THE ROLE OF FOLATE STATUS IN FORMATE METABOLISM AND ITS RELATIONSHIP TO ANTIOXIDANT CAPACITY DURING ALCOHOL INTOXICATION.

A Thesis Submitted to the College of Graduate Studies and Research in Partial Fulfillment of the Requirements For the Degree of Doctor of Philosophy in the Toxicology Graduate Program University of Saskatchewan, Saskatoon, SK, Canada.

AbdulRazaq Abubakar Hamud Sokoro

PERMISSION TO USE

In presenting this thesis in partial fulfillment of the requirements for a postgraduate degree from the University of Saskatchewan, I agree that the Libraries of this university may make it freely available for inspection. I further agree that permission for copying of this thesis in any manner, in whole or in part, for scholarly purposes may be granted by the professor or professors who supervise my thesis work or, in their absence, by the head of the department or the Dean of the College in which my thesis work was done. It is understood that any copying or publication or use of this thesis or parts thereof for financial gain shall not be allowed without my written permission. It is also understood that due recognition shall be given to me and to the University of Saskatchewan in any scholarly use which may be made of any material in my thesis.

Requests for permission to copy or to make other use of material in this thesis in whole or part should be addressed to:

Chair of the Toxicology Group

Toxicology Centre

University of Saskatchewan

Saskatoon, Saskatchewan, S7N 5B3

ABSTRACT

Alcohol abuse during pregnancy has been associated with Fetal Alcohol Spectrum Disorder (FASD). Research to date has focused on the role played by ethanol in the development of this disorder. In addition to ethanol, alcoholic drinks also contain methanol. Hence, consumption of alcohol can also lead to methanol accumulation. Methanol is metabolized to formaldehyde, which is then rapidly metabolized to formate, a toxic metabolite. Folate, a B-vitamin and antoxidant, is a cofactor in the metabolism of formate. This study assessed the relationship between formate and folate, formate kinetics in folate deficiency and, changes in antioxidant capacity during formate insult in folate deficiency. The findings of this study would lead to a better understanding of the role of formate in the development of the etiology of FASD and form the basis of future research.

The relationship between formate and folate was investigated in intoxicated human female subjects, sober drug rehabilitating females and, pregnant women. A negative (inverse) relationship was observed between plasma formate and folate in pregnant sober women (correlation coefficient = -0.4989). Such a relationship, however, was not observed in whole blood in alcohol intoxicated (correlation coefficient = 0.0899) and detox women (correlation coefficient = 0.2382). Because of the health promoting ingredients in grain and fruit based alcoholic drinks, antioxidant B-vitamins were higher during intoxication while homocysteine levels were lower.

Formate kinetics during folate deficiency and changes in the body antioxidant capacity was investigated in folate deficient young swine. Folate deficiency altered formate kinetics leading to decreased systemic clearance (by

approximately 2.3 fold), increased half-life (by 2.5 fold) and, consequently increased exposure (by 2.7 fold). Folate deficiency alone compromised antioxidant capacity. However, the combination of folate deficiency and formate insult further compromised antioxidant capacity.

In conclusion, methanol accumulates after alcohol intoxication, which can lead to formate build up in the body. During folate deficiency formate kinetics is altered leading to reduced formate clearance and increased exposure. Exposure to formate coupled to folate deficiency compromises antioxidant capacity, which can have deleterious effects on the fetus.

ACKNOWLEDGEMENTS

First and foremost, I wish to thank my supervisor, Dr. Denis C. Lehotay, for what has been a wonderful opportunity and grand adventure in the field of Clinical Biochemistry and Analytical Toxicology. Thank you for being my mentor and guide. I would like to express my gratitude to my advisory committee, Dr. Barry Blakley, Dr. Jane Alcorn, Dr. Gordon Zello, and Dr. Ed Krol for their invaluable contribution to this research during the course of my degree.

While I am grateful to a variety of people who have in some way contributed towards my research during my studies at the Saskatchewan Disease Control Laboratory, special thanks go to Jeff Eichhorst, Manager of Screening and Reference Testing Section, and Michele Etter, Toxicology Research Supervisor, for the opportunity to develope skills in Analytical Chemistry and Mass Spectrometry and, always had time to mentor me in these techniques no matter how many times I broke the instruments! A special thank you goes to Dr. James House and his group of the Department of Animal Science, University of Manitoba for providing the folate deficient pigs, hosting the experiments, and us and helping with the animal experiments. Particular thanks to Dr. Gordon Zello for, not only accompanying me to the experiments, but also helping with the actual animal experiments.

Finally, I would like to thank my mother and close friends for their patience, support and encouragement. A special thank you goes out to my wife, Fatima, for her patience, love, and moral support over the years. Without the support of my wife and children, I could not and would not have made it.

TABLE OF CONTENTS

Permission to use ..... **i**

Abstract ..... **ii**

Acknowledgements ..... **iv**

Table of Contents ..... **v**

List of Tables ..... **xi**

List of Figures ..... **xiii**

List of Abbreviations ..... **xviii**

Preface ..... **xx**

CHAPTER 1

Introduction 1

1.1 Alcohol Consumption and Abuse 1

1.1.1 Types of Alcoholic Beverages and their various alcohol contents 2

1.1.2 Definition of binge drinking 5

1.1.3 Prevalence of alcohol consumption 7

1.1.5 Social/Cultural Values and Alcohol Consumption 9

1.1.6 Biochemistry and Clinical Toxicology of Alcohol Consumption 10

1.1.6.1 Ethanol and Acetaldehyde 10

1.6.3.1 Methanol and Formate 11

1.2 Effects of Alcohol Abuse: Fetal Alcohol Spectrum Disorder (FASD) 13

1.2.1 Definition of Fetal Alcohol Spectrum Disorder 13

1.2.2 Prevalence of Fetal Alcohol Spectrum Disorder 14

1.2.3 Fetal Alcohol Spectrum Disorder and Binge Drinking....16
1.2.4 Biochemical Aspects of Fetal Alcohol Spectrum Disorder....17
1.2.5 Clinical Diagnosis and Treatment....19
1.3 Role of Vitamins and Other Cofactors in Formate Metabolism....22
1.3.1 Folic Acid....22
1.3.2 Vitamin B6....29
1.3.3 Vitamin B12 and Methylmalonic Acid....32
1.3.4 Homocysteine....38
1.3.5 Methionine, S-adenosylmethionine and S-Adenosylhomocysteine....40
1.3.6 Methylglyoxal....42
1.4 Role of Free Radicals and Antioxidant Capacity....43
1.5 Synopsis....47
1.6 Thesis Hypothesis and Objectives....49
1.6.1 Overall Hypothesis....49
1.6.2 Hypothesis regarding the relationship between formate and folate levels 49
1.6.2.1 Specific Objectives....50
1.6.3 Hypothesis regarding relationship between formate pharmacokinetics and folate status....50
1.6.3.1 Specific Objectives....50
1.6.4 Hypothesis regarding relationship between formate pharmacokinetics and Antioxidant capacity in folate deficiency state....51
1.6.4.1 Specific Objective....51
CHAPTER 2
Quantitative Endogenous Formate Analysis in Plasma and Whole Blood Using Headspace Gas Chromatography without Head Space Analyzer....52
2.1 Introduction....52

2.2 Experimental 53

2.2.1 Materials 53

2.2.2 Analysis of clinical sample and Recovery experiments 54

2.2.3 Sample Preparation 54

2.2.4 Instrumentation 55

2.3 Results 56

2.3.1 Linearity 57

2.3.2 Precision and Recovery 59

2.3.3 Clinical Samples 61

2.4 Discussion and Conclusion 62

CHAPTER 3

A Simple Method for the Quantitative Analysis of Endogenous Folate Catabolites P-aminobenzoylglutamate(p-ABG) and Its Acetamido (ap-ABG) Derivative in Biological Samples by Liquid Chromatography-Tandem Mass Spectrometry 64

3.1 Introduction 64

3.2 Experimental 68

3.2.1 Materials and Methods 68

3.2.2 Recovery experiments 69

3.2.3 Analysis of clinical samples 69

3.2.4 Instrumentation 70

3.3 Results 72

3.3.1 Linearity 80

3.3.2 Precision and Recovery 81

3.3.3 Clinical Samples 83

3.4 Discussion and Conclusions 86

CHAPTER 4

Relationship between Formate and RBC Folate Levels in Alcohol Intoxicated Females 89

4.1 Introduction 89

4.2 Experimental 92

4.2.1 Chemicals 92

4.2.2 Subject Recruitment 92

4.2.3 Formate Analysis 93

4.2.4 Instrumentation and Data Analysis 93

4.2.5 Ethanol and Methanol Analysis 94

4.2.6 Plasma and Red Blood Cell (RBC) Folate 95

4.2.7 Homocysteine (Hcy) 96

4.2.1 Vitamins B6 & B12 96

4.2.9 Data Analysis 97

4.3 Results 98

4.4 Discussion and Conclusion 101

CHAPTER 5

Formate Pharmacokinetics During Formate Insult in Folate Deficient Young Swine. 105

5.1 Introduction 105

5.2 Experimental 107

5.2.1 Chemicals 107

5.2.2 Animals and Husbandry 107

5.2.3 Formate Formulation and Administration 108

5.2.4 Formate Analysis 109

5.3 Results 109

5.4 Discussion and Conclusions 113

CHAPTER 6

Folate Utilization During Formate Insult in Folate Deficient Pigs 119

6.1 Introduction 119

6.2 Experimental 121

6.2.1 Animals and Husbandry 121

6.2.2 Formate Formulation and Administration 121

6.2.3 Formate Analysis 121

6.2.4 Folate utilization 122

6.2.5 Plasma and Red blood cell (RBC) Folate 122

6.2.6 Methionine 122

6.2.7 Homocysteine (Hcy) 123

6.2.8 Vitamins B6 & B12 123

6.2.9 Methylmalonic Acid (MMA) 124

6.2.10 *S*-adenosylmethionine (SAM) & *S*-adenosylhomocysteine(SAH) 125

6.2.11 Methylglyoxal 126

6.2.12 Glutathione (GSH) and Glutathione Disulphide (GSSG) 127

6.2.13 Data Analysis 129

6.3 Results 129

6.4 Discussion and Conclusion 137

CHAPTER 7

Discussion and Concusions 142

7.1 Background 142

7.2 Human Experiments 143
7.3 Pig Experiments 145
7.4 Conclusions 146
CHAPTER 8
References 148

LIST OF TABLES

Table 1.1 Types of alcoholic beverages and their alcohol contents 4

Table 1.2 Methanol and ethanol levels following consumption of 1L of beverage 5

Table 1.3 Diagnostic criteria for fetal alcohol spectrum disorder (FASD) 21

Table 1.4 Suggested mechanisms for the promotion of oxidative stress by ethanol 46

Table 2.1 Parameter values for the gas chromatograph 56

Table 2.2 Relationship between mean and CV of various aqueous standards 59

Table 2.3 Recovery and precision of aqueous spiked plasma samples 60

Table 2.4 Summary Statistics of plasma formate in pregnant women (n = 69) 61

Table 2.5 Mean and natural log plasma formate concentrations in young pigs (n = 4) administered 237 mg/kg formate by bolus injection through left jugular vein. 62

Table 3.1 Turbo ion source parameters in the tandem mass spectrometer (MS/MS) for the analysis of p-aminobenzoylglutamate (*p*-ABG), ap-acetamidobenzoylglutamate (*ap*-ABG) and d_3-glutamic acid 72

Table 3.2 Recovery and precision results of p-aminobenzoylglutamate (*p*-ABG) and ap-acetamidobenzoylglutamate (*ap*-ABG) in spiked serum and urine samples 82

Table 4.1 Parameter values for analysis of methylformate in the gas chromatograph 94

Table 4.2 Results of various analytes in alcohol intoxicated and detox rehabilitation females 98

Table 4.3 Results of various analytes in pregnant females (n = 69) 99

Table 4.4 Amount of B-vitamins present in 1 L of pilsner beer (pilsner urquel) 103

Table 5.1 Pharmacokinetic parameter estimates of formate in folate deficient (FFD) & folate control (FFC) male pigs 113

Table 5.2 Levels of hepatic folate intermediates in various species and subspecies 114

Table 5.3 Formate elimination half-life in various species 117

Table 6.1 Source parameters for analysis of glutathione (GSH) and glutathione disulphide (GSSG) in the tandem mass spectrometer (MS/MS) 129

Table 6.2 Summary of various analytes during formate insult in pigs (n = 4/group) 131

LIST OF FIGURES

Figure 1.1 The harmful effects of alcohol consumption and folate deficiency 7

Figure 1.2 Metabolic pathway of methanol 12

Figure 1.3 Mechanism of action of the deleterious effects of alcohol in the developing fetus 18

Figure 1.4 Deprotonated molecule of folic acid 22

Figure 1.5 Overview of one-carbon metabolism and transsulfuration pathways 23

Figure 1.6 An overview of one-carbon metabolism and transsulfuration pathways 25

Figure 1.7 Structures of different forms of vitamin B6 29

Figure 1.8 A simplified overview of vitamin B-6 metabolism and transport 31

Figure 1.9 Structure of cobalamin (vitamin B-12) 34

Figure 1.10 Digestion, absorption and transport of vitamin B-12 35

Figure 1.11 Metabolic pathway of adenosylcobalamin (AdenosylB12) and methylcobalamin (MethylB12) 36

Figure 1.12 Structure of the amino acid methionine 40

Figure 1.13 Metabolic pathway of methionine and its utilization in the synthesis of cysteine 41

Figure 1.4 Reactions of some antioxidant enzymes 44

Figure 1.15 The generation of free radicals through the oxidation of formate 47

Figure 2.1 Chromatographic spectrum of 1000 μL headspace of 1 mM aqeous standard of ethylformate 56

Figure 2.2 Standard curve of low aqueous calibrators of formic acid run on Hewlett Packard Series II 5890 fitted with a DB-ALC1 capillary column (30 m x 0.53 mm ID, 3.0 μm) and Flame ionization detector using a gastight syringe..... 58

Figure 2.3 Standard curve of high aqueous calibrators of formic acid run on Hewlett Packard Series II 5890 fitted with a DB-ALC1 capillary column (30 m x 0.53 mm ID, 3.0 μm) and Flame ionization detector using a gastight syringe..... 58

Figure 2.4 Precision profile of various aqueous formate concentrations injected into Hewlett Packard Series II 5890 fitted with a DB-ALC1 capillary column (30 m x 0.53 mm ID, 3.0 μm) and flame ionization detector using a gastight syringe. 60

Figure 2.5 A Rectilinear plot of plasma formate vs time plot in young pigs (n = 4) administered 237mg/kg formate by bolus injection through left jugular vein.. 61

Figure 3.1 The chemical structure of folic acid and the most important natural folates.... 66

Figure 3.2 The catabolism of folic acid in the body..... 67

Figure 3.3 Chemical structure of 2,4,4-d3-glutamic acid..... 68

Figure 3.4 Product ion scan (Q3) of 1 mM aqueous folate catabolite p-aminobenzoylglutamate, *p*-ABG (m/z = 267) in the Q3 compartment of the mass spectrometer..... 73

Figure 3.5 Product ion scan (Q3) of 1 mM aqueous folate catabolite ap-acetamidobenzoylglutamate, *ap*-ABG (m/z = 309) in the Q3 compartment of the mass spectrometer..... 74

Figure 3.6 Proposed positive ion fragmentation of d3-glutamic acid (d_3-Glu) in the Q3 compartment of the mass spectrometer..... 75

Figure 3.7 Chromatographic spectrum of p-aminobenzoylglutamate (*p*-ABG), ap-acetamidobenzoylglutamate (*ap*-ABG) and d3-glutamic acid in 100 nM spiked pooled serum..... 75

Figure 3.8 Chromatographic spectrum of p-aminobenzoylglutamate (*p*-ABG), ap-acetamidobenzoylglutamate (*ap*-ABG) and d3-glutamic acid in unspiked pooled serum. 76

Figure 3.9 Chromatographic spectrum of p-aminobenzoylglutamate (*p*-ABG), ap-acetamidobenzoylglutamate (*ap*-ABG) and d3-glutamic acid in 100 μM spiked pooled urine 77

Figure 3.10 Chromatographic spectrum of p-aminobenzoylglutamate (*p*-ABG), ap-acetamidobenzoylglutamate (*ap*-ABG) and d3-glutamic acid in pooled unspiked urine 78

Figure 3.11 Standard curve of aqueous calibrators of p-aminobenzoylglutamate (*p*ABG) transition (267>120) 79

Figure 3.12 Standard curve of aqueous calibrators of ap-acetamidobenzoylglutamate (*ap-ABG*) transition (309>162) 80

Figure 3.13 Box plot of serum folate levels (mean ± SD) in PTH female patients (n = 11), pregnant (prenatal, n = 18) women and lab female volunteers (n = 5) 83

Figure 3.14 Box plot of serum folate catabolites p-aminobenzoylglutamate (*p*-ABG) and ap-acetamidobenzoylglutamate, (*ap*-ABG) (mean ± SD) in PTH female patients (n = 11), pregnant (prenatal, n = 18) women and lab female volunteers (n = 5) 84

Figure 3.15 Box plot of total urinary folate catabolites p-aminobenzoylglutamate (*p*-ABG) and ap-acetamidobenzoylglutamate (*ap*-ABG) (mean ± SD) in catecholamine-tested female patients (n = 19) and lab female volunteers (n = 5) 85

Figure 4.1 An overview of the folate pathway 91

Figure 4.2 Linear regression plot of natural logarithmic of blood formate vs RBC folate levels in alcohol intoxicated females (Age < 45 years, n = 28 after removal of outliers) 100

Figure 4.3 Linear regression plot of natural logarithmic of blood formate vs RBC folate levels sober females in Detox program (Age < 45 years, n = 15 after removal of outliers) 100

Figure 4.4 Linear regression plot of natural logarithmic of plasma formate vs plasma folate levels sober pregnant females (n = 59 after removal of outliers) 101

Figure 5.1 Box plot of log mean plasma formate levels at the end of infusion in male pigs (n = 12) after infusion of 351mg/kg sodium formate (237 mg/kg formate; n= 4/group) and physiological saline (111 – 130 mL; n = 2/group) 110

Figure 5.2 Semilogrithmic plot of plasma formate (mean ± SE) concentration (mM) vs time of folate deficient (FFD) and folate control (FFC) male pigs (n = 4; 31 - 38 kg) 112

Figure 6.1 Box plot of mean plasma formate levels before (baseline) and at end of a short intravenous infusion (10 min) in male pigs (n=12) of 351 mg/kg sodium formate (237 mg/kg formate; n=4/group) or saline (111-130 mL solution; n=2/group) 132

Figure 6.2 Box plot of mean plasma homocysteine levels in folate control group (FFC) and folate deficicent (FFD) before and after a short intravenous infusion (10 minutes) in male pigs (n = 4) of 351 mg/kg sodium formate (237 mg/kg formate) 133

Figure 6.3 Box plot of mean plasma methylglyoxal levels in folate control group (FFC) and folate deficicent (FFD) before and after a short intravenous infusion (10 minutes) in male pigs (n=4) of 351 mg/kg sodium formate (237 mg/kg formate) 134

Figure 6.4 Box plot of mean plasma SAM to SAH ratio in folate control group (FFC) and folate deficicent (FFD) before and after a short intravenous infusion (10 minutes) in male pigs (n=4) of 351 mg/kg sodium formate (237 mg/kg formate) 135

Figure 6.5 Box plot of mean plasma folate catabolites (*p*-ABG & *ap*-ABG) levels in folate control group (FFD) before and after a short intravenous infusion (10 minutes) in male pigs (n=4) of 351 mg/kg sodium formate (237 mg/kg formate)..... 136

Figure 6.6 Box plot of mean plasma folate catabolites (*p*-ABG & *ap*-ABG) levels in folate deficient group (FFD) before and after a short intravenous infusion (10 minutes) in male pigs (n=4) of 351 mg/kg sodium formate (237 mg/kg formate)..... 137

LIST OF ABBREVIATIONS

***p*-ABG**	p-aminobenzoylglutamate
***ap*-ABG**	ap-acetamidobenzoylglutamate
ARBD	Alcohol-Related Birth Defects
ARND	Alcohol-Related Neurodevelopmental Disorder
AUC	Area under the curve
BAC	Blood alcohol content
Cend-inf	Concentration at end of infusion
Cl_s	Systemic clearance
EtOH	Ethanol
FAE	Fetal Alcohol Effect
FASD	Fetal Alcohol Spectrum Disorder
FFC	Folate Control Diet
FFD	Folate Deficient Diet
GSH	Glutathione
GSSG	Glutathione Disulphide
Hcy	Homocysteine
K	Elimination rate constant
MeOH	Methanol
MG	Methylglyoxal

MMA	Methylmalonic acid
MRT	Mean residence time
MTHFR	Methyltetrahydrofolate reductase
MS	Methionine synthase
PTH	Parathyroid Hormone
PLP	Pyridoxal 5'-phosphate
SAH	*S*-adenosylhomocyateine
SAM	*S*-adenosylmethionine
SHMT	*S*-hydroxymethyltransferase
SRM	Selective reaction monitoring
$\mathbf{t_{1/2}}$	Elimination half-life
THBQ	Tetrahydro-beta-carboline
THF	Tetrahydrofolate
TIQ	Tetrahydroisoquinoline
$\mathbf{V_{ss}}$	Volume of distribution under steady state

PREFACE

This thesis describes the effects of folate deficiency on formate kinetics during intoxication and its relationship to antioxidant capacity in folate deficient young swine and human females as well as the methodologies used. Currently, two chapters have been published (Chapters 2 and 3), two have been submitted for publication in scientific journals (Chapter 5 and 6) and, two others (Chapters 1 and 4) are in the process of submission.

Chapter 1 reviews background information, hypotheses, and objectives. Chapters 2 and 3 describe the development of methods of analysis of formate and folate catabolites p-aminobenzoylglutamate and ap-acetamidobenzoylglutamate for use in formate levels and folate utilization, respectively. Chapter 4 describes methanol and formate levels in alcohol intoxicated females, sober drug rehabilitating females and, preganant women (formate levels only). It also describes the relationship between formate and folate in these subjects as well as homocysteine (Hcy) and B-vitamin (B6 and B12) status. Chapters 5 and 6 describe the kinetics of formate and folate utilization during formate insult in young swine, respectively. Chapter 7 discusses the results of entire thesis study and the conclusions derived therein.

CHAPTER 1

Introduction

1.1 Alcohol Consumption and Abuse

The World Health Organization (WHO) estimates that there are about 2 billion people worldwide who consume alcoholic beverages and 76.3 million with diagnosable alcohol use disorders (World Health Organization, 2004). The 1998-99 National Population Health Survey (Statistics Canada, 2002) found that 78% of Canadians 15 years and older had consumed alcohol in the past year, 12.6% were former drinkers, and 9.5% were abstainers. Since the 1980s rates of past-year use had generally declined. Men, younger people and those with higher incomes and education had the highest rates of drinking. Rates of alcohol use also varied among the provinces, from a low of 59% in Newfoundland and Labrador to a high of 78% in Alberta. The 2000-01 Canadian Community Health Survey (Statistics Canada, 2003) reports a significant gender difference in the prevalence of heavy drinking (i.e., drinking 5 or more drinks per occasion 12 or more times in the past year), with 29.0% of current male drinkers 15 years and over, and only 11.4% of their female counterparts drinking to this extent. The rates of past-year use among high school students in Canada ranged from 48% to 65%, with rates increasing considerably with age (e.g., 80% in grade 12 in Ontario). There is evidence that rates of binge drinking have increased among high school and university students through the past decade. It was estimated that 4% of Canadians were alcohol-

dependent in 1994. Among Canadians 16 years and older who were current drinkers, 10% (about 1.5 million Canadians) had driven a vehicle after consuming "too much alcohol" by their own account.

Alcohol consumption during pregnancy has been associated with a variety of disorders in the developing fetus. These disorders consist of a wide spectrum of effects ranging from mild to extreme manifestations known as Fetal Alcohol Spectrum Disorder (FASD). Smith and Jones (Jones & Smith, 1973) first described the distinct dysmorphic condition associated with maternal alcohol abuse during pregnancy now known as FASD. Fetal alcohol spectrum disorder consists of a pattern of anomalies occurring in the babies born to drinking women and consists of (a) pre- and/or postnatal growth retardation, (b) morphological anomalies, and (c) central nervous system (CNS) dysfunction (1998b; Hannigan *et. al.*, 1992; Rosett, 1980; Streissguth, 1986; Hannigan *et. al.*, 1992).

1.1.1 Types of Alcoholic Beverages and Their Various Alcohol Contents

The alcoholic beverage market includes the three traditional forms of alcoholic beverages – beer, wine, and distilled spirits as well as a more recent fourth category called flavored alcoholic beverages (FABs), which is a cross between alcohol, fruit juices, flavorings, and soft drinks that the alcohol industry has given a variety of labels: “low alcohol coolers”, “flavored alcoholic beverages”, “flavored malt beverages”, “alcopops”, “malternatives”, “ready-to-drink beverages”, and “low alcohol refreshers”. Non-distilled beverages (wines and beers) have a maximum alcohol concentration of about 14 % by volume while distilled beverages (liquors and whiskeys) can have a maximum alcohol

concentration of 100%, though most are in the 40-50% range. Therefore, one glass of beer (12 oz beer; 4.5% alcohol) is equivalent to one glass of wine (4.5 oz glass of wine; 12% alcohol), which is similar to one mixed drink (1.5 oz mixed drink with 40% alcohol). In Canada, a standard drink of alcohol (ethyl alcohol or ethanol) should contain 13.6 grams or 17 ml of absolute alcohol-the amount contained in a 12-ounce (341ml) bottle of regular (5%) beer, five ounces (142 ml) of (12%) table wine or 1.5 ounces (43 ml) of 80-proof liquor. Table 1.1 shows a summary of the types of alcohol in the markets and the various alcohol contents. However, actual mean ethanol content of alcoholic drinks in the market is much higher (approximately 11.7% translating to about 0.67 oz) (Kerr *et. al.*, 2005). For example, spirits contain 0.89 oz (48.3% larger than a std drink), wine = 0.66 oz (10% larger), beer = 0.56 oz (6.7% less) and malt liquor = 7.23%v/v (Bluthenthal *et. al.*, 2005; Logan *et. al.*, 1999).In addition to ethanol, alcoholic beverages also contain methanol (Sprung *et. al.*, 1988; Caldwell, 1986; Kapur & Maziar, 1993; Tintinalli, 1995). The methanol in such drinks is congeners, a by-product of fermentation and distillation. Congeners are present in distilled or fermented drinks (wine, scotch, brandy, rum, and, to a lesser extent, vodka and gin) and give liquor its unique taste and are often not removed for fear of altering the characteristic taste. It is, therefore, without doubt that consumption of such alcoholic beverages would result in significant blood levels of ethanol and methanol. Table 1.2 lists the methanol contents of various beverages and blood levels obtained.

Table 1.1: Types of alcoholic beverages and their alcohol contents.

Beverage	Alcohol content (Percentage by volume)
Brandy	40 - 50
Whisky	40 - 55
Rum	40 - 55
Wines (Port, Sherry Champagne, etc)	10 - 22
Beer	4 - 8
Flavored Alcoholic Beverages	5 - 8

Table 1.2: Methanol levels in various alcoholic drinks and blood following consumption of 1L of beverage.

Beverage	MeOH in beverage (mg/L) *	MeOH (mM) in blood**
Beer	4-50	0.002-0.030
White wine	15-45	0.009-0.027
Red wine	70-130	0.042-0.078
Cognac	180-370	0.10-0.22
Calvados	310-640	0.18-0.38
Kirsch	1900-2500	1.1-1.50
Plum	3000-4500	1.80-2.70
Slivovitz	1500-4000	0.907-2.40
Liquor	10-560	0.006-0.33
Rum	6-70	0.004-0.042
Scotch whisky	100-130	0.06-0.078
Irish whisky	10-110	0.006-0.066
US whisky	200-330	0.120-0.199
Cornwine	5-100	0.003-0.06
Aquavit	5-650	0.003-0.391
Gin	10-1350	0.006-0.812
Vodka	5-170	0.003-0.102

*from Sprung *et. al.*, 1988. **Maximum blood MeOH levels following consumption of 1 litre of beverage in a 150 lb, 5'8", 25 years old male.

1.1.2 Definition of Binge Drinking

The national institute on alcohol abuse and alcoholism (NIAAA) (National Advisory Council on Alcohol Abuse and Alcoholism, 2004) defines binge drinking as "a pattern of drinking alcohol that brings blood alcohol concentration (BAC) to 0.08 gram percent or above". For the typical adult, this pattern corresponds to consuming 5 or

more drinks for males, or 4 or more drinks for females in about 2 hours. A "drink" refers to half an ounce of alcohol (e.g., one 12–oz. beer, one 5–oz. glass of wine, or one1.5–oz. shot of distilled spirits). It is important to note that the number of drinks needed to reach a binge–level BAC is lower for some individuals such as older people or people taking other drugs or certain medications, than for the "typical adult."

Therefore, binge describes an extended period of time (typically at least two days) during which time a person repeatedly becomes intoxicated. Binge drinking can result in acute intoxication, hangovers, headaches, nausea, shakiness and possible vomiting and memory loss. Other short-term risks of binge drinking include assaults, car accidents and risky behaviors such as swimming, driving, unsafe or unwanted sex, verbal or physical abuse. More important, binge drinking could pose significant risk to the fetus such as miscarriage and health effects (Figure 1.1) associated with elevated maternal ethanol and methanol levels such as FASD (Chaudhuri, 2000a; Day & Richardson, 1991; Zuba *et. al.*, 2002).

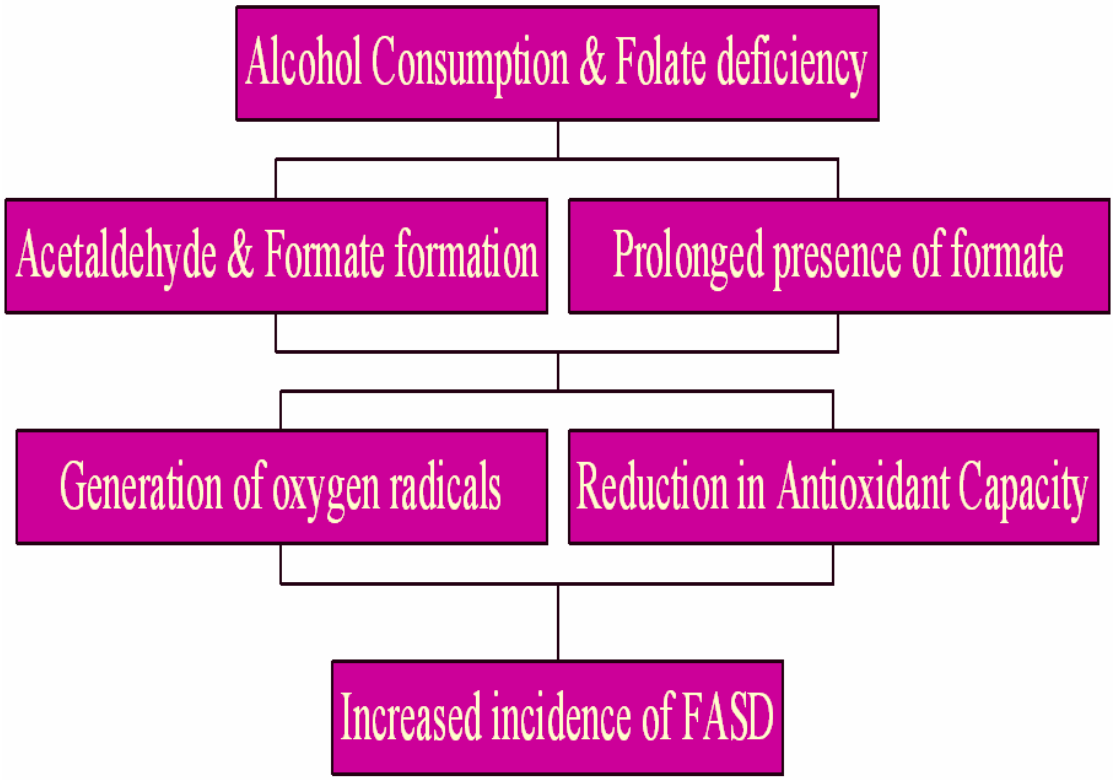

Figure 1.1: The harmful effects of alcohol consumption and folate deficiency.

1.1.3 Prevalence of Alcohol Consumption

Several studies have shown that alcohol consumption amongst women of childbearing age is high with 4% being considered alcohol dependent (Chambers *et. al.*, 2005; Center for Disease Control, 2004; Ebrahim *et. al.*, 1998). In the study conducted by Cambers and coworkers (Chambers *et. al.*, 2005), 43% of 100 pregnant low-income Latinas surveyed reported some alcohol use in the 3-months prior to pregnancy. Twenty percent (20%) reported at least one binge episode of 4 or more standard drinks during that time frame. Five percent (5%) reported drinking 7 or more drinks per week. In another study of women aged 18 - 44 years from the 2002 behavioral risk factor

surveillance system (BRFSS) survey, by the center for disease control (CDC) (CDC, 2002), indicated that approximately 10% of pregnant women use alcohol, and approximately 2% engage in binge drinking or frequent use of alcohol. The results further show that more than half of women who do not use birth control (and therefore might become pregnant) report alcohol use with 12.4% reporting binge drinking.

A study conducted by Ebrahim and coworkers found an increase in alcohol consumption in pregnant women in the U. S. from 9.5% in 1992 to 15.3% by 1995. They found that 14.6% of pregnant women consumed alcohol, and 2.1% consumed alcohol frequently (at least five drinks per occasion or at least seven drinks per week) (Ebrahim *et. al.*, 1998). In another study (Ebrahim *et. al.*, 1999) they found that pregnant women were one fifth (prevalence rate ratio 0.2, 95% confidence interval 0.1-0.2) likely to binge drink as would nonpregnant women. Pregnancy-related reduction in binge drinking was largest among women aged less than 30 years and among those who had quit smoking and smallest among black women. Binge drinking among pregnant women was independently associated with being unmarried, being employed, and smoking. Nonpregnant, unmarried, nonblack, college level education women aged 30 years or younger, who are employed, or a student, and currently smoking are more likely to binge drink (Ebrahim *et. al.*, 1999).

Consumption of high alcohol content drinks is also high among certain groups. For example, 88% of 329 African-Americans surveyed by Bluthenthal and co-workers (Bluthenthal *et. al.*, 2005) preferred malt liquor beer (MLB) to regular beer. Four percent (4%) of daily or near-daily drinkers were MLB drinkers compared with 48% for regular beer (RB) and 29% for hard liquor (HL). Average alcohol consumption per occasion was

higher in MLB group (5.2 drinks, as compared with 4.2 for RB and 3.1 for HL), translating to daily average EtOH consumption of 6.97 oz, as compared with 2.13 oz for RB drinkers and 6.13 oz for HL drinkers.

1.1.5 Social/Cultural Values and Alcohol Consumption

Alcohol is one of the oldest social drinks known in many communities. A significant amount of alcohol consumption occurs in bars, taverns (accounting for 27% of total consumption among 20-24 year olds, compared to only 2% among those 65 and older) and social gatherings (accounting for 27% of drinkers aged 15-19 years) despite legal age restrictions. Friends are by far the dominant drinking companions for young people (Bobo & Husten, 2000). Several studies have shown alcohol consumption rates increase with age and that initiation and continued use is primarily influenced by sociocultural factors such as peer and family influences, demographics/socioeconomic status, advertising and, availability of alcohol itself (Bobo & Husten, 2000; O'Connor & Whaley, 2006; Pirkle & Richter, 2006).

Alcohol use in adolescents is associated with drinking by parents, siblings and peers (Brook *et. al.*, 1986; Rittenhouse & Miller, 1984; Scheier *et. al.*, 1997; Williams *et. al.*, 1998). Males are more likely to report current and binge drinking compared to females (Substance abuse and mental health services administration, SAMHSA, 1998) with a disproportionate number being from socioeconomically disadvantage background (D'Onofrio, 1997). Grube and co-workers (Grube & Wallack, 1994) observed that awareness of beer commercials among children in fifth and sixth grade was correlated with intentions to drink as adults.

1.1.6 Biochemistry and Clinical Toxicology of Alcohol Consumption

1.1.6.1 Ethanol and Acetaldehyde

Alcoholism is usually understood as an addiction to ethanol. Evidence suggests that it is the oxidation product of ethanol, acetaldehyde, which condenses with endogenous amines to form tetrahydroisoquinoline (TIQ) and - tetrahydro-beta-carboline (THBC) alkaloids which ultimately might be responsible for addiction (Brooks, 1997;Sprung *et. al.*, 1988). Commercial alcoholic beverages are made from natural products, and therefore contain, in addition to ethanol (EtOH), trace to small amounts of hundreds, if not thousands, of constituents and contaminates (also called congeners). While some congeners make up the distinctive characteristic of the beverage, others are by-products of the manufacturing process that are never removed for fear of altering the unique taste and smell of the drink. One of these congeners is methanol (MeOH) (see table 1.2). It is evident, without exception, that methanol is present in all alcoholic beverages. Its metabolite, formaldehyde, is a much more potent reaction partner for TIQ and THBC formation than acetaldehyde (Sprung *et. al.*, 1988). It seems possible that methanol and its metabolites, formaldehyde and formate, play a more significant role in the pathophysiology and possibly the etiology of chronic alcoholism and FASD than ethanol.

The specific pathophysiology of alcohol teratogenesis on the developing fetus is not known, but may involve free radical formation leading to oxidative stress and cellular damage. Acetaldehyde and formate, the metabolites of ethanol and methanol, respectively, have been implicated in DNA damage, and cellular death, as well as fetal malformations and death of the individual (Chaudhuri, 2000c; Chaudhuri, 2000b; Cook *et.*

al., 2001; Eriksson, 2001b; Hard *et. al.*, 2001; Kitson, 1996; Riley *et. al.*, 2001; Warren & Foudin, 2001). Excess alcohol consumption tends to result in compromised dietary habits and poor health maintenance behaviours. This may lead to maternal malnutrition, which in turn negatively affects fetal nutrition. Alcohol also decreases maternal intestinal absorption of nutrients, decreases placental uptake and availability of the nutrients to the fetus, and increases renal and hepatic metabolism of nutrients. Thus, it affects the metabolism of essential nutrients.

Exposure of the fetus to acetaldehyde in the first trimester affects organogenesis and craniofacial development, leading to malformations due to excessive cell death in the midline of the developing embryo (Brooks, 1997; Chaudhuri, 2000c; Chaudhuri, 2000b; Eriksson, 2001a; Hard *et. al.*, 2001; Kitson, 1996; Menegola *et. al.*, 2001). Formate, a metabolite of methanol, is a much more potent toxic compound than acetaldehyde that has been implicated in cell death (Gonzalez-Quevedo *et. al.*, 2002).

1.6.3.1 Methanol and Formate

Methanol is a hydrophilic, low molecular weight substance that freely diffuses, and therefore, is not concentrated in, or excluded from, any particular cell or body compartment. Methanol toxicity is an important public health concern because of the selective actions of formate on the retina, optic nerve and central nervous system (Eells *et. al.*, 2000). The toxicity of methanol is mediated through formate. Thus, for methanol toxicity to occur, it must first be metabolized by alcohol dehydrogenase (ADH) to formaldehyde and thence to formate (Figure 1.2). Human ADH has an affinity about 20-fold higher for ethanol than for methanol (Haffner *et. al.*, 1997; Tephly, 1991).

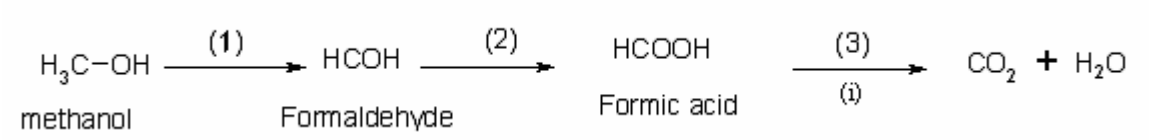

Figure 1.2: Metabolic Pathway of methanol. (1) Alcohol Dehydrogenase (ADH); (2) Aldehyde Dehydrogenase (ALDH); (3) Catalase-Peroxidase; (i) Tetrahydrofolate dependent

Consequently, a simultaneous consumption of ethanol and methanol will result in considerable inhibition of methanol metabolism, the extent of which is dependent on blood ethanol levels. In chronic alcoholics, the activity of hepatic cytochrome P450 2E1, a key enzyme in the metabolic elimination of ethanol (Klotz & Ammon, 1998), is increased. This could accelerate the rate of elimination of ethanol and thereby reduce its protective effect in methanol-containing alcoholic drinks. The half-life of elimination of methanol is about 140 min, its oral absorption 100%, and its volume of distribution approximately equal to the total body water at equilibrium (Paine & Dayan, 2001). Small amounts of methanol are excreted, unchanged, in urine and expired in air. About 10% of the dose ingested is excreted in urine as formate.

A blood ethanol level greater than 22 mM (100mg%) will totally inhibit the active catabolism of methanol. This will take a consumption of 36g of ethanol over a period of 1 hour, followed by the continued consumption of about 6.5g/hr for about 40 hrs for methanol levels to be halved by normal physiological processes in the body as described earlier (Haffner *et. al.*, 1997). This is, however, only seen in non-drinkers and average consumers of alcohol including binge drinkers. Empirical observations indicate that a

blood methanol level exceeding 3.24 mM (20mg%) is high enough to produce a clinical toxicity to justify treatment. A concentration of 3.24 mM is at the border of severe illness. A level of 0.16 mM (1mg%) is normal and levels of 0.810 mM (5mg%) are tolerated without evidence of any clinical disorder (Jacobsen *et. al.*, 1988; Paine & Dayan, 2001).

The body produces methanol as well. Several workers have described an endogenous production of methanol (Iffland & Staak, 1990; Roine *et. al.*, 1989; Sarkola & Eriksson, 2001). Endogenously produced methanol levels are sufficiently low (0.016 mM to 0.045 mM; 0.07 – 0.2 mg/100 ml) that they would normally go undetected by analytical procedures routinely employed in clinical laboratories (Gilg *et. al.*, 1987). Kapur and Maziar (Kapur & Maziar, 1993) observed significantly higher formate levels in three methanol positive patients. This would imply a continued presence of methanol and formate in the plasma of alcoholics. Such an exposure would probably increase the risk of organ and tissue damage including that of the fetus.

1.2 Effects of Alcohol Abuse: Fetal Alcohol Spectrum Disorder (FASD)

1.2.1 Definition of Fetal Alcohol Spectrum Disorder

The disorders associated with alcohol consumption during pregnancy known as fetal alcohol spectrum disorder (FASD) consist of a wide spectrum of effects ranging from mild, known as fetal alcohol effects (FAE) or alcohol related neuro-developmental disorders (ARND), to extreme manifestations known as full blown fetal alcohol spectrum disorder (FASD). Smith and Jones (Jones & Smith, 1973) first described the distinct dysmorphic condition associated with maternal alcohol abuse during pregnancy known as

FASD. Fetal alcohol spectrum disorder (FASD) consists of a pattern of anomalies occurring in the babies born to drinking women and consists of (a) pre- and/or postnatal growth retardation, (b) morphological anomalies, and (c) central nervous system (CNS) dysfunction (Hannigan *et. al.*, 1992; Rosett, 1980; Streissguth, 1986; Hannigan *et. al.*, 1992). The mild form of FASD includes an incomplete picture of nonspecific physical and psychological manifestations of neurodevelopmental deficits following alcohol exposure and was originally termed as fetal alcohol effect (FAE), also known as atypical FASD. This (FAE) was later replaced by the term alcohol-related birth defects (ARBD) and/or alcohol-related neurodevelopmental disorder (ARND) (Stratton and Battaglia, 1996). The effects of ARBD and ARND can be just as severe as full blown FASD (Abel, 1999b). The term FASD is a new umbrella term that encompasses the full range of effects and conditions that range from mild, such as FAE, ARBD and ARND, to severe (full-blown FASD).

