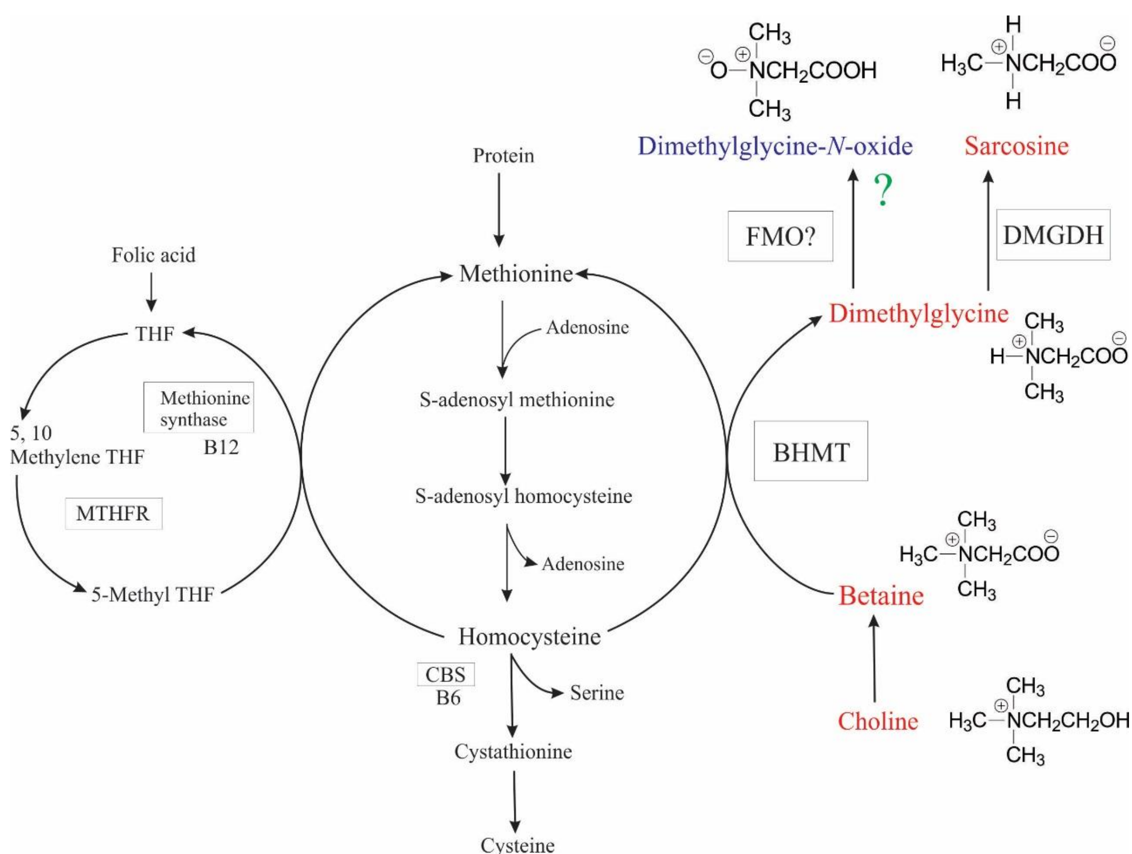


Fig. 6.18. Proposed metabolic pathway showing the metabolism of betaine to DMG, then to sarcosine and DMGO.

It was previously thought that DMG was only metabolized by demethylation to sarcosine by dimethylglycine dehydrogenase.[4b] However, the discovery of DMGO in human plasma and urine is likely to have important implications for one-carbon metabolism. Plasma concentrations of DMGO in the healthy subjects (median = 0.57) are lower, but comparable, to plasma concentrations that have been reported for sarcosine (range 0.60 to 2.67 μmol/L).[176] The strong correlations observed for plasma and urine DMGO with plasma DMG and betaine, particularly in the group with metabolic syndrome, provide solid evidence that DMGO is a metabolite of DMG.

