DEVELOPMENT OF LABORATORY TESTS FOR ASSESSING VITAMIN STATUS

Malina Kate Storer

A thesis submitted for the degree of Doctor of Philosophy at the University of Otago, Dunedin, New Zealand

Date: February 27, 2006
ABSTRACT

Aim
To develop assays for the determination of functional vitamin status and to determine the functional vitamin status of an elderly population. Specifically the focus has been to develop assays to measure the biological concentrations of \(N,N\)-dimethylglycine and glycine betaine as possible markers of functional folate status.

Background
Functional vitamin deficiencies arise when the tissue concentration of a vitamin derived coenzyme is inadequate this can occur despite normal concentrations of the vitamin in the blood or urine, and leads to lower activity of the vitamin dependent enzyme. The lowered enzyme activity causes changes in the concentrations of metabolites associated with the biochemical pathway catalysed by the enzyme. The amount of vitamin at a tissue level can be determined by measuring these changes. Functional vitamin deficiencies have been associated with many chronic diseases; the relationships between nutrients and disease can be investigated using appropriate assays.

Methods and results
Two new trifluromethanesulfonate reagents (2-phenanthrenacyl and 6-methoxynapthacryl) have been synthesised. These reagents form highly fluorescent derivatives with \(N,N\)-dimethylglycine, glycine betaine and propionylcarnitine. Using 2-phenanthrenacyl triflate as the derivatising reagent the detection limit for glycine betaine is improved from 0.2 µM to 0.04 µM. Optimisation is achieved by changing the solvent, base and water content of the reaction mixture. Polar aprotic solvents are used, with the presence of some water or alcohol tolerated. Suitable bases include the inorganic bases, magnesium hydroxide, silver oxide and lithium phosphate.

The cationic derivatives of \(N,N\)-dimethylglycine and glycine betaine in plasma are separated by HPLC on an alumina column within 50 minutes. Cation exchange HPLC is carried out using a polar organic solvent containing an aqueous buffer with an organic cation and a hydrophilic anion. Selectivity is affected by the choice of organic solvent and buffer. Increasing the water content and the buffer concentration reduces the retention of the derivatives. Propionylcarnitine can be quantified after separation by HPLC on a
non-endcapped strong cation exchange column however the use of this assay to detect functional biotin deficiency has not been validated.

$^{1}$H NMR can be used to measure $N,N$-dimethylglycine and glycine betaine in urine. The inter and intra-assay CV’s were < 10% and recoveries were ≥ 97% over a linear range from 50 µM to 1000 µM. Limits of detection using $^{1}$H NMR spectroscopy (15 – 25 µM) are higher than HPLC assays, though adequate for the detection of raised concentrations in urine.

Elderly hip fracture patients (aged 65-90) were investigated, as they are known to have poor nutrition compared to health elderly, and would be expected to have associated vitamin deficiencies. A greater percentage of hip fracture patients had insufficient vitamin B12 and folate concentrations compared to age matched healthy elderly controls (Folate 55% and 32% <120 pM, Vitamin B12 7% and 5% <8.5 nM). The results for other analytes are difficult to interpret because of the affects of recent trauma. In the control population glycine betaine predicts total homocysteine concentrations (multiple linear regression –0.055 P = 0.099) and is a stronger predictor of folate than total homocysteine. The ratio of the concentrations of $N,N$-dimethylglycine/glycine betaine was not significantly associated with folate status.

**Conclusion**

The concentrations of $N,N$-dimethylglycine and glycine betaine in plasma can be measured by HPLC, and in urine by $^{1}$H NMR. In healthy elderly there is a high prevalence of vitamin deficiency. The ratio of $N,N$-dimethylglycine/glycine betaine is not an appropriate marker of functional folate status, however the associations between glycine betaine and homocysteine metabolism require further investigation.


ACKNOWLEDGEMENTS

The work described in this thesis would not have been accomplished without guidance, support and encouragement from a large number of people. In particular I owe a huge thanks to my supervisors; Dr Alan Happer for advice on all things chemistry, Professor Peter George for supporting my research at Canterbury Health Laboratories and his clinical biochemistry expertise, Professor Stephen Chambers for his enthusiasm regarding the clinical study and Dr Michael Lever for being a wealth of information, guidance and advice regarding all aspects of this project. You have all had a huge part to play in this accomplishment and I am extremely grateful.

I would like to thank John Lewis and Suman Mishra in the Steroid Laboratory at Canterbury Health Laboratories for the biotin assay development, Tim Wilkinson for consultation during the initial conception of the hip fracture study and Elisabeth Wells for invaluable statistical advice. Phil Zakaria, Paul Haddad and all of the team at the Australian Centre for Research on Separation Science, deserve thanks for helping me come to terms with capillary electrophoresis. My thanks to the “Men of Steel” for their assistance during my time in the Chemistry department. I would also like to thank Steve Brennan for his assistance with mass spectrometry and Martin for his NMR spectroscopy expertise.