1.2.2 Prevalence of Fetal Alcohol Spectrum Disorder

Fetal alcohol spectrum disorder (FASD) is a medical, social and economical catastrophe of great magnitude. Fetal Alcohol Spectrum Disorder in newborns is under-recognized due to the absence of many of the manifestations of fetal alcohol exposure, which usually appear with age (Stoler and Holmes, 1999; Canadian Pediatric Society, 2002). It is now recognized as a leading nongenetic cause of mental retardation (Abel & Sokol, 1986). It has been estimated that the incidence of full-blown FASD is between 2.8/1000 and 4.8/1000 live births. The incidence of a combination of FASD is estimated

to be at least 9.1/1000 live births (Sampson *et. al.*, 1997). Abel (Abel & Hannigan, 1995; Abel, 1995) estimated the incidence of FASD among "heavily" drinking women (as defined by consumption of 5 or more drinks per occasion, an average of 2 or more drinks per day, a positive MAST score, or a clinical diagnosis) at about 4.3% of all live births. In one community in British Columbia, Robinson *et. al.,* identified 22 of 116 children (16%) as having FASD (Robinson *et. al.*, 1987). MacDonald (1991) suggested a rate of 3.3/1000 children in British Columbia and a rate of atypical FASD of up to five times as high if older children with the syndrome were included. This means that based on 42,909 births per year in 1998 (BC vital Statistics, 1998) 142 infants were born with FASD and 710 infants were born with atypical FASD per year in British Columbia. In northern Manitoba, Williams and coworkers (Williams *et. al.*, 1999) estimated incidence of FASD to be 7.2/1000 children based on physical findings at birth. However, he points out that a number of cases might have been missed. Lupton, Burd and Harwood (Lupton *et. al.*, 2004) found that physical signs of FASD are not as physically apparent until 4 years of age, and therefore, diagnoses made based on birth records will only catch the most severe cases and will miss the others. Although all races are susceptible, FASD is found in disproportionately higher frequencies in Native American Populations (Aase, 1981; Macdonald, 1991; Williams *et. al.*, 1999). The incidence of FASD is much higher in other communities outside of North America. For example, the rate of FAS found in South Africa is 65.2-74.2 per 1,000 children in the first grade population (33-148 times greater than U.S. estimates). This is the highest yet reported in any overall community in the world (Viljoen *et. al.*, 2005).

Many women who drink continue to do so into the pregnancy. This could be due to lack of understanding of alcohol's detrimental effect to the fetus, whereas alcohol dependent women may be unable to abstain. Those who intend to abstain during pregnancy, however, may consume alcohol in early gestation before they realized were pregnant. In Canada there is increasing awareness of the extent of FASD in Native communities.

1.2.3 Fetal Alcohol Spectrum Disorder and Binge Drinking

Binge drinking is defined as a pattern of drinking alcohol that brings blood alcohol concentration (BAC) to 0.08 gram percent (g%) or above (National Institutes of Health, 2004). For the typical adult, such a pattern corresponds to consuming 5 or more drinks for males, or 4 or more drinks for females, in approximately 2 hours. A drink is defined as one containing the equivalent of 0.6 oz (17.74 mL; 14g) of pure alcohol (National Advisory Council on Alcohol Abuse and Alcoholism, 2004). It is evident that binge drinking results in higher BAC than with peak BAC achieved with continuous drinking.

Since Alcohol dehydrogenase is a saturable enzyme at higher alcohol levels, metabolism of alcohol does not keep up with intake (Jones & Sternebring, 1992; Kitson, 1996). Therefore, binge drinking not only leads to elevated BAC but also prolonged exposure, factors that could lead to fetal adverse effects (Abel & Hannigan, 1995). This is particularly important in unrecognized pregnancies where the mother might be engaged in binge drinking during a time in which the fetus is most susceptible to the harmful effects of alcohol (Abel, 1999b; Chaudhuri, 2000b; Day & Richardson, 1991).

Alcohol consumption amongst women is high with 4% being considered alcohol dependent (Bloomfield *et. al.*, 2006; Bluthenthal *et. al.*, 2005; Chambers *et. al.*, 2005). A study conducted by Chambers *et. al.* (Chambers *et. al.*, 2005) found 43% of 100 pregnant low-income Latinas surveyed reported some alcohol use in the 3 months prior to pregnancy with 20% reporting at least one binge episode of 4 or more standard drinks during that time frame; 5% reported drinking 7 or more drinks per week; 8% continued drinking alcohol after recognition of pregnancy. Frequency of periconceptional alcohol use did not differ between women who planned or did not plan the pregnancy in the study. The study also found that awareness of alcohol warning messages and/or knowledge of the FASD was significantly correlated with alcohol consumption in the periconceptional period. The investigators also found that predictors of alcohol use in the periconceptional period included English language/higher level of acculturation, younger maternal age, lower parity, higher level of education and, younger age at first drink and having ever smoked.

1.2.4 Biochemical Aspects of Fetal Alcohol Spectrum Disorder

Evidence from epidemiological studies has shown that there is a selective presence of FASD in certain populations indicating that fetal alcohol spectrum disorder is not an equal opportunity disorder (Abel & Hannigan, 1995; Abel, 1995; Abel & Sokol, 1991; Abel & Sokol, 1987). On the basis of the disparities in the occurrence of FASD, Abel and Hannigan drew inferences about potential socio-behavioral risk factors for FASD that they called "permissive" factors. These are alcohol intake pattern, race and socioeconomic status, culture, and smoking. They argued that these factors provide the context within

which vulnerability to alcohol's teratogenic effects are increased. The permissive factors create a biological milieu that is "provocative" for FASD. Examples of "provocative" factors are increased blood alcohol levels (BAL), under-nutrition, environmental pollutants, psychological and physical stress, and increased drug levels in the body. The provocative factors increase cellular susceptibility to alcohol's toxic effects, which are thought to occur through hypoxia and free radical damage (Abel & Hannigan, 1995).

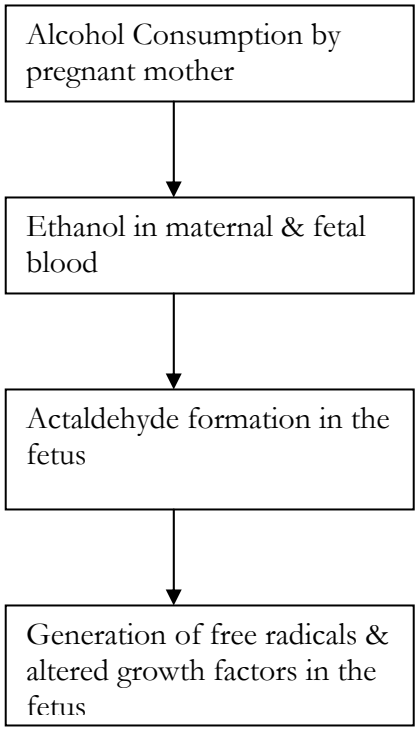

Figure 1.3: Mechanism of action of the deleterious effects of alcohol in the developing fetus.

Prenatal alcohol exposure can have a variety of consequences ranging from death or FASD at one end of the spectrum, to relatively normalcy at the other end. Evidence from autopsy and magnetic resonance imaging studies have illustrated microcephaly, with tissue loss, cerebral dysgenesis, and abnormal neural migration and glial cells in children

with full-blown FASD (Roebuck *et. al.*, 1998). Holoprosencephaly, a condition associated with the failure of the brain to divide into two hemispheres leading to neurodevelopmental and facial abnormalities, is a characteristic feature of full-blown FASD. Other abnormalities include that of the brainstem, cerebellum (especially of the anterior vermis), corpus callosum such as agenesis and hypoplasia, absence of olfactory lobes, absent or hypoplastic caudate nuclei and, hypoplasia of the hippocampus accompanied by abnormal or absent basal ganglia. There are findings from positron emission tomography that have demonstrated glucose metabolism abnormalities, especially in the anterior caudate nucleus and vermis of the cerebellum, even in the absence of overt structural abnormalities (Indian and Inuit Health Committee, 2002).

1.2.5 Clinical Diagnosis and Treatment

The diagnosis of FASD is based on a triad of clinical features (Table 1.3) in an individual exposed to alcohol in utero. This triad consists of pre- and postnatal growth deficiency, a characteristic pattern of CNS dysfunction and facial abnormalities (Astley & Clarren, 2002; Indian and Inuit Health Committee, 2002; Roebuck *et. al.*, 1998). Perhaps the most devastating sequelae of FASD are the neurobehavioural manifestations, associated with alcohol's effect on the CNS (Roebuck *et. al.*, 1998). Central nervous system dysfunction leads to primary disabilities which are manifested in a number of ways, including: compromised intelligence (IQ 50-115), language, learning and memory; abnormal activity, attention (in the form of Attention Deficit Disorder), and behaviour (Indian and Inuit Health Committee, 2002). Secondary disabilities in FASD individuals

include mental health problems, disrupted school experiences, trouble with the law and incarceration, inappropriate sexual behavior, and drug and alcohol abuse problems.

Considerable neurological development occurs postnatally. If child care, nutrition, and surrounding environment are adequate, it is probable that FASD children can make considerable progress in leading a normal life. This is particularly true in mildly affected individuals. For example, treatment regiments enable the clinical management of FASD and include behavioral interventions (Klintsova *et. al.*, 1998), special education programs and stimulants. Intensive case management of drinking mothers has been shown to prevent women from having additional children with drug and/or alcohol exposure (Ernst *et. al.*, 1999; Grant *et. al.*, 1999).

Therefore, the most desirable way of dealing with FASD is through abstinence before, and after, conception. However, once a diagnosis of FASD is made measures must be put in place, as described earlier, that involves coordination of several levels of identification, intervention, and treatment in order to maximize the child's postnatal development. With adequate education and protection from negative child rearing environments and attention to predictable crises at various developmental stages, can make the difference between achieving a reasonable degree of independence and life satisfaction and more negative outcomes in FASD individuals (Campbell & Ramey, 1994).

Table 1.3: Diagnostic Criteria for Fetal Alcohol Spectrum Disorder. (Modified from Stratton & Battaglia, 1996 and Warren & Foudin, 2001.)

1 - FASD with confirmed maternal alcohol exposure*
1. Confirmed maternal alcohol exposure* 2. Characteristic pattern of facial and premaxillary zone anomalies that include short palpebral fissures, flat upper lip, flattened philtrum and flat midface. 3. Growth retardation 4. Neurodevelopmental abnormalities of the CNS such as structural brain anomalies, small head size at birth, poor eye-hand coordination, neurosensory hearing loss, impaired fine motor skills and poor gait.
2 - FASD without confirmed maternal alcohol exposure*
1. Growth retardation 2. Neurodevelopmental abnormalities of the CNS such as structural brain anomalies, small head size at birth, poor eye-hand coordination, neurosensory hearing loss, impaired fine motor skills and poor gait. 3. Characteristic pattern of facial and premaxillary zone anomalies that include short palpebral fissures, flat upper lip, flattened philtrum and flat midface.
3 - Partial FASD with confirmed maternal alcohol exposure*
1. Confirmed maternal alcohol exposure* 2. Growth retardation *and one of the following characteristics:* 3. Neurodevelopmental abnormalities of the CNS such as structural brain anomalies, small head size at birth, poor eye-hand coordination, neurosensory hearing loss, impaired fine motor skills and poor gait. 4. Some componenets of facial anomalies 5. Behavioural or congnitive abnormalities inconsistent with developmental level such as learning difficulties, poor impulsive control, problems in memory, attention and/or judgment.
4 - Alcohol-related birth defects (ARBD)
1. Confirmed maternal alcohol exposure* 2. One or more congenital defects (malformations and dysplasia of the heart, kidney, bone, vision or hearing systems.
5 - Alcohol-related neurodevelopmental disorder (ARND)
1. Confirmed maternal alcohol exposure* 2. Neurodevelopmental abnormalities of the CNS such as structural brain anomalies, small head size at birth, poor eye-hand coordination, neurosensory hearing loss, impaired fine motor skills and poor gait. *and/or:* 3. Behavioural or congnitive abnormalities inconsistent with developmental level such as learning difficulties, poor impulsive control, problems in memory, attention and/or judgment.

*Defined as a pattern of excess alcohol intake characterized by substantial, regular intake or by binge drinking; CNS = Central Nervous System; FASD = Fetal Alcohol Spectrum Disorder.

1.3 Role of Vitamins and Other Cofactors in Formate Metabolism

1.3.1 Folic Acid

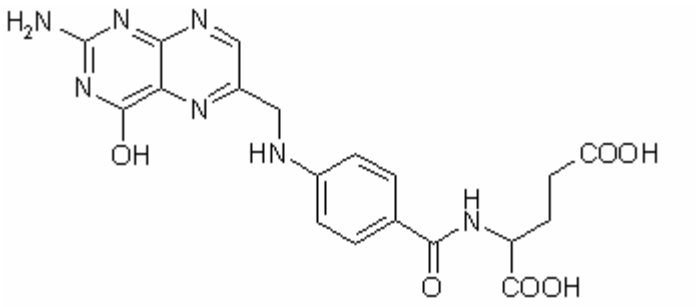

Figure 1.4: Deprotonated molecule of folic acid

Folic acid (Figure 1.4) is a vitamin and a cofactor in the elimination of formate. It plays a major coenzymatic role in one carbon metabolism. The principal function of folate coenzymes is to accept or donate one-carbon units in folate-requiring metabolic pathways. Thus, folic acid is a key participant in the biosynthesis of DNA and RNA, and the methylation of transfer RNA and certain amino acids (Wagner, 1995) (Figures 1.5 and 1.6). Thus, the requirement for folate by the body is related to the cellular reproduction occurring at any particular time (Wagner, 1995). One such time of an increased folate requirement is during pregnancy when there is an enormous increase in cellular proliferation as a result of uterine enlargement, expansion of blood volume, placental development and fetal growth. The folate molecule that acts as an acceptor of carbon in the one-carbon cycle is in the polyglutamyl form of tetrahydrofolate (THF) (Wagner, 1995)

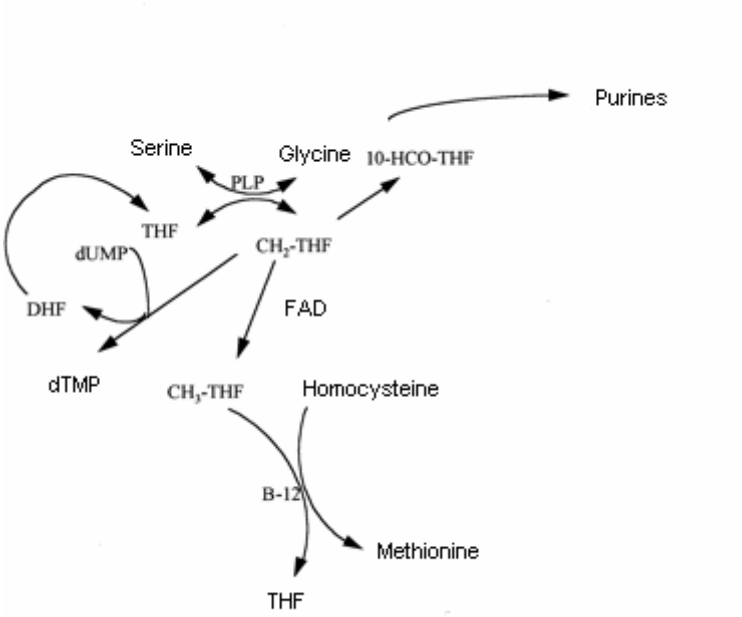

Figure 1.5: Overview of one-carbon metabolism and transsulfuration pathways. Abbreviations: Ado, adenosyl; CH_3-THF, 5-methyl-THF; DHF, dihydrofolic acid; dTMB, deoxythymidine monophosphate; dUMP, deoxyuridine monophosphate; FAD, flavin adenine dinucleotide; 10-HCO-THF, 10-formyl-THF; PLP, pyridoxal phosphate; THF, tetrahydrofolate.

The 3-carbon of serine acts as a major carbon source in the conversion of THF to 5,10-methylene-THF, a crucial first step in the metabolism of folate (Figure 1.5). This one-carbon unit is transferred from serine to THF via pyridoxal phosphate (PLP)-dependent serine hydroxymethyltransferase (SHMT) to form 5,10-methylene-THF and glycine. Thus, a portion of the 5,10-methylene-THF produced undergoes irreversible enzymatic reduction to the methyl oxidation state (as 5-methyl-THF) by methylene tetrahydrofolate reductase (MTHFR). The N-5 methyl group of 5-methyl-THF can only be used metabolically for transfer to homocysteine, which results in the generation/regeneration of methionine. The enzyme MTHFR plays a key role in one-carbon metabolism. Its catalytic action results in the conversion of methylene-THF to 5-

methyl-THF, thus irreversibly directing the one-carbon moiety to methylation of homocysteine synthesis. Between 50 and 80% of the homocysteine generated is re-methylated, depending on the dietary content of methionine and choline (Bailey & Gregory, III, 1999).

The methionine synthase reaction involves removal of a methyl group from 5-methyl-THF, which is sequentially transferred to the vitamin B-12 and then to homocysteine (HCY) to form methionine. Other than for protein synthesis, methionine serves as a methyl group donor through conversion to S-adenosylmethionine (SAM), a key biological methylating agent involved in over 100 methyltransferase reactions with a wide variety of acceptor molecules. This methionine synthase reaction also regenerates THF, which is required for the formation of 5,10-methylene-THF and 10-formyl-THF. These substances are used directly in thymidylate and purine synthesis, respectively. In the thymidylate synthase reaction, 5,10-methylene-THF donates its methyl unit (becoming the thymidine methyl group). In the de novo purine synthesis pathway, two separate steps utilize 10-formyl-THF (Bailey & Gregory, III, 1999).

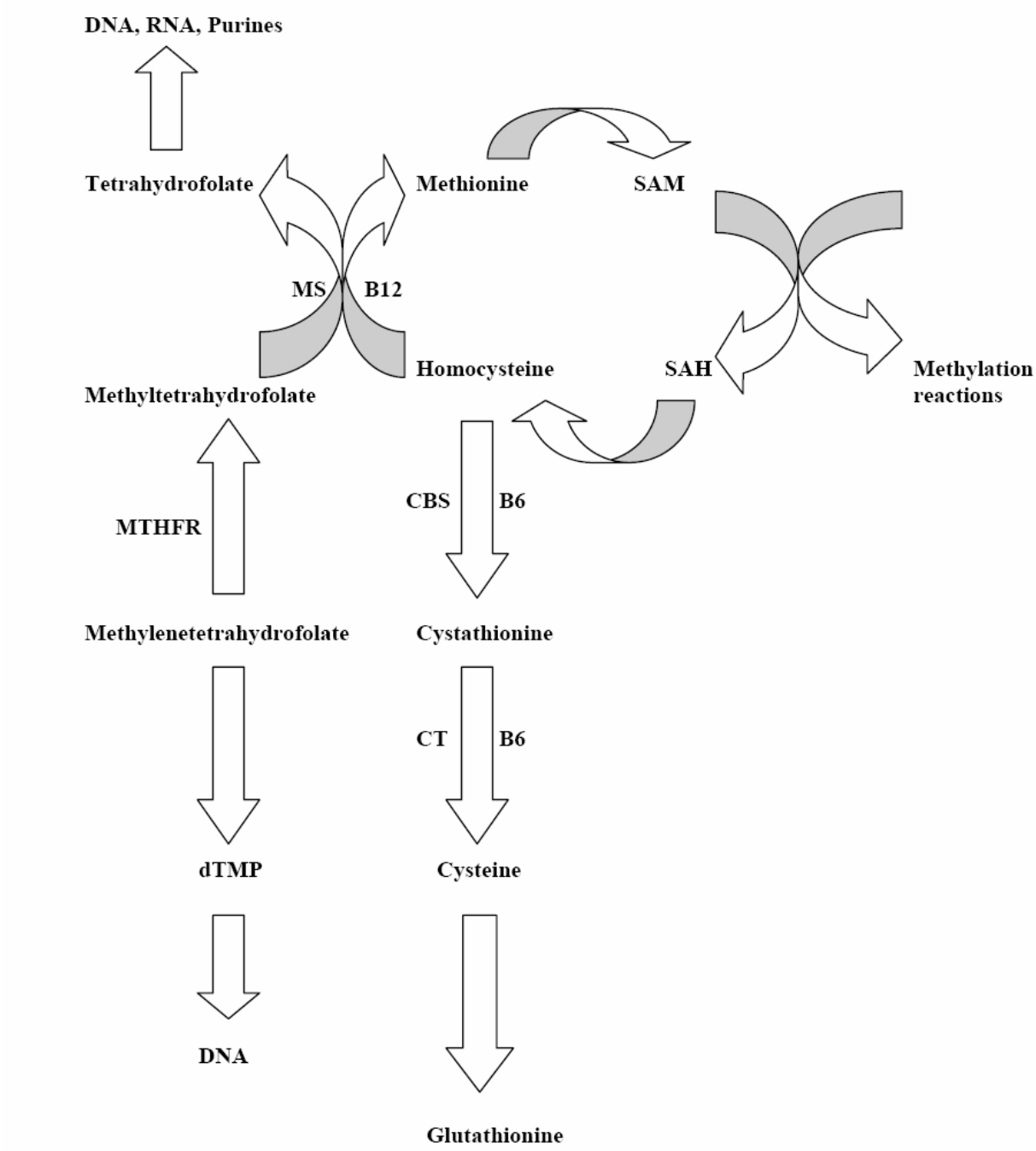

Figure 1.6: An overview of one-carbon metabolism and transsulfuration pathways. SAM, adenosylmethionine; SAH, adenosylhomocysteine; dTMP, deoxythymidine monophosphate; B12, vitamin B 12; B6, vitamin B6; MS, Methionine synthase; CBS, Cystathionine β-synthase; CT, Cystathionase; MTHFR, Methylenetetrahydrofolate reductase.

Since the synthesis of methyl groups and other one-carbon units is tightly controlled (Shane, 1995; Wagner, 1995), the coenzymes involved in folate metabolism are compartmentalized between the cytosol and the mitochondria. While the metabolic products of these reactions are readily transported between compartments, the folate coenzymes are not. Under most circumstances the cytosolic form of *S*-hydroxymethyltransferase (SHMT) is thought to function in the transfer of carbons from serine to THF, yielding 5,10-methylene-THF. The mitochondrial form of SHMT is thought to convert glycine to serine and to serve as a source of mitochondrial THF (Garcia-Martinez & Appling, 1993; Kastanos *et. al.*, 1997). In contrast to one-carbon metabolism in cytosol, the mitochondrial one-carbon metabolism derives much of the one-carbon transfer from folate-mediated serine oxidation (to formate) rather than via SHMT (Graham *et. al.*, 1997). The carbon transfer from serine to THF by SHMT is inhibited by 5-methyl-THF and by 5-formyl-THF (Stover & Schirch, 1991). The 5-formyl-THF is formed in a secondary reaction catalyzed by SHMT (Stover & Schirch, 1990). *S*-adenosylmethionine regulates this pathway by inhibiting MTHFR, which suppresses the production of 5-methyl-THF. Therefore, the intracellular presence of excess methyl groups (such as 5-methyl-THF and SAM during periods of high of methionine and choline intake, and adequate supply of other relevant coenzymes) prevents the further *de novo* biosynthesis of methyl groups (Bailey & Gregory, III, 1999).

The influence of intracellular folate concentrations (as governed largely by dietary intake) on the entry and processing of substrate in one-carbon metabolism is uncertain (Bailey & Gregory, III, 1999). Since folates in the body serve as carbon acceptors, carriers, and donors, a severe folate deficiency can impair one-carbon metabolism (Miller *et. al.*,

1994). However, because of the role of 5-methyl and 5-formyl-THF as SHMT inhibitors, marginal folate deficiency may (at least transiently) have little adverse effect on carbon flux because the effects of these physiologic inhibitors are diminished. Flux through SHMT can be predicted to be a function of pyridoxal 5'-phospahte (PLP) concentrations (Jones, III & Priest, 1978). In situations of poor folate status, *S*-adenosylhomocysteine (SAH) concentrations increase due to the impairment of methyl group synthesis and homocysteine re-methylation. Inhibition by the resulting product (SAH) suppresses many of the SAM-dependent methyltransferase reactions (Selhub & Miller, 1992). This illustrates the far-reaching effects of impaired one-carbon metabolism during nutritional deficiency. Although other sources of one-carbon units exist (e.g., choline, formate, glycine or betaine), serine appears to be the primary carbon donor for the diverse processes of one-carbon metabolism (Pasternack *et. al.*, 1996; Shane, 1995; Wagner, 1995).

The important role of folate in one carbon metabolism is underscored by the fact that maternal folate status can modulate the developmental toxicity of methanol (Fu *et. al.*, 1996; Sakanashi *et. al.*, 1996). Sakanashi and coworkers (Sakanashi *et. al.*, 1996) also found a correlation between fetal body weights and increased incidence of cleft palate in methanol treated CD-1 mice with low folic acid levels. Thus, raising folic acid consumption can reduce the risk of having a neural-tube defect (NTD) pregnancy (Wald *et. al.*, 2001), although evidence points to the fact that maternal Hcy levels are the primary predictor of fetal blood Hcy (Molloy *et. al.*, 2002).

In 1996 the United States Food and Drug Administration (FDA) issued a regulation requiring all enriched grain products to be fortified with folic acid to reduce

the risk of neural-tube defects in newborns. This led to a fortification of 140 μg of folic acid per 100g of grain. Canada introduced a similar law in 1998. Wald and coworkers (Wald *et. al.*, 2001) studied the effect of folic acid supplementation on serum folate. They found an increase in serum folate level of 0.94ng/ml (95% CI 0.77-1.10) for every 0.1 mg/day increase in folic acid intake in women aged 20-35 years, and about double that in women aged 40-65. They concluded that women planning a pregnancy should take 5mg folic acid tablets daily, instead of the 0.4mg dose presently recommended.

The need for increased folic acid intake is concurred by other investigators (Feinleib *et. al.*, 2001; Siega-Riz *et. al.*, 2002) who state that a large proportion of women of childbearing age are still not consuming folic acid at recommended levels. Supplemental folic acid (monoglutamate form) (Figure 5.2) has a higher bioavailability (1.7 times) compared to folate from dietary sources (Feinleib *et. al.*, 2001).

1.3.2 Vitamin B6

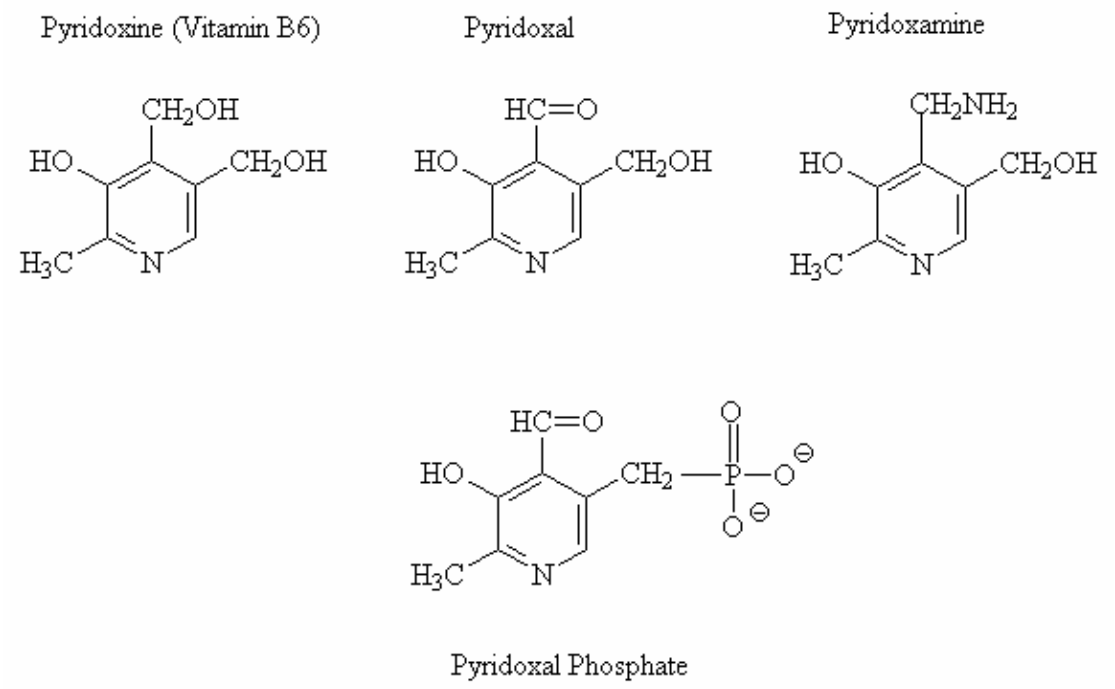

Figure 1.7: Structures of different forms of vitamin B6

Vitamin B-6 is a water-soluble vitamin that exists in six forms: pyridoxal (PL), pyridoxine (PN), pyridoxamine (PM); and their phosphate derivatives, pyridoxal 5'-phosphate (PLP), pyridoxine 5'-phosphate (PNP), and pyridoxamine 5'-phospate (PNP) (Figure 1.7). Pyridoxine is found in foods as a glycoside or in free form. The glycoside form is hydrolyzed by luminal enzymes and/or microflora before absorption in the jejunum by a process that is non-saturable. Pyridoxal 5'-phosphate is the active coenzyme form and also the most significant in human metabolism (Leklem, 1991). Pyridoxal 5'-

phosphate plays a vital role in the functions of more than 100 enzymes that catalyze essential chemical reactions (Leklem, 1991; Leklem, 1999). Vitamin B-6 is an essential vitamin because the body cannot synthesize it and its coenzyme. However, severe deficiency of vitamin B-6 is uncommon. Due to a poor diet and impaired metabolism of vitamin B-6, alcoholics are thought to be most at risk of deficiency of vitamin B-6 (Holman, 1995).

The conversion of PN, PL and PM to their respective phosphate forms is a crucial step in the metabolism of vitamin B-6. A single kinase enzyme catalyzes the conversion, which in the brain and liver is dependant on zinc. The erythrocyte kinase is most active with cobalt or manganese. A flavin-dependent oxidase converts PNP and PMP to PLP (Figure 1.7).

The liver is the major source of plasma PLP. During conditions of increased muscle turnover such as in moderately severe exercise, circulating levels of PLP can increase as well. Pyridoxal 5'-phosphate must be dephosphorylated before being taken by cells and then rephosphorylated within the cell (Figure 1.8). This occurs because PLP does not cross membranes. The major determinant of plasma PLP is the balance of two pathways: the liver formation of PLP and, its breakdown by alkaline phosphatase. Tissues use PL released in the circulation as needed. The liver takes up the excess where it is degraded to pyridoxic acid for excretion by the kidneys. About half of the dietary intake of B-6 is excreted in this way. Urinary excretion, therefore, reflects vitamin intake rather than body reserves.

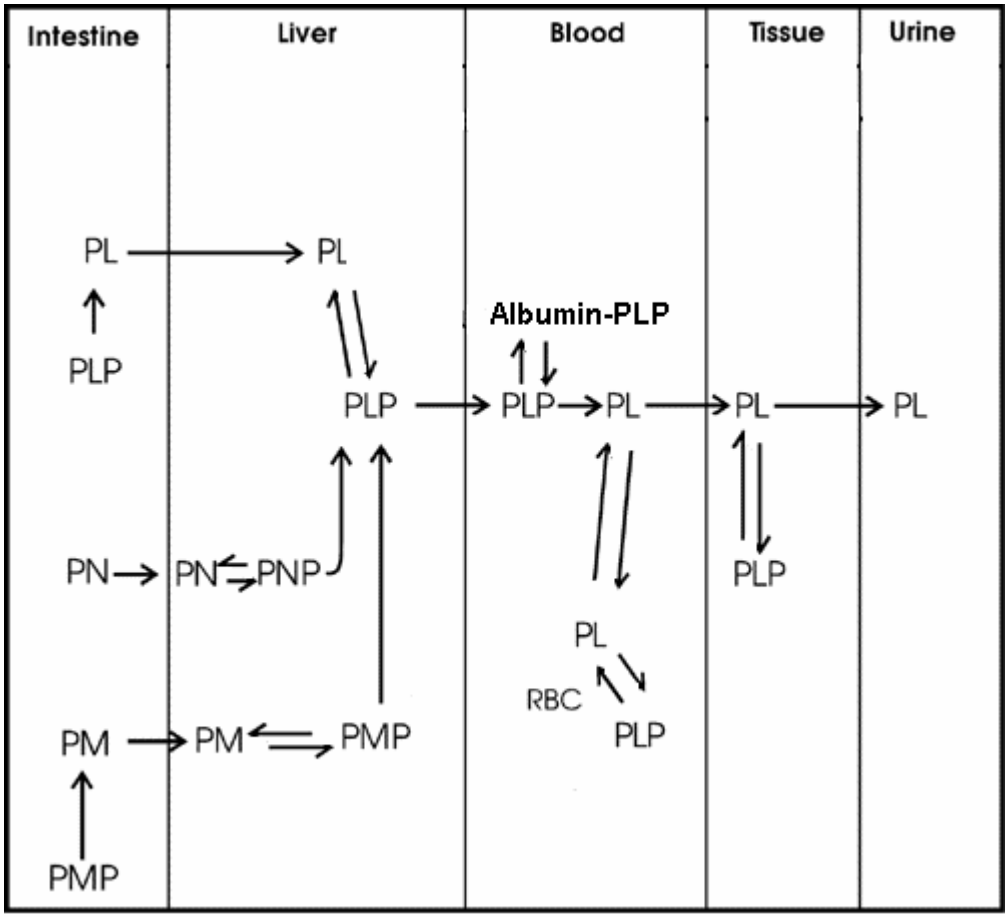

Figure 1.8: A simplified overview of vitamin B-6 metabolism and transport. PL = Pyridoxal; PN = Pyridoxine; PM = Pyridoxamine; PLP = Pyridoxal 5'-phosphate; PNP = Pyridoxine 5'- phosphate; PMP = Pyridoxamine 5'-phosphate; RBC = Redblood cell. (Modified from Holman, 1995)

Increased levels of PLP are encountered in patients with hypophosphatasia, an inborn error of metabolism characterized by deficient alkaline phosphatase activity (Holman, 1995). Abnormally low levels of PLP occur in patients with chronic renal failure and those on dialysis or with kidney transplants. Alcohol also reduces hepatic reserves of PLP, most likely due to accelerated hydrolysis by alkaline phosphatases. Deficiency of pyridoxine can also cause sideroblastic anemia.

Homocystinuria, an inborn error of vitamin B-6 dependent metabolism, is one of the disorders of amino acid metabolism characterized by increased concentration of Hcy in the plasma (Holman, 1995). Symptoms of the disease, due to the accumulation of homocysteine, in adults include dislocation of the eye lens, thrombosis, mental retardation and abnormal skeletal formation. About 50% of the patients, however, respond to vitamin B-6 doses of up to 1500 mg/day. For some unresponsive patients, administration of Betaine, which is a methyl donor in the methylation of homocysteine to methionine, may be helpful (Holman, 1995). Other vitamin B-6 dependent inborn errors of metabolism include cystathioninuria, xanthurenic aciduria, hyperglycinemia and histidinemia.

Normal plasma or serum values of vitamin B-6 range from 20-121 nM, depending on body requirements and method of detection. Values of < 14 nM are indicative of a deficiency, and patients with levels of 15 – 20 nM are considered to be marginally low in vitamin B-6 (Deitrick et. al., 2001; Holman, 1995). In pregnancy, levels of vitamin B-6 are often lower (Contractor & Shane, 1970). Cases of maternal deficiency may be excerbated by toxemia or hypermesis gravidum (severe vomiting) (Shane & Contractor, 1980). Moretti and coworkers (Moretti *et. al.*, 1982) found that high doses of the vitamin are antilactogenic, although a typical multivitamin supplementation elevates plasma and milk PLP without reducing prolactin or lactation (Andon *et. al.*, 1976).

1.3.3 Vitamin B12 and Methylmalonic Acid

Vitamin B-12 consists of a family of substances composed of tetrapyrrole rings surrounding a central cobalt atom with nucleotide side chains attached to the cobalt

(Figure 1.9). The name cobalamin is the name given to the overall group, with each of the different upper axial ligand links of cobalt conferring a different name (e.g., methylcobalamin for the methyl moiety; hydrocobalamin for hydroxyl moiety; aquacobalamin for H_2O moiety; cyanocobalamin for cyanide; 5-deoxyadenosylcobalamin for 5-deoxyadenosine). The human body is incapable of synthesizing the corrin ring structure (see Figure 1.9), and so humans are completely dependent upon dietary sources of vitamin B12. One of the most commonly supplemented forms of vitamin B-12 is cyanocobalamin (CN-Cbl). Humans have to remove and detoxify the cyanide molecule, although at this level it's toxicologically insignificant. The resulting cobalamin, (Cbl), from the cleavage of CN-Cbl, is then reduced to its usable +1 oxidation state. This is followed by conversion to one of the two metabolically active coenzyme forms: adenosylcobalamin (AdenosylB12) and methylcobalamin (MethylB12) (Pezacka *et. al.*, 1990).

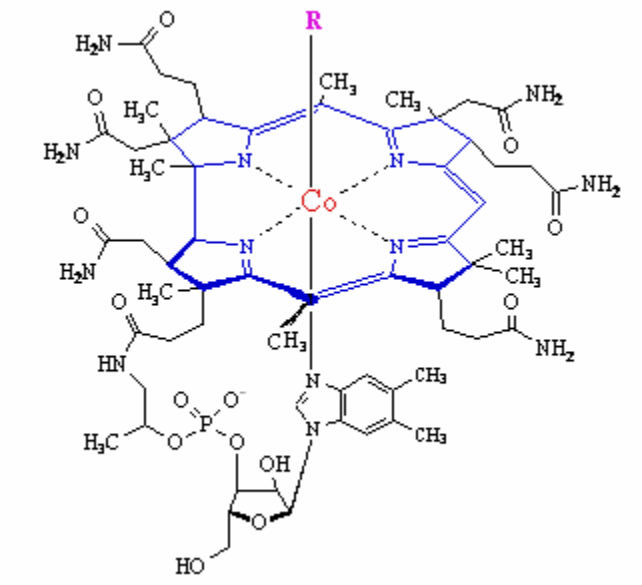

Figure 1.9: Structure of Cobalamin (vitamin B-12)

Absorption of vitamin B-12 from the gastrointestinal tract requires the formation of a complex between dietary cobalamins and R-proteins, and also the secretion of intrinsic factor by the stomach parietal cells. Pancreatic enzymes in the small intestine cleave the cobalamin-R-protein complex. The released cobalamin molecule binds with intrinsic factor where it is absorbed in the distal ileum. Cobalamin is then detached from intrinsic factor in the enterocyte cells of the small intestine, and is bound to transcobalamin II for transport into tissues (Figure 1.10).

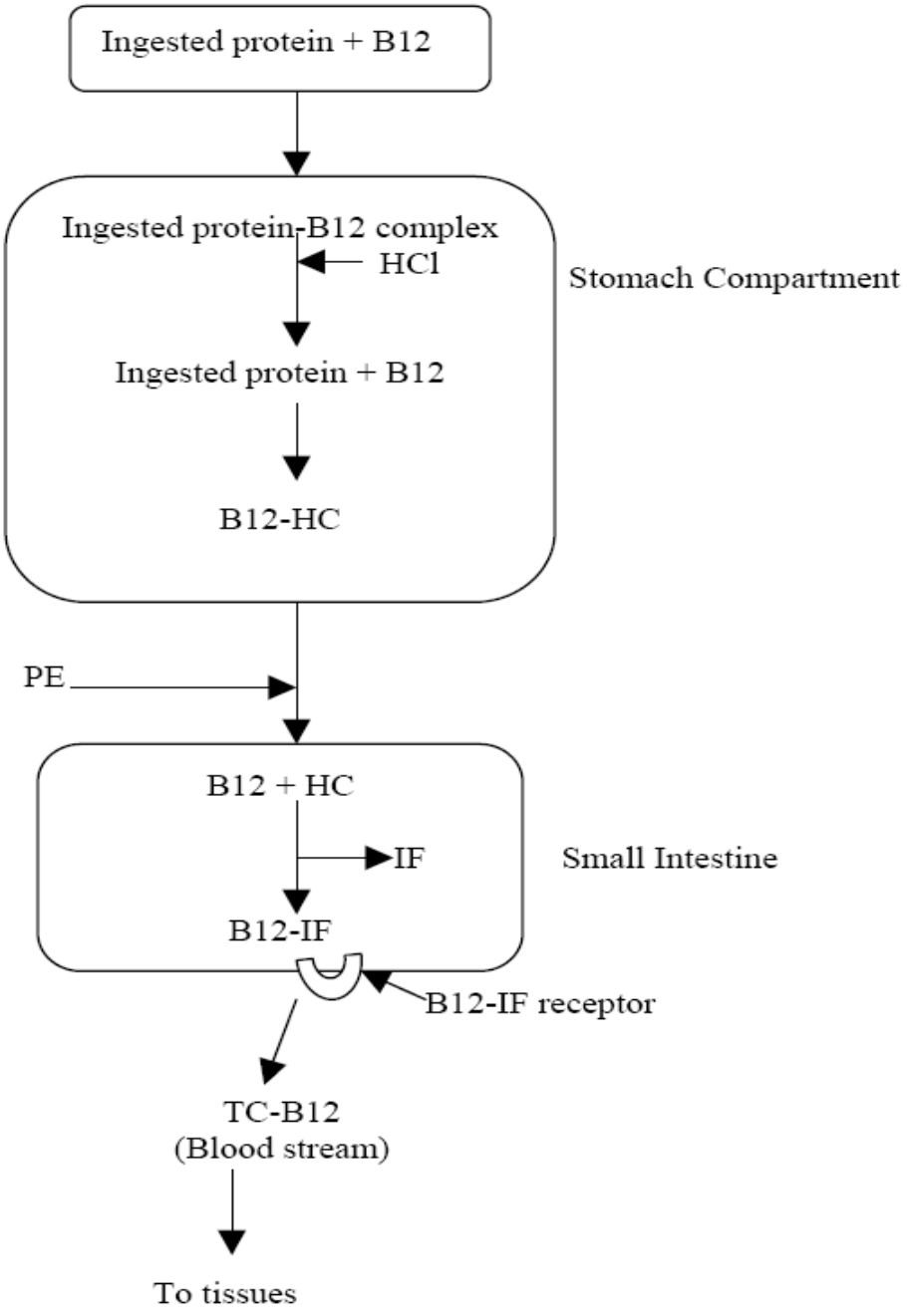

Figure 1.10: Digestion, absorption and transport of vitamin B-12. B12 = vitamin B12; HCl = Hydrochloric acid; IF = Intrinsic factor; HC = Haptocorrin; PE = Pancreatic enzyme; TC = Transcobalamin. (modified from Axis-Shield, 2002. www.active-b12.com/b12-deficiency/general.php)

Adenosylcobalamin (also referred to as coenzyme B12, cobamamide, cobinamide, or dibencozide) is the major form of vitamin B-12 in cellular tissues, and is retained in the mitochondria. MethylB12 is predominantly found in plasma, certain other body fluids (such as cerebral spinal fluid), and cytosol (Gregory Kelly, 2002). AdenosylB12 is important in carbohydrate and lipid metabolism as well as in the synthesis of porphyrin. It mediates the mitochondrial conversion of methylmalonyl-CoA to succinyl-CoA. This reaction is catalyzed by methylmalonyl-CoA Mutase, which then enters the tricarboxylic acid (TCA) cycle (Qureshi *et. al.*, 1994). Hence, deficiency of adenosylB12 leads to accumulation of methylmalonic acid, MMA (Figure 1.11).