The observation that DMGO is significantly lower in the plasma of people with the metabolic syndrome and people with type 2 diabetes compared to healthy controls suggests that it may be an important risk marker in disease. The negative correlation observed for plasma DMGO with LDL cholesterol may indicate that DMGO could be a useful risk marker, in the same way that betaine and choline have been reported to be for metabolic syndrome and diabetes.[73, 84] A long term study relating DMGO concentrations to health outcomes, such as cardiovascular events and death, would help to determine the value of DMGO concentrations as a diagnostic marker. The proposed metabolism of DMG to DMGO *in vivo* needs to be confirmed. Whether or not DMGO is metabolized further in the body, and how it might be metabolized, also requires further investigation. The high fractional clearances observed in the groups with metabolic syndrome and diabetes suggests that DMGO is poorly retained by the kidneys, and if DMGO plays a role in modulating one-carbon metabolism, it is less likely to be effective in people with these health conditions. The strong correlation observed between plasma DMGO and urine DMGO in metabolic syndrome (r^2 = 0.74) also indicates that DMGO is readily excreted, or not resorbed by the kidneys. It may also be possible that DMG is converted to DMGO in order to remove it from the body, therefore increasing the ability for BHMT to lower homocysteine. DMG is a feedback inhibitor for BHMT pathway.[177] However, DMG can also be removed by demethylation to sarcosine *via* DMGDH.

7. Chapter Seven - Summary

7.1. Thesis Summary

At the start of this project, LC-MS/MS instruments were not found in most New Zealand clinical laboratories. However, in recent years the technology has become more affordable, and many clinical laboratories now have several instruments. This project has shown that liquid chromatography mass spectrometry (LC-MS and LC-MS/MS) methods are not perfect, but provide many advantages over previous methods for the measurement of betaine, other osmolytes, and other one-carbon metabolites. Mass spectrometry has increased the number of osmolytes (and related metabolites) that can be reliably measured in order to provide more information about their metabolism and potential use as risk markers in disease.

Re-addressing the research hypotheses

- Hypothesis: *Tandem mass spectrometry (LC-MS/MS) can be used to measure betaine, other osmolytes, and other one-carbon metabolites in plasma and urine with greater efficiency and improved performance than conventional HPLC-UV methods.*

Mass spectrometry improves efficiency for the analysis of methylamines in plasma and urine by decreasing sample preparation time and sample run times, therefore improving sample throughput compared with previous HPLC-UV methods. While the precision and accuracy was not significantly improved for many analytes using mass spectrometry compared to traditional HPLC-UV methods, the accuracy for the measurement of some analytes was greatly improved using mass spectrometry due to superior selectivity. Like other methods, the quality of results obtained by mass spectrometry is limited by sources of error such as: sample treatment; sample stability; and accuracy of pipetting, particularly when preparing calibration standards. Other problems encountered using mass spectrometry include: a narrow linear range; variable accuracy and precision when suitable isotopic standards could not be used; interference from closely related structural isomers; and insufficient sensitivity for some analytes. The advantages of mass spectrometry techniques over traditional

HPLC techniques are: simple and efficient sample preparation; a greater number of analytes can be measured within a shorter sample run time; isotopic internal standards help to correct for extraction and ionization differences between the samples and calibration standards; and there is a high degree of assurance that interfering compounds do not affect the accuracy of the results. Mass spectrometry methods also have the advantage that they save time and money compared to previous HPLC-UV methods. Sample preparation, and run times were reduced. Solvent flow rates were also reduced, leading to much lower consumption of acetonitrile in the mobile phase.

- *Hypothesis:* *Polyols (including sorbitol and* myo-*inositol) can be measured using derivatization or complexation with boronic acids or transition metals in combination with techniques such as HPLC, mass spectrometry, or NMR spectroscopy.*

The measurement of polyols was found to be challenging. Attempts to derivatize sorbitol and inositol with isocyanates or *N*-methylisotoic anhydride produced many chromatographic peaks for each compound which were difficult to distinguish in complex sample matrices. Complexing polyols with boronic acids was not successful either. These complexes are most stable under alkaline conditions which are not compatible with silica based columns. *myo*-Inositol as well *scyllo*-inositol and *chiro*-inositol could be measured by LC-MS after separation on an amide column, either directly with APCI, or by detection as an iodide adduct using electrospray ionization. Unfortunately, a suitable method to quantify sorbitol was not developed in time to measure inositols in the DEWL study samples. The detection limits for inositols using LC-MS techniques were around 1 to 2 µmol/L. This is approximately the expected concentration range reported for plasma sorbitol in healthy people.[61a] If sorbitol could be detected without significant interference, then these methods would most likely lack the required sensitivity. However, the elevated sorbitol concentrations expected in people with diabetes may have been detectable.

