INVESTIGATION OF EFFECTS OF RESTRICTED VITAMIN B6 SUPPLY ON THE METABOLISM OF CULTURED HEPG2 CELLS USING ANALYSIS OF AMINO ACID PATTERNS AND THE IN VIVO KINETICS OF THE TRANSSULFURATION PATHWAY

By

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To my grandfather, James DeRatt Sr.

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AA	Amino acids
ASR	Absolute synthesis rate
B6	Vitamin B6
CBS	Cystathionine β-synthase
CO_2	Carbon dioxide
CoA	Coenzyme A
CSE	Cystathionine γ-lyase
Csn	Cystathionine
CVD	Cardiovascular disease
Cys	Cysteine
DHF	Dihydrofolate
DNA	Deoxyribonucleic acid
EAR	Estimated Average Requirement
EDTA	Ethylenediaminetetraacetic acid
Ep	Enrichment plateau
FSR	Fractional synthesis rate
GABA	Gamma-aminobutyric acid
GC/MS	Gas chromatography/mass spectroscopy
Gly	Glycine
GSH	Glutathione
H_2S	Hydrogen sulfide
HBSS	Hank’s Balanced Salt Solution
HCl	Hydrochloride
Hcy	Homocysteine

HEPES	4-(2-hydroxyethyl)-1-piperarzineethanesulfonic acid
HFBA	heptafluorobutyric anhydride
HPLC	High performance liquid chromatography
I	Initial rate
IOM	Institute of Medicine
LPH	Lactase-phlorizin hydrolase
MEM/EBSS	Minimum Essential Medium with Earle's Balanced Salts
Met	Methionine
µL	microliter
mg	milligram
NH_4OH	Ammonium hydroxide
nM	nanomolar
PL	Pyridoxal
PLP	Pyridoxal phosphate
PM	Pyridoxamine
PMP	Pyridoxamine phosphate
PN	Pyridoxine
PNG	Pyridoxine 5'-β-D-glucoside
PNGH	Pyridoxine 5'-β-D-glucoside hydrolase
PNP	Pyridoxine phosphate
RDA	Recommended dietary allowance
RM	Remethylation
SAH	S-adenosylhomocysteine
SAM	S-adenosylmethionine
Ser	Serine

SHMT	Serine hydroxylmethyl transferase
TCA	Trichloroacetic acid
TCEP	Tris (2-carboxyethyl)phosphine
THF	Tetrahydrofolate
TM	Transmethylation
TS	Transsulfuration pathway
UL	Upper limit

Abstract of Thesis Presented to the Graduate School
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Pyridoxal phosphate (PLP) functions as a coenzyme in cellular one carbon metabolism and many other roles in amino acid interconversion and catabolism. The PLP-dependent transsulfuration enzymes, cystathionine beta-synthase (CBS) and cystathionine gamma-lyase (CSE), have been implicated in hydrogen sulfide (H_2S) production concurrent with the formation of lanthionine and homolanthionine. Recent research has identified H_2S as an endogenously produced gasotransmitter that modulates physiological functions in the cardiovascular and central nervous systems. My objective was to investigate the effects of restricted vitamin B6 supply on the metabolism of cultured HepG2 cells by analyzing the amino acid patterns and the *in vivo* kinetics of the transsulfuration pathway. Cells were cultured for 6 weeks in media containing concentrations of PL that represented severe (10 nM), marginal (50 nM), adequate (200 nM) and supraphysiological (2000 nM) conditions. Severely deficient cells had greater concentrations of valine, threonine, glycine, glutamine, asparagine, and alanine compared to marginal deficiency or adequacy, while lanthionine concentration was lower in deficiency ($P<0.002$). Extracellular homocysteine and

cysteine concentrations were greater in severely deficient cells ($P<0.002$). Metabolic flux studies using [U-$^{13}C_5$] L-methionine and (3,3 D2) L-cysteine indicated significantly lower fractional synthesis rates of lanthionine and homolanthionine at 10 and 50 nM PL, whereas that of cystathionine was higher in cells cultured in 50 nM PL. Remethylation rates and homocysteine synthesis were not affected by vitamin B6 restriction. Overall, these findings suggest CSE was impaired in severe and marginal deficiency while CBS was only impaired in severe deficiency, indicating a decrease in H_2S production. These results and observations of H_2S biomarker production suggest a mechanism by which vitamin B6 inadequacy influences cardiovascular disease risk.