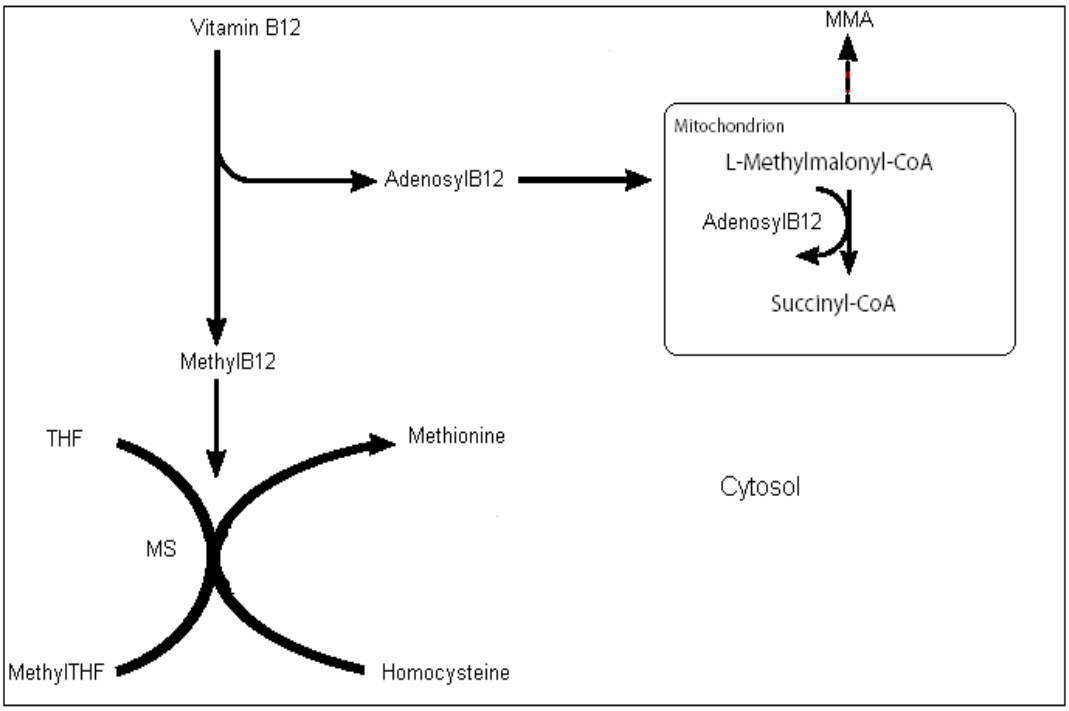

Figure 1.11: Metabolic pathway of adenosylcobalamin (AdenosylB12) and methylcobalamin (MethylB12). THF = Tetrahydrofolate; MethylTHF = Methyltetrahydrofolate; MS = Methionine synthase; MMA = Mehtylmalonic acid. (modified from Axis-Shield, 2002. www.active-b12.com/b12-deficiency/general.php)

Under normal conditions, MMA is present in physiological fluids at very low concentrations. In methylmalonic acidemia, a condition arising due to an inborn error of metabolism leading to disorders of vitamin B12 metabolism, the concentration of MMA becomes significantly elevated. Normal serum methylmalonic acid levels are 0.08 to 0.56 μmoles/L. Urine levels are less than 4.7mg/mg creatinine Values greater than these normal range suggest vitamin B12 deficiency or a genetic disease resulting in high levels of methylmalonic acid (methylmalonic aciduria).

The metabolism of MMA occurs in the mitochondria and is catalyzed by methylmalonyl CoA mutase, one of only two known B12-requiring reactions, and the only one to occur in mitochondria (Figure 1.11). Methylmalonic acidemia has been attributed to the disruption of this reaction, resulting in associated neurological symptoms and learning deficiencies. Another contributing factor to methylmalonic aciduria may be the secondary accumulation of propionyl CoA. Propionyl CoA acts as a substitute for acetyl CoA in the citrate synthase reaction and forms methyl citrate, a TCA cycle poison. Due to their high oxidative demands, neural tissues are highly sensitive to the build-up of methyl citrate (Bralley & Lord, 1999).

In humans methylB12 acts as a cofactor for the enzyme methionine synthase (see Figure 1.6) found in the cytosol. Therefore, elevated levels of homocysteine and methylmalonic acid, MMA (leading to methylmalonic acidemia) can be used as biomarkers of decreased levels of the coenzyme forms of vitamin B12 (TC-B12), or the presence of a genetic enzyme defect. Since methylB12 is a potential donor of the methyl group in the regeneration of methionine from homocysteine, this argument can be used to justify this coenzyme form of vitamin B12 as a part of the nutritional protocol for

lowering homocysteine. Araki et al demonstrated this point by administering 1000 μg of MethylB12 i.m. daily for three weeks to ten diabetic patients with elevated plasma homocysteine levels. The plasma levels of homocysteine decreased following treatment from a mean value of 14.7 to 10.2 nmol/ml ($P < 0.01$) (Araki *et. al.*, 1993). Bhatt and coworkers (Bhatt *et. al.*, 1986) also suggested that adenosylB12 be the cobalamin therapy of choice for individuals with biochemically uncharacterized methylmalonic acidemia after successful treatment of methylmalonic patients with AdenosylB12. Although it is not unusual to have high blood levels of vitamin B12, patients with hepatitis, cirrhosis, and other liver diseases have low liver concentrations of vitamin B12 and its coenzymes. This is due to an impaired ability of the liver to absorb vitamin B12 from the portal circulation (Glass *et. al.*, 1958).

1.3.4 Homocysteine

Biochemical signs of subclinical folate deficiency can be demonstrated by measurement of plasma total homocysteine (tHcy). This is because plasma tHcy is a functional marker of the activity of the enzymes involved in the retroconversion of Hcy to methionine and its conversion to cystathione, which is dependent on vitamin B6 status (Van der Dijs *et. al.*, 2002) (Figure 1.6). Folate and vitamin B12 act as a substrate for methionine synthase and 5, 10-methylenetetrahydrofolate reductase (MTHFR). Thus, homocysteine is an intermediate product in the metabolism of methionine. In healthy individuals, two different pathways are utilized to metabolize homocysteine. One pathway leads to the conversion of homocysteine back to methionine and is dependent on folate and vitamin B 12. The other pathway results in the conversion of homocysteine

to cysteine. This pathway requires two vitamin B-6 (PLP)-dependent enzymes. Therefore, the level of homocysteine in the blood is regulated by at least three vitamins: folic acid, vitamin B-12, and vitamin B-6 (See Figure 1.6). Studies have shown a link between elevated Hcy and vascular disorders, a link that is biologically plausible since Hcy promotes oxidant injury to the vascular endothelium, impairs endothelium-dependent vasomotor regulation and may also alter the coagulant properties of the blood. The high plasma Hcy levels can be reduced by dietary supplements of folic acid and other B vitamins (Maxwell, 2000).

Bonnette and co-workers (Bonnette *et. al.*, 1998) found that plasma Hcy concentrations were significantly lower (mean Hcy concentration 60% lower) in normal pregnant women during the second trimester than in non-pregnant women, independent of folate intake (5.4 μM compared to 8.7 μM). Possible explanations for these lower levels include dilution of maternal blood secondary to plasma expansion. While the increased gestational blood volume begins around the sixth (6^{th}) week of pregnancy and peaks at 30-34 weeks, hematocrit decreases during the second week to 33-39% (Blackburn & Loper, 1992). Another explanation is that the hormonal milieu might contribute to lower plasma Hcy concentrations, especially in the second trimester. This explanation is based on the fact that plasma Hcy levels are lower in premenopausal women compared to postmenopausal women (Van der Mooren *et. al.*, 1997; Wouters *et. al.*, 1995).

1.3.5 Methionine, S-adenosylmethionine and S-Adenosylhomocysteine

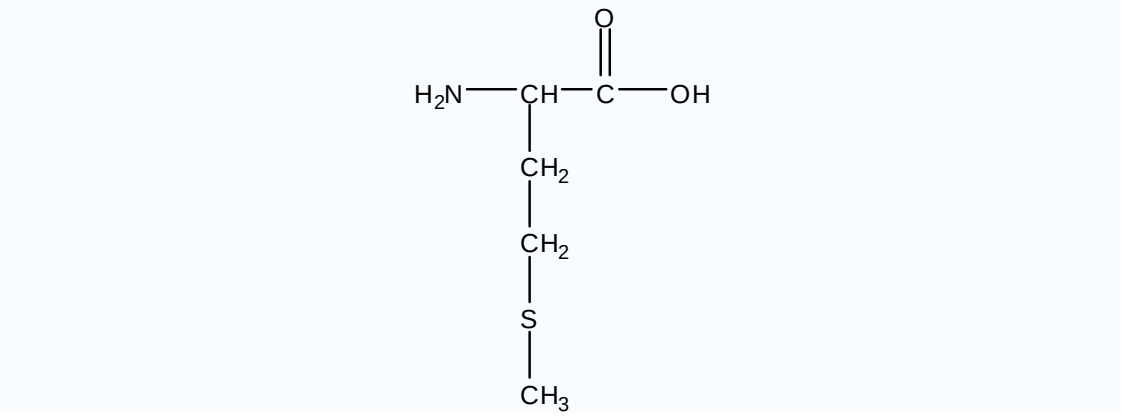

Figure 1.12: Structure of the amino acid Methionine.

Methionine (Figure 1.12) is a sulfur-containing essential amino acid. It reacts with adenosine triphosphate (ATP) to form S-adenosyl methionine (SAM), a potent donor of methyl groups (Figures 1.6). *S*-adenosylhomocysteine (SAH) is formed as a result of methyl transfer to other R-CH_3 groups (in catecholamines, DNA, RNA, etc) (Figure 1.11). *S*- adenosylhomocysteine (SAH) is then cleaved by the enzyme adenosylhomocyteinase to yield homocysteine and adenosine. The retroconversion of Hcy to methionine is catalyzed by methionine synthase, a reaction that occurs under methionine-sparing conditions and requires N^5-methyl-tetrahydrofolate as methyl donor.

Regulation of the methionine metabolic pathway is based on the availability of methionine and cysteine. If both amino acids are present in adequate quantities, SAM accumulates and is a positive effector on cystathionine synthase, encouraging the production of cysteine and α-ketobutyrate (both of which are glucogenic). However, if methionine is scarce, SAM will form only in small quantities, thus limiting cystathionine synthase activity. Under these conditions accumulated homocysteine is re-methylated to methionine, using N^5-methylTHF and other compounds as methyl donors.

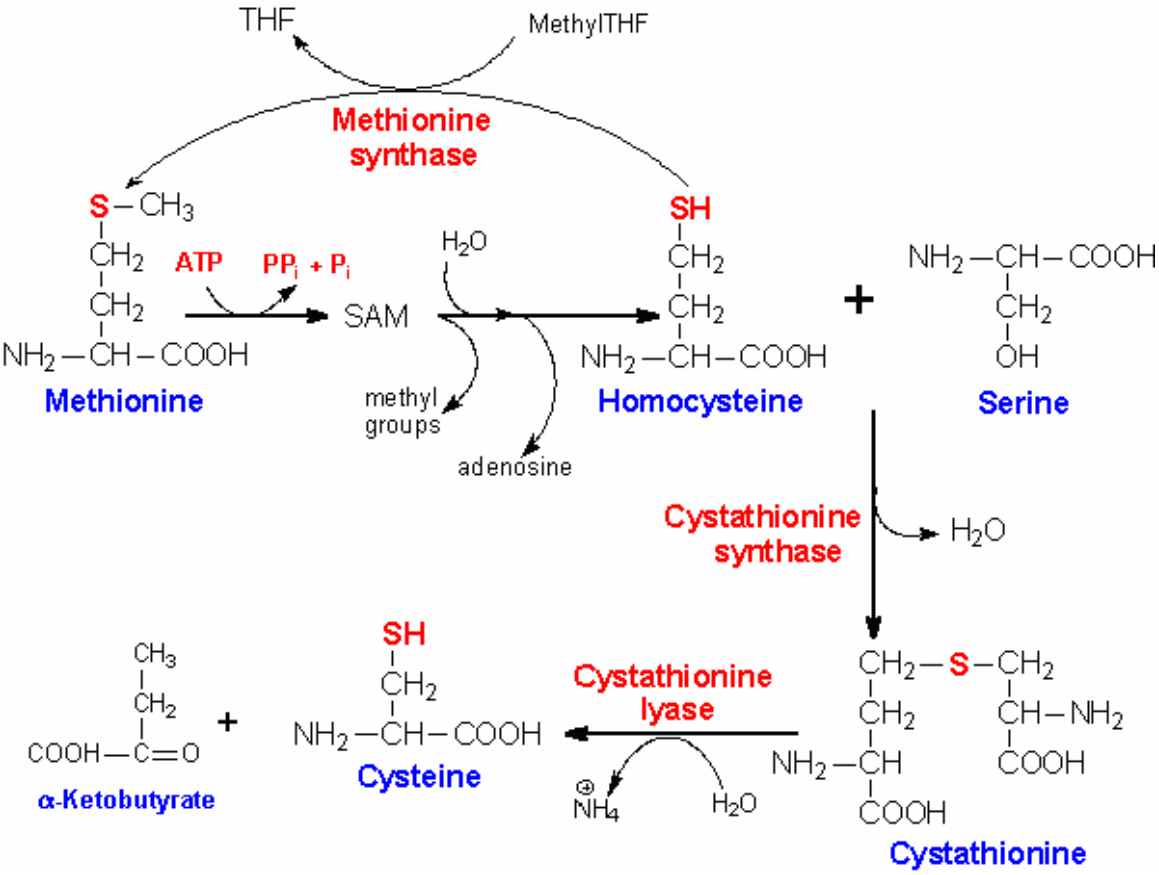

Figure 1.13: Metabolic pathway of methionine and its utilization in the synthesis of cysteine. SAM = *S*-adenosylmethionine; THF = Tetrahydrofolate; MethylTHF = Methyltetrahydrofolate.

Circulating levels of 5-MTHF regulate hepatic methionine metabolism. As illustrated in Figures 1.6 and 1.13, this is achieved through the retro conversion of Hcy to methionine. Folate deficiency in the presence of ethanol consumption enhances the imbalance in methionine metabolism and DNA damage (Halsted *et. al.*, 2002b). Prior studies have shown that methionine synthase and SAM are reduced with compensatory increase in betaine-homocysteine methyltransferase (BHMT) in animals chronically fed ethanol (Barak *et. al.*, 1987; Halsted *et. al.*, 2002d; Trimble *et. al.*, 1993).

1.3.6 Methylglyoxal

Methylglyoxal (MG) and glyoxal (GL) are reactive α-oxoaldehydes formed as a by-product of glycolysis, lipid and amino acid metabolism (Thornalley, 1993). Both MG and GL undergo glycation with proteins by forming a covalent bond in a nonenzymatic reaction with free amino groups to form a schiff base (Kasper *et. al.*, 2000; Thornalley *et. al.*, 1999). The Schiff base then undergoes rearrangement to form relatively stable ketoamines known as Amadori products. The glycated biomolecules then undergo progressive dehydration, cyclization, oxidation and rearrangement to form advanced glycation end-products (AGEs) (Raj *et. al.*, 2000). The glyoxals also react nonenzymatically with sulfhydryl (–SH) groups of membrane proteins, metabolic enzymes and membrane ion channels inhibiting their function. For example, GL reacts with arginine leading to imidazolium formation, while it reacts with lysine to form an AGE oxidative adduct, *N*-(carboxymethyl) lysine (Lee *et. al.*, 1999; Wells-Knecht *et. al.*, 1995). Both GL and MG react non-enzymatically with guanyl nucleotides of DNA/RNA to form 6,7-dihyro-6, 7-dihydroxy-imidazo [2,3-b] purine-9 (8) one derivatives (Yamaguchi & Nakagawa, 1983).

Under normal physiological conditions, methylglyoxal is kept at a low level by binding to cysteine and being excreted in bile and urine or through catabolism via the glutathione-dependent glyoxalase system (Schauenstein & Esterbauer, 1979; Schauenstein *et. al.*, 1977). Therefore, any deficiencies in cysteine and/or glutathione would result in elevated MG.

1.4 Role of Free Radicals and Antioxidant Capacity

Free radicals are chemical species that possess one or more unpaired electrons. The term *Reactive Oxygen Species (ROS)* collectively describes free radicals such as $O_2^{\bullet-}$, $OH^{\bullet}$, and the non-radical oxygen derivatives such as hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl). Cellular components are attacked by unstable free radicals, resulting in damage to lipids, proteins and DNA, thereby initiating a chain of events that leads to the onset of disease condition. Free radicals originate in various ways. For the most part, they originate from biochemical redox reactions involving oxygen, which occur as part of normal metabolism, *e.g.*, $O_2^{\bullet-}$, $NO^{\bullet}$, H_2O_2. They may also be produced as part of a controlled inflammatory reaction by phagocytes, *e.g.*, HOCl, $O_2^{\bullet-}$ Occasionally, free radicals may be formed in response to ionizing radiation, UV light, environmental pollution, cigarette smoke, hypoxia, excessive exercise, and ischemia, *e.g.,* $O_2^{\bullet-}$, $OH^{\bullet}$, $ROO^{\bullet}$.

The body has a number of mechanisms that control the production of ROS and limit or repair their damage to tissues. These mechanisms consist of preventative actions and scavenging by antioxidants species. The former prevent the formation of new ROS and comprise of proteins such as ceruloplasmin, metallothionine, albumin, transferrin, ferritin, and myoglobin. The latter group of antioxidants remove formed ROS, hence preventing the formation of radical chain reactions. This group consists of certain enzymes such as superoxide dismutase, glutathione peroxidase, glutathione reductase, catalase, metalloenzymes, and small molecules such as glutathione, ascorbic acid,

tocopherol, bilirubin, uric acid, caroteniods and flavonoids. Figure 1.14 below shows a typical reaction of the antioxidant enzymes.

Superoxide Dismutase (SOD) $2O_2^{\bullet-} + 2H^+ \longrightarrow H_2O_2 + O_2$

Glutathione Peroxidase (GPx) $ROOH + 2GSH \longrightarrow ROH + H_2O +$ GSSG

Glutathione Reductase (GR) $NADPH + H^+ + GSSG \longrightarrow NADP^+ + 2GSH$

Figure 1.14: Reactions of some antioxidant enzymes.

Alcohol induces in the liver a hyper metabolic state that is marked by increased mitochondrial respiration. This leads to a high rate of reoxidation of NADH, which is produced during the metabolism of alcohol by cytosolic alcohol dehydrogenase. The subsequent decrease in the NAD+/NADH ratio favours mitochondrial superoxide generation by increasing electron flow along the respiratory electron transport chain (Ishii *et. al.*, 2003). Other reports (Colell *et. al.*, 1998; Fernandez-Checa *et. al.*, 1998; Fernandez-Checa *et. al.*, 2002) have shown that mitochondrial glutathione and SAM are important antioxidants in the prevention of the free radical chain reaction.

Evidence exists that shows ethanol to have similar effects on fetal tissue. Henderson and coworkers (Henderson *et. al.*, 1995) have shown that exposure to 2 mg/mL ethanol in cultured fetal rat hepatocytes increased total and mitochondrial membrane lipid peroxidation products, malondialdehydes (MDA), dienes, and fluorescent products, as well as H_2O_2 production, while decreasing GSH levels. They also showed that some measures of this lipid peroxidation could be reversed by pre-treatment with vitamin E or compounds that normalized GSH stores, such as SAM and *N*-

acetylcysteine. This group has shown that the same effects are evident in *in vivo* experiments. The greatest sensitivity to lipid peroxidation due to in-utero ethanol exposure in rats seems to reside in the earliest gestational stage (day 14). Exposure to ethanol at this point results in a 30% increase in membrane lipid peroxidation within 1-hour of maternal ethanol consumption (Henderson *et. al.*, 1995).

Ethanol is metabolized in the liver through two pathways. The primary pathway is by alcohol dehydrogenase. Oxidation of ethanol by microsomal ethanol oxidizing system (MEOS) is the other, but minor pathway (Cederbaum, 1989). The significance of the MEOS pathway increases under conditions of elevated concentrations of ethanol and in situations of chronic alcohol consumption. In fact, Cederbaum (Cederbaum, 1989) indicates that the induction of this pathway is of importance from a toxicological perspective. This is due to the fact that the induction of the MEOS system results in the generation of reactive oxygen species leading to lipid peroxidation. Acetaldehyde, produced from the metabolism of ethanol, results in reduction of the hepatic redox state. It is then oxidized by the two closely related molybdo-flavo enzymes, xanthine oxidase and aldehyde oxidase. Both of these enzymes carry out the univalent and divalent reduction of O_2 and the release of $O_2^{\bullet-}$ and H_2O_2 (Fridovich, 1989).

Table 1.4: Suggested mechanisms for the promotion of oxidative stress by ethanol. (from Cederbaum, 1989. Reprinted with permission from Elsevier).

1. Depletion of Glutahione (GSH)
2. Altered antioxidative defence
3. Direct toxic effect of ethanol on cell membranes and membrane fluidity
4. Metabolic effects of ethanol via:
    i - Acetaldehyde (direct effect on cellular components)
    ii - Altered redox state
    iii - Ethanol radicals – $CH_3CH_2O^{\bullet}$, $CH_3C^{\bullet}HO$, ethanol scavenges $^{\bullet}OH/OR$
5. Ethanol induced cytochrome P450 as a Fenton/Haber-Weiss catalyst
6. Chronic effect of ethanol via:
    i - Mitochondria injury
    ii - Microsomal proliferation and induction
    iii - Hypermetabolic state
7. Increased hepatic iron levels
8. Induction of release of chemoattractants

Formate, the metabolite of methanol, also produces free radicals through a Fenton-like reaction (Dikalova *et. al.*, 2001). In this reaction, a hydroxyl radical ($^{\bullet}OH$) will be formed through the Fenton-like reaction, which will, in turn, oxidize formate (HCO_2^-), forming the carbon dioxide anion radical ($^{\bullet}CO_2^-$). The carbon dioxide anion radical then reacts with molecular oxygen, forming carbon dioxide and the superoxide radical (Figure 1.15).

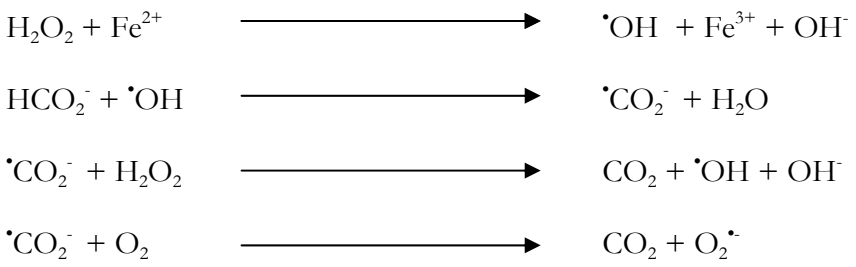

Figure 1.15: The generation of free radicals through the oxidation of formate.

In addition to the induction of $O_2^{\bullet-}$, alcohol itself is able to interact with the hydroxyl radical, and thus, reduce the hydroxyl radical-induced toxicity (West *et. al.*, 1994). Ethanol also causes a decrease in hepatic GSH levels by a modest 30 to 50% decrease even after the short-term administration of large doses of ethanol (simulating binge drinking). The reduction in GSH has been observed in association with ethanol-induced lipid peroxidation (Fridovich, 1989; Videla *et. al.*, 1985; Videla & Valenzuela, 1985; Macdonald *et. al.*, 1977; Comporti *et. al.*, 1973; Shaw, 1989). There is increased excretion of oxidized glutathione following chronic alcohol exposure (Sies *et. al.*, 1979). Table 1.4 and Figure 1.1 summarize the suggested mechanisms by which oxidative stress is promoted by alcohol.

1.5 Synopsis

Methanol is present in alcoholic beverages as a congener from the manufacturing process. Methanol toxicity, mediated through formate, is reduced in the presence of ethanol due to the preferential utilization of the primary alcohol-metabolizing enzyme, alcohol dehydrogenase. Due to the increased activity of hepatic cytochrome P450 2E1 in alcoholic drinkers, the elimination of ethanol from the body is enhanced, thereby,

reducing the protective effect of ethanol in methanol toxicity, while increased free radicals such as $O_2^{\bullet-}$ and reduced antioxidants (*e.g.,* GSH) enhance oxidative stress and tissue injury. This could lead to fetal brain injury and development of FASD in pregnant women.

Folic acid is a vitamin cofactor that is required, among other things, in the elimination of formate. The status of folate metabolism in the body is as equally important as its bioavailability in the successful elimination of formate. Folate metabolism, however, is also dependent upon vitamins B-6 and B-12. Monitoring the levels of vitamin B-6, vitamin B-12, homocysteine, methylmalonic acid, and the folate catabolite *p*-aminobenozylglutamate and its acetamido derivative ap-acetamidobenzoylglutamate, methionine, and its related intermediates can evaluate the overall status of folate metabolism. Measurements of markers of oxidative stress will elucidate the extent to which the toxicity of methanol and formate are mediated in the body in the various folate states.

Binge alcohol drinkers may suffer from inadequate folate intake and elevated BAC. Inadequate folate intake, coupled to alcohol's impairment of enterohepatic circulation of folic acid, could put such individuals, and especially the fetus, at risk of the toxic effects of formate (Figure 1.1). However, no data exists as to the true nature of the relationship between formate metabolism and folate status in binge drinking individuals, and how this relationship may affect the development of FASD. This study aimed at establishing the relationship between formate levels and folate status in intoxicated females. The study

also investigated how formate metabolism is affected during formate insult in folate deficient young pig.

This study involved the recruitment of human female subjects presenting to the emergency room of the Royal university hospital, Saskatoon for alcohol intoxication, the Regina detox centre for drug and alcohol rehabilitation and the prenatal clinic of various hospitals of the Calgary health region. For these groups, the relationship between formate and folate levels was determined. Because of the difficulties and ethical considerations involved in studying formate kinetics in folate deficient human, an experimental folate deficient young swine was utilized to meet this objective. A group of these animals, together with their folate replete counterparts, were administered formate to assess formate pharmacokinetics and antioxidant capacity.

1.6 Thesis Hypothesis and Objectives

1.6.1 Overall Hypothesis

The overall null hypothesis of this study was as follows:

Ho: *Formate does not accumulate significantly during alcohol intoxication and is unaffected by circulating folate levels in drinkers. Hence, antioxidant capacity is also unaffected in these individuals.*

This hypothesis was broken down into two separate (but related) components:

1.6.2 Hypothesis regarding the relationship between formate and folate levels

I. Ho: *Formate levels are not increased and correlated with folate levels during intoxication in human females.*

1.6.2.1 Specific Objectives

Using human female subjects, the objectives were to determine whether:

- blood methanol and formate levels were increased during intoxication
- blood formate levels correlated with blood folate levels in alcohol intoxicated females.
- blood formate levels correlated with blood folate levels in sober drug and alcohol rehabilitating females.
- plasma formate levels correlated with plasma folate levels in pregnant females.
- blood levels of vitamins B12 and B6, and Hcy various among the above mentioned groups.

1.6.3 Hypothesis regarding relationship between formate pharmacokinetics and folate status

II. Ho: *Formate pharmockinetics is not altered during formate insult in folate deficient state.*

1.6.3.1 Specific Objectives

Using folate deficient young pigs, the objectives were to determine whether:

- formate kinetics is altered in folate deficiency during formate insult.

- folate utilization is altered during formate insult in folate deficiency state by measuring plasma levels of folate catabolites *p*-aminobenzoylglutamate and its acetylated derivative ap-acetamidobenzoylglutamate.

1.6.4 Hypothesis regarding relationship between formate pharmacokinetics and Antioxidant capacity in folate deficiency state

III. Ho: *Antioxidant capacity is not altered during formate insult in folate deficiency.*

1.6.4.1 Specific Objective

Using folate deficient young pigs, the objective was to determine whether:

- plasma levels of vitamins B12 and B6, Hcy, methionine, SAM, SAH, MG and MMA, as markers of oxidative stress, changed during formate insult in folate deficient pigs.

CHAPTER 2

Quantitative Endogenous Formate Analysis in Plasma and Whole Blood Using Headspace Gas Chromatography without Head Space Analyzer.

2.1 Introduction

Formic acid and its conjugate base formate are essential endogenous one-carbon metabolites in most living organisms participating in vital one-carbon pool of intermediary metabolism (Cook et. al., 2001; Fu et. al., 2001). Studies have shown that formate is the metabolite of methanol (MeOH) and responsible for the toxicity observed in methanol poisoning (Barceloux *et. al.*, 2002; Dorman *et. al.*, 1994; Sejersted *et. al.*, 1983). Another source of formate is through the diet, environmentally through inhalation of methanol vapours, production by intestinal microflora and certain dietary supplements (Hanzlik *et. al.*, 2005; Li *et. al.*, 2001). For methanol toxicity to occur, it first has to be metabolized by alcohol dehydrogenase (ADH) to formaldehyde and thence to formic acid (Figure 1.2). The toxicity of formate includes optic nerve damage, an increased anion gap and metabolic acidosis (Medinsky *et. al.*, 1997; Smith & Taylor, 1982).

Early laboratory diagnosis of methanol poisoning as well as monitoring of formate levels, whether for occupational health or clinical research purposes, is essential. In the case of methanol poisoning, analysis by gas chromatographic (GC) based methods are the most common. Most hospitals use osmolal and anion gap to aid in their diagnosis of alcohol ingestion. Methanol increases the osmolal gap while formate increases the anion gap (Barceloux *et. al.*, 2002; Hovda *et. al.*, 2004; Jacobsen *et. al.*, 1982). Metabolic acidosis

and increased anion and osmolal gaps might not be seen at lower concentrations of plasma methanol (< 20 mM) and formate (Aabakken *et. al.*, 1994; Hovda *et. al.*, 2004; Hovda *et. al.*, 2005). Furthermore, increases in osmolal and anion gap could also be due to ethylene glycol ingestion (Ellenhorn *et. al.*, 1997). Given the 'ubiquitous' nature of formate and its deleterious effect at higher levels, it is important to have a reliable quantitative method for the measurement of plasma formate at all levels (subclinical and clinical). Most routine clinical methods involve enzymatic analysis and/or GC with flame ionisation detection (FID). Although these methods are reliable, their sensitivity at lower endogenous levels is poor. Mass spectrometry may provide a better sensitivity but is expensive and has longer turn around due to sample preparation. We describe here a simple quantitative GC-FID method that is sensitive at lower formate levels and provides a faster turn around time.

2.2 Experimental

2.2.1 Materials

Pure formic acid Standard (88% v/v) was obtained from Fisher Scientific (Fair Lawn, NJ.). Sulphuric acid and anhydrous ethyl alcohol were obtained from J. T. Baker (Phillipsburg, NJ., USA) and Commercial Alcohols Inc. (Brampton, ON.), respectively. A Gas tight syringe (Hamilton 1.25 mL; 22/2"/2) was purchased from Hamilton Company (Reno, NV.). A working stock solution of 100 mM formic acid was prepared in deionized water. Aqueous serial dilutions were made from each of the stock at concentrations of 20.0 mM, 10.0 mM, 4.0 mM, 2.0 mM, 1.0 mM, 0.5 mM, 0.25 mM, 0.125 mM and blank.

A 10% (v/v) ethanol in deionised water was prepared as an internal standard. These working standards and the ethanol were prepared fresh each time.

2.2.2 Analysis of clinical sample and Recovery experiments

Pooled plasma was spiked with formic acid to required concentrations (0.125 and 1 mM). Limits of detection (LOD) and quantitation (LOQ) were determined in aqueous samples due to the ubiquitous nature of formate in biological samples. Five levels (ten replicates of each) were prepared at 0.010, 0.020, 0.125, 0.250 and 1.000 mM. Data obtained was analysed using EP Evaluator software version 7 (David G. Rhoads Associates, Kennett Square, PA., USA).

Plasma samples (personal information identifications removed) were obtained from 69 pregnant women whose plasma samples were sent for prenatal panel (rubella Ig G, syphilis, hepatitis B virus antigen and HIV antigen). Another 16 and 11 serial plasma samples were collected from a female patient admitted into the intensive care unit for methanol poisoning. A further four samples were obtained from a pharmacokinetic study using pigs (31-38 kg, n=4), which were injected intervenously with 237 mg/kg formate.

2.2.3 Sample Preparation

Two linear calibration curves were constructed. One consisting of 0 mM, 0.125 mM, 0.25 mM, 0.5 mM, 1.0 mM and 4.0 mM, for low formate samples. The second curve was constructed from standards prepared at 0 mM, 2 mM, 4 mM, 10 mM and 20.0 mM for high formate samples. Two hundred microlitres (200 uL) plasma samples and aqueous

standards were each placed in 1 mL glass vial. Fifty microlitres (50 uL) of aqueous ethanol (10% v/v) was added as internal standard and as a derivatizing agent. The formation of ethylformate was enhanced by addition to the reaction mixture of 200 uL concentrated sulphuric acid as a catalyst. The vials were then sealed immediately and placed in a waterbath for 15 minutes at 60ºC to allow the ethylformate formed to equilibrate in the headspace above the reaction mixture in the vial. One millilitre (1 mL) of this headspace gas was siphoned using a gastight syringe and injected into the gas chromatograph.

2.2.4 Instrumentation

Analysis of ethylformate was done on a gas chromatograph fitted with a flame ionisation detector (Hewlett Packard Series II 5890) fitted with a DB-ALC1 capillary column (30 m x 0.53 mm ID, 3.0 μm, part number J125-9134, J & W Scientific available in Canada from Chromatographic Specialities Inc., Brockville, ON.). Other parameters were set as outlined in table 2.1.

Integration of the internal standard peak (ethanol) and ethylformate was done using HP 3365 Series II Chemstation (version A.03.21, Hewlett-Packard Co., 1989-1992). Quantitation of the ethylformate was done using the ratio of the ethylformate peak area to that of ethanol using standard curves (Chemistry Software for Windows®, ChemSW™, Inc., 1993-1998).

Table 2.1: Parameter values for the Gas chromatograph.

Parameter	Type	Value
Carrier Gas	Helium	80 cm/sec, measured at 40° C
Oven	Isothermal	40° C
Injector	Headspace splitless	250° C
Detector	FID	300° C
Detector gas	Hydrogen	23 mL/min
Column	DB-ALC1 capillary column	30 m x 0.53 mm ID, 3.0 μm,

2.3 Results

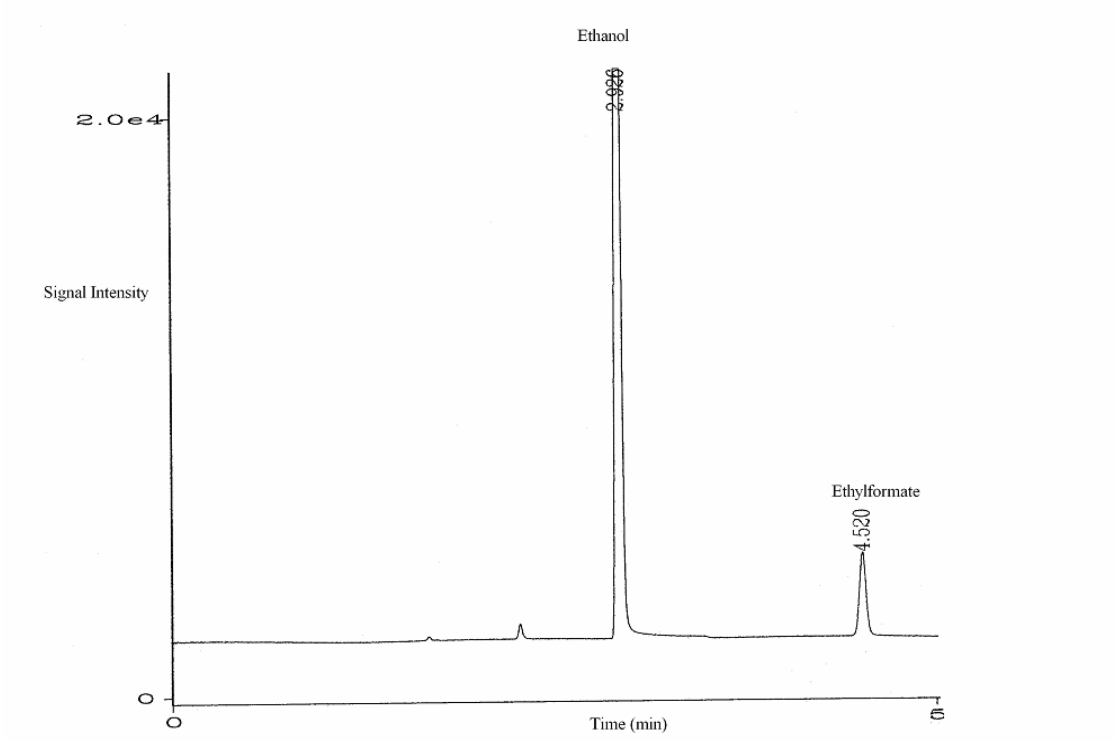

Figure 2.1: Chromatographic spectrum of 1000 μL headspace of 1 mM aqueous standard of ethylformate. Sample was injected into a Hewlett Packard Series II 5890 fitted with a DB-ALC1 capillary column (30 m x 0.53 mm ID, 3.0 μm) and Flame ionization detector using a gastight syringe.

Figure 2.1 is a representative chromatogram showing the internal standard (ethanol) eluting at 2.9 min and ethylformate at 4.5 min. A six-point linear calibration curve was constructed for the aqueous standards. The chromatography of these aqueous standards showed specificity of the method for ethylformate and its complete separation from the internal standard (ethanol). Spiked samples also showed chromatography consistent with that seen in the aqueous standards. The amount of ethanol added as an internal standard resulted in very high levels (final concentration 311 mM; 14.29g). Thus, any ethanol present in the sample would not have an effect on the quantitation.

2.3.1 Linearity

A six-point calibration curve was constructed for the low standards (Figure 2.2) and five-point for the high standards (Figure 2.3). The dynamic range selected was based on physiological levels of formate (Buttery & Chamberlain, 1988;Dorman *et. al.*, 1994;Jacobsen et. al., 1988;Medinsky *et. al.*, 1997). The low standard curve calibration points included a blank, 0.125, 0.250, 0.500, 1.000, 2.000 and 4.000 mM. The linear regression equation was $y = 0.0537x + 0.00075$ ($r = 0.9992$; $r^2 = 0.9985$). The high standard curve calibration points included a blank, 2, 4, 10 and 20 mM. The linear regression equation was $y = 0.00502x + -0.00034$ ($r^2 = 0.9999$).

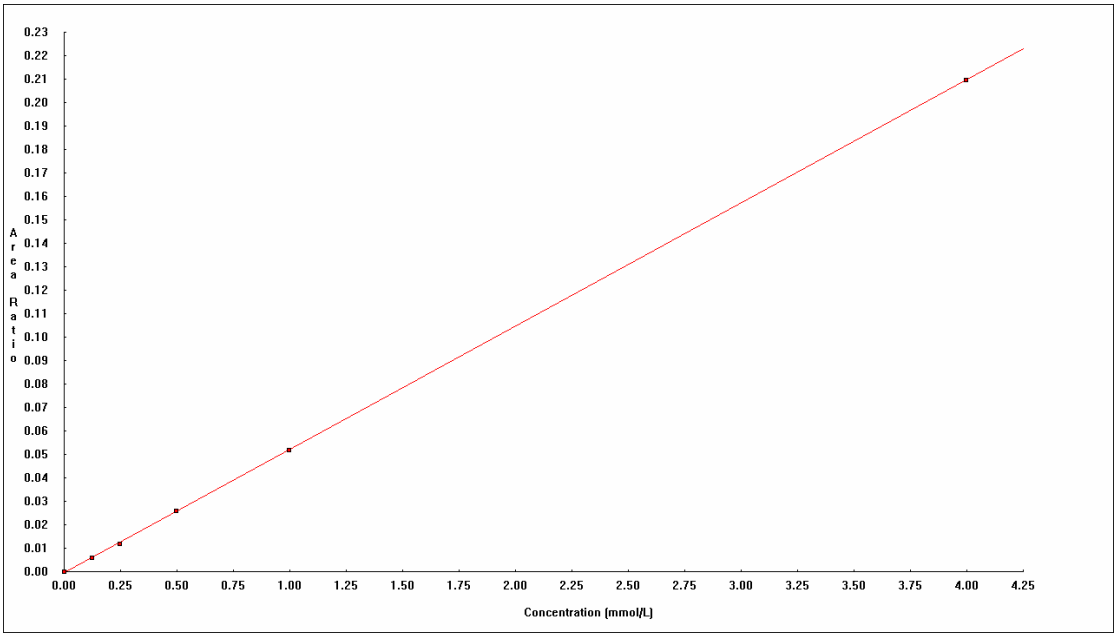

Figure 2.2: Standard curve of low aqueous calibrators of formic acid run on Hewlett Packard Series II 5890 fitted with a DB-ALC1 capillary column (30 m x 0.53 mm ID, 3.0 μm) and Flame ionization detector using a gastight syringe.

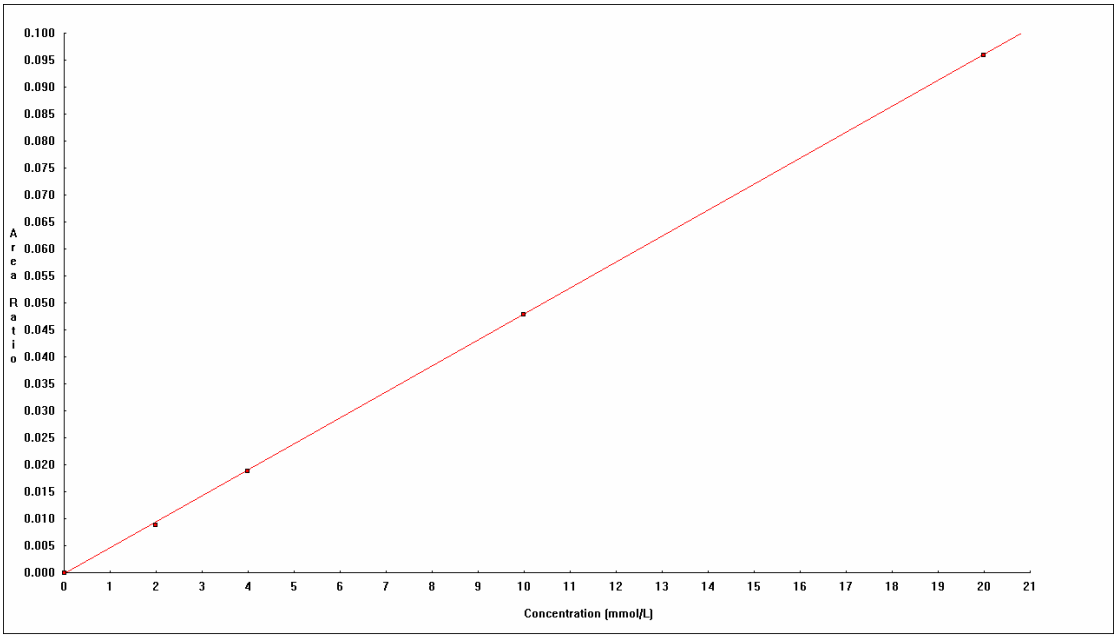

Figure 2.3: Standard curve of high aqueous calibrators of formic acid run on Hewlett Packard Series II 5890 fitted with a DB-ALC1 capillary column (30 m x 0.53 mm ID, 3.0 μm) and Flame ionization detector using a gastight syringe.

2.3.2 Precision and Recovery

The limit of detection (LOD), determined in aqueous standards, was 0.02 mM corresponding to imprecision (coefficient of variation, CV) of 24%. Limit of quantification (LOQ) was 0.026 mM (CV = 20%). The LOQ was obtained by fitting a curve using the equation CV = A + B*(1/mean) to estimate the relationship between mean and CV. Based on this model the concentration at whose upper 95% confidence interval for the curve has a CV of 20% is determined as the LOQ (Figure 2.4 and Table 2.2).

Recovery and intra-assay precision of formate in plasma was determined at two levels (0.125 mM and 1.000 mM). Recovery and precision at both spiked levels were acceptable (101-108%). Imprecision was 10.5% and 8.6% at 0.125 mM and 1 mM levels, respectively (Table 2.3).

Table 2.2: Relationship between mean (replicates of 10) and coefficient of variation (CV) of various aqueous formate standards (mmol/L).

Sample	Target Conc.	N	Mean	SD	Meas. CV(%)	Fitted CV(%)	95% CI for Fitted Low	95% CI for Fitted High
0.010	0.010	10	0.0043	0.0039	90.4	90.2	85.9	94.5
0.020	0.020	10	0.0202	0.0041	20.5	21.8	19.9	23.8
Estimated LOQ		-	0.026	-	-	18.0	16.0	20.0
0.125	0.125	10	0.1249	0.0102	8.2	6.3	4.1	8.6
0.25	0.25	10	0.2519	0.0122	4.8	4.8	2.5	7.2
1	1	10	1.0423	0.0319	3.1	3.7	1.4	6.1

Note: LOQ = Limit of Quantitation; Conc. = Concentration; N = Number of replicates; SD = Standard deviation; CV = Coefficient of variation; CI = Confidence interval.