I am grateful to the willing patients and volunteers who participated in the clinical study. Additionally I would like to thank the blood collection staff and the staff of wards 18 and 19 at Christchurch Hospital for their cooperation. I am indebted to Chris Budgen, Lesney Stuart, Charles Hawes, Sue Carnoutsos, Linda Pike and the other staff in the respective sections at Canterbury Health Laboratories for many of the analyses performed on the samples.

Financial assistance is invaluable in an undertaking such as this and I am grateful to Canterbury Health Laboratories, the Foundation for Research Science and Technology, and the Todd Foundation for their contributions.

Many thanks to the people that have put up with me every day in the lab; Sarah M for tolerating me in the confined space of our office at all times but especially during the stressful writing-up process, Chris M for keeping the place running, Wendy, Madhu, Martin, and Sandy S for the laughs and the moral support: it’s been a pleasure to work with you all. Sandy W, Chris S, Julia and Linda thanks for sharing your lab space. To my summer students Sarah
L, Catherine, and Ange thank you for your great work and entertainment over the summer months.

Thanks to the people who helped me stay sane; Marie, Sarah L, and Anna for understanding exactly what I was going through, along with Kate and Jasmine for distracting me, entertaining me, planning with me and counseling me. My thanks to Matt for putting up with me, and Michaela, and everyone else who’s flatted with me for their support.

My biggest thanks and all my love to my family; to Heath and Briar for your support, even if you didn’t think I was ever going to finish University and to my parents - thanks for having me, for your unwavering support, encouragement and love. I couldn’t have done this without you.

In closing I would like to thank the anonymous author of my email quote… thank you for keeping it all in perspective!

"Life should NOT be a journey to the grave with the intention of arriving safely in an attractive and well-preserved body, but rather to skid in sideways, Champagne in one hand, strawberries in the other, body thoroughly used up, totally worn out & screaming 'WOO HOO - What a ride!'"
### TABLE OF CONTENTS

Abstract ................................................................................................................................. ii
Publications arising from this thesis ........................................................................................ iv
Acknowledgements ................................................................................................................... v
Table of contents ...................................................................................................................... vii
Table of illustrations ................................................................................................................. xii
List of abbreviations ................................................................................................................. xvii

1 AN OVERVIEW OF VITAMIN DEFICIENCIES AND METHODS FOR THE
DETERMINATION OF VITAMIN STATUS ........................................................................... 2
1.1 History and significance of vitamins ............................................................................... 2
  1.1.1 The history of vitamins ............................................................................................... 4
1.2 Causes of vitamin deficiency .......................................................................................... 7
1.3 Functional vitamin deficiencies and the consequences .................................................... 9
  1.3.1 Vitamins and the risk of cardiovascular disease ......................................................... 9
  1.3.2 Vitamins concentrations and cancer risk ................................................................. 12
  1.3.3 Vitamin status and the connection with pregnancy complications ....................... 13
  1.3.4 The influence of vitamins status on neurological disorders ................................... 14
  1.3.5 Vitamins and the risk of hip fracture ....................................................................... 15
1.4 Determining vitamin status .......................................................................................... 16
  1.4.1 Determining thiamine status ................................................................................... 20
  1.4.2 Measuring biotin concentrations in biological samples .......................................... 23
  1.4.3 Measuring folate concentrations in biological samples ......................................... 26
  1.4.4 Determining vitamin B\textsubscript{12} concentrations ................................................ 29
  1.4.5 Correcting vitamin deficiencies .............................................................................. 33
1.5 Aims of this work ........................................................................................................... 34
1.6 References for chapter one ............................................................................................. 35
3.3.9 Precision and accuracy studies ................................................................. 100
3.4 Discussion .................................................................................................. 101
3.5 Experimental ............................................................................................. 102
3.6 References for chapter three ...................................................................... 104

4 VALIDATION OF A ¹H NMR ASSAY FOR GLYCINE BETAINE AND N,N-DIMETHYLGLYCINE IN URINE ................................................................. 108
4.1 Use of ¹H NMR spectroscopy to determine biological concentrations of betaines ...... 108
4.2 Validation of an ¹H NMR assay for glycine betaine and N,N-dimethylglycine ....... 110
4.2.1 Methods .................................................................................................. 110
4.3 Results ....................................................................................................... 111
4.3.1 Precision study ..................................................................................... 112
4.3.2 Accuracy ............................................................................................... 112
4.3.3 Linearity ............................................................................................... 113
4.4 Discussion .................................................................................................. 114
4.5 Experimental ............................................................................................. 116
4.6 References for chapter four ........................................................................ 117