CHAPTER 1
INTRODUCTION

Overt B6 deficiency is almost never medically diagnosed in today's population due to high bioavailability in natural and fortified foods. However, B6 insufficiency is widespread in specific sub-populations such as the elderly and young women as well as with chronic alcohol abuse. Epidemiological evidence suggests low dietary intake or plasma concentrations of B6 are associated with increased CVD risk, although recent trials demonstrated the ineffectiveness of B6 supplementation on the prevention of cardiovascular incident recurrence. Determining specific mechanistic relationships has proven challenging due to the magnitude of critical functions that require B6 and the many stages of CVD. To date, mechanisms of B6 modulation on CVD risk are unknown.

Inadequate B6 status has been related to inflammation, immune function, and thrombosis: all pivotal components in the progression of CVD. Elevated homocysteine, hypercholesterolemia, and many other risk factors have been related to CVD, however even in these studies, B6 status remained an independent risk factor for CVD. Due to the necessity of B6 in many metabolic reactions, it is likely B6 is a limiting factor in many reactions affecting CVD risk. The recent discovery of endogenously produced H_2S, which acts as a neuromodulator and smooth muscle relaxant, provides another possible mechanism of B6 modulation against CVD. H_2S, once thought to be toxic, is produced endogenously via the reduction of thiols and thiol-containing molecules. The two enzymes responsible for the majority of endogenously produced H_2S are PLP-dependent, thus providing a hypothetical connection between H_2S production and B6 status.

This study was designed to investigate biomarkers of H_2S production as well as other amino acids during restriction of B6 in addition to kinetics in the transsulfuration pathway in HepG2 cells. The first study sought to examine the effects of B6 concentration, ranging from severe to marginal deficiency into adequacy, on intracellular and extracellular amino acid concentrations. The second study used stable isotopes, (3,3 D2) L-cysteine and [U-$^{13}C_5$] L-methionine, to determine synthesis rates of amino acids in the transsulfuration pathway and H_2S biomarkers under B6 constraints. The synthesis rates of these biomarkers suggest a parallel relationship with H_2S production. Labeled products of [U-$^{13}C_5$] L-methionine such as homocysteine and cystathionine can be used to determine the extracellular concentration in which inhibition of CBS and CSE occurs in cultured HepG2 cells. By determining a connection between H_2S production and B6 status, it will provide preliminary data for a possible mechanism in which B6 is related to CVD risk.

CHAPTER 2
LITERATURE REVIEW

Vitamin B6

History

The first report of a water-soluble nutritional factor later identified as B6 was made in 1934 (1, 2). Purification and crystallization of pyridoxine was reported by five laboratories in 1938 (3-8). A year later, synthesis confirmed the proposed structure. In the 1940s, studies of the nutritional requirements of lactic acid bacteria led to the identification and recognition of pyridoxal and pyridoxamine as natural forms of the vitamin, as well as demonstrating the active coenzyme form of B6 was pyridoxal 5-phosphate (6, 9-12). Since its discovery, the understanding of B6 properties, metabolic function, and role in maintaining health have continuously expanded.

Chemistry and Function

B6 denotes the family of water soluble vitamins with the shared structure of 2-methyl, 3-hydroxyl, 5-hydroxymethyl-pyridines exhibiting the nutritional activity of pyridoxine. The three main derivatives of B6 differ in one carbon substituent at position 4 of 2 methyl-3-hydroxy-5-hydroxymethyl-pyridines as seen in Figure 2-1. Pyridoxine (PN) contains an alcohol substituent, for pyridoxal (PL) it is an aldehyde substituent, and for pyridoxamine (PM) it is an amine substituent. Each can each be phosphorylated at the 5'-hydroxymethyl group yielding the additional three members; pyridoxine 5'-phosphate (PNP), pyridoxal 5'-phosphate (PLP), and pyridoxamine 5'-phosphate (PMP). The structural differences cause pyridoxine to have a hydroxymethyl function, pyridoxamine, an aminomethyl function, and pyridoxal, a formyl function (13).

The pyridine ring system of B6 molecules exist mainly as zwitterions at neutral pH due to the basic character of the pyridinium N and the acidic nature of the 3-OH. Thus the net charge of B6 is dependent on environmental pH. The 4'-amino group of PM and PMP and the 5'-phosphate ester of PLP and PMP will contribute to the charge of the vitamin. The complex ionization at several ionic sites explains the reactivity in enzymatic processes (14).