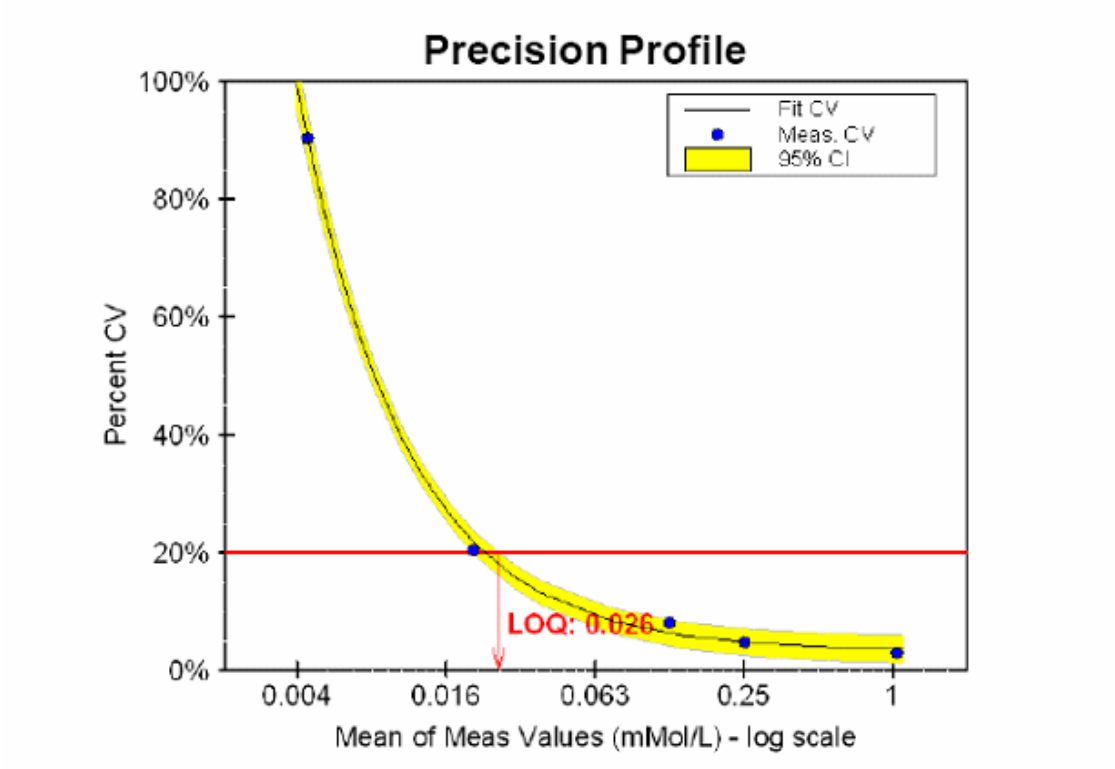

Figure 2.4: Precision profile of various aqueous formate concentrations injected into Hewlett Packard Series II 5890 fitted with a DB-ALC1 capillary column (30 m x 0.53 mm ID, 3.0 µm) and flame ionization detector using a gastight syringe. N = 10 per calibrator level; Coefficient of variation (CV) = [(3.357 ± 0.742 + (0.373 ± 0.007)]*(1/mean); r^2 = 0.999; Std Error of Estimate = 1.38. Limit of Quantitation (LOQ) = 0.026 mM. CI = Confidence interval.

Table 2.3: Recovery and precision of aqueous spiked plasma samples.

Sample Type	Concentration (mmol/L)	N	Mean (mmol/L)	SD	CV (%)	Recovery (%)
Plasma	1	10	1.075	0.092	8.60	108
	0.125	10	0.126	0.013	10.49	101
	0.125	10	0.125	0.010	8.16	100
Aqueous	0.020	10	0.020	0.004	20.45	101
	0.010	10	0.004	0.004	90.44	43

SD = Standard Deviation; CV = Coefficient of variation; N = number of replicates.

2.3.3 Clinical Samples

Table 2.4: Summary Statistics of Plasma formate in pregnant women (n = 69).

Statistic	Value (mM)	95% Confidence Interval
Mean	0.202	0.154 – 0.249
Standard Deviation (SD)	0.198	0.170 – 0.238
Range	0.011 – 0.957	
Median	0.108	

The results of formate analysis on 69 pregnant women are summarized in Table 2.4. The mean ± SD was 0.202 ± 0.198 mM. This was consistent with that found by other workers (Buttery & Chamberlain, 1988; Dorman *et. al.*, 1994; Hanzlik *et. al.*, 2005;Medinsky *et. al.*, 1997; Tephly *et. al.*, 1992). Plasma formate profile with time in pigs (n=4) administered a bolus intravenous injection of formate (237 mg/kg, as a sodium formate) is shown in Figure 2.5.

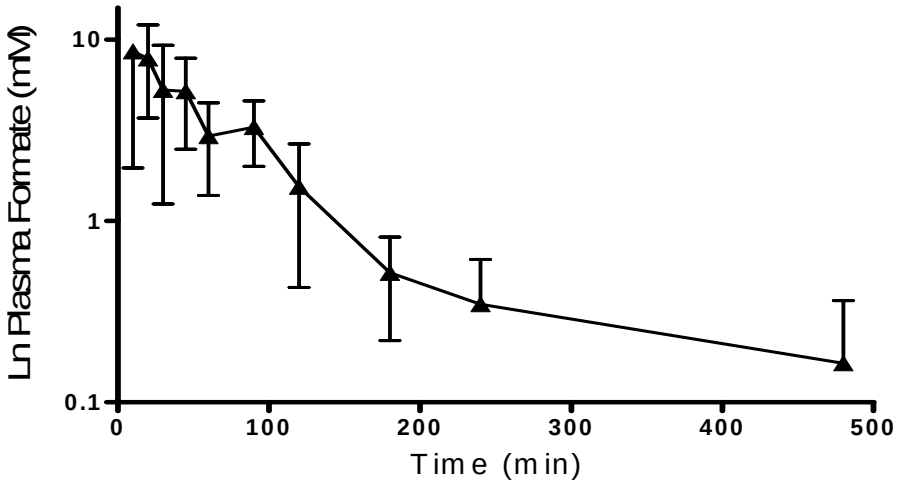

Figure 2.5: A semilogrithmic plot of plasma formate vs time plot in young pigs (n = 4) administered 237 mg/kg formate by bolus injection through left jugular vein. Each point represents mean±SD values.

Table 2.5: Mean and natural log plasma formate concentrations in young pigs (n = 4) administered 237 mg/kg formate by bolus injection through left jugular vein.

Time	CF2	CF3	CF4	CF6	Mean formate conc. (mM)	Ln mean conc. (mM)
10	5.00	16.55	11.93	1.43	8.73	2.17
20	3.27	11.75	9.06		8.03	2.08
30	2.71	9.72	8.16	1.02	5.40	1.69
45	2.19	7.78	6.08		5.35	1.68
60	1.84	3.11	4.19		3.01	1.10
90	1.82	4.61	3.95		3.46	1.24
120	0.98	1.58	2.98	0.82	1.60	0.47
180	0.73	0.65	0.68		0.69	-0.37
240	0.35	0.32	0.60	0.61	0.47	-0.76
480	0.35	0.29	0.35		0.33	-1.11

2.4 Discussion and Conclusion

Formate is an important compound that is present in humans, animals and plants. Most methods available in hospitals for diagnosis of methanol poisoning do not quantitate endogenous formate levels and rely instead on methanol levels. Gas chromatography-mass spectrometric (GC-MS) methods are able to quantitate such levels but require lengthy sample preparation. Such instruments are also expensive. Therefore, such a method is not suited for routine clinical settings. Our method combines both high sensitivity and simplicity accompanied by faster turnaround time. The instrument, GC-FID, is far cheaper than GC-MS, making it a more accessible instrument for both hospitals and research labs. Unlike the method developed by Abolin *et. al.* (Abolin *et. al.*, 1980), this method cannot be used to monitor ethanol levels encountered in ethanol intoxication or levels used in the treatment of methanol poisoning. However, our method is approximately four times more sensitive (LOQ of 26 μM, 1.2 mg/L) than that of Abolin *et. al.* (LOQ of 109 μM, 5 mg/L). This is probably due to the fact that we used a

capillary column (0.53 mm ID vs 3mm in Abolin *et. al.*) that was much longer (30 m vs 1.8 m in Abolin *et. al.*). Hence, we were able to achieve a better resolution in our peaks not only because of the column properties but also due to the fact that our esterification of formate produced ethylformate compared to methylformate in Abolin *et. al.* (Abolin *et. al.*, 1980). Unlike ethylformate, methylformate elutes very close to methanol, thus, making it impossible to separate completely from the methanol peak. In fact, this was the reason we chose ethylformate as our esterification product.

We have demonstrated the quantitative assay of plasma formate at various levels as seen in the pig and pregnant human population experiments described. The method can also be used in research settings as demonstrated by the data on formate disappearance in pigs administered formate as well as in the methanol-poisoned patient. The turnaround time from sample receipt to results was approximately 25 minutes.

CHAPTER 3

A Simple Method for the Quantitative Analysis of Endogenous Folate Catabolites *P-aminobenzoylglutamate*(*p*-ABG) and Its Acetamido (*ap-ABG*) Derivative in Biological Samples by Liquid Chromatography-Tandem Mass Spectrometry.

3.1 Introduction

The amount of biologically active folate excreted by mammals is considerably less than that which is ingested. It has long been suggested that the major daily turnover for folate is via excretion of the catabolites p-aminobenzoylglutamate (*p*-ABG) and ap-acetamidobenzoylglutamate (*ap*-ABG) (McPartlin *et. al.*, 1992; McNulty *et. al.*, 1993). Thus, the obligatory requirement of folic acid like with any other vitamin can be related to this catabolism. Certain conditions such as pregnancy, periods of rapid growth, during drug therapy regimes and in a variety of clinical conditions can increase folate catabolism and requirements.

The principal function of folate coenzymes is to accept or donate one-carbon units in folate-requiring metabolic pathways. Cellular folates in their reduced form function conjugated to a polyglutamate chain, which are a mixture of unsubstituted polyglutamyl tetrahydrofolates and various substituted one-carbon forms of tetrahydrofolate (such as 10-formyl, 5,10-methylene, and 5-methyl) (Figure 3.1).

The reduced forms of the vitamin, particularly the unsubstituted dihydro and tetrahydro forms, are unstable chemically and are easily split between the C-9 and N-10

bond to yield a substituted pteridine and p-aminobenzoylglutamate, which have no biologic activity (Figure 3.2) (Blakley, 1969; Murphy & Scott, 1979). Substituting a carbon group at N-5 or N-10 decreases the tendency of the molecule to split. The substituted forms, however, are also susceptible to oxidative chemical rearrangements and, consequently, loss of activity (Blakley, 1969). The major catabolite, however, consists of *ap*-ABG whereas *p*-ABG exists in small amounts (McPartlin *et. al.*, 1992;Murphy & Scott, 1979).

Early studies of folate catabolism in humans and laboratory animals have involved the administration of radiolabelled tracers, such as ^{14}C pteroylmonoglutamate (^{14}C PteGlu) and ^{3}H pteroylmonoglutamate (^{3}H PteGlu), and the subsequent analysis of the excreted catabolite in urine. McNaulty and co-workers (McNaulty, 1993) developed a Reverse-phase high performance liquid chromatographic method for the quantitation of endogenous folate catabolites independent of *in vivo* administration of radiolabelled tracers. The method, like the earlier methods, required extensive sample preparation and cleanup to achieve detection and quantification of the urinary catabolites.

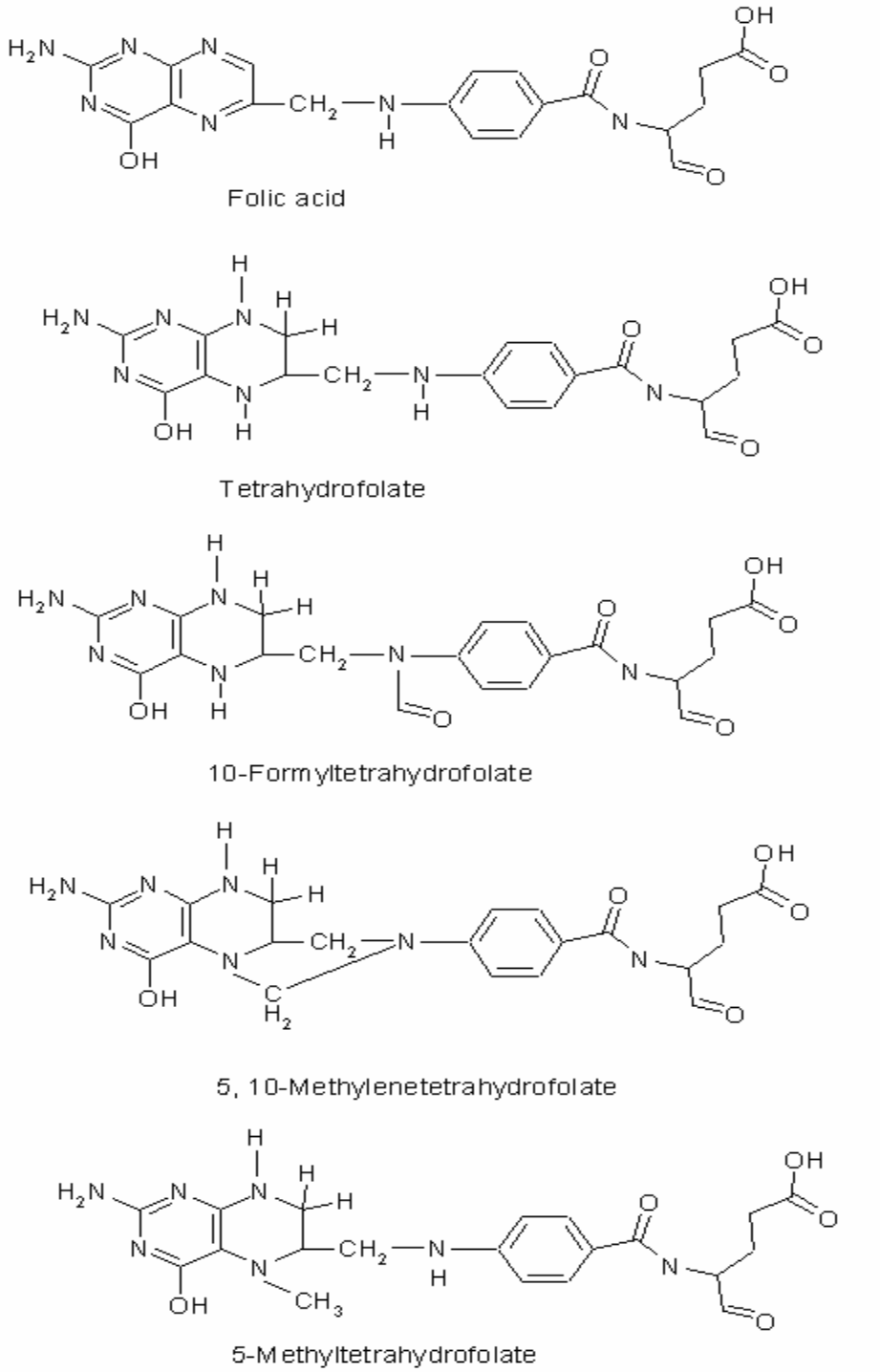

Figure 3.1: The chemical structure of folic acid and the most important natural folates.

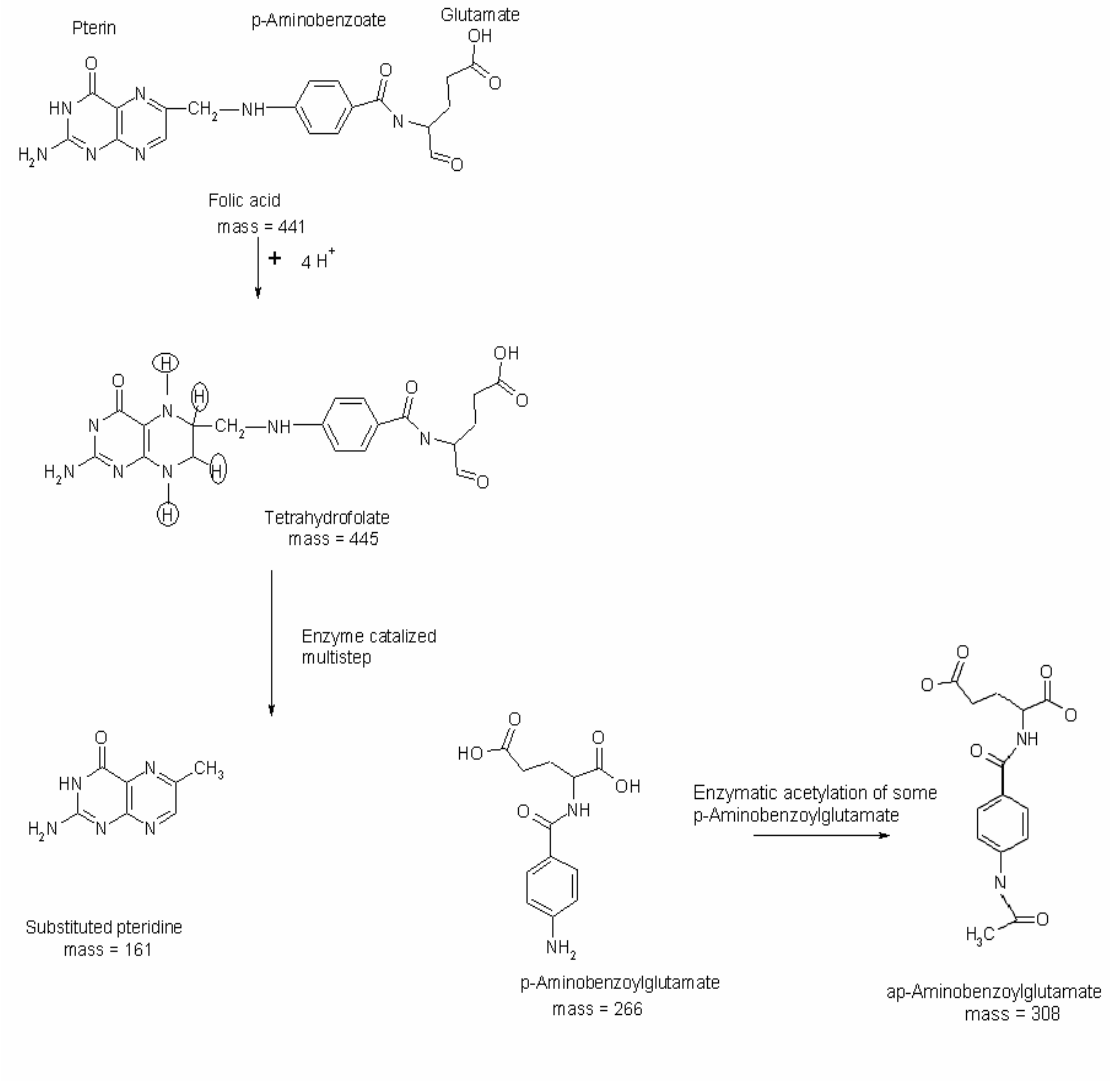

Figure 3.2: The catabolism of folic acid in the body.

We have developed a novel, versatile, simple method for the detection and quantification of the folate catabolites p-aminobenzoylglutamate (*p*-ABG) and its acetamido derivative ap-acetamidobenzoylglutamate (*ap*-ABGlu) in serum, plasma and urine using liquid chromatography-tandem mass spectrometry (LC/MS/MS). Sample preparation and cleanup is achieved in one step involving precipitation with acetonitrile. The resulting supernatant is injected into a Biorad CAT/MET analytical column fitted on

Agilent 1100 liquid chromatograph coupled to a Sciex API 4000 Mass Spectrometer with an electrospray ionization source. Detection of the catabolites was by selective reaction monitoring (SRM) of the respective ions.

3.2 Experimental

3.2.1 Materials and Methods

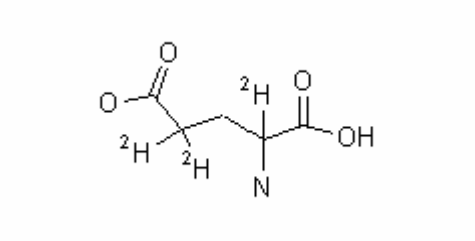

Figure 3.3: Chemical structure of 2,4,4-d3-glutamic acid.

Pure p-ABG standard was obtained from Sigma-Aldrich (Oakville, ON.). Acetamido derivative was prepared from the p-ABG by adding 140 μL of 50% acetic acid (VWR Canlab, Toronto, ON.) in deionized water (Arctic Glacier, Regina, SK.) (v/v) and 20 μL acetic anhydride (Sigma-Aldrich, Oakville, ON., Canada) to 10 mg of pABG. The reaction mixture was mixed vigorously and incubated in the dark at room temperature for 1 hour. The remaining acetic acid was allowed to evaporate under a stream of nitrogen at room temparature. Stock solutions of both the p-ABG and the ap-ABG were prepared in deionized water and contained 1.5 mM each, respectively. Aqueous serial dilutions were made from each of the stocks at concentrations of 100 nM, 50 nM, 10 nM and blank for serum and 200 μM, 100 μM, 50 μM, 10 μM and blank for urine specimens. These, together with the working stock solutions were stored at –20ºC.

d3-glutamic acid (d_3-Glu) (Figure 3.3) was purchased from CDN isotopes and used as internal standard. A working stock solution of 1 mM was prepared in deionized water. HPLC grade acetonitrile (Anachemia), ammonium acetate (BDH), formic acid (Fisher Scientific) and, deionized water was used to prepare the HPLC mobile phase.

3.2.2 Recovery experiments

Pooled serum (from patients that are being tested for thyroid hormones) and urine samples (from patients being tested for drugs of abuse) were spiked with p-ABG and ap-ABG to required concentrations (100 nM, 10 nM, and blank for serum and 100 μM, 10 μM and blank for urine samples). Aqueous standards were prepared as described above. 2 μL of d3-glutamic acid (1mM) was added to each 200 μL specimen as internal standard. Each sample was then acidified with 4 μL 6N HCl (Fisher Scientific, Ottawa, ON.) and vigorously vortexed. Precipitation/dilution (x3 for serum samples and x30 for urines) was achieved by addition of 100% acetonitrile, vortexing and spinning at 1900*g* for 10 minutes. The resulting supernatant was then pipetted into a standard 96-well plate and placed on the Agilent 1100 well-plate autosampler.

3.2.3 Analysis of clinical samples

Blood and urine samples were collected after informed consent from a group of 5 healthy volunteers working in our lab. Blood was collected in a serum separator tube to yield serum, which was immediately analysed for serum folate on the Architect automated immunoanalyzer (Abbott Laboratories, Abbott Park, IL.) as described in 4.2.6. The remainder of the sample was analysed for p-ABG and ap-ABG within the hour as

described above. Urine samples were collected in sterile urine containers, labelled, acidified to pH 3 with 6N HCl and analysed for p-ABG and ap-ABG; also within an hour.

Serum samples (n = 11) collected at various collection centres, frozen within six hours and sent to our lab for parathyroid hormone assay were analysed for p-ABG and ap-ABG on the same day as PTH assay. A group of 18 serum samples from pregnant women was collected from a batch of serum samples sent to this lab for analysis of prenatal panel (rubella Ig G, syphilis, hepatitis B virus antigen and HIV antigen). Urine samples (24-hr urine samples stored at 4°C during collection) were collected from samples sent for urinary catecholamine assay in our lab (n = 19) after being acidified to pH 3 with 6N HCl. These urine samples were also analysed for p-ABG and ap-ABG on the same day as received from the urinary catecholamine bench.

The results obtained were subjected to a modified Levene equal-variance test and subsequent two-tailed, two sample t-test using NCSS 2000/PASS 2000 Dawson Edition statistical software package (NCSS, Kaysville, UT.).

3.2.4 Instrumentation

Liquid chromatography-tandem mass spectrometry of the samples was done on an Agilent 1100 LC system, which includes a vacuum degasser, a binary pump, a well-plate auto-sampler and a heated column compartment. This system was coupled to an Applied Biosystems MDS Sciex API 4000 triple quadrupole tandem mass spectrometer. The chromatographic column consisted of a normal phase CAT/MET analytical column (catalog # 195-6033, Bio-Rad Laboratories, Hercules, CA.). The mobile phase

composition consisted of 2 mM aqueous ammonium acetate buffer with 0.1% formic acid (pH 3.0) and 100 % acetonitrile as the organic phase. Chromatography consisted of an isocratic run of 50:50 buffer to organic phase. Flow rate was set at 300 μL per minute, column temperature at 35ºC and an injection volume of 10 μL was used. A 15-second flush-port needle wash was incorporated into the procedure to prevent cross-contamination between injections.

Selective reaction monitoring (SRM) was done on the mass spectrometer using the Sciex Turbo ion spray source in positive ion mode. Resolution in quadrupole 1 (Q1) and quadrupole 3 (Q3) chambers were at unit mass. Other source parameters were as indicated in Table 3.1. The Multiple SRM mass transitions monitored were 309>162, 309>180, both for ap-ABG and 267>120 for pABG. The dwell time for all the analytes was set at 500 msec. Run time for each LC injection was set at 10 minutes. Data was acquired and analyzed using Analyst software 1.4 (Applied Biosystems MDS Sciex).

Table 3.1: Turbo ion source parameters in the tandem mass spectrometer (MS/MS) for the analysis of p-aminobenzoylglutamate (*p*-ABG), ap-acetamidobenzoylglutamate (*ap*-ABG) and d_3-glutamic acid.

Instrument parameter	***Value***
Source temperature	450ºC
Collision associated dissociation (CAD) gas	6 p.s.i.
Curtain gas	25 p.s.i.
Gas source 1 & 2 (GS 1 & GS 2)	30 p.s.i.
Ion spray voltage	5500 V
Declustering potential (DP) for both transitions 309>162 & 309>180 (ap-ABG)	38 V
Declustering potential (DP) for transition 267>120 (pABG)	35 V
Declustering potential (DP) for transition 151>87 (d_3-glutamic acid)	34 V
Electrode potential (EP)	10 eV
Collision energy (CE) for transition 309>162	19 eV
Collision energy (CE) for transition 309>180	14 eV
Collision energy (CE) for transition 267>120	12 eV
Collision energy (CE) for transition 151>87 (d_3-glutamic acid)	23 eV

3.3 Results

Figures 3.4 and 3.5 show the spectral analysis of *p-ABG* and *ap-ABG* and the proposed fragmentation chemistries for each as well as the internal standard d_3-glutamic acid, d_3-Glu (Figure 3.6). Mass spectral data were achieved through infusion of aqueous methanolic standards directly into the MS/MS. The respective ions and fragment ions were used to determine which selective reaction monitoring (SRM) would be monitored.

The chromatography of these aqueous standards showed complete separation of the analyte and the internal standard (d_3-glutamic acid). Spiked serum and urine samples showed chromatography consistent with that seen in the aqueous standards (Figures 3.7-3.10). The acetylation process of p-ABG to ap-ABG resulted in a 100% conversion within the 1-hour incubation period as determined by the Q1 scan data before and after derivatization of p-ABG (data not shown).

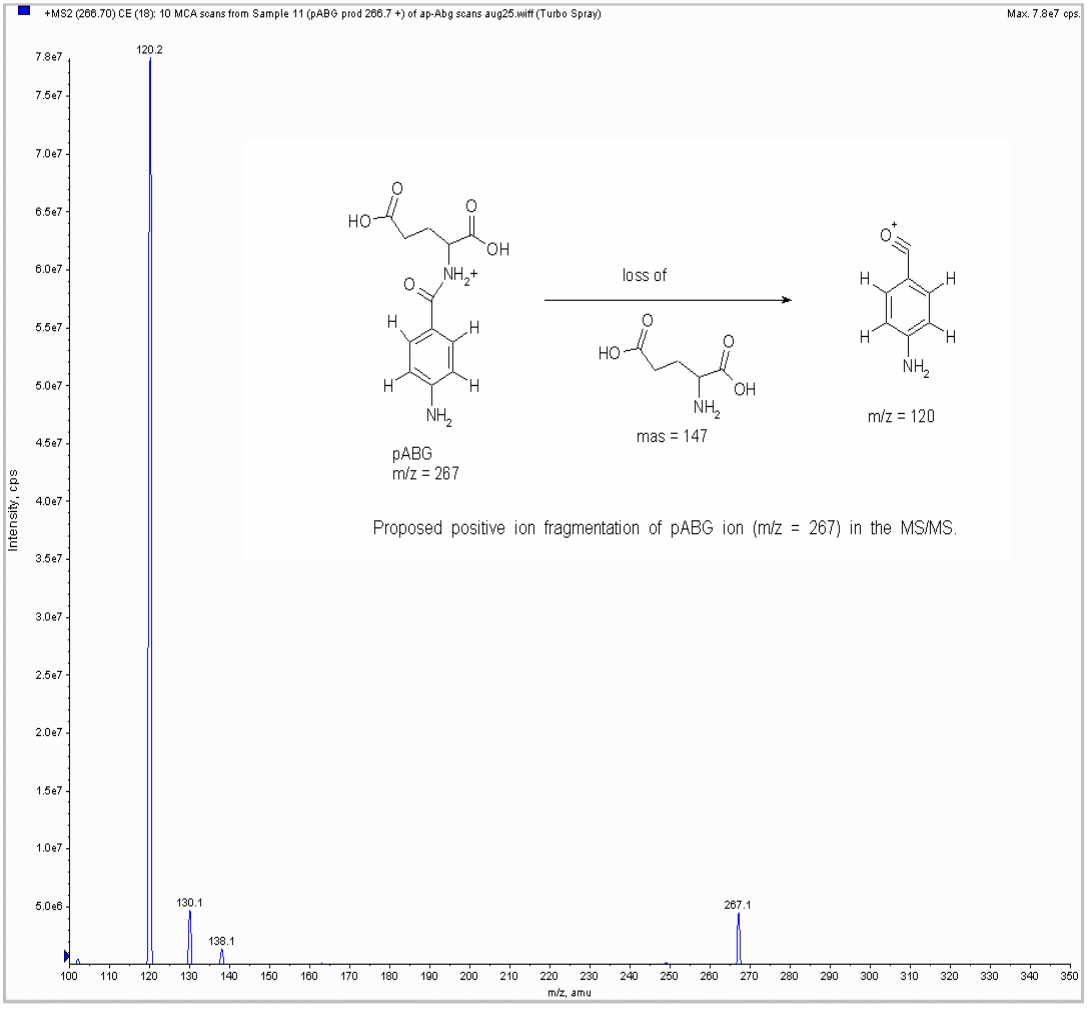

Figure 3.4: Product ion scan (Q3) of 1 mM aqueous folate catabolite p-aminobenzoylglutamate, *p*-ABG (m/z = 267) in the Q3 compartment of the MS/MS.

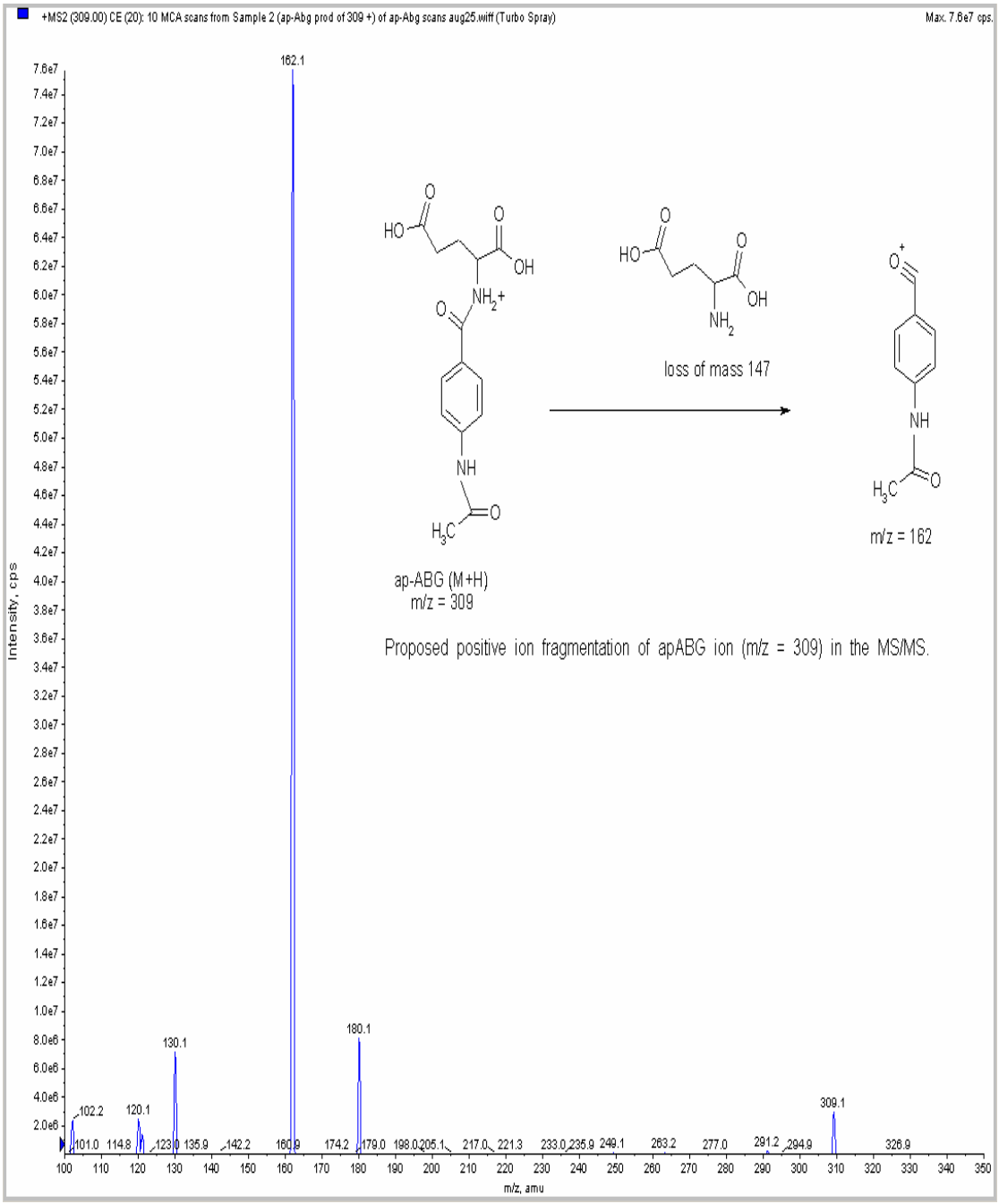

Figure 3.5: Product ion scan (Q3) of 1 mM aqueous folate catabolite ap-acetamidobenzoylglutamate, *ap*-ABG (m/z = 309) in the Q3 compartment of the MS/MS.

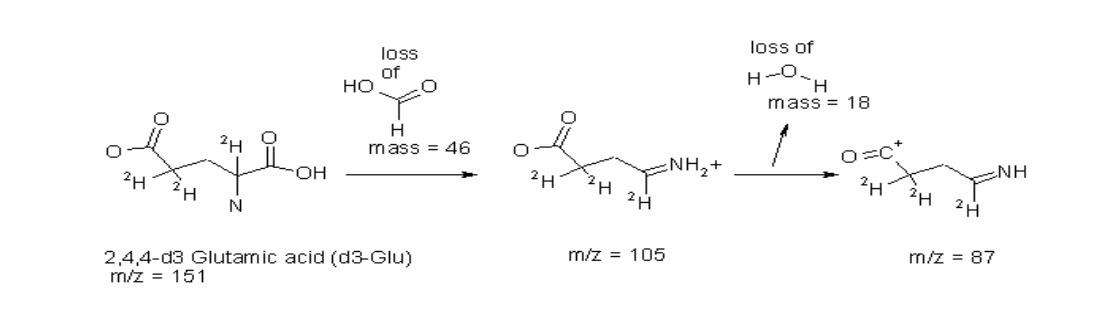

Figure 3.6: Proposed positive ion fragmentation of d_3-glutamic acid (d_3-Glu) in the Q3 compartment of the mass spectrometer.

Figure 3.7: Chromatographic spectrum of p-aminobenzoylglutamate (*p*-ABG), ap-acetamidobenzoylglutamate (*ap*-ABG) and d_3-glutamic acid in 100 nM spiked pooled serum.

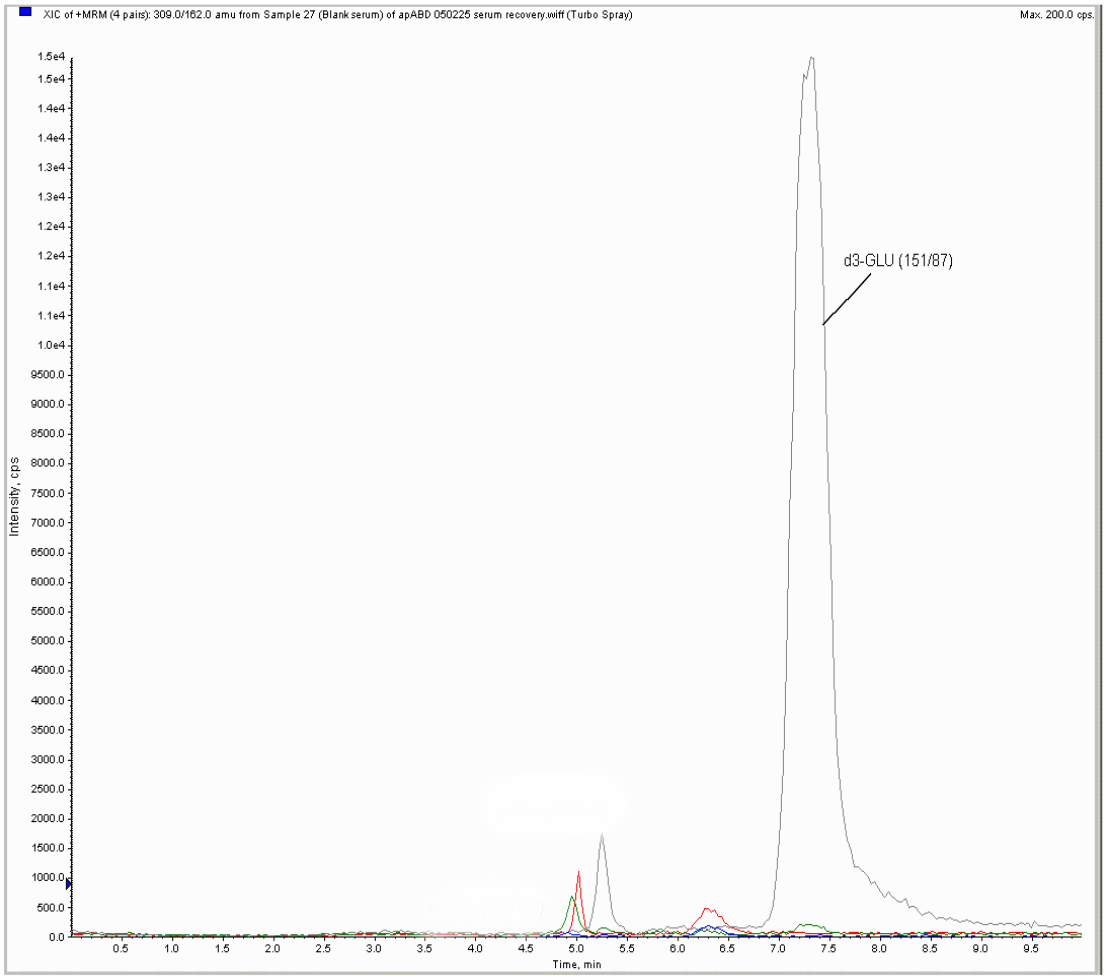

Figure 3.8: Chromatographic spectrum of p-aminobenzoylglutamate (*p*-ABG), ap-acetamidobenzoylglutamate (*ap*-ABG) and d3-glutamic acid in unspiked pooled serum. The green and red peaks at approximately 4.9 and 5.0 min, respectively, are probably artifacts (unidentified co-eluting compounds).

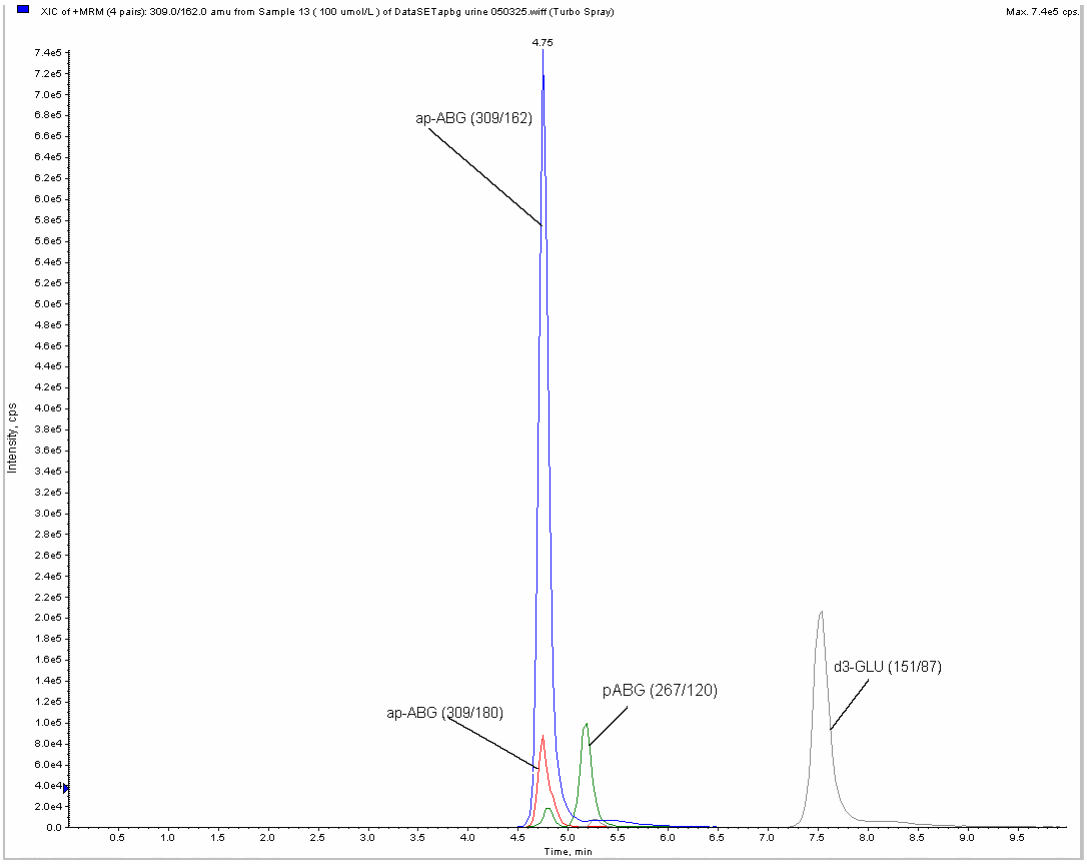

Figure 3.9: Chromatographic spectrum of p-aminobenzoylglutamate (*p*-ABG), ap-acetamidobenzoylglutamate (*ap*-ABG) and d3-glutamic acid in 100 μM spiked pooled urine.