- *Hypothesis: The analytical methods developed in this thesis can be used to investigate concentrations of betaine, other osmolytes, and other one-carbon metabolites in order to provide new information on their metabolism, and the usefulness of these metabolites as risk markers in disease.*

The methods developed in this thesis were used to investigate the metabolism of betaine, other osmolytes, and other one-carbon metabolites in an overweight population with type 2 diabetes. Important information was obtained about: how concentrations of these metabolites are different to other populations; the biological variability of these metabolites; how these metabolites correlate with markers of glycemic control and renal function; and the effect of diet treatment and drug treatment on concentrations of these metabolites.

The concentrations of many osmolytes and related compounds were shown to be higher in the urine of people with diabetes compared to a group of overweight men without diabetes (with metabolic syndrome). The high individuality of betaine (and other one-carbon metabolites) in the group with diabetes show that single measurements are a useful representation of a person's status, and do not vary greatly with time. The observation that plasma and urine TMAO measurements have a high intra-individual variation in diabetes is useful information, especially considering the current focus on using TMAO as a risk marker in disease. Despite the low reliability of single measurements of TMAO, it has still shown to be a strong predictor of health outcomes in disease, including diabetes.[98] Urine betaine and urine *myo*-inositol correlated strongly with plasma glucose and HbA_{1c}, showing that glycemic control has an effect on the urinary excretion of these osmolytes. Plasma *myo*-inositol was found to correlate strongly with markers of renal function, and homocysteine, a risk factor for cardiovascular disease. To determine whether osmolyte concentrations are predictive of outcomes, such as cardiovascular events or kidney failure in diabetes, would require the DEWL study participants to be identified and their medical records examined. However, ethics approval was not able to be obtained to do this. Proving the usefulness of osmolytes as diagnostic markers is beyond the scope of this project, and would require further research studies to relate osmolyte concentrations to health outcomes.

Betaine metabolism has previously been shown to be affected by fibrate therapy.[15] However, it has been shown here that only betaine and its metabolite,

DMG, were found to be elevated in the urine of people on fibrate therapy. The other osmolytes, *myo*-inositol, GPC, and taurine were not significantly elevated. This suggests that the mechanism causing betaine to be increased in the urine of people taking fibrates in unlikely to be related to the role of betaine as an osmolyte.

The observation that betaine concentrations in the urine were significantly higher in healthy people after they began treatment on fenofibrate is strong evidence that the relationship with fibrate is causative. It was previously unknown whether people who were on fibrates had elevated urine betaine because fibrates affect betaine metabolism, or simply that the people were prescribed fibrates because they had high blood lipids and less healthy. However, the observation that healthy people had a significant increase in urine betaine after starting on the drug fenofibrate is strong evidence that fibrates cause the loss of betaine from the body. The reason why fibrates cause people to lose betaine remains unknown, but it is possible that fibrates may affect the ability of betaine transporters such as BGT1 to function normally. This is the first study to show an association between a fibrate other than bezafibrate and increased urine betaine. Betaine was the only osmolyte that was significantly affected in the urine by fenofibrate, confirming the findings from the group from the DEWL study that were on bezafibrate.

No differences in osmolyte (or other metabolite) concentrations were observed between the two diets prescribed in the DEWL study. This may be because many of the DEWL study participants were not thought by the DEWL researchers to have followed dietary advice properly over the two years of the study.[146]

The presence of DMGO in plasma and urine was a novel finding that was confirmed by using 2 different chromatography systems, and 4 different mass transitions. These mass transitions were based on mass fragments that are expected from the structure of DMGO.