The coenzymatic action of PLP in most B6 dependent reactions involves a carbonyl-amine condensation. PLP readily condenses with the uncharged amino group of amines while PMP will react with aldehydes or ketones to form Schiff bases (aldimines) at the 4-formyl substituent. While Schiff bases can exist devoid of metal ions, their stability is greatly increased by covalent bonding with a metal ion. PL and PM can form Schiff bases, however, the phosphorylated forms do so more readily because the phosphate group prevents the formation of an internal hemiacetal bridge between the C-5 hydroxymethyl group and the C-4 aldehyde of PL, thereby maintaining the carbonyl in a reactive form (15). Alkaline environments offer optimum pH for Schiff base formation and stability (16).

B6 affects nearly all aspects of metabolic function and cellular homeostasis while specifically acting as a coenzyme in processes including amino acid metabolism, one-carbon metabolism and nucleotide synthesis, neurotransmitter metabolism, heme synthesis, gluconeogenesis, and glycogenolysis. Though involved in carbohydrate and lipid metabolism, PLP is most extensively linked to amino acid metabolism by functioning as a coenzyme in synthesis, degradation, and interconversion of amino acids. The pathways constituting one-carbon metabolism contain four enzymes that

require PLP, without, the remethylation cycle and DNA synthesis would be affected. Amino acid decarboxylases form hormones and neurotransmitters such as epinephrine, serotonin, dopamine, and GABA by irreversibly breaking the α-carboxyl group from an amine. PLP forms a heme precursor, δ-aminolevulinate, through a reaction with glycine and succinyl CoA. Glycogenolysis reactions also require PLP for enzymatic cleavage of glycogen and general acid catalysis.

Food Sources and Bioavailability

B6 exists in food in different chemical forms. Plants also contain glycosylated forms of B6, generally as pyridoxine-5'-β-D-glucoside (PNG), although other glycosylated forms exist. PNG comprises 5-75% of total B6 and accounts for 15-20% of B6 in mixed diets (17). The obligatory step in nutritional utilization of PNG is the intestinal hydrolysis of its β-glucosidic bond which releases PN (18, 19). PLP and PMP make up >80% of the B6 found in animal origin food such as meats, fish, eggs, and dairy products. Today, breakfast cereals and beverages are fortified with B6 as PN-HCl because of its stability (20).

Dietary PL, PN, PM, PLP, PMP, and PNP if present, are approximately 75% bioavailable in a typical mixed diet (21). The acidic environment of the stomach dissociates Schiff bases thus freeing the vitamers prior to absorption. The bioavailability of PNG is 50-60% relative to PN, however variation exists among individuals (22, 23). B6 from animal sources is approximately 10% more digestible than plant sources (24).

Food processing can affect B6 absorption. Non-enzymatic interconversion between B6 compounds occurs when the double bond of the Schiff base migrates causing subsequent hydrolysis and dissociation (25). Foods that contain aldehyde and

amine forms of B6 are especially susceptible to transamination during thermal processing. For example, concentrations of PM and PMP increased during cooking or thermal processing of meat and dairy products (26, 27). This exchange between PLP and PMP is not detrimental to the nutritional status of B6 in foods since both are readily absorbed.

Degradation of B6 in food is dependent on the form of B6, temperature, pH of the solution, and the presence of other reactive compounds. B6 is susceptible to photochemical oxidation resulting in 4-pyridoxic acid phosphate formation, a nutritionally inactive derivative. Light-induced degradation will cause B6 losses in food processing, storage, preparation, and analysis (28, 29). Ink et al used intrinsic and extrinsic labeling in rats to show thermal processing of food reduces the bioavailability of B6 from animal tissue by 25-30%(30). PNG co-administered with PN antagonistically affects the utilization and metabolism of PN and other non-glycosylated forms of B6 in humans and rats (22, 31, 32).

Absorption and Metabolism

Absorption of B6 in humans occurs in the small intestine, primarily in the jejunum, and from bacterial production in the large intestine (33). Alkaline phosphatase in the small intestine hydrolyzes the phosphorylated forms at the brush border membrane enabling absorption. Glycoslyated forms, specifically PNG, were found to be effectively absorbed in humans and rats but not completely hydrolyzed in the intestine by the cytosolic enzymes PNGH or the brush border membrane enzyme LPH(19, 22, 23, 30, 34-36). Many kinetic studies show intestinal absorption of PL, PM, and PN occurs by simple, non-saturable diffusion (37-39), although evidence exists for a carrier-mediated

absorption mechanism that is dependent upon pH and concentration of the vitamin (33, 40).