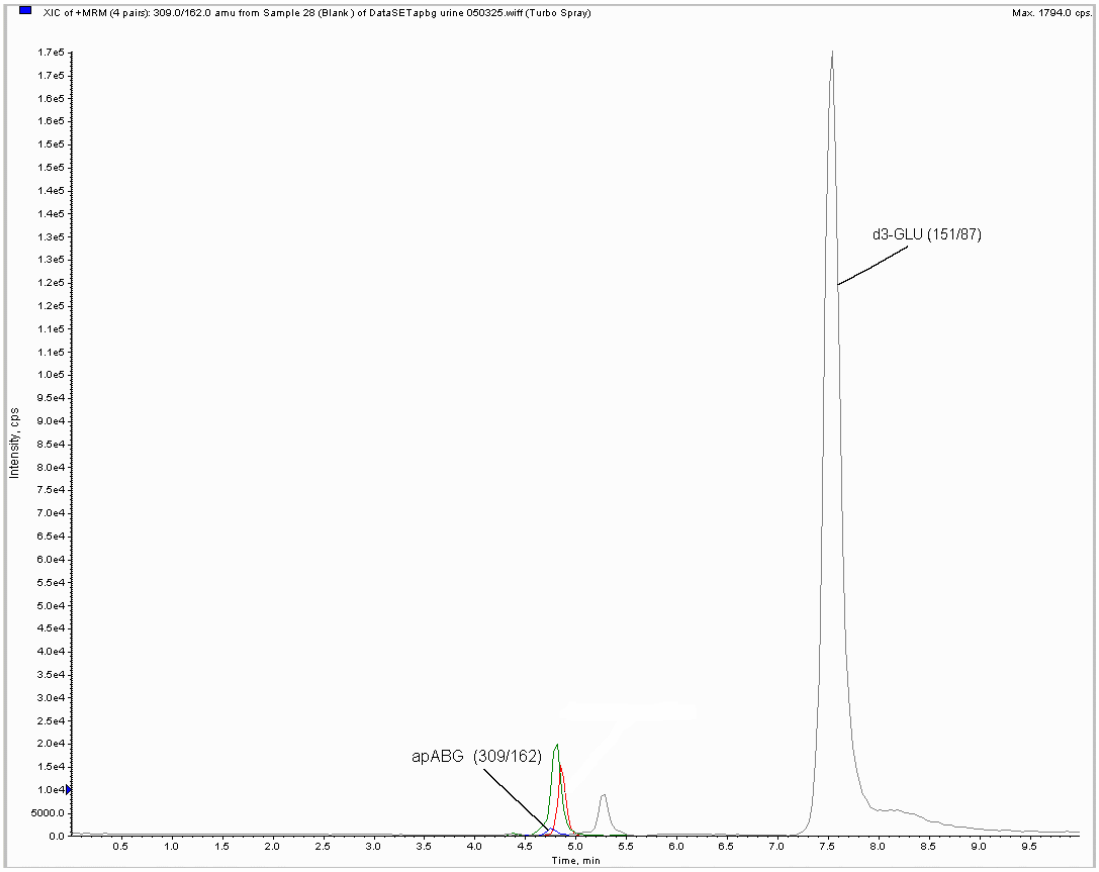

Figure 3.10: Chromatographic spectrum of p-aminobenzoylglutamate (*p*-ABG), ap-acetamidobenzoylglutamate (*ap*-ABG) and d3-glutamic acid in pooled unspiked urine. The green and red peaks at approximately 4.8 and 4.9 min, respectively, are probably artifacts (unidentified co-eluting compounds).

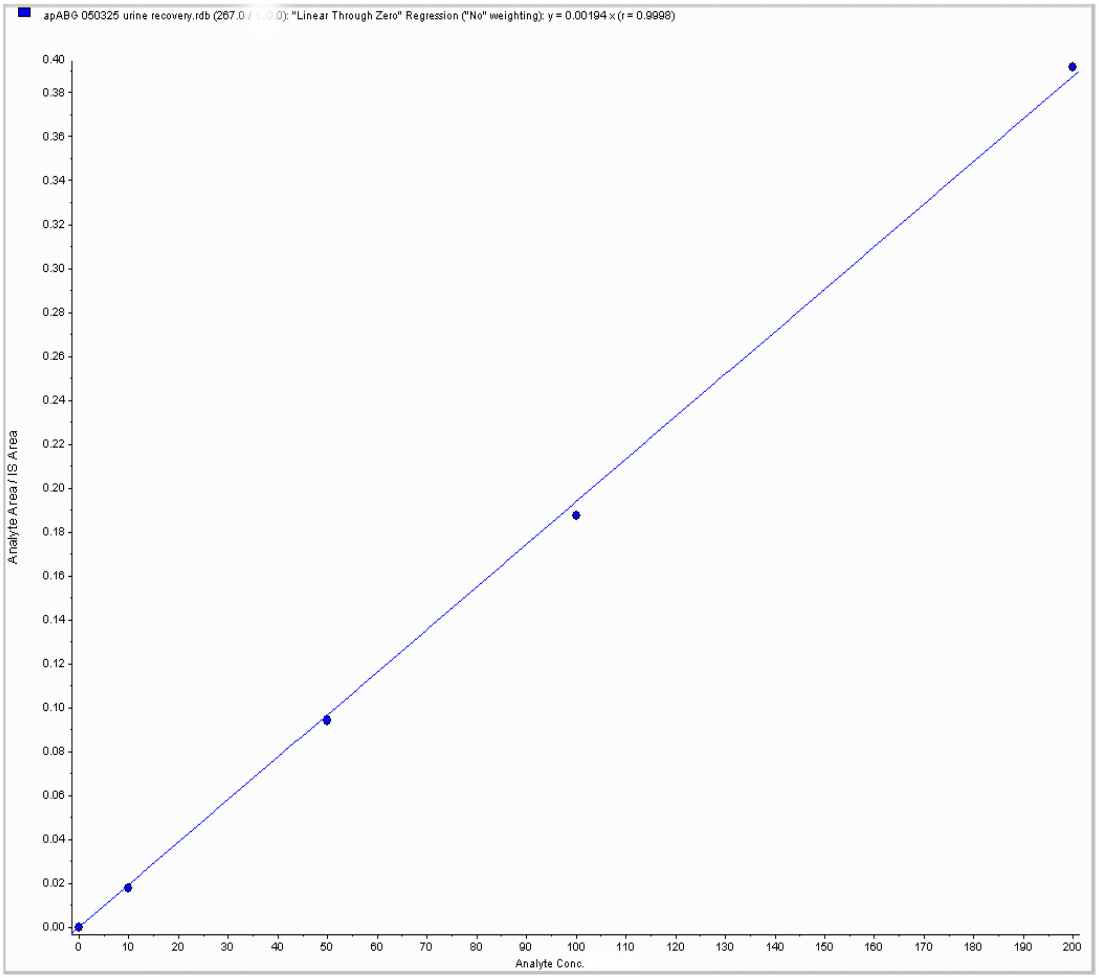

Figure 3.11: Standard curve of aqueous calibrators of p-aminobenzoylglutamate (*p*ABG) transition (267>120). The regression of the line graph is linear through zero (no weighting) resulting in a regression equation of $y = 0.00194x$ ($r^2 = 0.9996$).

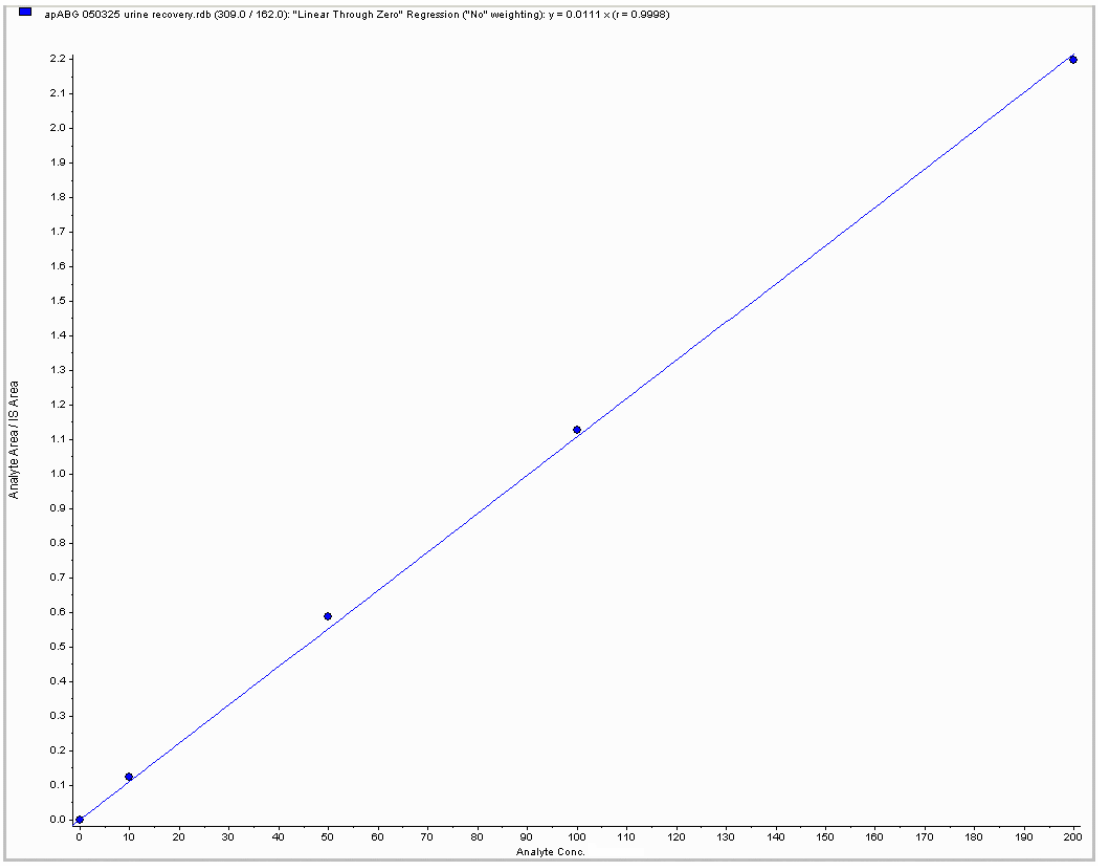

Figure 3.12: Standard curve of aqueous calibrators of *ap-acetamidobenzoylglutamate* (*ap-ABG*) transition (309>162). The regression of the line graph is linear through zero (no weighting) resulting in a regression equation of y = 0.0111x (r^2 = 0.9996).

3.3.1 Linearity

A four-point calibration curve for serum and five-point curve for urine were constructed for the standards (Figures 3.11 and 3.12). The dynamic range selected was based on both the limits of detection and the findings of Caudill and co-workers in which the baseline concentrations of the p-ABG was around 24 nM in non-pregnant women with folate supplementation of 450 μg/day of folic acid and around 63 nM in pregnant

women with 850 μg/day of folic acid supplementation (Caudill *et. al.*, 1998). The standard curve calibration points included a blank, 10 nM, 50 nM and 100 nM for serum and blank, 10 μM, 50 μM, 100 μM and 200 μM for urine. 2 μL of 1 mM d_3-Glu was added to 200 μL of specimen as an internal standard. Linear through zero regression (no weighting) equations were $y = 0.0111x$ ($r^2 = 0.9996$) for ap-ABG 309>162 transition, $y = 0.00131x$ ($r^2 = 0.9998$) for ap-ABG 309>180 transition and $y = 0.00194x$ ($r^2 = 0.9996$) for the p-ABG 267>120 transition.

3.3.2 Precision and Recovery

Intra-assay precision of ap-ABG and p-ABG for serum and urine specimens was determined at two levels (10 nM and 100 nM for serum and 10 μM and 100 μM for urine). Pooled serum and urine samples from various patients (sent for various assays to our reference lab) were divided into ten different aliquots and spiked with ap-ABG and p-ABG at the above two levels. The limit of detection (LOD) was 0.1 nM in serum corresponding to a signal to noise ratio of 3:1. Limit of quantification (LOQ) was 1 nM, which equals 33.3 femtomole (fmol) (absolute amount of sample). Table 3.2 shows the results of the precision and recovery assay.

Although all transitions showed good recovery, two transitions (267>120 at 100 nM serum and 309>180 at 100 nM serum) had higher CV values (>20%). These high CV values were contributed by two samples and may also be due to an unidentified co-eluting substance. While we did not exclude these results, we recommend that transition 309/162 be used as a specific marker of ap-ABG.

Table 3.2: Recovery and precision results of p-aminobenzoylglutamate (*p*-ABG) and ap-acetamidobenzoylglutamate (*ap*-ABG) in spiked serum and urine samples.

Sample	Analyte	Transition	Concentration	Precision	Recovery
Urine	*p*ABG	267>120	10 μmol/L	CV = 6.06%	96.90%
				n = 10	
			100 μmol/L	CV = 12.66%	100.35%
				n = 10	
	ap-ABG	309>180	10 μmol/L	CV = 2.89%	91.84%
				n = 10	
			100 μmol/L	CV = 4.03%	95.48%
				n = 10	
		309>162	10 μmol/L	CV = 3.59%	102.40%
				n = 10	
			100 μmol/L	CV = 3.07%	102.35%
				n = 10	
Serum	*p*ABG	267>120	10 nmol/L	CV = 4.80%	81.90%
				n = 10	
			100 nmol/L	CV = 21.72%	80.40%
				n = 10	
	ap-ABG	309>180	10 nmol/L	CV = 2.54%	122.80%
				n = 10	
			100 nmol/L	CV = 20.04%	96.84%
				n = 10	
		309>162	10 nmol/L	CV = 2.02%	88.94%
				n = 10	
			100 nmol/L	CV = 12.91%	92.82%
				n = 10	

Note: CV = Coefficient of variation

3.3.3 Clinical Samples

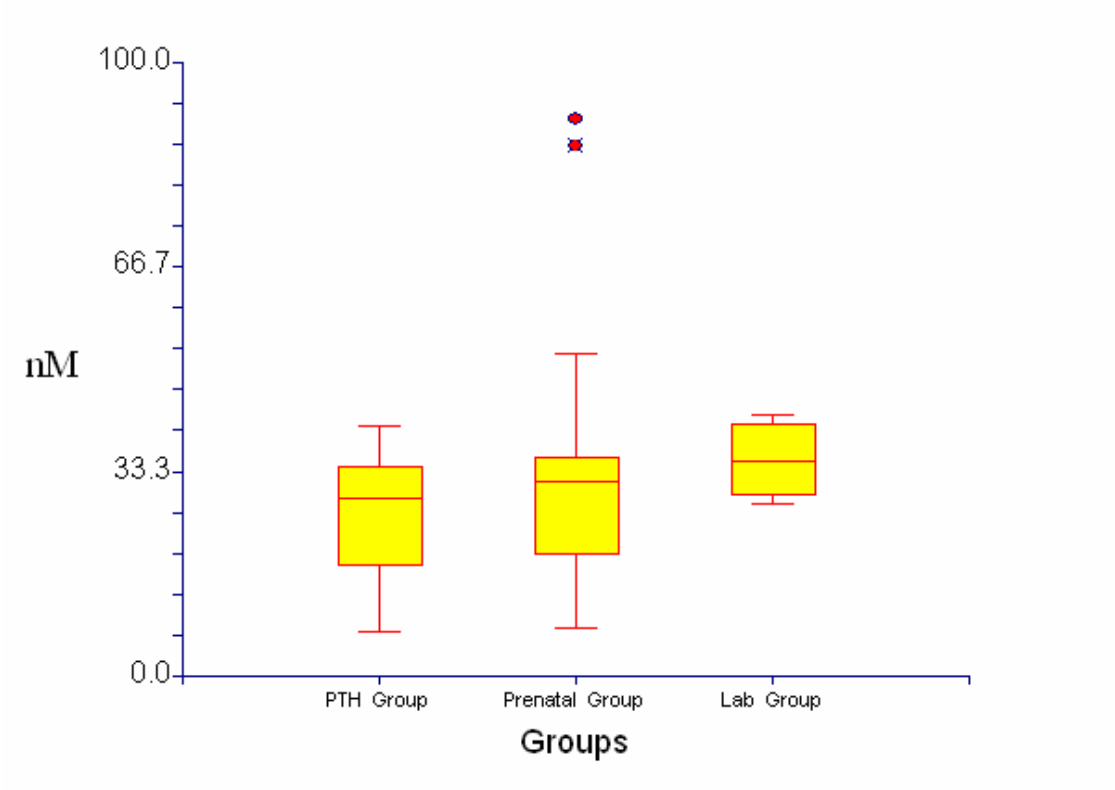

Figure 3.13: Box plot of serum folate levels (mean ± SD) in parathyroid hormone female patients (n = 11), pregnant (prenatal, n = 18) women and lab female volunteers (n = 5). Solid red dots represent extreme outliers.

The mean age of the group for the lab volunteers was 40 years. Serum folate levels (mean ± SD) in this group were 35.3 ± 2.4 nM (Figure 3.13). Total serum catabolites (p-ABG + ap-ABG) were 11.9 ± 3.1 nM with 75% of it being in the form of p-ABG and 25% in the form of ap-ABG (Figure 3.14). Total urinary catabolites were 2.9 ± 0.9 μM with *p-ABG* consisting of 27% and 73% made up of *ap-ABG*.

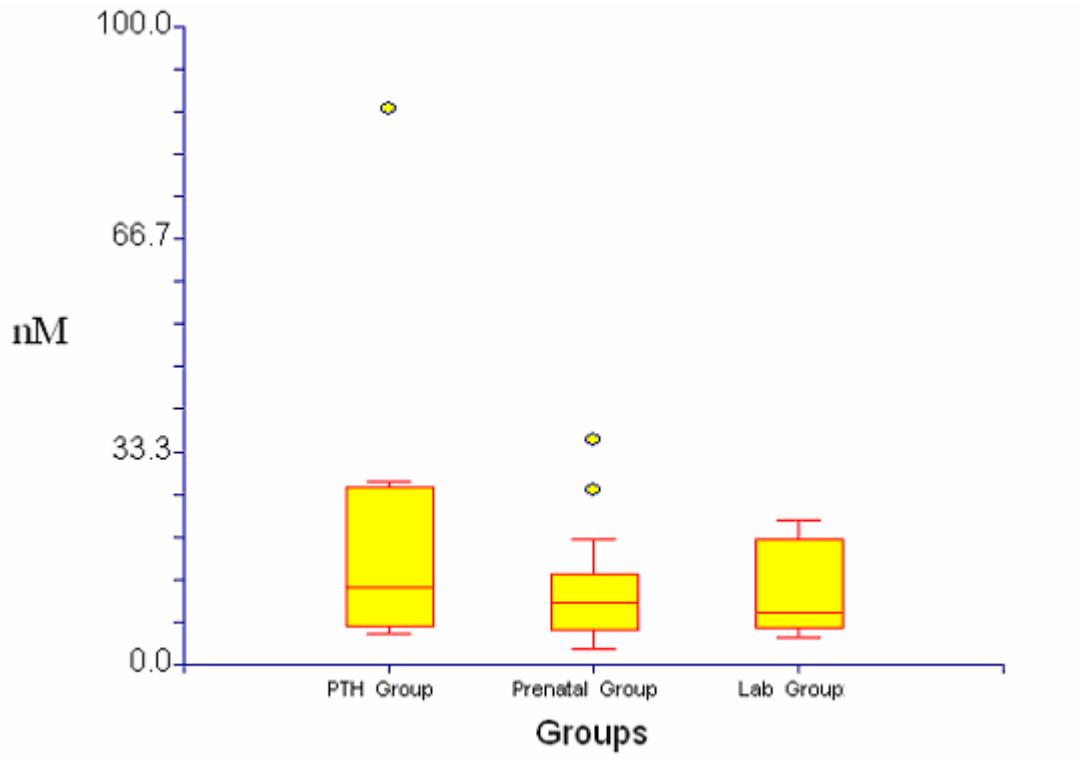

Figure 3.14: Box plot of serum folate catabolites p-aminobenzoylglutamate (*p*-ABG) and ap-acetamidobenzoylglutamate (*ap*-ABG) (mean ± SD) in parathyroid hormone female patients (n = 11), pregnant (prenatal, n = 18) women and lab female volunteers (n = 5). Solid yellow dots represent mild outliers.

The urinary catecholamines group (n = 19) had a mean age of 50 years. Total urine catabolites in this group were 581.8 ± 84.5 nM. *ap*-ABG made up 76% while *p*-ABG consisted of 24% (Figure 3.15). The values and relative ratios of the urine catabolites seen here resemble that seen in the volunteers group despite the significant age difference in the means.

Mean age for the PTH group (n = 11) was 55 years. Mean serum folate levels were 27.0 ± 3.1 nM. Total serum catabolite levels in this group were 20.4 ± 7.2 nM. 37% of

the total folate catabolites were made up of *ap-ABG*. The remaining 64% was made up of *p*ABG.

Mean age for the pregnant group (n = 18) was 27 years. Serum folate levels were 33.9 ± 5.4 nM. Total serum catabolites were 11.4 ± 2.1 nM, with ap-ABG making 17% and p-ABG 83% of the total catabolites.

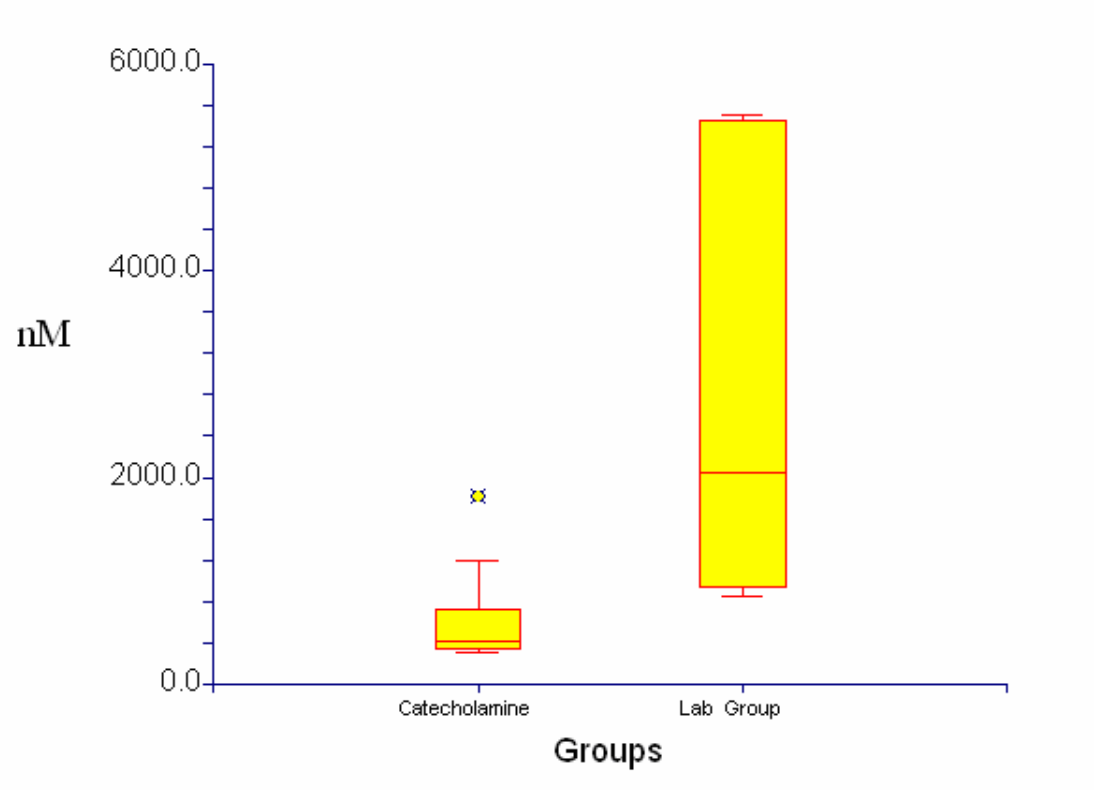

Figure 3.15: Box plot of total urinary folate catabolites p-aminobenzoylglutamate (*p*-ABG) and ap-acetamidobenzoylglutamate (*ap*-ABG) (mean ± SD) in catecholamine-tested female patients (n = 19) and lab female volunteers (n = 5). Solid yellow dot represents mild outlier.

3.4 Discussion and Conclusions

Evidence that folate catabolism proceeds by splitting of folate molecule at C9-N10 bond was initially provided by Futterman and Silverman (Futterman & Silverman, 1957) who demonstrated the enzymatic reduction of folate to tetrahydrofolates followed by spontaneous cleavage to *p*-ABG and a pteridine moiety. Murphy and Scott (Murphy & Scott, 1979) analysed ^{3}H-labeled compounds excreted in rat urine each day over a 10-day period following an intramuscular administration of [3', 5', 7, 9-^{3}H] pterylglutamate. They found a number of pteridines and *p*-ABG, with the major catabolite consisting of *ap*-ABG. To demonstrate the suitability of *ap*-ABG as an indicator of folate catabolism, Geoghegan and co-workers (Geoghegan *et. al.*, 1995) showed that reverse-phase HPLC of radiolabeled urinary products of rats given ^{3}H and ^{14}C folic acid contained a variety of intact folate as well as products of C9-N10 scission of the folate molecule, pteridines, *p*-ABG and ap-ABG. Of all the folate metabolites present in the urine, only *ap*-ABG persisted at high levels for the 10-day period of the experiment. This finding is corroborated by the works of Connor and co-workers (Connor *et. al.*, 1979) and Pheasant and co-workers (Pheasant *et. al.*, 1981;Caudill *et. al.*, 1998).

Serum folate levels in the lab volunteers (35.3 ± 5.8 nM), PTH (27.0 ± 10.4 nM) and prenatal (33.9 ± 22.7 nM) groups were not significantly different from each other ($p<0.05$) (Figure 3.13). These levels are consistent with levels found by the National Health and Nutritional Examination Survey (NHANES), 1999-2000 (Wagner, 1995). This study found that median serum folate concentrations for women increased from 10.9 nM (4.8 ng/mL) to 29.5 nM (13.0 ng/mL) from 1988 - 1994 to 1999 - 2000.

Serum catabolite levels (Figure 3.14) in the lab volunteers, PTH and prenatal groups were also not significantly different form each other ($p < 0.05$). Serum samples collected for prenatal analysis are collected and shipped to the lab at 4°C. It takes up to 24-hrs for the sample to reach the lab. Once in the lab, the samples were analysed for *p*-ABG and *ap*-ABG after the designated tests (prenatal panel) were done. Thus, analysis of serum catabolites was done approximately 48 hours after collection and storage at 4°C. We believe the degradation of *p*-ABG and possible dilution effect of the increased blood volume during pregnancy could have contributed to the lower levels of serum catabolites seen in this group despite increased folate utilization anticipated in pregnancy (Suh *et. al.*, 2001;Gregory, III *et. al.*, 2001).

Urinary folate catabolites levels (Figure 3.15) in the lab volunteers were not significantly different from the catecholamine group ($p< 0.05$) (mean of 581.8 ± 368.4 nM in catecholamine group vs. 2.9 ± 2.3 μM in the lab volunteers). This is probably due to the greater standard deviation in the catecholamine group and the fact that catecholamine urine samples are 24-hr urine collection. While urine samples collected from lab volunteers were frozen immediately upon collection, the catecholamine samples were stored at 4 ºC for 24-hr before being frozen. We believe that this storage condition would have led to loss of some catabolites. The levels seen in the lab volunteers, however, are similar to that seen *in vivo* kinetic studies of folate metabolism (Lin *et. al.*, 2004) and others (Caudill *et. al.*, 1997). There was no urine sample available for the other groups.

We have developed a novel, rapid test for *p*-ABG and *ap*-ABG that can be used in routine clinical assessment of folate catabolism. In this method, both *p*-ABG and *ap*-ABG

are assayed simultaneously within the same sample. The samples are acidified with a small amount of 6N HCl (4 μL HCl to 200 μL sample) and precipitated with acetonitrile. Deuterated glutamic acid (d_3-Glu) is added to the samples before HCl acidification and acetonitrile precipitation. It is used as an internal standard because of its similarity to the folate catabolite molecules. Separation of the two compounds is achieved by HPLC and, detection by Multiple SRM of specific ions in MS/MS. Sensitivity of this method is very high with detection of femtomolar (fmol) amounts.

To date, all the methods available for the analysis of folate catabolism are based on extensive sample clean up and use of HPLC. Not only are these methods labour intensive and time consuming, rendering them impractical for use in routine clinical analysis, but they also have limited sensitivity. The objective of this paper was to develop a routine method for quantitative measurement of the folate catabolites p-aminobenzoylglutamate (*p*ABG) and acetamido-*para*--aminobenzoylglutamate (*ap*-ABG) in serum and urine using liquid chromatography-tandem mass spectrometry (LC-MS/MS). Our method provides ease of analysis with much higher sensitivity, specificity, and versatility regardless of the type of biological sample used, and faster turnaround times.

CHAPTER 4

Relationship between Formate and RBC Folate Levels in Alcohol Intoxicated Females.

4.1 Introduction

Alcohol consumption is associated with a variety of health problems that include malnutrition, gastrointestinal and, in pregnant women, risk of having a child with Fetal Alcohol Spectrum Disorder (FASD) (Abel & Hannigan, 1995; Sokol *et. al.*, 2003). Approximately 10% of pregnant women use alcohol, with 2% engaged in binge drinking (defined by the National Institute on Alcohol Abuse and Alcoholism as 4 or more drinks in about 2 hours) (Center for Disease Control, 2004). More than half of women who do not use contraceptives consume alcohol regularly with 12.4% classified as binge drinkers (Center for Disease Control, 2004).

Alcohol consumption not only leads to increased ethanol but also increased methanol levels. This is due to methanol's presence in all alcoholic beverages (Table 1.2) as a congener and a by-product of the manufacturing process that is never removed for fear of altering the unique taste and smell of the drink (Sprung *et. al.*, 1988). Formaldehyde, a metabolite of methanol, is a much more potent reaction partner for tetrahydroisoquinoline (TIQ) and tetrahydro-beta-carboline (THBC) formation than acetaldehyde (Sprung *et. al.*, 1988). Formic acid, another metabolite of methanol, is cytotoxic and capable of inducing free radical mediated damage (Dikalova *et. al.*, 2001; Ellenhorn *et. al.*, 1997; McMartin, 1977). In primates, metabolism of formate is via its

oxidation to CO_2 (Figure 1.2), a reaction dependent on tetrahydrofolate, the metabolically active form of folic acid (Dorman *et. al.*, 1994; Halsted *et. al.*, 2002b; Larroque *et. al.*, 1992; Friedmann *et. al.*, 1954; Malorny, 1969; McMartin, 1977). During oxidation of formate a formyl group (CHO) is transferred to tetrahydrofolate resulting in the formation of CO_2 and H_2O. Tetrahydrofolate levels depend on folic acid intake as well as on the overall status of the folate pathway (Figures 1.6 and 4.1). The vitamins B12 and B6 are important cofactors in this pathway (Bosy-Westphal *et. al.*, 1945; Kalter, 2000; Lucock, 2000). Hence, any change in the folate levels is bound to have a negative effect on this pathway. Increased formate, decreased folate and altered one-carbon metabolism could lead to disastrous consequences in vulnerable groups, particularly in pregnant women where it could lead to the development of fetal alcohol spectrum disorder (FASD) fetus.

In this experiment, we determined the relationship between red blood cell (RBC) folate and blood formate in alcohol intoxicated females. We also assessed what effect such a relationship would have on homocysteine, Hcy (a thrombogenic, atherogenic, neurotoxic non-proteinogenic amino acid whose remethylation to methionine is catalyzed by methionine synthase in the presence of vitamin B12), vitamin B12, and vitamin B6 levels.

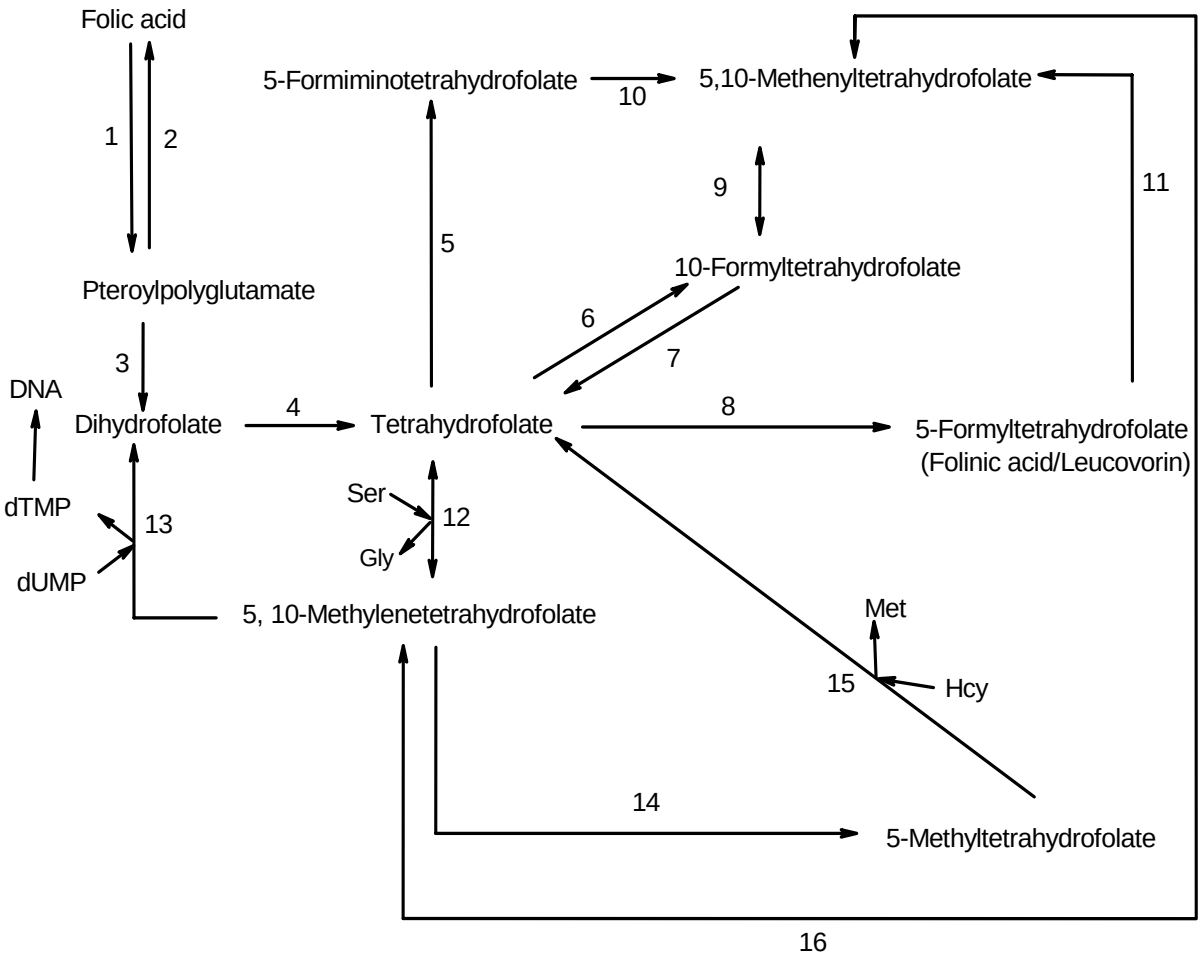

Figure 4.1: An overview of the folate pathway. Gly = Glycine; Ser = Serine; Hcy = Homocysteine; Met = Methionine; 1= Pteroylpolyglutamate synthetase; 2 = γ-Glutamyl hydrolase; 3 & 4 = Dihydrofolate reductase; 5 = N-Formimino-L-glutamate:tetrahydrofolate 5-formiminotransferase (a Bifunctional enzyme in mammals); 6 = 10-Formyltetrahydrofolate synthetase; 7 = 10-Formyltetrahydrofolate dehydrogenase; 8 = N-Formylglutamate:tetrahydrofolate 5-formyltransferase; 9 = 5, 10-Methenyltetrahydrofolate cyclohydrolase; 10 = 5- Formiminotetrahydrofolate cyclodeaminase; 11 = 5-Formyltetrahydrofolate cyclodehyrase; 12 = Serine hydroxymethyltransferase; 13 = Thymidylate synthase; 14 = 5, 10-Methylenetetrahydrofolate reductase; 15 = Methionine synthase; 16 = 5, 10-Methylenetetrahydrofolate dehydrogenase.

4.2 Experimental

4.2.1 Chemicals

Pure standard of formic acid (88% v/v), acetonitrile and methanol were obtained from Fisher Scientific (Fair Lawn, NJ.). Concentrated sulphuric acid (H_2SO_4) was purchased from J. T. Baker (Phillipsburg, NJ.) while Chromosorb 101™ packed column was purchased from Chromatographic specialties Inc. (Brockville, ON.). Human whole blood control (Biorad® #1, Bio-Rad Laboratories, Inc., Hercules, CA.) was used as the quality control for ethanol.

4.2.2 Subject Recruitment

Blood samples (500) collected from alcohol intoxicated patients presenting to the emergency room (ER) of the Royal university hospital (RUH), Saskatoon, for ethanol (EtOH) analysis were recruited for the study on anonymous basis. Only subjects who were females of less than 45 years with EtOH levels of greater than 5 mM were included in the study resulting in 266 samples. This group was designated as the intoxicated group. Apart from the EtOH already done at the hospital, methanol (MeOH), formic acid, RBC folate, Hcy, vitamins B12 and B6 were analyzed.

Eighty-three (83) subjects fitting the inclusion criteria described above were recruited from alcohol and drug rehabilitation program center in Regina. After informed consent, blood samples were collected for the analysis of the same analytes as the intoxicated group. This group was designated as the detox group. Another group (pregnant group) of 69 plasma samples were collected from Calgary health region on anonymous basis from pregnant females presenting to the prenatal clinic for a first trimester prenatal screen.

Ethics approval for the study was obtained from institutional ethics review board of the univesity of Saskatchewan, Regina Qu'Appelle health region and the Calgary Health Region.

4.2.3 Formate Analysis

Blood formate analysis was done using a headspace analysis technique on a gas chromatograph (GC) fitted with a flame ionization detector and Chromosorb 101™ column (GC-FID) without a headspace analyzer. A stock solution of 100 mM formic acid was prepared in deionized water. A seven-point linear calibration curve was constructed from the stock solution at 4, 2, 1, 0.5, 0.25, 0.125, and 0 mM. The samples and the calibrators were treated the same way as below.

Formate present in the sample (200 μL) was methylated to methylformate by addition to the sample of 50 μL of MeOH in a 1 mL glass vial. Catalysis of the reaction was facilitated by the addition of 100 μL of concentrated H_2SO_4 into the reaction vial and sealing it. Methyl formate vapors formed were allowed to equilibrate into the headspace above the reaction solution in the vial for 30 minutes at room temperature before injecting 1 mL into the GC. Because the amount of MeOH added is in excess (250 mM) of what is required for methylation as well as that found in MeOH overdose, it also serves as an internal standard.

4.2.4 Instrumentation and Data Analysis

Analysis of methylformate was performed on a gas chromatograph fitted with a flame ionisation detector (Varian 3300) and a Chromosorb 101™ packed column column

(6' × 1/8", J & W Scientific, Chromatographic Specialties Inc., Brockville, ON.). Other parameters were set as outlined in Table 4.1.

Table 4.1: Parameter values for analysis of methylformate in the gas chromatograph.

Parameter	Type	Value
Carrier Gas	Nitrogen	30 mL/min
Oven	Isothermal	40ºC
Injector	Headspace to splitless	220ºC
Detector	Flame ionization detector	300ºC
Detector gas	Hydrogen	23 mL/min
Column	Chromosorb 101™	105ºC

Quantitation of the methylformate was done by plotting the peak area ratios of methylformate to MeOH against the calibration concentrations using Chemistry Software for Windows® software (ChemSW™ Inc., 1993-198). Linearity of the calibration curve was established in a similar manner. Linear regression analysis of standard curve produced $r^2 > 0.999$. Assay limit of quantification was 0.05 mM, and intra-assay and inter-assay accuracy and precision had coefficient of variation values <5%. Quality control samples at low, medium and high concentrations were analyzed in duplicate for each run as acceptance criteria for the analysis.

4.2.5 Ethanol and Methanol Analysis

Analysis of EtOH and MeOH was done using the same instrument as for formate with minor modifications. The GC was fitted with Poropak Q™ packed column (6' ×

1/8", J & W Scientific, Chromatographic Specialties Inc., Brockville, ON.) set at a temperature of 125°C (isothermal). A protein free filtrate of the sample (500 μL) diluted with 500 μL acetonitrile (total volume 1000 μL) was prepared by precipitation of the proteins using 0.3N barium hydroxide and 5% w/v zinc sulfate (500 μL, each), followed by centrifugation at 2600 rpm for 10 minutes. The supernatant (2 μL) was injected in to the GC.

Ethanol and methanol levels were quantitated in a similar manner as formate using acetonitrile as internal standard. Assay limit of quantification was 0.1 mM for EtOH and 0.03 mM for MeOH. Intra-assay and inter-assay accuracy and precision had coefficient of variation values <5%. Quality control samples at low, medium and high concentrations were analyzed in duplicate for each run as acceptance criteria for the analysis.

4.2.6 Plasma and Red Blood Cell (RBC) Folate

Plasma and RBC folates were determined by an immunoassay technique run on an Architect™ system (Abbott Laboratories, IL.). This assay was divided into two steps to determine the presence of folate in human serum, plasma, and red blood cells (RBC) using chemiluminescent microparticle immunoassay (CMIA) technology with flexible assay protocols, referred to as chemiflex. Two pre-treatment steps mediated the release of folate from endogenous folate binding protein. In pre-treatment step 1, sample and pre-treatment reagent 2 (Dithiothreitol or DTT) were aspirated and dispensed into a reaction vessel (RV). In pre-treatment step 2, an aliquot of sample/pre-treatment reagent 2 mixture was aspirated and dispensed into a second RV. Pre-treatment reagent 1 (potassium hydroxide or KOH) was then added. An aliquot of the pretreated sample was

transferred into a third RV, followed by the addition of folate binding protein (FBV) coated paramagnetic microparticles and assay specific diluent. Folate present in the sample was bound to the FBV coated microparticles. After washing, pteroic acid-acridinium labeled conjugate was added and bound by unoccupied sites of the FBV coated microparticles. Pre-trigger and trigger solutions were then added to the reaction mixture; the resulting chemiluminescent reaction was measured as relative light units (RLUs). An inverse relationship existed between the amount of folate in the sample and the RLUs detected by the Architect optical system.

4.2.7 Homocysteine (Hcy)

Plasma homocysteine was determined by an immunoassay technique run on an Immulite® 2000 system (DPC Los Angeles, CA). In this assay, the sample was pretreated with S-adenosyl-L-homocysteine (SAH) hydrolase and Dithiothreitol (DTT) by incubating the mixture for 30 minutes in a tube containing no bead. After the incubation period, the treated sample was then transferred to a second tube containing a SAH-coated polystyrene bead and an alkaline phosphatase-labeled antibody specific for SAH and incubated for another 30 minutes. During this incubation period, the converted SAH from the pretreated sample competed with immobilized SAH for binding to the antibody-labeled enzyme. Unbound enzyme was removed by centrifugal wash. The reaction mixture was then analyzed in the immulite® 2000 Analyzer.

4.2.1 Vitamins B6 & B12

Plasma vitamin B-6 (PLP) levels were analyzed using HPLC system utilizing the method developed by Deitrick and co-workers (Deitrick *et. al.*, 2001). Briefly, 500 μL of

plasma was deproteinated with 500 μL 0.8 M perchloric acid. The mixture was vortexed vigorously for 1 minute and centrifuged at 1900*g* for 5 minutes. The supernatant (50 μL) was injected into an HPLC fitted with a Purospher C18 column and a diode array detector. Mobile phase A was 0.1 mol/L potassium dihydrogen phosphate buffer (*p*H = 3.5) and, mobile phase B (the column clean-up phase) was 100% acetonitrile. For quantification, the peak area from each sample was compared with standard calibration curve of PLP.

Plasma vitamin B12 was analyzed using immunoassay technique run on an Architect™ system (Abbott Laboratories, IL.). This system utilizes a chemiluminescent microparticle immunoassay (CMIA) technology with flexible assay protocols. The pretreated sample was mixed with a diluent and intrinsic factor coated with paramagnetic microparticles. Vitamin B12 present in the sample was bound to the intrinsic factor. The complex is washed and then had B12 acridium-labeled conjugate added to it. After addition of pre-trigger and trigger solution to the final mixture, a chemiluminescent reaction took place, which was measured as relative light units (RLUs). An inverse relationship existed between amount of vitamin B12 present in the sample and RLUs detected by the ARCHITECT *i2000* optical system.

4.2.9 Data Analysis

Mean±SE values of each analyte were calculated for each group. Two-sample t-test was done to compare means between the two groups using NCSS 2007 software (NCSS, Kaysville, UT.).

4.3 Results

Table 4.2: Results of various analytes in alcohol intoxicated and detox rehabilitation females.

Parameter	Intoxicated (n = 266) Result (mean ± SE)	Detox (n = 83) Result (mean ± SE)	p-value	Conclusion
Age (yrs)	34 ± 1	28 ± 1	0.001	H_1
Ethanol (mM)	38.84 ± 1.77	0.11 ± 0.00	0.000	H_1
Methanol (mM)	0.13 ± 0.01	0.04 ± 0.00	0.003	H_1
Blood formate (mM)	0.10 ± 0.00	0.10 ± 0.01	1.000	H_0
RBC folate (nM)	575 ± 23	643 ± 30	0.151	H_0
Hcy (μM)	7.08 ± 0.23	8.23 ± 0.32	0.012	H_1
Vit B12 (pM)	287 ± 15	221 ± 10	0.000	H_1
Vit B6 (nM)	43.97 ± 3.26	20.13 ± 2.01	0.000	H_1

Note: H_0 = no statistical difference between means at p=0.05; H_1 = the means are statistically different at p=0.05; SE = Standard Error.