DMGO was found to be lower in the plasma of people with the metabolic syndrome and people with type 2 diabetes compared to healthy controls. Fractional clearances of DMGO, DMG, and betaine were significantly higher in the subjects with the metabolic syndrome due to their low plasma concentrations. The observation that DMGO has a moderately low fractional clearance (median = 15%) in healthy subjects suggests that it may be retained for further metabolism or has a specific purpose. Despite plasma DMGO concentrations being low (typically < 1 μmol/L), the correlations with DMG and betaine were highly significant. This is suggestive that

DMGO is a one-carbon metabolite that is formed from the oxidation of DMG. This will be confirmed by future studies.

These osmolytes and other metabolites were found to be significantly different in various blood components. White cells in particular appear to accumulate high concentrations of betaine, taurine, and *myo*-inositol. Blood enriched with white cells were considerably higher in *myo*-inositol and taurine compared to whole blood and plasma. Further investigation is required to identify what blood samples are best for indicating the tissue status for osmolyte and other metabolite concentrations. The measurement of osmolytes in blood cells is problematic because traditional techniques for isolating blood cells require the use of buffers that are likely to change the osmotic status of cells and therefore affect the intracellular osmolyte concentrations.

Further research

Most osmolytes and related metabolites could be reliably measured by LC-MS techniques. However, further work is required to measure sorbitol using LC-MS techniques. Separation of sorbitol from mannitol and galactitol is challenging, but may be possible using interactions with reagents such as boronic acids if practical problems can be over-come and the right analytical conditions can be achieved. The analysis of GPC would be more reliable if an isotopic standard was available. The detection of TMA in plasma was also challenging. Derivatization of TMA with alkyl halides was useful. However, the cross reaction of TMAO with alkyl halides to form the same derivative as TMA was problematic, especially considering the much lower concentrations of TMA present in plasma. If a reliable LC-MS method for TMA can be developed, it would allow for further research on how TMA relates to its metabolite, TMAO as risk marker in disease.[87b] A reliable method for TMA in plasma is required to determine whether or not it also has value as a risk marker.

There are still unresolved questions regarding the role that osmolytes play in health and disease. The accelerated loss of the osmolytes: betaine; *myo*-inositol; and GPC in people with type 2 diabetes of concern considering the importance of osmolytes in maintaining osmotic control for cells. However, the long term health effects of a chronic deficiency of these osmolytes are still poorly understood. Supplementing people who have diabetes with these osmolytes may prove to be a

useful therapy, and could potentially help to reduce the incidence of secondary complications associated with diabetes, such as circulation problems, eye problems, and renal and cardiovascular disease.

Two fibrate drugs (bezafibrate and fenofibrate) have been associated with increased urinary betaine excretion. However, it is not known if other fibrate drugs, such as gemfibrozil, have the same effect. If other fibrates do not increase betaine loss from the body, then they might be a better treatment option, particularly for people who have type 2 diabetes and are already at risk of betaine depletion.

The identification of *N*,*N*-dimethylglycine-*N*-oxide (DMGO) in plasma and urine raises a number of unanswered questions. It is likely, but not yet proven, that DMGO is formed by the oxidation of DMG. This could be shown experimentally using deuterium labelled isotopes in an animal model. This could also help to identify how DMGO is further metabolized. DMGO may be cleaved to form TMAO, it may be demethylated by either BHMT or DMGDH, or it may be converted back to DMG. The quaternary amine structure of DMGO suggests that it is more likely to be a substrate for BHMT than for DMGDH. However, the observation that *N*-hydroxy-*N*-methylglycine was undetectable in the plasma (and very low in the urine) suggests that demethylation is not a major fate for DMGO in the body. Once the metabolism of DMG to DMGO is confirmed *in vivo*, the enzyme responsible for the oxidation will need to be identified. Further work involving the measurement of DMGO in cohorts with metabolic syndrome, diabetes, and cardiovascular disease would help to identify its usefulness as a risk marker by monitoring the outcomes (such as cardiovascular events) for these people over time. While DMGO could not be measured in all of the DEWL study samples due to a time limit on the ethics approval, the biological variation of DMGO in people with diabetes and other populations still needs to be investigated. When measuring DMGO in future samples, it may be useful to also measure sarcosine, as they are both likely to be metabolites of DMG. A reliable method for the measurement of sarcosine still needs to be developed. The measurement of sarcosine is important as it is related to choline and betaine metabolism, and has been shown to also be a risk marker of diabetes.[61b] However, sarcosine lacks the sensitivity to be measured in the same run as betaine and the other methylamines, and there was not time to measure sarcosine by another method in this project. Ueland *et al*.[178] measured sarcosine by a GC-MS/MS method after derivatization, and it was measured along with a number of amino acids. They also