Once absorbed into the enterocyte, PL, PM, and PN are metabolically trapped by phosphorylation catalyzed by pyridoxal kinase. The addition of the negatively charged phosphate group hinders diffusion across the cell membrane while promoting protein binding. Dephosphorylation allows for movement across the basolateral membrane and into portal circulation (39). Vitamers are metabolized mainly in the liver to the metabolically active form, PLP, as shown in Figure 2-2. PL, PN, and PM are phosphorylated by PL kinase using ATP-zinc as a cofactor and phosphoryl donor (41). Hepatic PL kinase activity is ten-fold higher than that of phosphatase causing PLP to be the predominate form of B6. PMP and PNP interconversion into PLP is catalyzed by FMN-dependent pyridoxine phosphate oxidase (42, 43). While extrahepatic tissues utilize B6, few contain oxidase activity to yield PLP from other forms. PNP oxidase prevents excess PLP production by its susceptibility to product inhibition by PLP (44). If excess PLP is produced, it is catabolized in the liver by FAD-dependent aldehyde oxidase and NAD-dependent aldehyde dehydrogenase into 4-PA, then excreted in quantifiable concentrations in urine (45, 46). Non-specific phosphatases dephosphorylate PLP releasing PL from the liver into circulation where it is bound to albumin in the plasma or hemoglobin in erythrocytes for transport (47-50).

Tissue uptake requires circulating PLP to be dephosphorylated by plasma membrane phosphatases, thereby allowing carrier-mediated transport across cellular membranes (51, 52). In tissue, B6 is again trapped by phosphorylation and concentrated into the mitochondria and cytosol. Total body concentrations of B6 are

approximately 170 mg (1000 µmol) in adults (50). The largest pool is located in skeletal muscle, constituting 70-80% of the total pool, bound to glycogen phosphorylase (50, 53-55). Research shows muscle tissue is resilient to B6 restriction and supplementation whereas plasma concentration is directly affected by intake (54).

Requirements and Allowances

Controlled dietary studies and observational investigations have concluded that plasma PLP concentration is dependent upon B6 intake and antagonistically affected by dietary protein intake (56-58). While the 1989 RDA was based on the B6-protein relationship, the current RDA was revised to account for average requirements(59, 60). The current RDA for adults, 1.3 mg, has been shown to maintain PLP concentration ≥20 nmol/L (61). Since the criteria for an adequate dietary intake is based on PLP status, increased intake is recommended for pregnant and lactating women and also elderly as seen in Table 2-1.

Current debate exists on the sufficiency of the RDA for B6. Recent NHANES data estimated the average B6 intake in the United States to be 1.86 mg/day for individuals not consuming supplements however plasma PLP concentrations were indicative of deficiency in all subgroups examined (61). Rimm et al. showed an increased risk of coronary heart disease in people consuming the current RDA (1.3 mg) compared to those consuming greater amounts (>1.7 mg), indicating additional protective effects could be attained at greater intake of B6 (62).

Deficiency and Toxicity

B6 deficiency may be caused by insufficient dietary intake, impaired absorption and interconversion, or unavailability due to drug interactions (41, 63-66). B6 deficiency can either be severe (<20 nmol/L PLP) or marginal (20-30 nmol/L PLP), each defined by

plasma PLP concentration ranges (67, 68). Adequate B6 status is defined by a PLP concentration of >30 nmol/L. The prevalence of marginally to severely deficient individuals in the population is between 9 and 30%, with significantly elevated indices of deficiency in women of childbearing age and in elderly (61).

Clinical manifestations of severe B6 deficiency are seizures, skin lesions, and microcytic anemia. Seizures in infants fed a commercial formula in which B6 degraded due to processing and storage were most likely a result of impaired neurotransmitter production (69, 70). Neurotransmitter levels were shown in rats to be depressed by B6 restriction (71). Some doctors have exploited the relationship between neurotransmitter production and B6 status by prescribing patients suffering from depression B6 supplements (72). The results from this prescription were varied at best. Other manifestations of B6 deficiency are abnormal red blood cell formation. Without PLP, δ-aminolevulinate cannot be produced, thus limiting heme synthesis. Limited hemoglobin synthesis causes red blood cell size to decreased resulting in mircocytic anemia (73). Skin lesions also appear in severe deficiency but are corrected with B6 supplementation (74).