Table 4.3: Results of various analytes in pregnant females (n = 69).

Parameter	Result (mean ± SE)
Plasma formate (mM)	0.202 ± 0.02
Plasma folate (nM)	40.53 ± 3.16
Homocysteine (μM)	5.57 ± 0.24
Vitamin B12 (pM)	395.00 ± 36.00

Note: SE = Standard Error

The results of the analysis of the intoxicated, detox and pregnant groups are summarized in Tables 4.2 and 4.3. Mean age (mean ± SE) was higher in the intoxicated group (34 ± 1 years vs 28 ± 1 years in the detox group, p = 0.001). Ethanol and methanol levels were also higher in the intoxicated group (38.84 ± 1.77 mM vs 0.11 ± 0.00 mM, p = 0.000 and 0.13 ± 0.01 vs 0.04 ± 0.00, p = 0.003, respectively). Plasma formate in the pregnant group (Table 4.2) was 0.173 ± 0.02 mM. Blood formate and red cell formate levels were not statistically different between the intoxicated and detox groups. However, Hcy levels were lowest in the pregnant group (5.57 ± 0.24 μM). Levels in the intoxicated and detox groups were 27% (7.08 ± 0.23 μM, p = 0.000) and 48% (8.23 ± 0.32 μM, p = 0.000) higher, respectively. Vitamin B12 levels were highest in the pregnant group (395 ± 36 pM) while the detox group had the lowest (44% lower; 395 ± 36 pM, p = 0.000). Vitamin B12 levels in the intoxicated group were 27% lower (287 ± 13 pM, p = 0.005). Vitamin B6 levels in the detox group were 54% lower compared to the intoxicated group (20.13 ± 2.01 μM vs 43.97 ± 3.26 μM, p = 0.000).

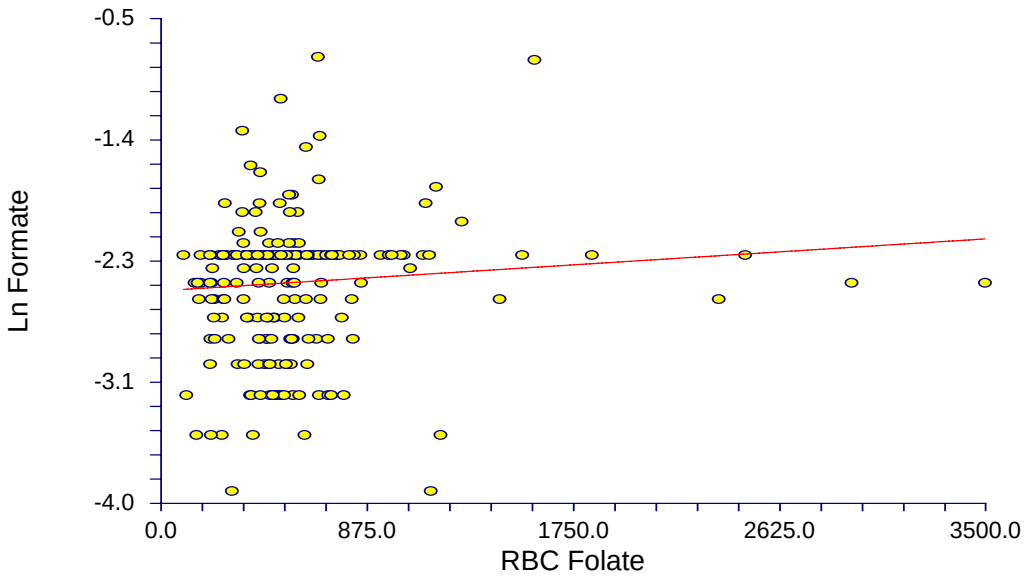

Figure 4.2: Linear regression plot of natural logarithmic of blood formate vs RBC folate levels in alcohol intoxicated females (Age < 45 years, n = 260). Correlation (r)= 0.0899.

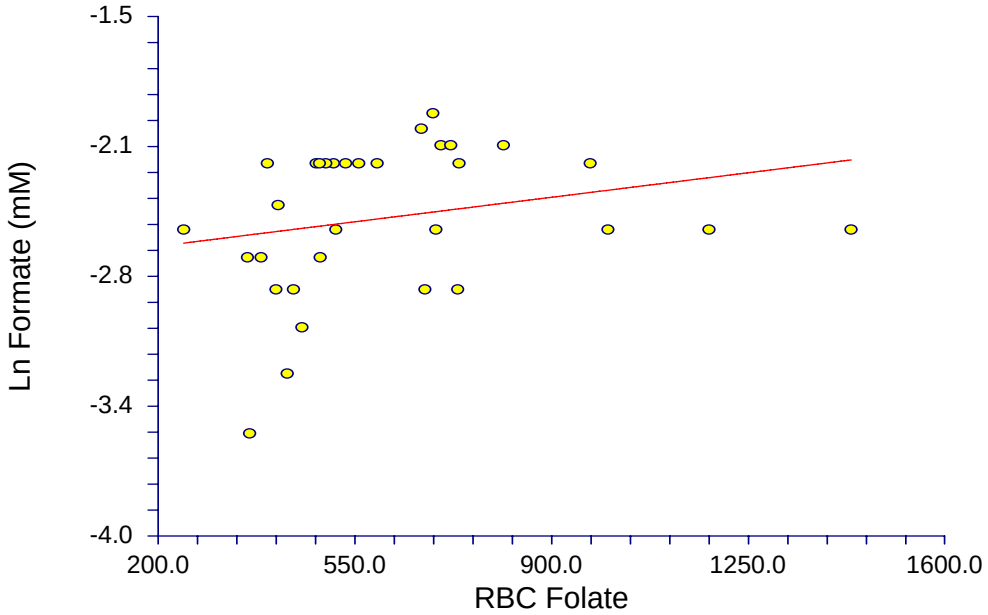

Figure 4.3: Linear regression plot of natural logarithmic of blood formate vs RBC folate levels sober females in Detox program (Age < 45 years, n = 80). Correlation (r) = 0.2382.

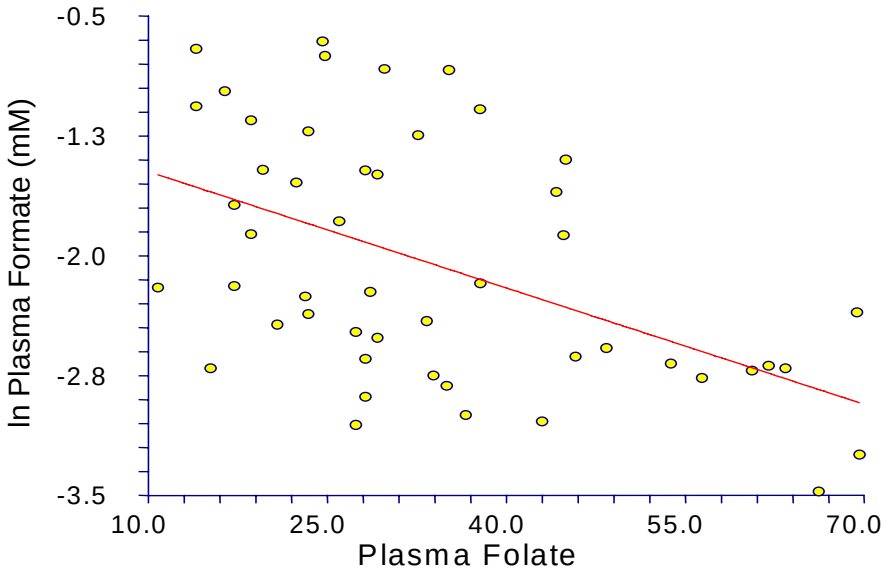

Figure 4.4: Linear regression plot of natural logarithmic of plasma formate vs plasma folate levels sober pregnant females (n = 67). Correlation (r) = -0.4989.

Linear regression analysis of natural log of blood formate against RBC folate in the intoxicated group (Figure 4.2) produced a correlation coefficient of 0.0899. A similar correlation for the detox group (Figure 4.3) produced a correlation of 0.2382. The correlation of natural log of plasma formate against plasma folate in the pregnant group (Figure 4.4) produced correlation of -0.4989.

4.4 Discussion and Conclusion

Formate is a toxic compound that is also normally present at low levels in humans, animals and plants. In the body it arises from intermediary metabolism of histidine and tryptophan. The major exogenous source of formate is through ingestion of MeOH and

MeOH-containing drinks. The toxicity of formate is well documented and includes metabolic acidosis, free radical damage and cytotoxicity (Clay *et. al.*, 1975; Dikalova *et. al.*, 2001; Friedmann *et. al.*, 1954; Hantson *et. al.*, 2005; Jacobsen *et. al.*, 1988; Jacobsen *et. al.*, 1982; Kerns *et. al.*, 2002; McMartin, 1977). Folic acid is an essential vitamin and cofactor required for a wide range of biochemical pathways involving one carbon metabolism (Wagner, 1995) including the metabolism of formate to CO_2 and H_2O during methanol/formic acid intoxication (Makar *et. al.*, 1990; Medinsky *et. al.*, 1997). Thus, body folate requirement is directly related to the amount of these biological processes (McPartlin *et. al.*, 1992; McNulty *et. al.*, 1993). Thus, in the absence of adequate folic acid supplementation one would expect folate levels to decrease as formate levels rise and vice versa.

In this study human female subjects of child-bearing age were recruited because of the susceptibility of developing fetus to formate toxicity. These groups were also chosen to represent alcohol intoxication state (intoxicated females), malnutrition state (as represented in recovering alcoholics in the detox program) and, pregnancy state (represented by the prenatal clinic recruits). As expected, we observed an inverse (negative) relationship between plasma formate and folate levels (Correlation = -0.4989; Figures 4.4). No meaningful relationship, however, was observed for both the intoxicated and detox groups. It is important to note that formate levels observed in the intoxicated group was not elevated and were consistent with those seen in non-methanol intoxicated individuals (Ellenhorn *et. al.*, 1997; McMartin *et. al.*, 1980; Osterloh *et. al.*, 1986; Sejersted *et. al.*, 1983; Yip & Jacobsen, 2003). This is probably due to sampling issues. The blood samples from these subjects were collected as part of their treatment protocol as they would have

presented to the hospital emergency room. Thus, significant time might not have elapsed for methanol to be metabolized to formate. This is supported by the elevated ethanol and methanol levels in this group compared to the detox group suggesting that blood was drawn before significant methanol was metabolized.

Low formate levels in the intoxicated group could also be explained by the fact that these individuals might have consumed grain/fruit based alcoholic beverage. Such alcoholic beverages contain levels of B-vitamin that meet, and in some cases exceed, the recommended daily allowance (RDA), Table 4.4 (Kellner & Cejka, 1999; Walker & Baxter, 2000). Because of the proximity of the recruiting institution (RUH) to the university, it is plausible that most of these subjects were university students on a binge-drinking spree. Most of the other subjects might also have been binge drinkers as opposed to chronic alcoholics. As a result they would have been inadvertently supplementing with B-vitamins.

Table 4.4: Amount of B-vitamins present in 1 L of Pilsner Beer (Pilsner Urquel).

Vitamin	Level (µg)	RDA (µg/day)*
Folate	400 - 600	400
B6	70 - 1700	1300
B12	3 - 140	2.4

Note: RDA=Recommended Daily Allowance; *values from Dietary Reference Intakes by Institute of Medicine, 1998, National Academy Press, Washington.

The argument that the intoxicated group could be mostly made up of binge beer drinkers is supported by the observation of lower Hcy and higher vitamin s B12 and B6

levels in this group compared to the detox group, an observation consistent with that of Beulens and coworkers (Beulens *et. al.*, 2005) in which they observed increased plasma vitamins B6 (11%) and B12 in non-alcoholic volunteers consuming alcoholic beer over a 3 week period. Lower vitamins B6 and 12 levels in the detox group would suggest that this group is primarily made up of chronic alcoholics, who often suffer from micronutrient deficiency, especially B-vitamins (Cravo & Camilo, 2000).

We, therefore, conclude from this study that a negative (inverse) relationship might exist between plasma formate and folate levels. However, there is insufficient evidence to make any meaningful conclusion in blood.

CHAPTER 5

Formate Pharmacokinetics During Formate Insult in Folate Deficient Young Swine.

5.1 Introduction

Formic acid and its conjugate base formate are essential endogenous one-carbon metabolites in most living organisms participating in vital one-carbon pool of intermediary metabolism. It is produced in the body as a result of catabolism of several amino acids including serine, glycine, histidine and tryptophan, as well as by the recycling of methylthioadenosine from the polyamine biosynthesis pathway (Cook *et. al.*, 2001; Fu *et. al.*, 2001) through the oxidation of formaldehyde generated during cytochrome P450-catalyzed *N*- and *O*-demethylation reactions (Keefer *et. al.*, 1987). Formate is also produced during the hydrolysis of certain prodrugs containing acyloxymethyl substituents as protecting groups, such as Fosphenytoin and its analogs (Stella, 1996). Exogenous sources of formate include methanol containing alcoholic beverages, fragrances, roasted coffee, food flavours (especially fruit and honey mixtures) and industrial products such as decalcifiers, acidulating agents in textile dying and finishing, and mold inhibitors in grain and silage. Formic acid, a metabolite of methanol (MeOH), is responsible for the toxicity observed in methanol poisoning (Figure 1.2) (Barceloux *et. al.*, 2002; Dorman *et. al.*, 1994; Sejersted *et. al.*, 1983). During methanol intoxication methanol is first metabolized by alcohol dehydrogenase (ADH) to formaldehyde and thence to formic acid by aldehyde dehydrogenase (Figure 1.2). Excess formate production may then lead to formate

accumulation and toxicity such as optic nerve damage, increased anion gap and metabolic acidosis (Medinsky *et. al.*, 1997; Smith & Taylor, 1982). Urinary excretion of formic acid is low (about 2%-7%) (Friedmann *et. al.*, 1954; Malorny, 1969; Sperling *et. al.*, 1953).

The vitamin folic acid plays a major coenzymatic role in one carbon metabolism and is also a critical cofactor in the elimination of formic acid. Folate coenzymes function to accept or donate one-carbon units in folate-requiring metabolic pathways (Wagner, 1995). During formate metabolism folate coenzymes transfer a formyl group (CHO) to tetrahydrofolate resulting in the formation of CO_2 and H_2O (Medinsky *et. al.*, 1997; Makar *et. al.*, 1990). Thus, adequate tetrahydrofolate levels are important for efficient formate metabolism. The importance of maintaining optimal folate levels in reducing the detrimental effects of formate in the body cannot be ignored given the ubiquitous nature of methanol and formate, formate's role in free radical damage (Dikalova *et. al.*, 2001) and the dependency of its metabolism on folate status.

Species differences exist in the metabolism of formate. This is due to differences in the activities of formyltetrahydrofolate synthetase, the enzyme catalyzing the transfer of CHO group to tetrahydrofolate, methylenetetrahydrofolate dehydrogenase, which catalyzes the removal of H^+ from 5, 10-methylenetetrahydrofolate to form 5, 10-methenyltetrahydrofolate, and the tetrahydrofolate substrate (Black *et. al.*, 1985; Johlin *et. al.*, 1987; Johlin *et. al.*, 1989). Species susceptible to methanol/formate poisoning have lower hepatic tetrahydrofolate levels and thus slower formate metabolism than resistant species such as rats. Pigs have the lowest levels (3.3 ± 1.1 nmol/g, mean $\pm$ SE) compared to rats (11.4 ± 0.8 nmol/g, mean $\pm$ SE) (Tephly *et. al.*, 1992). Hence, mice and rats have the highest rate of formate metabolism and are less susceptible to formate toxicity while

humans and pigs tend to be more susceptible. This suggests that pigs may be an appropriate model system for investigations into the effects of formate toxicity and its relationship to folate status. The purpose of this experiment was to determine the influence of folate deficiency on the pharmacokinetics of exogenously administered formate in a pig model.

5.2 Experimental

5.2.1 Chemicals

Pure formic acid standard (88% v/v) was obtained from Fisher Scientific (Fair Lawn, NJ, USA) and sodium formate from Sigma-Aldrich (Oakville, ON.). Normal saline was obtained from Fluka (Oakville, ON.). Sulphuric acid and anhydrous ethyl alcohol were purchased from J. T. Baker (Phillipsburg, NJ.) and Commercial Alcohols Inc. (Brampton, ON.), respectively. Gas tight syringes (Hamilton 1.25 mL; 22/2"/2) were purchased from Hamilton Company (Reno, NV.). All other chemicals used were analytical grade.

5.2.2 Animals and Husbandry

The diets consisted of a semi-purified diet formulated to meet the nutrient requirements of the 5 - 20-kg pig. The basal diet (folate control diet, FFC) consisted of 48.6% corn starch, 20% lactose, 15% casein, 5% corn oil, 5% cellulose, 2.2% monocalcium phosphate, 1.5% limestone, 0.5% salt, and 0.2% cystine and a vitamin/mineral premix that provided 150% of the nutrient requirements of swine for micronutrients (National Research Council, 1998). Folic acid was provided at 0.6 mg/kg. The composition of folate deficient diet (FFD) was the same as that of FFC diet except

folic acid was eliminated. Additionally, 1% succinylsulfathiazole was added to both diets as an antibiotic to prevent the synthesis and absorption of folate produced by commensal microorganisms in the gastrointestinal tract. Diets were offered to piglets in the form of powder for 4 weeks before administration of formate. Folate deficiency was confirmed with plasma level determinations (<30 nmol/L).

Twelve pigs (all male, ~5 kg; Cotswold Canada Ltd.), weaned at 18 days of age, were randomly assigned to 1 of 2 treatment groups (n = 6/group). Pigs receiving the FFC diet were matched (by body weight) to corresponding folate-deficient pigs (FFD) and received equivalent diet allotment using a pair-feeding protocol. Feed intake was measured daily. Folate status was monitored weekly for 6 weeks. When plasma folate concentrations had reached a nadir (<30 nmol/L) (after 6 weeks), the pigs were subjected to the formate experiments.

Pigs were group housed on raised, plastic-coated mesh flooring (4 pigs/pen), in an environmentally controlled room with continuous lighting. Temperature was initially set at 30°C, and gradually reduced to 25°C over the 6-week experimental period. Each pen was equipped with a self-feeder and a water nipple, to permit *ad libitum* intake of feed and water. Fresh feed was offered daily and body weight was measured weekly. The study was approved by the institutional committee on animal care and conducted in accordance with the Canadian Council of Animal Care guidelines (Olfert *et. al.*, 1993).

5.2.3 Formate Formulation and Administration

Four pigs (31-38 kg) in each group were administered sodium formate (351 mg/kg; 237 mg/kg formate base) via a surgically inserted catheter in the left jugular vein. The

remaining two pigs in each group were administered sterile saline via the same vein. Formate infusion over a 10 min period (109-133 mL solution in each animal depending on the weight of the animal) was carried out according to good practice guidelines (Diehl *et. al.*, 2001). Saline infusion (111-130 mL depending on body weight) was also performed in a similar manner. Sodium formate was prepared in normal saline and sterilized by autoclaving (AMSCO Isothermal® operated at 17.1-19.5 p.s.i. at 121ºC for 15 minutes) before use. Blood samples (~10 mL) were collected using sterile syringes coated with sodium heparin before and at 10, 20, 30, 45, 60, 90, 120, 180, 240 and 480 min post infusion. For plasma, samples were kept on ice during the collection period and centrifuged soon after sample collection was over for that period to remove the cells. The resultant plasma was then stored at -80ºC until analysis. For RBC folate whole blood samples were stored at 4ºC till analysis.

5.2.4 Formate Analysis

Formate analysis was done as described earlier in Sections 2.2.2 to 2.2.4.

5.3 Results

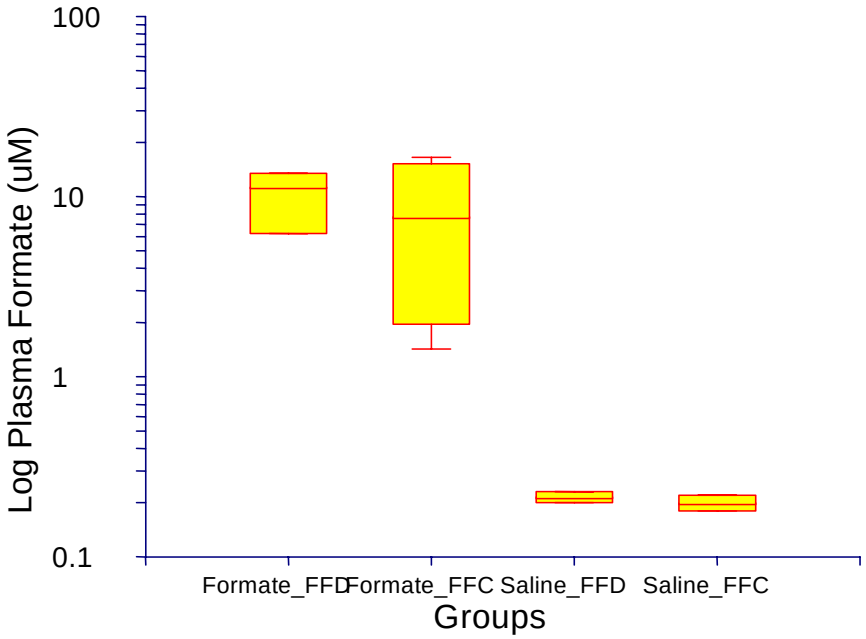

Figure 5.1: Box plot of Log mean plasma formate levels at the end of infusion in male pigs (n = 12) after infusion of 351 mg/kg sodium formate (237 mg/kg formate; n= 4/group) and physiological saline (111 – 130 mL; n = 2/group). Infusion was done over 10 min. Formate_FFD = Formate infused folate deficient group; Formate_FFC = Formate infused folate control group; Saline_FFD = Saline infused folate deficient group; Saline_FFC = Saline infused folate control group.

To reach folate deficiency status, the designated group (FFD) of pigs was fed a folate deficient diet for six weeks. Folate deficiency was successfully induced in this group at the end of 6 weeks as confirmed by plasma measurements (26 ± 5 nmol/L, Mean ± SE), which were at nadir (<30 nmol/L) and statistically different from the folate control diet fed pigs ($p = 0.007$). To determine whether folate deficiency altered formate pharmacokinetics, pigs were administered formate or saline via a short intravenous infusion. At the end of the infusion, plasma formate levels rose from a baseline value of 0.16 ± 0.01 mM (mean ± SE), in folate deficiency (FFD) and 0.12 ± 0.05 mM in folate

control (FFC), to a maximum concentration (Cend-inf) of 9.63 ± 0.57 mM and 10.04 ± 0.71 mM, respectively. Formate levels in saline administered animals did not rise significantly (Figure 5.1). Folate deficient diet pigs (FFD) accumulated more formate than FCC pigs, as indicated by the ~2.5-fold higher AUC values of FFD pigs relative to the FFC pigs (Figure 5.2 and Table 5.1). The plot of formate concentration as a function of time indicated first order elimination kinetics for both folate replete and deficient groups (Figure 5.2), but the slope of the log-linear terminal phase was much shallower in folate deficient pigs (FFD) (Figures 5.2). The half-life ($t_{1/2}$) of formate in FFD pigs was approximately 2.5-fold higher relative to the FFC pigs. Systemic clearance of formate was about 44% lower in FFD than FFC pigs, but volume of distribution (V_{SS}) remained approximately 18 L in both groups.

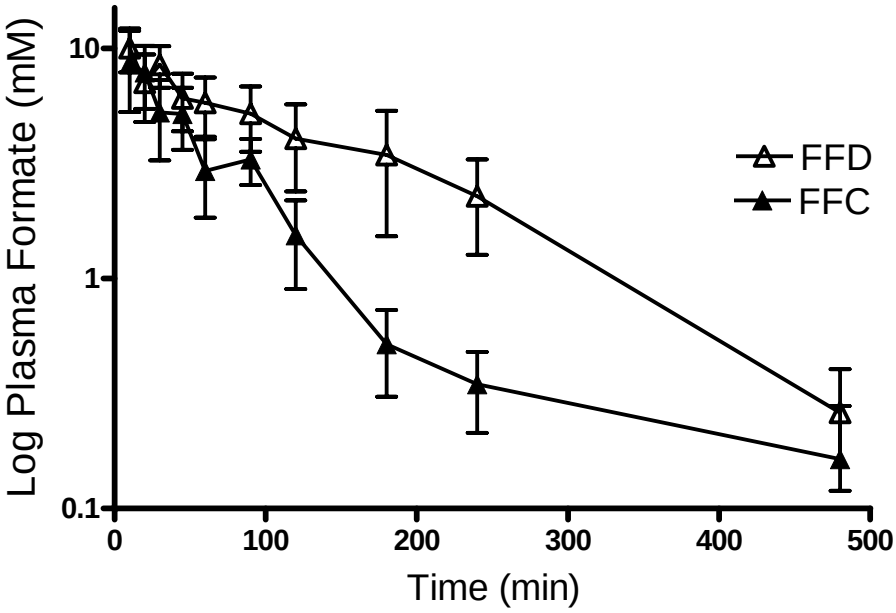

Figure 5.2: Semilogrithmic plot of plasma formate (mean ± SE) concentration (mM) vs time of folate deficient (FFD) and folate control (FFC) male pigs (n = 4; 31 - 38 kg). Animals were given a short intravenous infusion (~10 min) via the left jugular vein with 351 mg/kg sodium formate (237 mg/kg formate).

Table 5.1: Pharmacokinetic parameter estimates of formate in folate deficient (FFD) & folate control (FFC) male pigs.

Parameter	Units	FFD ± SE	CV (%)	FFC ± SE	CV (%)	p-value	Conclusion
Cend-inf	mM	9.63 ± 0.57	5.87	10.04 ± 0.71	7.04	0.668	H_0
Cend-inf	Mg/L	443 ± 26	5.88	462 ± 32	7.04	0.661	H_0
AUC	g min L^{-1}	72.37 ± 8.29	11.4	30.08 ± 2.58	8.58	0.003	H_1
K	min^{-1}	0.0061 ± 0.0009	14.9	0.0154 ± 0.0020	12.88	0.005	H_1
$t_{1/2}$	min	113 ± 17	14.9	45 ± 6	12.85	0.009	H_1
MRT	min	163 ± 24	14.9	65 ± 8	12.8	0.008	H_1
Cl_S	L min^{-1}	0.12 ± 0.01	11.5	0.27 ± 0.02	8.59	0.001	H_1
V_{SS}	L	18.84 ± 1.05	5.89	17.21 ± 1.35	7.04	0.377	H_0

Note: Pigs (31-38 kg) were administered 351 mg/kg sodium formate (237 mg/kg formate; n=4/group) via intravenous infusion (10 min) into the left jugular vein. Blood samples were collected over an 8-hour period. Values with the exception of CV (%) represent mean ± standard error (SE). CV = Coefficient of variation; H_0 = No statistical difference between means at p=0.05; H_1 = The means are statistically different at p = 0.05; AUC = Area under the curve; K = Elimination constant; $t_{1/2}$ = Elimination half-life; Cend-inf = Concentration at end of infusion; Cl_S = Systemic clearance; MRT = Mean residence time; V_{SS} = Volume of distribution under steady state

5.4 Discussion and Conclusions

Formate is a toxic compound that is normally present at low levels in humans, animals and plants. Endogenous formate arises from intermediary metabolism through such metabolic pathways as histidine and tryptophan degradation. Exogenous sources of formate include methanol containing alcoholic beverages, fragrances, roasted coffee, food

flavours (especially fruit and honey mixtures) and industrial products such as decalcifiers, acidulating agents in textile dying and finishing and, mold inhibitors in grain and silage. Folic acid, an essential vitamin and building block of tetrahydrofolate, is required in the metabolism of formate. Thus, efficient formate metabolism depends upon the activities of formyltetrahydrofolate synthetase and methylenetetrahydrofolate dehydrogenase, enzymes involved in folate metabolism and tetrahydrofolate levels (Black *et. al.*, 1985; Johlin *et. al.*, 1987; Johlin *et. al.*, 1989). Species and individual differences exist in tetrahydrofolate levels. Consequently, variable rates of formate metabolism are encountered across species and individuals (Table 5.2). Thus, mice and rats have the highest rate of formate metabolism and are less susceptible to formate toxicity while humans and pigs tend to be more susceptible. Consequently, folate status may have a marked impact on formate levels and predisposition to formate toxicity.

Table 5.2: Levels of hepatic folate intermediates in various species and subspecies.

Folate Intermediate	**Species/Subspecies concentration (nmol/g)**						
	Mouse[1]	**Rat**[1]	**Monkey**[1]	**Human**[1]	**Outbreed swine**[2]	**Micropigs**[3]	**Yucatan Mininpigs**[4]
Tetrahydrofolate	42.9±1.2	11.4±0.8	7.4±0.8	6.5±0.3	3.3±1.1	1.6±0.2	
5-Methyltetrahydrofolate	11.6±0.4	9.3±0.6	7.6±0.6	6.0±0.7	1.0±0.2	5.4±0.5	
Formyltetrahydrofolate	6.4±0.6	4.6±1.3	10.5±0.8	3.3±0.5	0.7±0.1	1.3±0.2	
Total	60.9±2.1	25.3±0.9	25.5±1.2	15.8±0.8	5.1±1.2	8.2±0.6	17.5±2.2

Note: Values represent mean ± Standard Error (SE). [1]Data from Johlin *et. al.*, 1987; [2]Data from Makar *et. al.*, 1990; [3]Data from Tephly *et. al.*, 1992; [4]Data from Dorman *et. al.*, 1993.

In the present study, formate levels increased ~70-fold following formate administration relative to the saline controls (Figure 5.2). The amount of formate administered (237 mg formate/kg) and the resultant formate levels have not been associated with overt metabolic crisis (Makar *et. al.*, 1990; Tephly *et. al.*, 1992; Dorman *et. al.*, 1993; Jacobsen & McMartin, 1986; Sophia Dyer, 1998) and no physical distress was observed in our animals during the experiment. This is consistent with Dorman and co-workers (Dorman *et. al.*, 1993), Makar (Makar *et. al.*, 1990), and Tephly (Tephly *et. al.*, 1992) who reported only minimum metabolic acidosis or bicarbonate depletion following administered doses of 425 mg/kg or 500mg/kg sodium formate (338 mg/kg formate).

Folate deficiency resulted in a significant decrease (55%) in the systemic clearance of formate but had no affect on the volume of distribution (~18 L; 0.53L/kg, $p=0.377$). Hence, the significantly lower elimination rate constant (2.5-fold) and longer half-life (2.5-fold) in folate deficient pigs was due to changes in systemic clearance alone. This is consistent with folate's critical role in formate metabolism. Consequently, folate deficiency state results in higher formate exposure as observed by the 2.5-fold higher AUC and MRT values in FFD pigs relative to FFC pigs (72.37 ± 8.29 g min L^{-1} vs 30.08 ± 2.58 g min L^{-1} and 163 ± 24 min vs 65 ± 8 min, respectively).

Increased formate exposure can lead to metabolic acidosis, free radical damage and neurotoxicity (Medinsky *et. al.*, 1997; Hantson *et. al.*, 2005; Jacobsen *et. al.*, 1988; Jacobsen & McMartin, 1986; McMartin, 1977; McMartin *et. al.*, 1980). Formaldehyde, a highly reactive intermediary metabolite of methanol and precursor of formic acid, can combine with a variety of cellular constituents forming relatively stable adducts (Medinsky *et. al.*,

1997). For example, formaldehyde combines with glutathione to form *S*-formylglutathione. This may significantly alter glutathione levels leading to reduced antioxidant capacity. The formic acid molecule dissociates completely at physiological pH into its conjugate base, formate, and a hydrogen ion. Formate distributes freely into total body water and is found in blood and CSF. The increased amounts of hydrogen ions formed as a result of the dissociation eventually overwhelm the normal acid-base balance of the body resulting in acidosis. Formate is also neurotoxic leading to edema and atrophy of the optic nerve (resulting in permanent vision loss). Damage to the putamen of the basal ganglia of the brain (the area that controls gross intentional motor activities that are normally performed unconsciously) is often seen in autopsies of victims of methanol poisoning. These adverse effects are reportedly associated with prolonged blood formate elevation (> 7 to 10 mM) of greater than 24 hours. Although endogenous levels of formate are low, ranging form 0.12 to 0.28 mM (Buttery & Chamberlain, 1988), it is conceivable to obtain much higher levels due to exposure from various sources (as a result of extensive use of formate in industry) in a folate deficiency state. Other conditions such as pregnancy, disease, malnutrition and convalescence can also aggravate folate deficiency and formate accumulation.

Table 5.3: Formate elimination half-life in various species.

Animal	Minipig[1]	Outbreed swine[2]	Micropig[2]	Human[4,5]	Rat[6]	Monkey[7]
Formate $t_{1/2}$ (min)	50 - 112	87 ± 18	74.1 ± 6	69 - 120	12 - 23	31 - 51

Note: [1, 4, 5, 6] and [7] values represent ranges while [2] values represent Mean ± Standard Error (SE). [1]Data from Dorman *et. al.*, 1993; [2]Data from Makar *et. al.*, 1990; [3]Data from Tephly *et. al.*, 1992; [4]Data from McMartin *et. al.*, 1980; [5]Data from Hanzlik *et. al.*, 2005; [6]Data from Clay *et. al.*, 1975; [7]Data from McMartin, 1977.

Our observations of formate kinetics as demonstrated by $t_{1/2}$, in normal pigs seem to support that of other workers (Table 5.3) with the exception of Vss. The Vss obtained of approximately 18 L (0.53 L/kg) is much lower than that observed by others. For example, Hanzlik (Hanzlik *et. al.*, 2005) found the Vss of formate in human females given an oral dose of 3900 mg calcium formate to be 2.36 L/kg. By extrapolating the kinetic plots (lnC versus t) of Clay *et. al.* (Clay *et. al.*, 1975) to time 0 and dividing by the dose, Hanzlik and colleagues calculated the dose-independent Vss of formate as 2.25 L/kg. They also obtained a Vss of 2.58 L/kg by similar extrapolation of the kinetic plot of Eells *et. al.* (Eells *et. al.*, 1981). This difference between our value and that of others is probably due to the fact that Hanzlik *et. al.* (Hanzlik et. al., 2005) and Eells *et. al.* (Eells et. al., 1981) obtained Vss based on oral and intraperitoneal administration of formate. Thus, such an estimate of Vss is confounded by formate bioavailability (i.e. the estimate is actually Vd/F). Also, the lack of the formate bioavailability in their work and in the literature leaves much speculation on how our value for Vss compares to others. Another possible

reason for this difference may be due to our short-term infusion (~10 min). As a result, there may be certain error associated with a Vss derived in such a manner. The i.v. infusion over such a time (~10 min) was necessary to address concerns of formate toxicity that might arise in the folate deficiency animals (351 mg/kg sodium formate administered in a 109-133 mL solution to each animal depending on the weight) as well as to comply with safe infusion guidelines for such animals (Diehl *et. al.*, 2001).

We have demonstrated that folate deficiency significantly reduces systemic formate clearance resulting in increased risk of formate toxicity in folate deficient pigs. The experimental model described in this study (of folate deficient pig) can serve as the basis of further research on modulation of effects of formate toxicity in folate deficiency in humans. Further research is needed to elucidate the effects (subclinical and clinical) of formate during folate deficiency in tissues and on biochemical parameters, and how this relates to disease manifestations.

CHAPTER 6

Folate Utilization During Formate Insult in Folate Deficient Pigs.

6.1 Introduction

Folic acid (Figure 3.2) is an antioxidant and a vitamin that plays a major coenzymatic role in one carbon metabolism. The principal function of folate coenzymes is to accept or donate one-carbon units in folate-requiring metabolic pathways (Wagner, 1995), e.g., DNA methylation. The folate molecule that acts as an acceptor of carbon in the one-carbon cycle is in the polyglutamyl form of tetrahydrofolate (THF) (Wagner, 1995).

The amount of biologically active folate excreted by mammals is considerably less than the amount obtained from dietary sources. The major daily turnover for folate is via excretion of the folate catabolites p-aminobenzoylglutamate, *p*-ABG, and ap-acetamidobenzoylglutamate, *ap*-ABG (McPartlin *et. al.*, 1992; McNulty *et. al.*, 1993) (Figure 3.2). Thus, the obligatory requirement of folic acid, like with any other vitamin, can be related to this catabolism. Certain conditions, such as pregnancy, periods of rapid growth, during drug therapy regimes and in a variety of disease conditions, can increase folate catabolism and, hence, its requirements.

The overall status of the one carbon pathway is also dependant on the levels of methionine and vitamins B6 and B12 (Figure 1.6) (Bailey & Gregory, III, 1999; Barak *et. al.*, 1991; Barber *et. al.*, 1999; Halsted *et. al.*, 2002a; Lucock, 2000; Scott *et. al.*, 1993). In situations of poor folate status, *S*-adenosylhomocysteine (SAH) concentration increases due to the impairment of methyl group synthesis and homocysteine remethylation.

Inhibition by resulting product (SAH) suppresses many of the SAM-dependent methyltransferase reactions (Selhub & Miller, 1992). This illustrates the far-reaching effects of impaired one-carbon metabolism during such a nutritional deficiency.

Folic acid is also a cofactor in the elimination of formic acid. Formic acid and its conjugate base, formate, are essential endogenous one-carbon metabolites in most living organisms participating in vital one-carbon pool of intermediary metabolism (Cook et. al., 2001; Fu et. al., 2001). However, formate is also a metabolite of methanol (MeOH) and is responsible for the toxicity observed in methanol poisoning (Barceloux *et. al.*, 2002; Dorman *et. al.*, 1994;Sejersted *et. al.*, 1983) (Figure 1.2). Methanol is first metabolized to formaldehyde by alcohol dehydrogense, which is then rapidly converted to formic acid by aldehyde dehydrogenase. The formate ion (from the dissociation of formic acid) is metabolized to CO_2 and H_2O via the folate-requiring pathway. In this reaction, a formyl group (CHO) is transferred to tetrahydrofolate resulting ultimately in the formation of CO_2 and H_2O (Makar *et. al.*, 1990; Medinsky *et. al.*, 1997). Excess production of formate leads to toxicity, which can include optic nerve damage, increased anion gap and metabolic acidosis (Medinsky *et. al.*, 1997; Smith & Taylor, 1982).

Species difference in the metabolism of formate is due to differences in the activities of formyltetrahydrofolate synthetase (the enzyme catalyzing the transfer of CHO group to tetrahydrofolate), methylenetetrahydrofolate dehydrogenase (which catalyzes the removal of H^+ from 5, 10-methylenetetrahydrofolate to form 5, 10-methenyltetrahydrofolate), and the tetrahydrofolate substrate (Black *et. al.*, 1985; Johlin *et. al.*, 1987; Johlin *et. al.*, 1989). Species susceptible to methanol/formate poisoning have lower hepatic tetrahydrofolate levels and thus slower formate metabolism than resistant

species such as rats. Pigs have the lowest levels of tetrahydrofolate (3.3 ± 1.1 nmol/g, mean ± SEM) compared to rats (11.4 ± 0.8 nmol/g, mean ± SEM) (Tephly *et. al.*, 1992). The maximal observable rate of formate metabolism in rats is approximately two-fold faster than in monkeys (McMartin, 1977). Thus, adequate tetrahydrofolate levels are important for efficient metabolism of formate.

In this experiment we determine the effect of formate insult on folate utilization during folate deficiency in male pigs. We also assess changes in antioxidant and free radical levels as in these animals as demonstrated by homocysteine, methylmalonic acid, methylglyoxal, *S*-adenosylmethionine, *S*-adenosylhomocysteine levels.

6.2 Experimental

6.2.1 Animals and Husbandry

Animal husbandry was done as described in 5.2.2.

6.2.2 Formate Formulation and Administration

Formate formulation and administration was done as described in 5.2.3.

6.2.3 Formate Analysis

Blood samples (~10 mL) were collected using sterile syringes coated with sodium heparin before and at 10, 20, 30, 45, 60, 90, 120, 180, 240 and 480 min post infusion. Samples were processed as described in 5.2.3 and analyzed for formate as described earlier in Sections 2.2.2 to 2.2.4.

6.2.4 Folate utilization

Folate utilization was assessed by measuring plasma levels of p-aminobenzoylglutamate (*p*-ABG) and its acetamido derivative, ap-acetamidobenzoylglutamate (*ap*-ABG) as decribed in 3.2.3.

6.2.5 Plasma and Red blood cell (RBC) Folate

Plasma and RBC folates were determined by an immunoassay technique run on an Architect™ system (Abbott Laboratories, IL.) as described in 4.2.6.

6.2.6 Methionine

Plasma methionine levels were determined using a electrospray ionization tandem mass spectrometric (ESI-MS/MS) method. This method involves derivatizing methionine to its dibutyl ester before detecting the levels using its mass to charge ratio (m/z = 209.1) and that of its product ion after fragmentation (m/z = 107.1). $^{2}H_{3}$-Methionine (Cambridge Isotopes Laboratories, Inc., Andover, MA.; 2.5 μmol/L stock solution in methanol) was used as an internal standard. Methionine Standard (Sigma-Aldrich, St. Louis, MO.) was used to make a standard curve in methanol at 0, 25, 50, 125, 250, 500 μmol/L levels. ERNDIM samples (www.erndim.unibas.ch) were used as a quality control (QC) sample in addition to the in-house QC made by spiking plasma with methionine at 25 μmol/L and 125 μmol/L. Each QC was run in duplicate in the middle and end of the run.

Briefly, 50 μL plasma sample was added to 200 μL of methanol, vortex mixed and spun down at 2500 rpm for 3 minutes. 25 μL of the supernatant was transferred to a

corresponding well in a 96 well plate that contains 180 μL of the internal standard solution. After gently mixing for 3 minutes, the solution in the well was evaporated to dryness under a stream of nitrogen air at 35°C. Dibutyl ester of methionine was obtained by adding 75 μL of butanolic HCl (3M HCl in butanol) to each well, gently mixing for 1 minutes and incubating for 20 minutes at 80°C. Excess butanolic HCl was evaporated under stream of nitrogen as before. Each dry well was then reconstituted with 120 μL of acetonitrile-water solution (4:1). This was then gently mixed for 1 minute before injecting into an API2000 tandem mass spectrometer (Applied Biosystems-MDS SCIEX, Concord, ON.) in positive ion mode. Mass spectrometer parameters were set as follows: Curtain gas = 25, Collision gas = 4, Ion Spray Voltage = 5300, Temperature = 300°C, Gas 1 = 30, Gas 2 = 40, Focusing Potential = 350, Entrance Potential = 10.

Quantitation of methionine levels was done using the by plotting the peak area ratios of methionine to $^{2}H_{3}$-Methionine against the calibration concentrations using Analyst 1.4 software (Applied Biosystems-MDS SCIEX, Concord, ON.).

6.2.7 Homocysteine (Hcy)

Plasma homocysteine was determined by an immunoassay technique run on an Immulite® 2000 system (DPC Los Angeles, CA) as describe in 4.2.7.

6.2.8 Vitamins B6 & B12

Plasma vitamin B-6 (PLP) levels were analyzed using HPLC system utilizing the method developed by Deitrick and co-workers (Deitrick *et. al.*, 2001b;Deitrick et. al., 2001) (see section 4.2.8).

Plasma vitamin B12 was analyzed using immunoassay technique run on an Architect™ system (Abbott Laboratories, IL.) as decribed in 4.2.8.

6.2.9 Methylmalonic Acid (MMA)

Methylmalonic acid (MMA) was determined using the method developed by Schmedes and coworkers (Schmedes & Brandslund, 2006). This is a liquid chromatography-tandem mass spectrometry (LC-MS/MS) method and involves a solid phase clean up of the plasma samples using a strong anion exchange SPE columns and detection by LC-MS/MS.

Aqueous calibrators were prepared at 0 μM, 0.2 μM, 0.4 μM, 0.75 μM, 1.0 μM, 1.5 μM, and, 2.0 μM. Plasma samples and calibrators were treated the same by adding 100 μL d_3-MMA to 500 μL sample/calibrator. This was then diluted with equal volume of sodium phosphate buffer (10 mM; pH = 7.4) before applying to a strong anion exchange SPE columns (strata-SAX; 100 mg; Phenomenex Corporation, San Diego, CA).