measured betaine, choline, and DMG and related metabolites on a different platform by LC-MS/MS.

Blood plasma may not be the best sample type for indicating the status of osmolytes in the tissues. For example, betaine is known to be higher in tissues than in the plasma,[168] and the kidneys have been shown to contain millimolar concentrations of betaine and other osmolytes.[32] Blood enriched with white cells has been shown here to contain betaine, *myo*-inositol, and taurine at significantly higher concentrations than in the plasma. Therefore osmolyte concentrations in white cells may prove to be a more useful indicator of tissue osmolyte concentrations. Purifying white cells without changing the osmolyte concentrations is likely to be difficult. However, it should be possible to relate the concentrations to cell counts in the samples to obtain an estimate of the concentrations within the individual cell types.

Conclusions

Liquid chromatography - mass spectrometry techniques have been shown to be suitable for the measurement of most osmolytes and related metabolites. The ability to measure the more analytically challenging osmolytes, such as *myo*-inositol, taurine, and TMAO, is considerably improved when using LC-MS/MS compared to traditional methods that involve derivatization followed by HPLC with UV or fluorescence detection. However, there are still unresolved problems that make the reliable measurement of some osmolytes difficult. These include finding suitable chromatography and mass spectrometry conditions to reliably measure sorbitol, and obtaining a more appropriate isotopic internal standard for GPC.

Betaine, other osmolytes, and other one-carbon metabolites were found to be affected by a range of factors in overweight people with type 2 diabetes, including glycemic control, renal function, and drug treatment. For example, the observed correlations of *myo*-inositol with other biological variables in diabetes suggest that it is a potentially useful risk marker. However, the longnitudinal studies that are required to relate *myo*-inositol concentrations to health outcomes are beyond the scope of this project.

This project has shown that betaine and the other one-carbon metabolite concentrations were consistent in people with type 2 diabetes over a 2 year period. The low intra-individual variability of betaine, DMG, and choline in people with type

2 diabetes adds credence as to the usefulness of single measurements of these metabolites as risk markers for health outcomes. Despite TMAO being reported to be an important risk marker for cardiovascular disease,[87b] the high intra-individual variability suggests that caution should be taken when considering the value of single TMAO measurements for a particular patient. TMAO concentrations can vary widely according to diet, and it is not retained by the kidneys. The relatively high intra-individual variation observed for the osmolytes GPC and taurine could be related to factors such as glycemic control, and indicates that caution should be taken when considering the diagnostic value of single measurements.

Because people who have type 2 diabetes and are taking fibrates lose excessive amounts of betaine in the urine, they are particularly likely to benefit from betaine supplementation, or by increasing the betaine content of their diet. Excessively high urinary betaine loss is likely to lead to a deficiency in tissue betaine concentrations, placing pressure on metabolic pathways to produce more betaine, and placing pressure on the body's choline reserves. Choline deficiency is in itself harmful and has been associated with liver disease.[179] Because the fenofibrate study has shown strong evidence that fibrates cause betaine to be lost from the body, the negative health consequences resulting from a potential betaine deficiency should be considered by clinicians before placing patients on these drugs to lower their blood lipids.

Because they accumulate osmolytes, white blood cells are likely to be more useful than plasma for representing the osmolyte status in tissues. However, obtaining pure white cells without changing the osmotic environment remains a challenge.

The use of LC-MS/MS has enabled the discovery of the previously unknown metabolite, DMGO, in human plasma and urine. This is a novel finding that, depending on the outcomes of future studies, may alter the currently accepted biochemical pathway for choline metabolism.

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