The upper limit (UL) for B6 was set at 100 mg per day due to reports of sensory neuropathy caused by pyridoxine supplements of over 500 mg per day (Table 2-2) (60, 75). Megadoses of B6 were given to treat premenstrual syndrome, asthma, and certain sensory neuropathies until reports stated neurotoxicity and photosensitivity were occurring at chronic doses of greater than 1 g/day (76). Toxic effects were not displayed in individuals consuming 100 mg per day(60). Toxicity of B6 has not been reported from

dietary intake alone. Currently, most daily vitamins provide approximately 2 mg B6 so toxicity by supplementation and dietary intake is not realistic in today's population.

Status Assessment

B6 can be measured through direct or indirect means. The most common direct measurement of B6 nutritional status is the quantification of PLP by HPLC or tyrosine decarboxylase. PLP concentration reflects tissues stores and is a reliable indicator of long term B6 status (77, 78). Urinary excretion of PLP as its catabolic product, 4-PA, can also quantified to determine B6 concentration from daily intake. Indirect measurements of B6 status assess the activity of PLP-dependent enzymes, such as kynurinase, after providing a bolus of precursor, in many cases tryptophan (79). Inhibition of enzyme activity is then related to B6 status. Indirect measurements of B6 are not specific and can often be affected by other unrelated factors.

Vitamin B6 and Cardiovascular Disease Risk

The pathogenesis of CVD involves the interplay between several genetic, nutritional, and life-style factors, thus the final outcome of this disease is due to many different contributory agents. Monkeys fed a pyridoxine deficient diet for 5 months developed atherosclerotic lesions, leading to further inquires of the relationship between B6 and CVD (80, 81). Furthermore, human studies have determined B6 deficiency is an independent risk factor for atherothrombotic and cerebrovascular disease (82-84). Many epidemiological studies reinstate this correlation in patients with coronary artery disease risk. The magnitude of the association between B6 and CVD is lower than other factors, such as blood lipid levels and homocysteine concentration, but it indicates the coenzymatic function of B6 in total body metabolism plays a compounding role in disease development.

There are many hypotheses of the mechanisms by which B6 is associated with CVD. The most published is the dependence of B6 in the transsulfuration pathway to degrade homocysteine. Elevated homocysteine in patients is a well-known predictor of CVD (85, 86). Robinson et al demonstrated through multivariate analysis that low PLP concentration is a risk factor of coronary artery disease independent of homocysteine concentration, suggesting a potential protective effect of B6 through mechanisms unrelated to homocysteine metabolism (87, 88). In addition to homocysteine elevation, B6 concentration inversely relates to C-reactive protein values (89). C-reactive protein is an inflammatory marker and also a predictor of coronary artery disease (90, 91). B6 plays a role in coagulation by inhibiting ADP receptors and prolonging bleeding by occupying the glycoprotein IIb/IIIa receptor or down-regulating its synthesis (92-98). B6 may induce hypercholesterolemia by inhibition of advanced glycation and lipooxidation of end-products (99-101). EPA and DHA production were hindered by B6 deficiency thus reducing the presence and protective effects of omega-3 fatty acids (102-105). T lymphocyte, macrophage differentiation, and interleukin production were shown to be affected by B6 status thereby possibly impairing immunological function (106-108). As briefly stated above, B6 has many possible mechanisms affecting the development of CVD. While these identify a few of the mechanisms, there are undoubtedly more unknown mechanisms by which B6 affects diseased states.

Amino Acids

Structure and Function

Amino acids utilized by humans are α-amino acids. All α-amino acids have the same core structure consisting of an amino group, carboxyl group, a hydrogen atom and a reactive group (Figure 2-3). The reactive group, R-group, gives each amino acid

its unique physical and chemical properties. The simplest amino acid is glycine, consisting of the central α-carbon bonded to a carboxyl group, an amino group, and two hydrogen atoms. Larger amino acids that have different substituents from each bond with the α-carbon can exist in two different molecular configurations; D and L isomers. L-amino acids are present in proteins and biological environments (109). Amino acids are categorized according to their R groups that dictate their chemical nature; aliphatic, acidic, basic, aromatic, sulfur-containing, and secondary amino acids. Each group has a similar structural component in the R group substituent (110). Peptide bonds are amide bonds between amino acids leading to the formation of peptides and proteins. The bond is formed between the carboxyl group of the first amino acid and the amino group of the second amino acid. The peptide bond appears to be a single bond but has double bond characteristics such as rigidity.

Requirements

Although there are many amino acids in nature, only about 20 are found in proteins. While forming proteins, amino acids can also control the initiation of mRNA translation and regulate protein synthesis (111). Essential amino acids are especially important to consume because they cannot be synthesized in sufficient amounts internally. Overloading one amino acid can cause a deficiency in another so maintaining a balance is essential. Current RDAs are based on nitrogen balance studies from Rose and others in the 1950s(112).