5 DEVELOPMENT OF CAPILLARY ELECTROPHORESIS ASSAYS FOR BETAINES, AND THIAMINE SPECIES IN BIOLOGICAL SAMPLES .............. 119
5.1 Introduction to capillary electrophoresis ........................................................ 119
5.1.1 Detection methods used in capillary electrophoresis assays ..................... 120
5.1.2 Capillary specifications ......................................................................... 121
5.1.3 Calculating analyte mobility..................................................................... 121
5.1.4 Selection of running voltage ................................................................... 122
5.1.5 Background electrolyte choice ................................................................. 122
5.1.6 Capillary coatings .................................................................................. 123
5.1.7 The use of additives .............................................................................. 123
5.1.8 Micellar electrokinetic chromatography .................................................. 124
5.2 Previous use of capillary electrophoresis for the separation of betaines ........... 126
5.3 Separation of betaines using capillary electrophoresis .................................. 128
5.3.1 Samples ................................................................................................. 128
5.3.2 Selection of coating for the capillary ....................................................... 129
5.3.3 Choice of buffer type and pH ................................................................. 131
5.3.4 Use of cyclodextrins ............................................................................. 133
7 COMPARISON OF FUNCTIONAL VITAMIN STATUS IN ELDERLY NEW ZEALANDERS WITH AND WITHOUT HIP FRACTURE

7.1 Vitamin deficiency in the elderly

7.2 Vitamin status of elderly hip fracture patients

7.3 Results

7.4 Discussion

7.5 Conclusions

7.6 Experimental

7.7 References for chapter seven

8 CONCLUSIONS AND FUTURE WORK
Figure 2.8 HPLC trace showing multiple N,N-dimethylglycine peaks in dimethoxyethane
.............................................................................................................................. 61
Table 2.7 Solvents used for derivatisation with triflate reagents................................. 62
Figure 2.9 HPLC trace showing derivatisation with alternative base.......................... 63
Figure 2.10 HPLC trace showing peaks resulting from impurities from resin bases ...... 63
Table 2.8 Relative efficiency of derivatisation with different bases ............................. 64
Table 2.9 Limits of detection .................................................................................... 66
Table 2.10 Best derivatisation conditions .................................................................. 67

3 OPTIMISATION OF AN HPLC ASSAY FOR THE SEPARATION OF GLYCINE
BETaine AND N,N-DIMETHYLGLYCINE IN PLASMA ...................................... 79
Figure 3.1 Structures of betaines and related compounds ......................................... 79
Figure 3.2 Homocysteine remethylation pathways ...................................................... 82
Table 3.1 Biological concentrations of glycine betaine and N,N-dimethylglycine in
plasma and urine ..................................................................................................... 87
Table 3.2 Limits of detection of common assays for glycine betaine and
N,N-dimethylglycine .............................................................................................. 88
Figure 3.1 Separation of betaine 2-naphthacyl ester derivatives by HPLC using four
different columns .................................................................................................... 92
Table 3.1 Choice of column ....................................................................................... 93
Table 3.2 Choice of base ........................................................................................... 95
Table 3.3 Effect of salt concentration in mobile phase ............................................... 96
Table 3.4 Effect of water in mobile phase .................................................................. 97
Figure 3.2 HPLC traces of separation of glycine betaine and N,N-dimethylglycine in
plasma with Alumina and strong cation exchange Phenosphere columns .............. 98
Table 3.5 Conditions for separation of glycine betaine and N,N-dimethylglycine in plasma
.................................................................................................................................. 99
Table 3.6 Linearity of the assay ................................................................................ 99
Table 3.7 Results of the precision studies ................................................................. 100
Table 7.11 Predictors of $N,N'$-dimethylglycine/glycine betaine ratio in control and #NOF population

...
LIST OF ABBREVIATIONS

#NOF fractured neck of femur

$^1$H NMR proton nuclear magnetic resonance

°C degrees celsius

CE capillary electrophoresis

EDTA $N,N,N,N$-ethylenediamine tetraacetic acid

ELISA enzyme linked immunosorbent assay

ESI MS/MS electrospray ionisation tandem mass spectrometry

GC-MS gas chromatography mass spectrometry

HIV human immunodeficiency virus

HPLC high pressure liquid chromatography

hr. hour

LC-MS/MS liquid chromatography tandem mass spectrometry

Lihep lithium heparin

M molL$^{-1}$

min minutes

NAD$^+$ nicotinamide adenine dinucleotide

NADH reduced nicotinaminde adenine dinucleotide

NMR nuclear magnetic resonance

pK$\alpha$ log of the acid dissociation constant

pH negative log of the hydrogen ion concentration

r correlation coefficient

RNA ribonucleic acid

SD standard deviation

UV ultra violet

v/v volume to volume

$\lambda$ wavelength