Selective reaction monitoring (SRM) was done on the mass spectrometer using the Sciex Turbo ion spray source in positive ion mode. Resolution in quadrupole 1 (Q1) and quadrupole 3 (Q3) chambers were at unit mass. Other source parameters were as indicated in Table 3.1. The Multiple SRM mass transitions monitored were 309>162, 309>180, both for ap-ABG and 267>120 for pABG. The dwell time for all the analytes was set at 500 msec. Run time for each LC injection was set at 10 minutes. Data was acquired and analyzed using Analyst software 1.4 (Applied Biosystems MDS Sciex).

6.2.10 *S*-adenosylmethionine (SAM) & *S*-adenosylhomocysteine(SAH)

S-adenosylmethionine (SAM) & *S*-adenosylhomocysteine(SAH) levels were determined using the method developed by Gellekink and coworkers (Gellekink *et. al.*, 2005) with some modifications. These modifications involved direction injection of the sample into the tandem mass spectrometer rather than using an HPLC column and the use of deutrated SAM ($^2H_3$-SAM) as an internal standard for both SAM and SAH. Precision and recoveries for both SAM and SAH were comparable to what the authors obtained.

The internal standard (1.5 μM $^2H_3$-SAM; 100 μL) was added to 500 μL of the calibrators (0 nM, 10 nM, 25 nM, 50 nM, 100 nM) and plasma samples. After mixing well the mixture was applied to an SPE column (SPE containing phenylboronic acid; BondElut® PBA 100 mg, Varian Inc. Lake Forest, CA.) preconditioned earlier with 5 mL of 0.1 M formic acid followed by 5 mL of 20 mM ammonium acetate (pH7.4). The column was then washed with 2 mL of 20 mM ammonium acetate (pH7.4) and dried for 2 min before eluting SAM and SAH with 1 mL 0.1 M formic acid. 15 μL of the eluate was injected directly into the triple quadrupole tandem mass spectrometer (API4000, Applied Biosystems MDS Sciex) connected to a pump delivering 0.2 mL/L acetic acid in water (buffer) and MeOH (solvent) at a rate of 200 μL/min and gradient of 0% - 0.3% buffer over a two minute run period.

Selective reaction monitoring (SRM) was done on the mass spectrometer using the Sciex Turbo ion spray source in negative ion mode. Resolution in quadrupole 1 (Q1) and quadrupole 3 (Q3) chambers were at unit mass. The Multiple SRM mass transitions monitored were 399>250 for SAM, 385>136 for SAH and 402>250 for $^2H_3$-SAM. The

dwell time for all the analytes was set at 200 msec. Data was acquired and quantitated using Analyst software 1.4 (Applied Biosystems MDS Sciex).

6.2.11 Methylglyoxal

Analysis of methylglyoxal (MG) was done using the method developed by Randell *et. al.* (Randell *et. al.*, 2005b) with significant author modifications (Randell *et. al.*, 2005a). Briefly, 500 μL of plasma was precipitated with 1000 μL trichloroacetic acid (12% v/v). After centrifugation, 400 μL of the supernatant was transferred to a clean glass tube to which 250 μL of the internal standard (2, 3-hexanedione; 50 ng/mL) was added. This mixture was made up to a final volume of 1000 μL using 10 mM disodium hydrogen phosphate, Na_2HPO_4, pH 7.4. 100 μL of 2, 3-diaminonaphthalene, 0.1% v/v in methanol (2,3-DAN) was then added to this mixture as a derivatizing agent and incubated at 4ºC for at least 24 hrs. The formed methylglyoxal-2, 3-diaminonaphthalene (MG-DAN) and 2, 3-hexanedione-DAN are extracted from the plasma mixture using 4000 μL (4 mL) of ethyl acetate, which is then blown down to dryness under nitrogen and reconstituted in 200 μL acetonitrile. Calibrators were prepared from pooled plasma by the standard addition method and treated as the samples.

Measurement of methylglyoxal levels was done using liquid chromatography-tandem mass spectrometry (LC-MS/MS) that consisted of Agilent 1100 series HPLC system and API 4000 tandem mass spectrometer (PE SCIEX, Applied Biosystems). Each run consisted of a 5 μL injection into the HPLC system pumping a solvent system made up of 0.1% formic acid in water (35%) and acetonitrile (65%) at a flow rate of 300 μL/min (3 mL/min) and a C8 column (Symmetry C8; 3.5 um; 2.1x100mm; Waters corporation,

WAT058961). Methylglyoxal-2, 3-diaminonaphthalene (MG-DAN) was detected in the MS/MS in ESI positive multiple reaction monitoring (MRM) ion mode by monitoring the transition 195 >168 while 2, 3-hexanedione-DAN by monitoring the transition 237>208 in the same ion mode.

6.2.12 Glutathione (GSH) and Glutathione Disulphide (GSSG)

Reduced (GSH) and oxidized (GSSG) glutathione levels in plasma were determined using an LC-MS/MS method developed for the study. Plasma (1 mL) samples and 1 mL aqueous calibrators (a mixture of GSH and GSSG; 0 mg/mL, 0.1 mg/L, 0.25 mg/mL, 0.5 mg/mL and 1.0 mg/mL) were treated similarly and, were diluted in 200 μL 1 N NaOH before adding 100 μL of (1 M Iodoacetic acid in 1 N NaOH) to quench and tie up the –SH groups of GSH. This was then mixed thoroughly and incubated at room temperature for 30 min. The internal standard (100 μL; 1 mM $^{2}H_{3}$-Glutamic acid; CDN Isotopes, Pointe-Claire, Qc.) was then added followed by 1 mL acetonitrile (Anachemia, Rouses Point, NY) to deproteinate proteins.

After spinning at 1600*g* for 5 minutes, the supernatant was placed on a SPE column (Strata X-AW 60 mg/3mL; Phenomenex, San Diego, CA) that was preconditioned with 3 mL 2% formic acid in MeOH (Fisher Scientific, Fair Lawn, NJ) followed by 3 mL deionized H_2O. Before elution, the column was washed with 3 mL 70% MeOH and dried under vacuum for 2 min. Elution of derivatized GSH (molecular weight = 365) and GSSG (underivatized) was by application to the column of 1 mL 2% NH_4OH (Fisher Scientific, Fair Lawn, NJ) in MeOH. The eluate was then dried under nitrogen gas and reconstituted in a solution containing 0.1% formic acid in water (90%) and MeOH

(10%). The reconstituted solution (15 μL) was injected into triple quadrupole tandem mass spectrometer (API2000, Applied Biosystems MDS Sciex) connected to a pump delivering 0.1% formic acid in water (90%) as the buffer and MeOH (10%) as solvent at a rate of 200 μL/min isocratic over a two minute run period.

Selective reaction monitoring (SRM) was done on the mass spectrometer using the Sciex Turbo ion spray source in negative ion mode. Resolution in quadrupole 1 (Q1) and quadrupole 3 (Q3) chambers were at unit mass. Other source parameters were as indicated in Table 6.1. The Multiple SRM mass transitions monitored were 364>143, 364>272, 364>128, for GSH, 611>272, 611>338, 611>306 for GSSG and, 149>105 for $^{2}H_{3}$-Glutamic acid. The dwell time for all the analytes was set at 200 msec. Data was acquired and quantitated using Analyst software 1.4 (Applied Biosystems MDS Sciex).

Table 6.1: Source parameters for analysis of Glutathione (GSH) and Glutathione Disulphide (GSSG) in the tandem mass spectrometer (MS/MS).

Instrument parameter	***Value***
Source temperature	500 ºC
Collision associated dissociation (CAD) gas	2 p.s.i.
Curtain gas	40 p.s.i.
Gas source 1 & 2 (GS 1 & GS 2)	40 p.s.i.
Ion spray voltage	-4200 V
Declustering potential (DP)	-15 V
Electrode potential (EP)	-10 eV
Collision energy (CE) for transition 364>143	-30 eV
Collision energy (CE) for transition 364>272	-18 eV
Collision energy (CE) for transition 364>128	-30 eV
Collision energy (CE) for transition 611>272	-34 eV
Collision energy (CE) for transition 611>338	-26 eV
Collision energy (CE) for transition 611>306	-30 eV
Collision energy (CE) for transition 149>105	-30 eV

6.2.13 Data Analysis

Mean ± SE values of each analyte were calculated for each group. Two-sample t-test was done to compare between the two groups using NCSS 2007 software (NCSS, Kaysville, UT.).

6.3 Results

To induce folate deficiency the designated group (FFD) was fed a folate deficient diet. After six weeks folate deficiency was successfully induced as confirmed by the

significantly lower ($p = 0.007$) plasma (26 ± 5 nM, Mean ± SE) folate levels in the FFD groupas compared with the FFC group (Table 6.2). To determine whether formate insult altered folate utilization during deficiency, pigs were administered formate via a short intravenous infusion. At the end of the infusion, plasma formate levels rose from a baseline value of 0.16 ± 0.01 mM (mean ± SE) in folate deficiency (FFD) and 0.12 ± 0.05 mM in folate control (FFC) to a maximum concentration (Cend-inf) of 9.63 ± 0.57 mM and 10.04 ± 0.71 mM, respectively (Figure 6.2).

As expected methionine, MMA, vitamin B6 and vitamin B12 levels were not statistically different between the two groups (folate-deficient, FFD, and folate-control, FFC). In the absence of formate (before formate administration), homocysteine (Hcy) ($p = 0.019$) and methylglyoxal (MG) ($p = 0.010$) levels were higher in folate deficiency. On formate insult, Hcy levels did not rise significantly in folate FFC and FFD groups ($p = 0.382$ and $p = 0.294$, respectively; Figure 6.2). However, MG levels increased in FFD group by 43% (from 3.20 ± 0.38 to 4.56 ± 0.40, $p = 0.049$) after formate administration. The increase in MG in FFC group was not significant (8%) (See Figure 6.3).

The ratio of SAM to SAH was lower in FFD group compared to FFC before formate infusion (2.69 ± 0.05 vs 4.23 ± 0.10 in FFC, $p = 0.000$). A decrease of 45% was observed after formate administration in FFC group (from 4.23 ± 0.10 to 2.31 ± 0.46, $p = 0.007$). The decrease was even greater in FFD after formate administration (58%, from 2.69 ± 0.05 to 1.13 ± 0.07, $p = 0.000$) (See Figure 6.4).

Table 6.2: Summary of various analytes during formate insult in pigs (n = 4/group).

Parameter	FFC	FFD	p-value	Conclusion
	Mean ± SE			
Formate Cend-inf (mM)	10.04 ± 0.71	9.63 ± 0.57	0.668	H_0
RBC Folate (nM)	171 ± 35	111 ± 6	0.067	H_0
Plasma Folate (nM)	100 ± 25	26 ± 5	0.007	H_1
Vit B6 (nM)	37 ± 6	49 ± 7	0.137	H_0
Vit B12 (pM)	260 ± 61	311 ± 52	0.450	H_0
Hcy before infusion (μM)	10 ± 3	31 ± 1	0.019	H_1
Hcy at end of infusion (μM)	14 ± 3	38 ± 6	0.000	H_1
MMA (μM)	0.12 ± 0.07	0.28 ± 0.16	0.069	H_0
Methionine (μM)	88 ± 15	93 ± 24	0.845	H_0
Methylglyoxal before infusion (μM)	2.54 ± 0.15	3.20 ± 0.38	0.160	H_0
Methylglyoxal at end of infusion (μM)	2.75 ± 0.25	4.56 ± 0.40	0.010	H_1
SAM before infusion (nM)	289 ± 4	253 ± 4	0.001	H_1
SAM at end of infusion(nM)	191 ± 25	127 ± 22	0.080	H_0
SAH before infusion (nM)	69 ± 2	94 ± 6	0.000	H_1
SAH at end of infusion(nM)	90 ± 18	116 ± 24	0.377	H_0
SAM:SAH before infusion	4.23 ± 0.10	2.69 ± 0.05	0.000	H_1
SAM:SAH at end of infusion	2.31 ± 0.46	1.13 ± 0.07	0.039	H_1
Baseline total folate catabolite (nM)	11.83 ± 2.13	5.33 ± 0.22	0.020	H_1
Catabolite at end of infusion (nM)	15.22 ± 1.60	8.86 ± 0.25	0.006	H_1

Note: FFD= folate-deficient pigs; FFC=folate-control pigs; Formate Cend-inf = Formate concentration at end of infusion; H_0=no statistical difference between means at p=0.05; H_1=the means are statistically different ($p<0.05$); RBC = Red blood cell; Vit = vitamin ; MMA = Methylmalonic Acid; SAM = S-adenosylmethionine (SAM); SAH = S-adenosylhomocysteine(SAH).

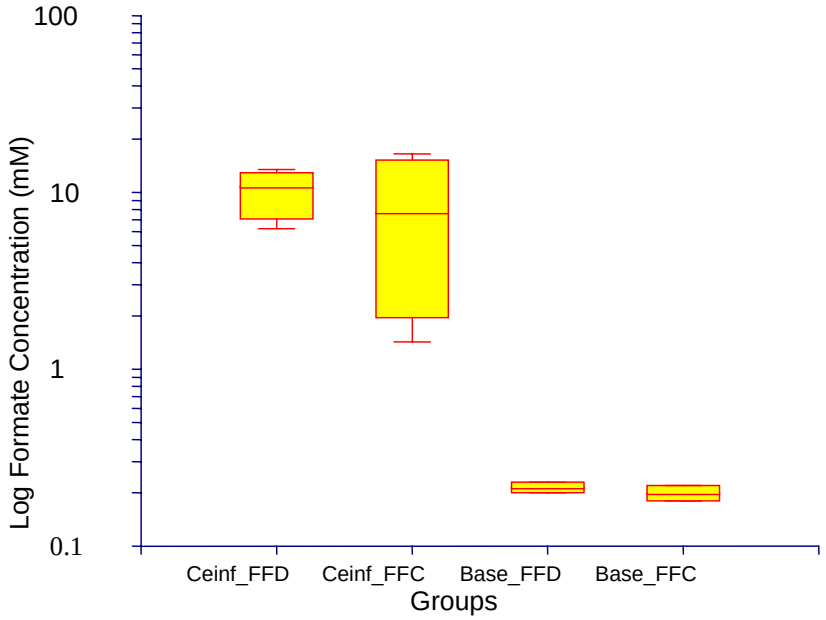

Figure 6.1: Box plot of mean plasma formate levels before (baseline) and at end of a short intravenous infusion (10 min) in male pigs (n=12) of 351 mg/kg sodium formate (237 mg/kg formate; n=4/group) or saline (111-130 mL solution; n=2/group). Ceinf FFD = Formate concentration at end of infusion in folate deficient group; Ceinf FFC = Formate concentration at end of infusion in folate control group; Base_FFD = Formate levels before formate infusion in folate deficient group.

Folate catabolite levels were higher in folate replete condition before ($p = 0.020$) and after ($p = 0.006$) folate insult. Folate utilization as indicated by total folate catabolites increased in both groups during formate insult by approximately 1.5 fold ($p = 0.014$ in FFC and $p = 0.000$ in FFD) (Figures 6.5 and 6.6).

Glutathione levels (both reduced and oxidized) in pig plasma were undetectable. Our method limit of quantitation (LOQ) was 0.1 mg/mL for both GSH and GSSG (326 μM for GSH and 163.3 μM for GSSG). Pig plasma glutathione levels are much lower than this LOQ (Villanueva *et. al.*, 2006).

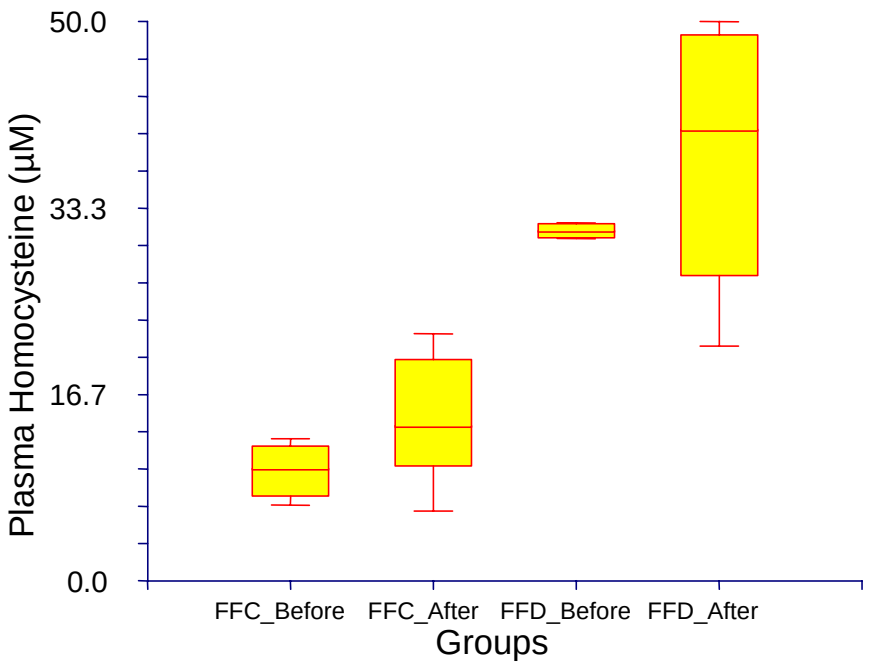

Figure 6.2: Box plot of mean plasma homocysteine levels in folate control group (FFC) and folate deficicent (FFD) before and after a short intravenous infusion (10 min) in male pigs (n=4) of 351 mg/kg sodium formate (237 mg/kg formate). FFC_Before = Folate Control group before infusion of sodium formate; FFC_After = Folate Control group after infusion of sodium formate; FFD_Before = Folate Deficient group before infusion of sodium formate; FFC_After = Folate Deficient group after infusion of sodium formate.

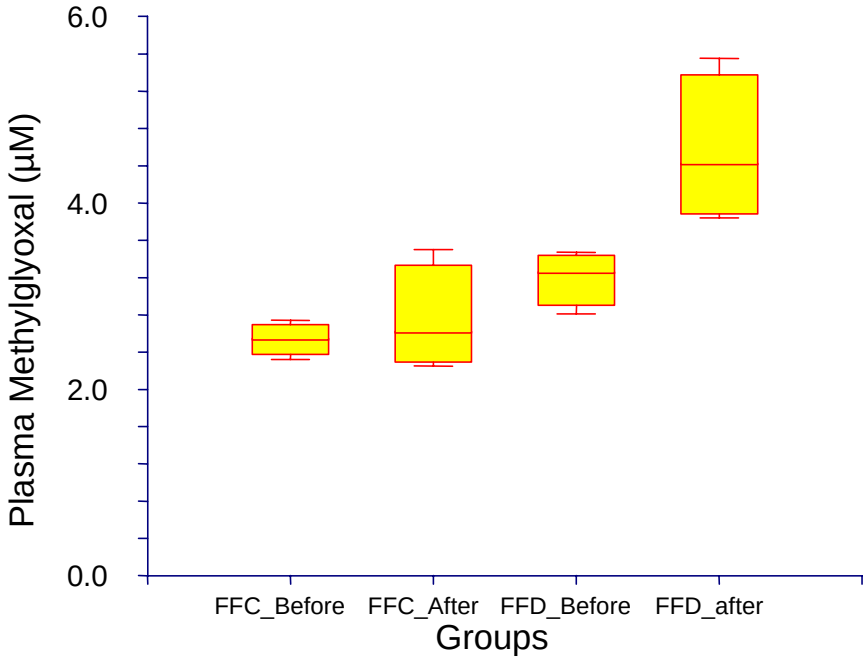

Figure 6.3: Box plot of mean plasma Methylglyoxal levels in folate control group (FFC) and folate deficicent (FFD) before and after a short intravenous infusion (10 min) in male pigs (n=4) of 351 mg/kg sodium formate (237 mg/kg formate). FFC_Before = Folate Control group before infusion of sodium formate; FFC_After = Folate Control group after infusion of sodium formate; FFD_Before = Folate Deficient group before infusion of sodium formate; FFC_After = Folate Deficient group after infusion of sodium formate.

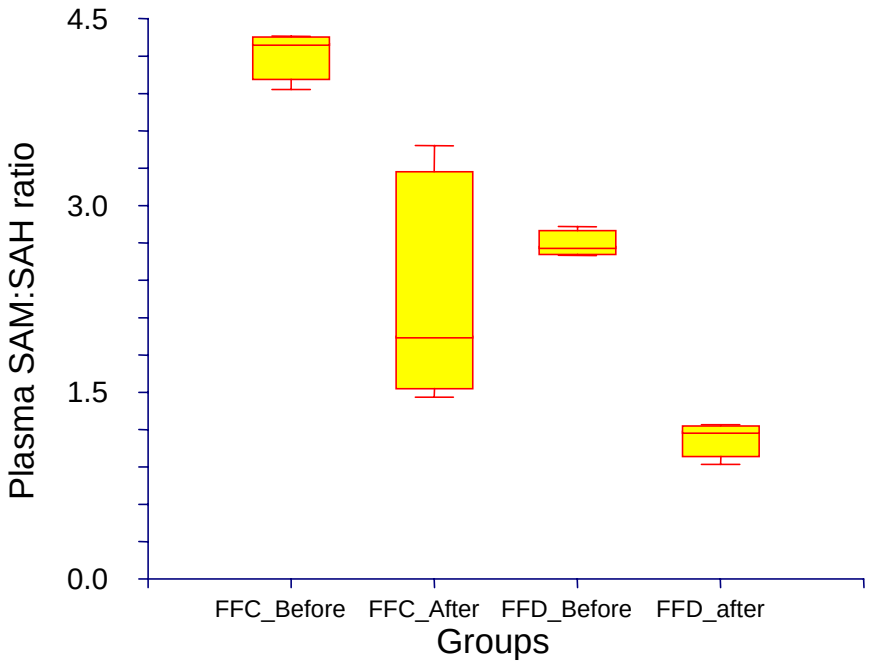

Figure 6.4: Box plot of mean plasma SAM to SAH ratio in folate control group (FFC) and folate deficicent (FFD) before and after a short intravenous infusion (10 min) in male pigs (n=4) of 351 mg/kg sodium formate (237 mg/kg formate). FFC_Before = Folate Control group before infusion of sodium formate; FFC_After = Folate Control group after infusion of sodium formate; FFD_Before = Folate Deficient group before infusion of sodium formate; FFC_After = Folate Deficient group after infusion of sodium formate; SAM = *S*-Adenosylmethionine: SAH = *S*-Adenosylhomocysteine.

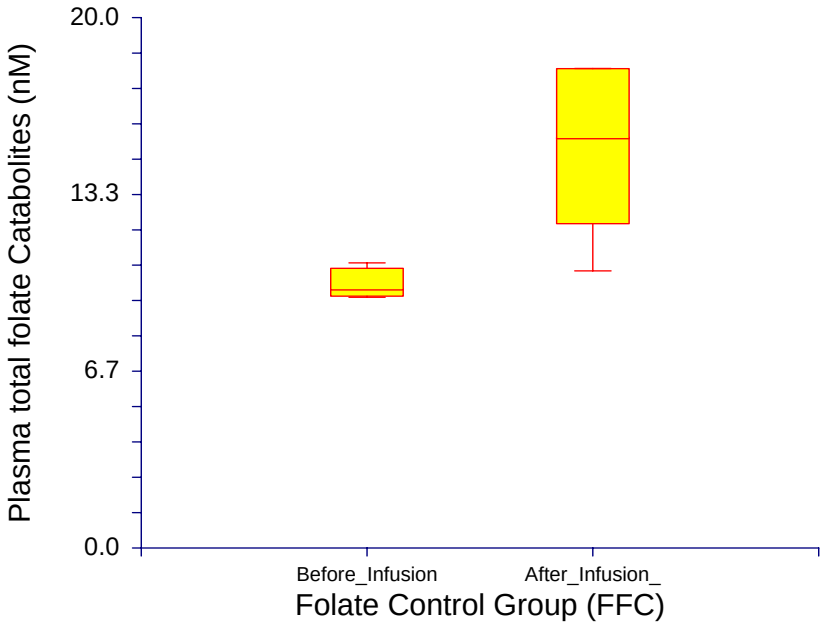

Figure 6.5: Box plot of mean plasma folate catabolites (*p-ABG* & *ap-ABG*) levels in folate control group (FFD) before and after a short intravenous infusion (10 min) in male pigs (n=4) of 351 mg/kg sodium formate (237 mg/kg formate). Before_Infusion = Plasma total folate catabolites levels before infusion of sodium formate; After_Infusion = Plasma total folate catabolites (*p*-ABG & *ap*-ABG) levels after infusion of sodium formate.

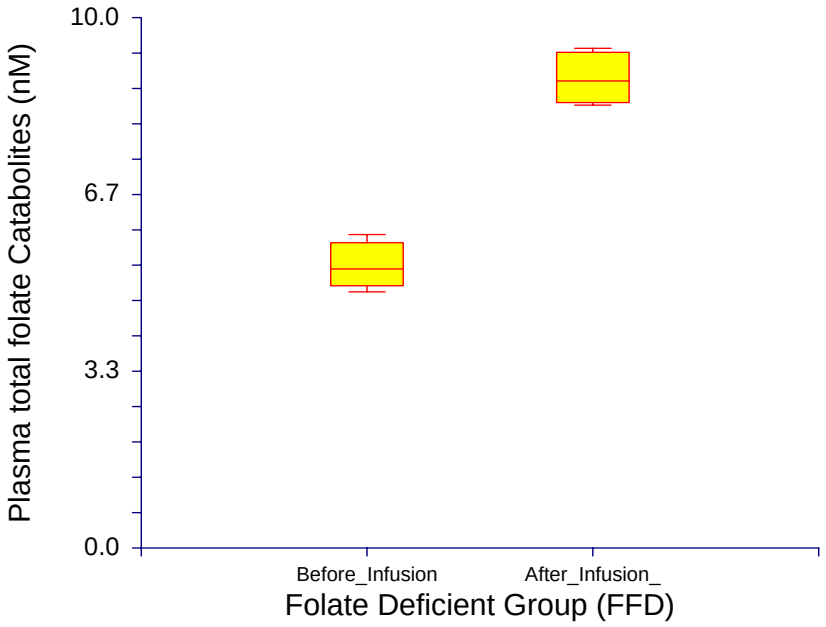

Figure 6.6: Box plot of mean plasma folate catabolites (*p-ABG* & *ap-ABG*) levels in folate deficient group (FFD) before and after a short intravenous infusion (10 min) in male pigs (n=4) of 351 mg/kg sodium formate (237 mg/kg formate). Before_Infusion = Plasma total folate catabolites levels before infusion of sodium formate; After_Infusion = Plasma total folate catabolites (*p*-ABG & *ap*-ABG) levels after infusion of sodium formate.

6.4 Discussion and Conclusion

Folic acid is an essential vitamin required for a wide range of biochemical pathways involving one carbon metabolism. It plays an essential role in cell replication and pregnancy because of its requirement in the synthesis of RNA, DNA and amino acids (Wagner, 1995). Folate is also required in the metabolism of formate to CO_2 and H_2O during methanol/formic acid intoxication (Makar *et. al.*, 1990; Medinsky *et. al.*, 1997). Thus, body folate requirement is directly related to amount of cellular reproduction occurring at any given time as well as the state of folate requiring pathways, and is

reflected by the plasma levels and urinary excretion of folate catabolites p-aminobenzoylglutamate (*p*-ABG) and ap-acetamidobenzoylglutamate (*ap*-ABG) (McPartlin *et. al.*, 1992; McNulty *et. al.*, 1993). In this study, we observed higher baseline folate utilization in folate replete condition (11.83 ± 2.13 nM) as compared with folate deficiency (5.33 ± 0.22 nM, p = 0.020). This observation is consistent with that made by Gregory and coworkers in which rate of folate turnover was increased proportionately to folate intake in young women (Gregory, III *et. al.*, 2000).

Regardless of folate status, folate utilization increases (by at least 29% in FFC) during peak formate insult (11.83 ± 2.13 to 15.22 ± 1.60 nM, p = 0.014 in FFC and 5.33 ± 0.22 to 8.86 ± 0.25 nM, p = 0.000 in FFD; Figures 6.2 and 6.3). Because of the lack of information on folate utilization during folate deficiency and formate insult it is not possible to compare this observation with others. Nevertheless, we have observed in our animals a significant increase in folate utilization during such conditions. This would indicate a possible redirection of folate resources from other folate requiring pathways to the formate elimination pathway. Such redirection would have profound effects on pathways such as DNA synthesis, repair and antioxidant capacity. Impaired cell division arising from impaired DNA synthesis may result in megaloblastic changes (Wickramasinghe, 1999) and is incompatible with normal fetal growth and development. Further evidence of such incompatibility is provided by data that show uracil incorporation into DNA instead of thymine with subsequent failure in normal repair to remove the misincorporated uracil. This leads ultimately to double strand breakage and chromosomal instability, which promotes apoptosis even in the absence of clinical symptoms of folate deficiency (Blount *et. al.*, 1997; Koury *et. al.*, 1997).

Folate deficiency contributes to the impairment of remethylation pathways, particularly that of homocysteine to methionine. We observed elevated Hcy levels in the FFD group (38 ± 6 μM vs 14 ± 3 μM in FFC, p = 0.000) and altered ratio of SAM to SAH (2.31 ± 0.46 in FFC vs 1.13 ± 0.07 in FFD, p = 0.039) although methionine levels were not different between the groups (88 ± 15 μM in FFC vs 93 ± 24 μM in FFD, p = 0.845). Formate insult also appears to reduce antioxidant capacity regardless of folate status as reflected by decreased SAM to SAH ratio. This is worsened by folate deficiency state with the decrease being 1.3 times greater in folate deficiency than folate replete. The reduced antioxidant capacity is complemented by an increase in methylglyoxal levels, despite folate status. The increase is more evident in folate deficiency (43%, p = 0.049) compared to folate replete, which was not statistically significant (8% increase, p = 0.495).

Homocysteine is a thrombogenic, atherogenic, neurotoxic non-proteinogenic amino acid. Elevated plasma homocysteine levels have been implicated in many pathological conditions (Refsum, 2001; Refsum *et. al.*, 1998). It mediates endothelial cell dysfunction that may contribute to the vascular complications of pregnancy via oxidative stress and altered nitric oxide function (Davidge, 1998; Chambers *et. al.*, 1999;Lentz, 1998). Increased Hcy has also been linked to congenital abnormalities due to its interaction with the N-methyl-D-asparate (NMDA) receptor system, which is involved in neuronal development and migration (Andaloro *et. al.*, 1998; Refsum, 2001; Rosenquist *et. al.*, 1996). *S*-adenosylmethionine is a compound that is known to activate homocysteine flux through the transsulfuration pathway and also is necessary for the utilization of the antioxidant glutathione via glutathione *S*-transferase. Folate deficiency and increased

demand for folate would lead to SAM depletion resulting in the reduction in the methylation of cytosine in DNA, proteins, phospholipids and neurotransmitters (Mattson & Shea, 2003).

In our study, formate insult and folate deficiency also caused elevations in levels of methylglyoxal and Hcy (markers of free radical injury). Methylglyoxal (MG), a reactive dicarbonyl compound formed as a by-product of glycolysis and lipid and amino acid metabolism (Thornalley, 1993), was increased by about 65% ($p = 0.010$) in folate deficiency (4.56 ± 0.40 μM) during formate insult as compared to the folate replete condition (2.75 ± 0.25 μM). Endogenous aldehydes including methylglyoxal are compounds of unusually high electrophilic reactivity and undergo nonenzymatic conjugation with with free amino ($–NH_2$) and sulfhydryl (–SH) groups of membrane proteins, metabolic enzymes and membrane ion channels, thereby, inhibiting their function (Schauenstein & Esterbauer, 1979). The autoxidation of methylglyoxal, its ability to generate more reactive oxygen species (ROS), and its toxicity, mediated via increased apoptosis and mutagenicity in the presence of superoxide anion and hydrogen peroxide, are well documented (Yamaguchi & Nakagawa, 1983; Kalapos, 1999; Shangari *et. al.*, 2004; Shangari *et. al.*, 2003). Under normal physiological conditions methylglyoxal levels are kept low in the body mainly by its catabolism via a glutathione dependent glyoxalase system or its conjugation to cysteine, the product of which is subsequently excreted in bile and urine (Schauenstein *et. al.*, 1977). The increased methylglyoxal levels seen in folate deficiency is probably due to the increased demand for glutathione to handle the increased in free radicals generated as a result of formate (Dikalova *et. al.*, 2001). As a result less is available to handle methylglyoxal.

In conclusion, we have demonstrated that formate insult increases folate utilization despite folate deficiency; increases free radical markers; and reduces antioxidant capacity as reflected by SAM to SAH ratio. These observations could have disastrous consequences on vital folate-dependent metabolic pathways, e.g., DNA synthesis and repair and antioxidant capacity of the body, during folate deficiency. The clinical implications of such findings require further investigation, particularly the effect of alcohol/methanol intoxication in pregnant women.

CHAPTER 7

Discussion and Concusions

7.1 Background

Fetal Alcohol Spectrum Disorder (FASD) is a preventable, devastating life-long developmental condition affecting children of women who abuse alcohol during pregnancy. The costs and consequences of FASD, both personally and financially, are tremendous to the affected individuals' families and the society at large. It is estimated that the annual average costs of caring for FASD affected children, once diagonized is US $ 2,342 per capita more than the annual average cost of care for children in who do not have FASD (US $500 per year) (Klug & Burd, 2003). The annual savings in health care costs alone, after 10 years of prevention, for one case of FASD would be US $23,420 dollars, US $ 128,810 dollars in 10 years and, US $491,820 dollars after 20 years. With an estimated annual birth rate of 1200 children diagonized with FASD, the estimated incremental annual cost of treating this disorder is US $74.6 million (Abel & Sokol, 1991). Approximately three-quarters of these costs are associated with mental health care.

Most of the studies linking prenatal alcohol consumption to FASD have identified ethanol as the culprit (Abel, 1999b; Chaudhuri, 2000b; Chaudhuri, 2000a; Jones & Smith, 1973; Jones *et. al.*, 1973), although the jury is still out on whether "alcohol" rather than "its abuse" is responsible for FASD (Abel, 1999b; Abel, 1999a). Despite the presence of methanol in alcoholic beverages (Caldwell, 1986; Gilg *et. al.*, 1987; Majchrowicz &

Mendelson, 1971; Paine & Dayan, 2001; Roine *et. al.*, 1989), no research to date has addressed the link that might exist between formate, a toxic metabolite of methanol, and FASD. This study attempts to address part of this question. It addresses, first, the question of the relationship between formate and folate levels in drinking women (see section 7.2), secondly, the kinetics of formate elimination in folate deficiency (see section 7.3); thirdly, changes in markers of free radicals and antioxidant capacity during formate insult in folate deficiency state (see section 7.3).

Although this study does not provide a link between the elevated formate, folate deficiency and occurrence of FASD/alcohol-related birth defects, it does provide evidence, identifying formate (probably as a result of increased alcohol intake) in folate deficiency state as a candidate alternative causative agent of FASD, and forms the basis for further research in that area.

In order to execute the study protocol novel methods had to be developed to determine formate levels (see chapter 2) and folate utilization (see chapter 3) in the experimental subjects. Together with other established methods, formate's presence, kinetics and role in altered antioxidant status was investigated.

7.2 Human Experiments

Methanol is present in alcoholic beverages (see chapter 1). To investigate whether alcohol consumption translated to elevated methanol and formate levels and, any correlation this might have on folate levels, intoxicated human female volunteers were recruited from the emergency room of a university hospital while two groups of sober

human females were recruited from a detox centre (non-pregnant females) and a prenatal clinic (pregnant females) (Chapter 4). Subjects were recruited to represent alcohol intoxication state (intoxicated females), malnutrition state (as represented in recovering alcoholics in the detox program) and, pregnancy state (represented by the prenatal clinic recruits).

In this study, we observed elevated methanol levels during intoxication compared to sober individuals (mean ± SE, 0.13 ± 0.01mM in intoxicated vs 0.04 ± 0.00 in sober, respectively; $p = 0.003$). The elevated methanol is due to the alcohol intake that was absent in the detox subjects. For a pregnant mother involved in binge drinking (consumption of 4 or more drinks in about 2 hours) such an elevation, in methanol levels, would prove disastarous for the fetus. Elevated methanol would, therefore, lead to higher formate levels. However, this was not the observational case where mean formate levels were not different between the intoxicated and detox groups (0.10 ± 0.00 mM in the intoxicated group vs 0.10 ± 0.01 mM in the detox group, $p = 1.000$). Methanol has to be metabolized to formaldehyde by alcohol dehydrogenase and then, by aldehyde dehydrogenase, to formate. During this metabolism, methanol levels fall as formate levels increase. In the presence of ethanol, alcohol dehydrogenase preferentially metabolizes ethanol. In our intoxicated subjects, blood sampling might have been done before significant methanol metabolism occurred, explaining the low formate levels. A definite negative (inverse), albeit weak, relationship exits between plasma formate and folate levels (correlation = -0.4989). However, this relationship could not be observed between whole blood formate and RBC folate in the intoxicated and detox females.

There might be some nutritional benefit from consumption of grain and fruit based alcoholic drinks (Kellner & Cejka, 1999; Mayer Jr. *et. al.*, 2001; Walker & Baxter, 2000)(see Table 4.4). This would explain the higher levels of folate and B-vitamins seen in the intoxicated group compared to the detox group. Higher folate, vitamin B6 and vitamin B12 have a Hcy lowering effect (Bleich *et. al.*, 2000; Lakshmi *et. al.*, 1910; Mayer Jr. *et. al.*, 2001; Pietrzik & Bronstrup, 157; Walker & Baxter, 2000), thus, could explain the lower levels seen in the intoxicated group. However, this could be a short-term benefit and is reversed in chronic alcoholism (Barak *et. al.*, 1987; Halsted *et. al.*, 2002b; Halsted *et. al.*, 2002d; Halsted *et. al.*, 2002c; Larroque *et. al.*, 1992; Mayer Jr. *et. al.*, 2001) as observed in the detox group. Therefore, adequate nutritional status, especially folate, and abstinence from alcohol would be beneficial in reducing methanol and, subsequently, formate accumulation.

7.3 Pig Experiments

This study also assessed the kinetics of formate during formate insult (as would be seen during binge drinking) in folate deficiency state in folate deficient pigs (Chapter 5). In this study formate accumulated due to formate administration regardless of folate status as observed in the mean end of infusion formate levels in folate deficient and folate replete pigs (mean ± SE, 9.63 ± 0.57 mM in folate deficiency vs 10.04 ± 0.71 in folate replete, $p = 0.668$). However, formate exposure is lower in folate replete condition (AUC = 30.08 ± 2.58 g min L^{-1} vs 72.37 ± 8.29 g min L^{-1} in folate deficiency, $p = 0.003$) due to the faster systemic clearance (0.27 ± 0.02 L min^{-1} vs 0.12 ± 0.01 L min^{-1} in folate deficiency, $p = 0.001$) resulting in shorter half-life (45 ± 6 min vs 113 ± 17 min in folate deficiency, p =

0.009) and mean residency time (65 ± 8 min vs 163 ± 24 min in folate deficiency, p = 0.008). It is evident that adequate folate status is important in preventing prolonged formate exposure.

Elevated formate coupled with folate deficiency enhanced free radical accumulation and compromised antioxidant status as observed in the increased MG levels (by 43%, p = 0.049) and reduced SAM to SAH ratio (by 58%, p = 0.000). Folate deficiency alone also appears to increase Hcy levels (by 210%, p = 0.019) and MG (by 26%). It also reduces SAM to SAH ratio by 57%, p = 0.000. A reduction in this ratio indicates alteration in the trans-sulphuration pathway and risk on oxidative stress.

Although the ratio of plasma GSH to GSSG is a good indicator of antioxidant capacity, we were unable to measure this despite setting up a method for this. This was due to the very low levels of glutathione in pigs and the sensitivity of our method (LOQ = 326 μM for GSH and 163.3 μM for GSSG). Despite this, we were able to show changes in antioxidant capacity using changes in methylglyoxal, homysteine, *S*-adenosylmethionine and *S*-adenosylhomocysteinelevels. However, we have been able to show, for the first time, real time changes in folate utilization during formate insult in these animals. This could form the basis for future studies in folate and formate kinetics in humans.

7.4 Conclusions

This study aimed at addressing some fundamental questions (as described in the hypothesis section 1.6) regarding formate presence in the body after alcohol consumption

and the role it plays in antioxidant capacity during folate deficiency. The conclusions of this study are:

1. methanol levels increase during alcohol consumption and might lead to increased formate levels.

2. plasma formate levels correlate negatively with plasma folate levels in the body.

3. formate kinetics are altered during formate insult in folate deficiency state leading to reduced clearance, longer half-life and mean residency time and, consequently, increased exposure.

4. folate utilization is increased during formate insult independent of folate status.

5. formate insult during folate deficiency reduces antioxidant capacity in the body.

CHAPTER 8

REFERENCES

Aabakken L, Johansen KS, Rydningen EB, Bredesen JE, Ovrebo S, & Jacobsen D (1994). Osmolal and anion gaps in patients admitted to an emergency medical department. *Hum. Exp Toxicol* **13**, 131-134.

Aase JM (1981). The fetal alcohol syndrome in American Indians: a high risk group. *Neurobehav Toxicol Teratol* **3**, 153-156.

Abel EL (1995). An update on incidence of FAS: FAS is not an equal opportunity birth defect. *Neurotoxicol Teratol* **17**, 437-443.

Abel EL (1999a). What really causes FAS: from the sublime to the ridiculous. *Teratol* **60**, 250.

Abel EL (1999b). What really causes FAS? *Teratol* **59**, 4-6.

Abel EL & Hannigan JH (1995). Maternal risk factors in fetal alcohol syndrome: provocative and permissive influences. *Neurotoxicol Teratol* **17**, 445-462.

Abel EL & Sokol RJ (1986). Fetal alcohol syndrome is now leading cause of mental retardation. *Lancet* **2**, 1222.

Abel EL & Sokol RJ (1987). Incidence of fetal alcohol syndrome and economic impact of FAS-related anomalies. *Drug Alcohol Depend* **19**, 51-70.

Abel EL & Sokol RJ (1991). A revised conservative estimate of the incidence of FAS and its economic impact. *Alcohol: Clin Exp Res* **15**, 514-524.

Abolin C, McRae JD, Tozer TN, & Takki S (1980). Gas chromatographic head-space assay of formic acid as methyl formate in biologic fluids: potential application to methanol poisoning. *Biochem Med* **23**, 209-218.

Andaloro VJ, Monaghan DT, & Rosenquist TH (1998). Dextromethorphan and other N-methyl-D-aspartate receptor antagonists are teratogenic in the avian embryo model. *Pediatr Res.* **43**, 1-7.

Andon MB, Howard MP, Moser PB, & Reynolds RD (1985). Nutritionaly relevant supplementation of vitamin B6 in lactating women: effect on plasma prolactin. *Pediatrics* **76:** 5 769-773.

Araki A, Sako Y, & Ito H (1993). Plasma homocysteine concentrations in Japanese patients with non-insulin-dependent diabetes mellitus: effect of parenteral methylcobalamin treatment. *Atherosclerosis* **103**, 149-157.

Astley SJ & Clarren SK (2002). Diagnostic Guide for Fetal Alcohol Syndrome and Related Conditions: *The 4-Digit Diagnostic Code*. FAS Diagnostic and Prevention Network, University of Washington, Seattle, WA.

Bailey LB & Gregory JF, III (1999). Folate metabolism and requirements. *J Nutr* **129**, 779-782.

Barak AJ, Beckenhauer HC, & Tuma DJ (1991). Hepatic transmethylation and blood alcohol levels. *Alcohol Alcohol* **26**, 125-128.

Barak AJ, Beckenhauer HC, Tuma DJ, & Badakhsh S (1987). Effects of prolonged ethanol feeding on methionine metabolism in rat liver. *Biochem Cell Biol* **65**, 230-233.

Barber RC, Lammer EJ, Shaw GM, & et.al. (1999). The role of folate transport and metabolism in neural tube defect risk. *Mol Genet Metab* **66**, 1-9.

Barceloux DG, Bond GR, Krenzelok EP, Cooper H, & Vale JA (2002). American Academy of Clinical Toxicology practice guidelines on the treatment of methanol poisoning. *J Toxicol Clin Toxicol* **40**, 415-446.

Beulens JW, Sierksma A, Schaafsma G, Kok FJ, Struys EA, Jakobs C, & Hendriks HF (2005). Kinetics of homocysteine metabolism after moderate alcohol consumption. *Alcohol: Clin Exp Res* **29**, 739-745.

Bhatt RH, Linnell JC, & Barltrop D (1986). Treatment of hydroxycobalamin-resistant methylmalonic acidemia with adenosylcobalamin. *Lancet* **2**, 465.

Black KA, Eells JT, Noker PE, Hawtrey CA, & Tephly TR (1985). Role of hepatic tetrahydrofolate in species differences in methanol toxicity. *Proc Natl Acad Sci , USA* **82**, 3854-3858.

Blackburn ST & Loper DL (1992). The hematologic and hemostatic systems, In Eoyang T, ed., *Maternal, Fetal, and neonatal physiology*, W. B. Saunders, Philadelphia, PA., pp. 159-200.

Blakley R (1969). The biochemistry of folic acid and related pteridines, In H.Newbergen and E.L.Taton, eds., *North Holland Research Monographs Frontiers of Biology,* **vol. 13**. North Holland Publishing Company, Amsterdam,Netherlands.

Bleich S, Degner D, Wiltfang J, Maler JM, Niedmann P, Cohrs S, Mangholz A, Porzig J, Sprung R, Ruther E, & Kornhuber J (2000). Elevated homocysteine levels in alcohol withdrawal. *Alcohol Alcohol* **35:** 4 351-354.

Bloomfield K, Gmel G, & Wilsnack S (2006). Introduction to special issue 'gender, culture and alcohol problems: a multi-national study'. *Alcohol Alcohol Suppl* **41**, i3-i7.

Blount BC, Mack MM, Wehr CM, & et.al. (1997). Folate deficiency causes Uracil misincorporation into human DNA and chromosomal breakage: implications for cancer and neuronal damage. *Proc Natl Acad Sci , USA* **94**, 3290-3295.

Bluthenthal RN, Browntaylor D, Guzman-Becerra N, & Robinson PL (2005). Characteristics of malt liquor beer drinkers in a low-income, racial minority community sample. *Alcohol: Clin Exp Res* **29**, 402-409.

Bobo JK & Husten C (2000). Sociocultural influences on smoking and drinking. *Alcohol Res Health* **24**, 225-232.

Bonnette RE, Caudill MA, Boddie AM, Hutson AD, Kauwell GP, & Bailey LB (1998). Plasma homocyst(e)ine concentrations in pregnant and nonpregnant women with controlled folate intake. *Obstet Gynecol* **92**, 167-170.

Bosy-Westphal A, Holzapfel A, Czech N, & Muller MJ (1945). Plasma folate but not vitamin B(12) or homocysteine concentrations are reduced after short-term vitamin B(6) supplementation. *Ann Nutr Metab 2001* 255-258.

Bralley JA & Lord RS (1999). Urinary Organic Acid Analysis. HealthChoice . The institute of Human Technologies Inc., Minneaolis, MN.

Brook JS, Whiteman M, Gordon AS, Nomura C, & Brook DW (1986). Onset of adolescent drinking: a longitudinal study of intrapersonal and interpersonal antecedents. *Adv Alcohol Subst Abuse* **5**, 91-110.

Brooks PJ (1997). DNA damage, DNA repair, and alcohol toxicity-a review. *Alcohol: Clin Exp Res* **21**, 1073-1082.

Buttery JE & Chamberlain BR (1988). A simple enzymatic method for the measurement of abnormal leels of formate in blood. *J. Anal Toxicol* **12**, 292-294..

Caldwell K (1986). Methanol levels in methylated spirit drinking alcoholics. *N Z Med J* **99**, 764-765.

Campbell FA & Ramey CT (1994). Effects of early intervention on intellectual and academic achievement: a follow-up study of children from low-income families. *Child Dev* **65**, 684-698.

Caudill MA, Cruz AC, Gregory JF, III, Hutson AD, & Bailey LB (1997). Folate status response to controlled folate intake in pregnant women. *J Nutr* 2363-2370.

Caudill MA, Gregory JF, Hutson AD, & Bailey LB (1998). Folate catabolism in pregnant and nonpregnant women with controlled folate intakes. *J Nutr* **128**, 204-208.

Center for Disease Control (2004). Alcohol consumption among women who are pregnant or who might become pregnant - United States, 2002. *MMWR Morb Mortal Wkly Rep* **53**, 1178-1181.

Center for Disease Control (2002). Behavioral Risk Factor Surveillance System. US Department of Health and Human Services, Atlanta, GA, USA.

Cederbaum AI (1989). Oxygen radical generation by microsomes: role of iron and implications for alcohol metabolism and toxicity. *Free Radic Biol Med* **7**, 559-567.

Chambers CD, Hughes S, Meltzer SB, Wahlgren D, Kassem N, Larson S, Riley EP, & Hovell MF (2005). Alcohol consumption among low-income pregnant Latinas. *Alcohol: Clin Exp Res* **29**, 2022-2028.

Chambers JC, McGregor A, Jean-Marie J, Obeid OA, & Kooner JS (1999). Demonstration of rapid onset vascular endothelial dysfunction after hyperhomocysteinemia: an effect reversible with vitamin C therapy. *Cir* **99**, 1156-1160.

Chaudhuri JD (2000a). A review of experimental evidences that could warn of possible dangers of alcohol consumption by pregnant mothers. *Indian J Med Sci* **54**, 545-554.

Chaudhuri JD (2000b). Alcohol and the developing fetus--a review. *Med Sci Monit* **6**, 1031-1041.

Chaudhuri JD (2000c). An analysis of the teratagenic effects that could possibly be due to alcohol consumption by pregnant mothers. *Indian J Med Sci* **54**, 425-431.

Clay RL, Murphy RC, & Watkins WD (1975). Experimental methanol toxicity in the primate: analysis of metabolic acidosis. *Toxicol Appl Pharmacol* **34**, 49-61.

Colell A, Garcia-Ruiz C, Miranda M, Ardite E, Mari M, Morales A, Corrales F, Kaplowitz N, & Fernandez-Checa JC (1998). Selective glutathione depletion of mitochondria by ethanol sensitizes hepatocytes to tumor necrosis factor. *Gastroenterol* **115**, 1541-1551.

Comporti M, Benedetti A, & Chieli E (1973). Studies on *in vitro* peroxidation of liver lipids in ethanol-treated rats. *Lipids* **8**, 498-502.

Connor MJ, Pheasant AE, & Blair JA (1979). The identification of *p*-acetamidobenzoate as a folate degradation product in rat urine. *Biochem J* **178**, 795-797.

Contractor SF & Shane B (1970). Blood and urine levels of vitamin B6 in the mother and fetus before and after loading of the mother with vitamin B6. *Am J Ob Gyn* 635-640.

Cook RJ, Champion KM, & Giometti CS (2001). Methanol toxicity and formate oxidation in NEUT2 mice. *Arch Biochem Biophys* **393**, 192-198.

Cravo ML & Camilo ME (2000). Hyperhomocysteinemia in chronic alcoholism: relations to folic acid and vitamins B(6) and B(12) status. *Nutr.* **16**, 296-302.

D'Onofrio CN (1997). The prevention of alcohol use by rural youth. *NIDA Res Monogr* **168,** 250-363.

Davidge ST (1998). Oxidative stress and altered endothelial cell function in preeclampsia. *Semin Reprod Endocrinol* **16**, 65-73.

Day NL & Richardson GA (1991). Prenatal alcohol exposure: a continuum of effects. *Semin Perinatol* **15**, 271-279.

Deitrick CL, Katholi RE, Huddleston DJ, Hardiek K, & Burrus L (2001). Clinical adaptation of a high-performance liquid chromatographic method for the assay of pyridoxal 5'-phosphate in human plasma. *J. Chrom. B* **751**, 383-387.

Desai N, Nawamongkolwattana B, Ranaweera S, Shrestha DM, & Sobhan MA, (2003). Prevention of harm from alcohol use: get high on life without alcohol, World Health Organization, Geneva, Switzerland.

Diehl K-H, Hull R, Morton D, Pfister R, Rabemampianina Y, Smith D, Vidal J-M, & Van de Vorstenbosch C (2001). A Good Practice Guide to Administration of Substances and Removel of Blood, including Routes and Volumes. *J Appl Toxicol* **21**, 15-23.

Dikalova AE, Kadiiska MB, & Mason RP (2001). An *in vivo* ESR spin-trapping study: free radical generation in rats from formate intoxication--role of the Fenton reaction. *Proc Natl Acad Sci U S A* **20:** 98, 13549-13553.

Dorman DC, Dye JA, Nassise MP, Ekuta J, Bolon B, & Medinsky MA (1993). Acute methanol toxicity in minipigs. *Fund Appl Toxicol* **20**, 341-347.

Dorman DC, Moss OR, Farris GM, Janszen D, Bond JA, & Medinsky MA (1994). Pharmacokinetics of inhaled ^{14}C methanol and methanol-derived ^{14}C formate in normal and folate-deficient cynomolgus monkeys. *Toxicol Appl Pharmacol* **128**, 229-238.

Ebrahim SH, Diekman ST, Floyd RL, & Decoufle P (1999). Comparison of binge drinking among pregnant and nonpregnant women, United States, 1991-1995. *Am J Ob Gyn* **180**, 1-7.

Ebrahim SH, Luman ET, Floyd RL, Murphy CC, Bennett EM, & Boyle CA (1998). Alcohol consumption by pregnant women in the United States during 1988-1995. *Obstet Gynecol* 187-192.

Eells JT, Henry MM, Lewandowski MF, Seme MT, & Murray TG (2000). Development and characterization of a rodent model of methanol-induced retinal and optic nerve toxicity. *Neurotox* **21**, 321-330.

Eells JT, Makar AB, Noker PE, & Tephly TR (1981). Methanol poisoning and formate oxidation in nitrous oxide-treated rats. *J Pharmacol Exp Ther* **217**, 57-61.

Ellenhorn MJ, Schonwald S, Ordog G, & Wasserberger J (1997). Alcohols and Glycols. In *Ellenhorn's Medical Toxicology: Diagnosis and Treatment of Human Poisoning,* Williams & Wilkins, Baltimore,MD., pp. 1127-1165.

Eriksson CJ (2001a). The role of acetaldehyde in the actions of alcohol (update 2000). *Alcohol: Clin Exp Res* **25**, 15S-32S.

Eriksson CJP (2001b). The Role of Acetaldehyde in the Actions of Alcohol (Update 2000). [Miscellaneous Article]. *Alcohol: Clin Exp Res May* **25**, 15S-32S.

Ernst CC, Grant TM, Streissguth AP, & Sampson PD (1999). Intervention with high-risk alcohol and drug–abusing mothers: II. Three-year finds from the Seattle model of paraprofessional advocacy. *J of Comm Psy* **27**, 19-38.

Feinleib M, Beresford SAA, Bowman BA, Mills JL, Rader JI, Selhub J, & Yetley EA (2001). Folate Fortification for the prevention of Birth Defects: Case Study. *Am J Epid* **154**, S60-S69.

Fernandez-Checa JC, Colell A, & Garcia-Ruiz C (2002). S-Adenosyl-L-methionine and mitochondrial reduced glutathione depletion in alcoholic liver disease. *Alcohol* **27**, 179-183.

Fernandez-Checa JC, Garcia-Ruiz C, Colell A, Morales A, Mari M, Miranda M, & Ardite E (1998). Oxidative stress: role of mitochondria and protection by glutathione. *Biofact* **8**, 7-11.

Fridovich I (1989). Oxygen radicals from acetaldehyde. *Free Radic Biol Med* **7**, 557-558.

Friedmann B, Nakada HI, & Weinhouse S (1954). A study of the oxidation of formic acid in the folic acid-deficient rat. *J Biol Chem* **210**, 413-421.

Fu SS, Sakanashi TM, Rogers JM, Hong KH, & Keen CL (1996). Influence of dietary folic acid on the developmental toxicity of methanol and the frequency of chromosomal breakage in the CD-1 mouse. *Reprod Toxicol* **10**, 455-463.

Fu TF, Rife JP, & Schirch V (2001). The role of serine hydroxymethyltransferase isozymes in one-carbon metabolism in MCF-7 cells as determined by (13)C NMR. *Arch Biochem Biophys* **393**, 42-50.

Futterman S & Silverman M (1957). The inactivation of folic acid by liver. *J Biol Chem* **224**, 31-40.

Garcia-Martinez LF & Appling DR (1993). Characterization of the folate-dependent mitochondrial oxidation of carbon 3 of serine. *Biochem* **32**, 4671-4676.

Gellekink H, van Oppenraaij-Emmerzaal D, van RA, Struys EA, den HM, & Blom HJ (2005). Stable-isotope dilution liquid chromatography-electrospray injection tandem mass spectrometry method for fast, selective measurement of S-adenosylmethionine and S-adenosylhomocysteine in plasma. *Clin Chem* **51**, 1487-1492.

Geoghegan FL, McPartlin JM, Weir DG, & Scott JM (1995). para-cetamidobenzoylglutamate is a suitable indicator of folate catabolism in rats. *J Nutr* **125**, 2563-2570

Gilg T, von Meyer L, & Liebhardt E, (1987). Formation and accumulation of endogenous methanol in relation to alcohol burden. *Blutalkohol* **24**(5), 321-332. 1987.

Glass GBJ, Boyd LJ, & Ebin L (1958). Radioactive vitamin B12 in the liver. *J Lab Clin Med* **52**, 849-859.

Gonzalez-Quevedo A, Obregon F, Urbina M, Rousso T, & Lima L (2002). Effect of chronic methanol administration on amino acids and monoamines in retina, optic nerve, and brain of the rat. *Toxicol Appl Pharmacol* **185**, 77-84.

Graham IM, Daly LE, Refsum HM, Robinson K, Brattstrom LE, Ueland PM, Palma-Reis RJ, Boers GH, Sheahan RG, Israelsson B, Uiterwaal CS, Meleady R, McMaster D, Verhoef P, Witteman J, Rubba P, Bellet H, Wautrecht JC, de Valk HW, Sales Luis AC, Parrot-Rouland FM, Tan KS, Higgins I, Garcon D, & Andria G (1997). Plasma homocysteine as a risk factor for vascular disease. The European Concerted Action Project. *JAMA* 1775-1781.

Grant TM, Ernst CC, & Streissguth AP (1999). Intervention with high-risk alcohol and drug–abusing mothers: I. Administrative strategies of the Seattle model of paraprofessional advocacy. *J Comm Psy* **27**, 1-18.

Gregory Kelly (2002). The Coenzyme Forms of Vitamin B12: Toward an understanding of their therapeutic potential. *Alt Med Rev* **2:** 6, 459-476.

Gregory JF, III, Caudill MA, Opalko FJ, & Bailey LB (2001). Kinetics of folate turnover in pregnant women (second trimester) and nonpregnant controls during folic acid supplementation: stable-isotopic labeling of plasma folate, urinary folate and folate catabolites shows subtle effects of pregnancy on turnover of folate pools. *J Nutr* **131**, 1928-1937.

Gregory JF, III, Swendseid ME, & Jacob RA (2000). Urinary excretion of folate catabolites responds to changes in folate intake more slowly than plasma folate and homocysteine concentrations and lymphocyte DNA methylation in postmenopausal women. *J Nutr* **130**, 2949-2952.

Grube JW & Wallack L (1994). Television beer advertising and drinking knowledge, beliefs, and intentions among schoolchildren. *Am J Pub Health* **84**, 254-259.

Haffner HT, Banger M, Graw M, Besserer K, & Brink T (1997). The kinetics of methanol elimination in alcoholics and the influence of ethanol. *Forensic Sci Int* **89**, 129-136.

Halsted CH, Villanueva JA, & Devlin AM (2002a). Folate deficiency, methionine metabolism, and alcoholic liver disease. *Alcohol* **27**, 169-172.

Halsted CH, Villanueva JA, Devlin AM, & Chandler CJ (2002b). Metabolic interactions of alcohol and folate. *J Nutr* **132**, 2367S-2372S.

Halsted CH, Villanueva JA, Devlin AM, & James SJ (2002c). Interactions of ethanol and folate deficiency in development of alcoholic liver disease in the micropig. *Trans Am Clin Climatol Assoc* **113:151-62; discussion 162-3.**, 151-162.

Halsted CH, Villanueva JA, Devlin AM, Niemela O, Parkkila S, Garrow TA, Wallock LM, Shigenaga MK, Melnyk S, & James SJ (2002d). Folate deficiency disturbs hepatic methionine metabolism and promotes liver injury in the ethanol-fed micropig. *Proc Natl Acad Sci USA* **99**, 10072-10077.

Hannigan JH, Welch RA, & Sokol RJ (1992). Recognition of Fetal Alcohol Syndrome and Alcohol-Related Birth Defects. In Mendelson J & Mello Ne eds, *Medical Diagnosis and Treatment of Alcoholism*, MaGraw-Hill, New York, NY., pp. 639-667.

Hantson P, Haufroid V, & Wallemacq P (2005). Formate kinetics in methanol poisoning. *Hum Exp Toxicol* **24**, 55-59.

Hanzlik RP, Fowler SC, & Eells JT (2005). Absorption and elimination of formate following oral administration of calcium formate in female human subjects. *Drug Metab Dispos* **33**, 282-286.

Hard ML, Einarson TR, & Koren G (2001). The role of acetaldehyde in pregnancy outcome after prenatal alcohol exposure. *Ther Drug Monit* **23**, 427-434.

Henderson GI, Devi BG, Perez A, & Schenker S (1995). In utero ethanol exposure elicits oxidative stress in the rat fetus. *Alcohol: Clin Exp Res* **19**, 714-720.

Holman P (1995). Pyridoxine. *J Austr Coll Nutur & Envl Med* **14**, 5-16.

Hovda KE, Hunderi OH, Rudberg N, Froyshov S, & Jacobsen D (2004). Anion and osmolal gaps in the diagnosis of methanol poisoning: clinical study in 28 patients. *Inten Care Med* **30**, 1842-1846.

Hovda KE, Urdal P, & Jacobsen D (2005). Increased serum formate in the diagnosis of methanol poisoning. *J Anal Toxicol* **29**, 586-588.

Iffland R & Staak M (1990). Methanol and isopropanol as markers of alcoholism. *Beitrage Zur Gerichtlichen Medizin* **48**, 173-177.

Indian and Inuit Health Committee CPS (2002). Fetal Alcohol Syndrome. *Paed Child Health* **7**, 161-174.

Ishii H, Adachi M, Fernandez-Checa JC, Cederbaum AI, Deaciuc IV, & Nanji AA (2003). Role of apoptosis in alcoholic liver injury. *Alcohol: Clin Exp Res* **27**, 1207-1212.

Jacobsen D, Bredesen JE, Eide I, & Ostborg J, (1982). Anion and osmolal gaps in the diagnosis of methanol and ethylene glycol poisoning. *Acta.Med.Scand.* **212**, 17-20.

Jacobsen D & McMartin KE (1986). Methanol and Ethylene Glycol poisonings: Mechanisms of toxicity, clinical course, diagnosis, and treatment. *Med Toxicol* **1**, 309-334.

Jacobsen D, Web R, Collins TD, & McMartin KE (1988). Methanol and formate kinetics in late diagnosed methanol intoxication. *Med Toxicol Adverse Drug Exper* **3**, 418-423.

Johlin FC, Fortman CS, Ngheim DD, & Tephly TR (1987). Studies on the role of folic acid and folate-dependent enzymes in human methanol poisoning. *Mol Pharmacol* **31**, 557-561.

Johlin FC, Swain S, Smith C, & Tephly TR (1989). Studies on the mechanism of methanol poisoning: Purification and comparison of rat and human liver 10-formyltetrahydrofolate dehydrogenase. *Mol Pharmacol* **35**, 745-750.

Jones AW & Sternebring B (1992). Kinetics of ethanol and methanol in alcoholics during detoxification. *Alcohol Alcohol* **27**, 641-647.

Jones CW, III & Priest DG (1978). Interaction of pyridoxal 5-phosphate with apo-serine hydroxymethyltransferase. *Biochim Biophys Acta* 369-374.

Jones KL & Smith DW (1973). Recognition of the fetal alcohol syndrome in early infancy. *Lancet* **2**, 999-1001.

Jones KL, Smith DW, Ulleland CN, & Streissguth P (1973). Pattern of malformation in offspring of chronic alcoholic mothers. *Lancet* **1**, 1267-1271.

Kalapos MP (1999). Methylglyoxal in living organisms: chemistry, biochemistry, toxicology and biological implications. *Toxicol Lett* **110**, 145-175.

Kalter H (2000). Folic acid and Human malformations: a summary and evaluation. *Rep Tox* **14**, 463-476.

Kapur B & Maziar Y (1993). Methanol: A Marker of Chronic Alcohol Use. *Clin Biochem* **26**, 124-125.

Kasper M, Roehlecke C, Witt M, Fehrenbach H, Hofer A, Miyata T, Weigert C, Funk RH, & Schleicher ED (2000). Induction of apoptosis by glyoxal in human embryonic lung epithelial cell line L132. *Am J Respir Cell Mol Biol* **23**, 485-491.

Kastanos EK, Woldman YY, & Appling DR (1997). Role of mitochondrial and cytoplasmic serine hydroxymethyltransferase isozymes in de novo purine synthesis in Saccharomyces cerevisiae. *Biochem* **36**, 14956-14964.

Keefer LK, Streeter AJ, Leung LY, Perry WC, Hu HS, & Baillie TA (1987). Pharmacokinetic and deuterium isotope effect studies on the metabolism of formaldehyde and formate to carbon dioxide in rats in vivo. *Drug Metab Dispos* **15**, 300-304.

Kellner V & Cejka P (1999). Yearbook of the Research Institute of Brewing and Malting. Research Institute of Brewing and Malting, Prague, pp 134-142.

Kerns W, Tomaszewski C, McMartin K, Ford M, & Brent J (2002). Formate kinetics in methanol poisoning. *J Toxicol Clin Toxicol* **40**, 137-143.

Kerr WC, Greenfield TK, Tujague J, & Brown SE (2005). A drink is a drink? Variation in the amount of alcohol contained in beer, wine and spirits drinks in a US methodological sample. *Alcohol: Clin Exp Res* **29**, 2015-2021.

Kitson KE (1996). Ethanol and acetaldehyde metabolism: past, present, and future. *Alcohol: Clin Exp Res* **20**, 82A-92A.

Klintsova AY, Cowell RM, Swain RA, Napper RM, Goodlett CR, & Greenough WT (1998). Therapeutic effects of complex motor training on motor performance deficits induced by neonatal binge-like alcohol exposure in rats . I. Behavioral results. *Brain Res* **800**, 48-61.

Klotz U & Ammon E (1998). Clinical and toxicological consequences of the inductive potential of ethanol. *Eur J Clin Pharmacol* **54**, 7-12.

Klug MG & Burd L (2003). Fetal alcohol syndrome prevention: annual and cumulative cost savings. *Neurotoxicol Teratol* **25**, 763-765.

Koury MJ, Horne DW, Brown ZA, Pietenpol JA, Blount BC, Ames BN, Hard R, & Koury ST (1997). Apoptosis of late-stage erythroblasts in megaloblastic anemia: association with DNA damage and macrocyte production. *Blood* **89**, 4617-4623.

Lakshmi AV, Maniprabha C, & Krishna TP (1910). Plasma homocysteine level in relation to folate and vitamin B6 status in apparently normal men. *Asia Pac J Clin Nutr 2001* 194-196.

Larroque B, Kaminski M, Lelong N, d'Herbomez M, Dehaene P, Querleu D, & Crepin G (1992). Folate status during pregnancy: relationship with alcohol consumption, other maternal risk factors and pregnancy outcome. *Eur J Obstet Gynecol Reprod Biol* **43**, 19-27.

Lee KW, Simpson G, & Ortwerth B (1999). A systematic approach to evaluate the modification of lens proteins by glycation-induced crosslinking. *Biochim Biophys Acta* **1453**, 141-151.

Leklem JE (1991). vitamin B-6. In Machlin L ed, *Handbook of Vitamins*, marcel Decker Inc., New York, NY., pp 341-378.

Leklem JE (1999). Vitamin B-6. In Shils M *et.al.* eds, *Nutrition in Health and Disease*, Williams & Wilkins, Baltimore, MD., pp 413-422.

Lentz SR (1998). Mechanisms of thrombosis in hyperhomocysteinemia. *Curr Opin Hematol* **5**, 343-349.

Li R, Bonham-Smith PC, & King J, (2001). Molecular characterization and regulation of formate dehydrogenase in *Arabidopsis thaliana*,. *Can.J.Bot.* **79**, 796-804.

Lin Y, Dueker SR, Follett JR, Fadel JG, Arjomand A, Schneider PD, Miller JW, Green R, Buchholz BA, Vogel JS, Phair RD, & Clifford AJ (2004). Quantitation of in vivo human folate metabolism. *Am J Clin Nutr* **80**, 680-691.

Logan BK, Case GA, & Distefano S (1999). Alcohol content of beer and malt beverages: forensic consideration. *J Forensic Sci* **44**, 1292-1295.

Lucock M (2000). Folic acid: nutritional biochemistry, molecular biology, and role in disease processes. *Mol Genet Metab* **71**, 121-138.

Lupton C, Burd L, & Harwood R (2004). Cost of fetal alcohol spectrum disorders. *Am J Med Genet* **127C**, 42-50.

Macdonald A (1991). Report to Communications and Education Branch, BC Ministry of Health and Minister Responsible for Seniors. BC Ministry of Health, Victoria, BC.

Macdonald CM, Dow J, & Moore MR (1977). A possible protective role for sulphydryl compounds in acute alcoholic liver injury. *Biochem Pharmacol* **26**, 1529-1531.

Majchrowicz E & Mendelson JH (1971). Blood methanol concentrations during experimentally induced ethanol intoxication in alcoholics. *J Pharmacol Exp Ther* **179**, 293-300.

Makar AB, Tephly TR, Sahin G, & Osweiler G (1990). Formate metabolism in young swine. *Toxicol Appl Pharmacol* **105**, 315-320.

Malorny G (1969). Metabolic experiments with sodium-formate and formic acid in man. *Z Ernahrungswiss* **9**, 340-348.

Mattson MP & Shea TB (2003). Folate and homocysteine metabolism in neural plasticity and neurodegenerative disorders. *Trends Neurosci* **26**, 137-146.

Maxwell SR (2000). Coronary artery disease: Free radical damage, antioxidant protection and the role of homocysteine. *Basic Res Card* **95**, 65-71.

Mayer Jr. O, Simon J, & Rosolova H (2001). A population study of the influence of beer consumption on folate and homocysteine concentrations. *Eur J Clin Nutr.* **55**, 605-609.

McMartin KE, Martin-Amat G, Makar AB, & Tephly TR (1977). Methanol poisoning. V. Role of formate metabolism in the monkey. *J Pharmacol Exp Ther* **201**, 564-572.

McMartin KE, Ambre JJ, & Tephly TR (1980). Methanol poisoning in human subjects. Role for formic acid accumulation in the metabolic acidosis. *Am J Med* **68**, 414-418.

McNulty H, McPartlin J, Weir D, & Scott J (1993). Reversed-phase high-performance liquid chromatographic method for the quantitation of endogenous folate catabolites in rat urine. *J Chromatogr* **614**, 59-66.

McPartlin J, Courtney G, McNulty H, Weir D, & Scott J (1992). The quantitative analysis of endogenous folate catabolites in human urine. *Anal Biochem* **206**, 256-261.

Medinsky MA, Dorman DC, Bond JA, Moss OR, Janszen DB, & Everitt JI (1997). Pharmacokinetics of methanol and formate in female cynomolgus monkeys exposed to methanol vapors. *Res Rep Health Eff Inst* 1-30.

Menegola E, Broccia ML, Di Renzo F, & Giavini E (2001). Acetaldehyde in vitro exposure and apoptosis: a possible mechanism of teratogenesis. *Alcohol* **23**, 35-39.

Miller JW, Nadeau MR, Smith J, Smith D, & Selhub J (1994). Folate-deficiency-induced homocysteinaemia in rats: disruption of S-adenosylmethionine's co-ordinate regulation of homocysteine metabolism. *Biochem J* 415-419.

Molloy AMP, Mills JLM, McPartlin JP, Kirke PNF, Scott JMS, & Daly SM (2002). Maternal and fetal plasma homocysteine concentrations at birth: The influence of folate, vitamin B12, and the 5,10-methylenetetrahydrofolate reductase 677C->T variant. [Miscellaneous Article]. *Am J Ob Gyn* March **186**, 499-503.

Moretti C, Fabbri A, Gnessi L, Bonifacio V, Fraioli F, & Isidori A (1982). Pyridoxine (B6) suppresses the rise in prolactin and increases the rise in growth hormone induced by exercise. *N Engl J Med* 444-445.

Murphy M & Scott JM (1979). The turnover catabolism and excretion of folate administered at physiological concentrations in the rat. *Biochim Biophys Acta* 535-539.

National Advisory Council on Alcohol Abuse and Alcoholism (2004). National Advisory Council on Alcohol Abuse and Alcoholism Summary of Meeting. *National Institute on Alcohol Abuse and Alcoholism*, Betheseda, MD.

National Institutes of Health, (2004). NIAAA Newsletter. Winter 2004 **3**, Bethesda, MD.

National Research Council (1998). Nutrient Requirements of Swine, 10th ed, National Academy of Sciences, Washington, DC.

O'Connor MJ & Whaley SE (2006). Health care provider advice and risk factors associated with alcohol consumption following pregnancy recognition. *J Stud Alcohol* **67**, 22-31.

Olfert ED, Cross BM, & McWilliam AA (1993). Guide to the Care and Use of Experimental Animals 2nd ed, Canadian Council on Animal Care, Ottawa.

Osterloh JD, Pond SM, Grady S, & Becker CE (1986). Serum formate concentrations in methanol intoxication as a criterion for hemodialysis. *Ann Intern Med* **104**, 200-203.

Paine A & Dayan AD (2001). Defining a tolerable concentration of methanol in alcoholic drinks. *Hum Exp Toxicol* **20**, 563-568.

Pasternack LB, Littlepage LE, Laude DA, Jr., & Appling DR (1996). 13C NMR analysis of the use of alternative donors to the tetrahydrofolate-dependent one-carbon pools in *Saccharomyces cerevisiae*. *Arch Biochem Biophys* 158-165.

Pezacka E, Green R, & Jacobsen DW (1990). Glutathionylcobalamin as an intermediate in the formation of cobalamin coenzymes. *Biochem Biophys Res Comm* **2**, 443-450.

Pheasant AE, Connor MJ, & Blair JA (1981). The metabolism and physiological disposition of radioactively labelled folate derivatives in the rat. *Biochem Med* **26**, 435-450.

Pietrzik K & Bronstrup A (1998). Vitamins B12, B6 and folate as determinants of homocysteine concentration in the healthy population. *Eur J Pediatr Apr* **157**, S135-S138.

Pirkle EC & Richter L (2006). Personality, attitudinal and behavioral risk profiles of young female binge drinkers and smokers. *J Adolesc Health* **38**, 44-54.

Qureshi AA, Rosenblatt DS, & Cooper BA (1994). Inherited disorders of cobalamin metabolism. *Crit Rev Oncol Hematol* **17**, 133-151.

Raj DS, Choudhury D, Welbourne TC, & Levi M (2000). Advanced glycation end products: a Nephrologist's perspective. *Am J Kidney Dis* **35**, 365-380.

Randell EW, Vasdev S, & Gill V (2005a). Measurement of methylglyoxal in human plasma. (Personal communication.)

Randell EW, Vasdev S, & Gill V (2005b). Measurement of methylglyoxal in rat tissues by electrospray ionization mass spectrometry and liquid chromatography. *J Pharmacol Toxicol Methods* **51**, 153-157.

Refsum H (2001). Folate, vitamin B12 and homocysteine in relation to birth defects and pregnancy outcome. *Br J Nutr 2001 May* S109-S113.

Refsum H, Ueland PM, Nygard O, & Vollset SE (1998). Homocysteine and cardiovascular disease. *Annu Rev Med* **49**, 31-62.

Riley EP, Thomas JD, Goodlett CR, Klintsova AY, Greenough WT, Hungund BL, Zhou F, Sari Y, Powrozek T, & Li TK (2001). Fetal Alcohol Effects: Mechanisms and Treatment. *Alcohol: Clin Exp Res* **25**, 110S-116S.

Rittenhouse JD & Miller JD (1984). Social learning and teenage drug use: an analysis of family dyads. *Health Psychol* **3**, 329-345.

Robinson GC, Conry JL, & Conry RF (1987). Clinical profile and prevalence of fetal alcohol syndrome in an isolated community in British Columbia. *CMAJ* **137**, 203-207.

Roebuck TM, Mattson SN, & Riley EP (1998). A review of the neuroanatomical findings in children with fetal alcohol syndrome or prenatal exposure to alcohol. *Alcohol: Clin Exp Res* **22**, 339-344.

Roine P, Eriksson P, Ylikahri R, Penttila A, & Salaspuro M (1989). Methanol as a marker of alcohol abuse. *Alcohol: Clin Exp Res* **13**, 172-175.

Rosenquist TH, Ratashak SA, & Selhub J (1996). Homocysteine induces congenital defects of the heart and neural tube: effect of folic acid. *Proc Natl Acad Sci USA* **93**, 15227-15232.

Rosett HL (1980). A clinical perspective of the Fetal Alcohol Syndrome. *Alcohol: Clin Exp Res* **4**, 119-122.

Sakanashi TM, Rogers JM, Fu SS, Connelly LE, & Keen CL (1996). Influence of maternal folate status on the developmental toxicity of methanol in the CD-1 mouse. *Teratol* **54**, 198-206.

Sampson PD, Streissguth AP, Bookstein FL, Little RE, Clarren SK, Dehaene P, Hanson JW, & Graham JM, Jr. (1997). Incidence of fetal alcohol syndrome and prevalence of alcohol-related neurodevelopmental disorder. *Teratol* **56**, 317-326.

Sarkola T & Eriksson CJ (2001). Effect of 4-methylpyrazole on endogenous plasma ethanol and methanol levels in humans. *Alcohol: Clin Exp Res* **25**, 513-516.

Schauenstein E & Esterbauer H (1979). Formation and properties of reactive aldehydes. Submolecular Biology and Cancer. **67**, 225-244. CIBA Foundation Symposium Excerpta Medica, London, UK.

Schauenstein E, Esterbauer H, & Zollner H (1977). Aldehydes in biological systems. In *Aldehydes in biological systems, their natural occurrence and biological activities*. Pion, London, UK, pp 1-7.

Scheier LM, Botvin GJ, & Baker E (1997). Risk and protective factors as predictors of adolescent alcohol involvement and transitions in alcohol use: a prospective analysis. *J Stud Alcohol* **58**, 652-667.

Schmedes A & Brandslund I (2006). Analysis of methylmalonic acid in plasma by liquid chromatography-tandem mass spectrometry. *Clin Chem* **52**, 754-757.

Scott JM, McPartlin J, Molloy A, McNulty H, Halligan A, Darling M, & Weir DG (1993). Folate metabolism in pregnancy. *Adv Exp Med Biol* **338**, 727-732.

Sejersted OM, Jacobsen D, Ovrebo S, & Jansen H (1983). Formate concentrations in plasma from patients poisoned with methanol. *Acta Med Scand* **213**, 105-110.

Selhub J & Miller JW (1992). The pathogenesis of homocysteinemia: interruption of the coordinate regulation by S-adenosylmethionine of the remethylation and transsulfuration of homocysteine. *Am J Clin Nutr* 131-138.

Shane B (1995). Folate Chemistry and Metabolism. In Bailey LB ed, *Folate in Health and Disease*, Marcel Dekker, New York, NY., pp 1-22.

Shane B & Contractor SF (1980). Vitamin B6 Metabolism and Role in Growth. In Tryfiates GP ed, *Food & Nutr.*, Westport, Connecticut.

Shangari N, Bruce WR, Poon R, & O'Brien PJ (2003). Toxicity of glyoxals--role of oxidative stress, metabolic detoxification and thiamine deficiency. *Biochem Soc Trans* **31**, 1390-1393.

Shangari N, O'Brien PJ, Poon R, & Bruce WR (2004). The cytotoxic mechanism of glyoxal involves oxidative stress. *Biochem Pharmacol* **68**, 1433-1442.

Shaw S (1989). Lipid peroxidation, iron mobilization and radical generation induced by alcohol. *Free Radic Biol Med* **7**, 541-547.

Siega-Riz AM, Bodnar LM, & Savitz DA (2002). What are pregnant women eating? Nutrient and food group differences by race. *Am J Ob Gyny* **186**, 480-486.

Sies H, Koch OR, Martino E, & Boveris A (1979). Increased biliary glutathione disulfide release in chronically ethanol-treated rats. *FEBS Lett* **103**, 287-290.

Smith EN & Taylor RT (1982). Acute toxicity of methanol in the folate-deficient acatalasemic mouse. *Toxicol* **25**, 271-287.

Sokol RJ, aney-Black V, & Nordstrom B (2003). Fetal alcohol spectrum disorder. *JAMA* **290**, 2996-2999.

Sophia Dyer (1998). Methanol: Update. *Clinical Toxicology Review* **20**:5, Massachusetts Poison Control System, Boston, MA.

Sperling F, Maxwell ES, & Von Oettingen WF (1953). Comparative excretion and distribution of C14-labeled carbonate and formate in large albino rats. *Am J Physiol* **174**, 33-38.

Sprung R, Bonte W, & Lesch OM (1988). Methanol--an up-to-now neglected constituent of all alcoholic beverages. A new biochemical approach to the problem of chronic alcoholism. *Wien Klin Wochenschr* **100**, 282-288.

Statistics Canada (2002). Canadian National Population Health Survey. Canadian Centre on Subsatance Abuse, Ottawa.

Statistics Canada (2003). Canadian community health survey: mental health and well-being. Statistics Canada, Ottawa.

Stella VS (1996). A case for prodrugs: fosphenytoin. *Adv Drug Delivery Rev* **19**, 311-330.

Stratton D, Howe C, & Battaglia F (1996). *Fetal Alcohol Syndrome: Diagnosis, Epidemiology, Prevention, and Treatment.* Institute of Medicine , National Academy Press, Washington, DC., pp 76-77.

Stover P & Schirch V (1990). Serine hydroxymethyltransferase catalyzes the hydrolysis of 5,10-methenyltetrahydrofolate to 5-formyltetrahydrofolate. *J Biol Chem* 14227-14233.

Stover P & Schirch V (1991). 5-Formyltetrahydrofolate polyglutamates are slow tight binding inhibitors of serine hydroxymethyltransferase. *J Biol Chem* 1543-1550.

Streissguth AP (1986). The Behavioral Teratology of Alcohol: Performance, Behavioral and Intellectual Deficits in Prenatally Exposed Children. In *Alcohol and Brain Development*, ed. West RJed, pp. 3-44. Oxford Press, New York.

Substance Abuse and Mental Health Services Administration (SAMHSA) (1998). Preliminary Results from the 1997 National Household Survey on Drug Abuse. *NHSDA Series H-6*, Rockville, MD, SAMHSA, **98** 3251.

Suh JR, Herbig AK, & Stover PJ (2001). New perspectives on folate catabolism. *Annu Rev Nutr* **21**, 255-282.

Tephly TR (1991). The toxicity of methanol. *Life Sci* **48**, 1031-1041.

Tephly TR, Green MD, & Gamble J (1992). Formate metabolism in micropigs. *Toxicol Appl Pharmacol* **116**, 142-145.

Thornalley PJ (1993). The glyoxalase system in health and disease. *Molecular Aspects of Medicine* **14**, 287-371.

Thornalley PJ, Langborg A, & Minhas HS (1999). Formation of glyoxal, methylglyoxal and 3-deoxyglucosone in the glycation of proteins by glucose. *Biochem J* **344 Pt 1**, 109-116.

Tintinalli JE (1995). Serum methanol in the absence of methanol ingestion. *Ann Emerg Med* **26**, 393.

Trimble KC, Molloy AM, Scott JM, & Weir DG (1993). The effect of ethanol on one-carbon metabolism: increased methionine catabolism and lipotrope methyl-group wastage. *Hepatol* **18**, 984-989.

Van der Dijs FPL, Fokkema MR, Dijck-Brouwer DAJ, Niessink B, Van der Wal TIC, Schnog JJB, Duits AJ, Muskiet FD, & Muskiet FAJ (2002). Optimization of Folic Acid, Vitamin B12, and Vitamin B6 Supplements in Pediatric Patients with Sickle Cell Disease. *Am J Hematol* **69**, 239-246.

Van der Mooren MJ, Demacker PNM, Blom HJ, de Rijke YB, & Rolland R (1997). The effect of sequential three-monthly hormone replacement therapy on several cardiovascular risk estimators in post-menopausal women. *Fertil Steril* **67**, 67-73.

Videla LA, Fernandez V, & Valenzuela A (1985). Effect of ethanol and iron on the hepatic and biliary levels of glutathione and lipid peroxidative indexes. *Alcohol* **2**, 457-462.

Videla LA & Valenzuela LA (1985). Alcohol ingestion, liver glutathione and lipoperoxidation. *Life Sci* **31**, 2395-2407.

Viljoen DL, Gossage JP, Brooke L, Adnams CM, Jones KL, Robinson LK, Hoyme HE, Snell C, Khaole NC, Kodituwakku P, Asante KO, Findlay R, Quinton B, Marais AS, Kalberg WO, & May PA (2005). Fetal alcohol syndrome epidemiology in a South African community: a second study of a very high prevalence area. *J Stud Alcohol* **66**, 593-604.

Villanueva JA, Esfandiari F, Wong DH, Ahmad I, Melnyk S, James SJ, & Halsted CH (2006). Abnormal transsulfuration and glutathione metabolism in the micropig model of alcoholic liver disease. *Alcohol: Clin Exp Res* **30**, 1262-1270.

Wagner C (1995). Biochemical role of folate in cellular metabolism. In Bailey LB ed, *Folate in Health and Disease*, Marcel Dekker, Inc., New York, NY., pp 23-42.

Wald NJ, Law MR, Morris JK, & Wald DS (2001). Quantifying the effect of folic acid. *Lancet* **358**, 2069-2073.

Walker C & Baxter ED (2000). Health-promoting ingredients in beer. *Tech Q Master Brewers Assoc Am* **37**, 301-305.

Warren KR & Foudin LL (2001). Alcohol-related birth defects--the past, present, and future. *Alcohol Res Health* **25**, 153-158.

Wells-Knecht KJ, Zyzak DV, Litchfield JE, Thorpe SR, & Baynes JW (1995). Mechanism of autoxidative glycosylation: identification of glyoxal and arabinose as intermediates in the autoxidative modification of proteins by glucose. *Biochem* **34**, 3702-3709.

West JR, Chen WJ, & Pantazis NJ (1994). Fetal alcohol syndrome: the vulnerability of the developing brain and possible mechanisms of damage. *Metab Brain Dis* **9**, 291-322.

Wickramasinghe SN (1999). The wide spectrum and unresolved issues of megaloblastic anemia. *Semin Hematol* **36**, 3-18.

Williams C, Epstein JA, Botvin GJ, Schinke SP, & Diaz T (1998). Psychosocial determinants of alcohol use among minority youth living in public housing developments. *J Dev Behav Pediatr* **19**, 145-154.

Williams RJ, Odaibo FS, & McGee JM (1999). Incidence of fetal alcohol syndrome in northeastern Manitoba. *Can J Pub Health* **90**, 192-194.

World Health Organization (2004). WHO Global Status Report on Alcohol 2004. WHO, Geneva.

Wouters MG, Moorrees MT, Van der Mooren MJ, Blom H.J., Schellekens LA, Thomas CM, & et.al. (1995). Plasma Homocysteine and menopausal status. *Eur J Clin Invest* **25**, 801-805.

Yamaguchi T & Nakagawa K (1983). Mutagenicity of and formation of oxygen radicals by trioses and glyoxal derivatives. *Agric Biol Chem* **47**, 2461-2465.

Yip L & Jacobsen D (2003). Endogenous formate elimination and total body clearance during hemodialysis. *J Toxicol Clin Toxicol* **41**, 257-258.

Zuba D, Piekoszewski W, Pach J, Winnik L, & Parczewski A (2002). Concentration of ethanol and other volatile compounds in the blood of acutely poisoned alcoholics. *Alcohol* **26**, 17-22.