Metabolism

Amino acids can be synthesized internally or must be acquired from dietary intake. This divides them into the categories of essential, nonessential and conditionally essential. The 9 essential amino acids are histidine, isoleucine, leucine, lysine,

methionine, phenylalanine, threonine, tryptophan, and valine. Nonessential amino acids are alanine, asparagine, aspartic acid, and glutamic acid. These can be derived from keto-acids in the transamination process. Conditional amino acids are only essential under certain conditions such as illness and stress.

For nonessential amino acids to be synthesized in the body, PLP must be present to serve as the coenzyme of transamination reaction. If the initial amino acid is not present then the “root” keto-acid is not available to make the final amino acid. For essential amino acids, there is no “root” keto-acid so they must be ingested. The keto-acid intermediates can be used for additional purposes such providing substrates for the citric acid cycle. Excess amino acids can be converted to pyruvic acid and acetyl CoA to be used in lipogenesis or synthesized into glucose and glycogen. This conversion to glucose is stimulated by the hormones cortisone, glucagon and cortisol. Nonessential amino acids can be made from alternative processes such as cysteine production from methionine or serine and glycine production from phosphoglyceric acid (113).

Transamination

Transamination is the transfer of an amine group from one molecule to another by a transaminase enzyme thereby producing nonessential amino acids *in-vivo*. A decreased rate of transamination occurred in correspondence to PLP deficiency (114). This double replacement reaction begins with an amino acid and a PLP-enzyme complex. PLP forms a Schiff base with the enzyme to link to the active site. Then a new Schiff base forms between the α-amino group of the amino acid and the PLP which has substituted from the enzyme-PLP linkage. This produces the intermediate aldimine. This linkage acts as an electron sink, weakening the bond. Once there is a break in the double bond, the intermediate is now called a ketamine. Hydrolysis then occurs and the

final product is an α-keto acid product and PMP-enzyme complex. Since this is a double replacement reaction, dehydrating a new α-keto acid substrate and the PMP-enzyme complex will reverse the reaction. The final result will be a new amino acid and a PLP-enzyme complex (115), as depicted in Figure 2-4.

The main keto-acids in this pathway are α-ketoglutarate and pyruvate, needed for glutamate and alanine production. Subsequently, glutamate can serve as the source of the amine group to make additional amino acids. Therefore there are many amino acids that can be produced from a few initially digested amino acids (113).

Amino Acids and Vitamin B6 Status

Vitamin B6 is an essential coenzyme in amino acid metabolism. Many studies conclude amino acid concentrations vary significantly in B6 deficiency. A human study by Park and Linkswiler in 1970, using a B6 deficient diet for 3 weeks, showed many changes in urinary excretion of free amino acids after a methionine load test. Plasma concentrations of glycine, serine, and threonine increased in the fasting and 2 hour postprandial states in marginally B6 deficient subjects. There was also an increase in the urinary excretion of serine and threonine before and after the methionine load. Plasma concentrations of alanine, isoleucine, leucine and valine decreased with B6 depletion (116). While many other amino acids use B6 as a coenzyme, regulatory pathways prevent amino acid imbalance in deficient states.

Studies conducted in this laboratory displayed the effects of B6 deficiency in conjunction with one carbon metabolism and the transsulfuration pathway. The concentration of glycine, which is an integral component of one-carbon metabolism, significantly increased in participants consuming a B6 deficient diet. Human studies have shown mild B6 depletion did not affect the concentrations of homocysteine,

methionine, and serine (117, 118). In the methylation cycle, fasting homocysteine increased slightly in B6 deficiency (119-121), however other studies showed moderate B6 deficiency had little effect on fasting homocysteine concentrations (119). The transsulfuration pathway is directly affected by B6 deficiency because of two PLP-dependent enzymes responsible for product formation. B6 restriction studies in humans have shown an increase in plasma cystathionine concentration (118, 121), as well as fractional synthesis rates (122). Glutathione synthesis rate, not concentration, was affected by B6 status in red blood cells in healthy men and women while rat studies showed an increase in glutathione concentration in B6 deficiency (123, 124).

One Carbon Metabolism

One carbon metabolism is comprised of; the folate cycle which acquires one-carbon units to support DNA synthesis, the methionine cycle which provides for methylation processes, regeneration of methionine and regulation of homocysteine, and the thymidylate cycle which synthesizes DNA. As seen in Figure 2-5, there are four PLP-dependent enzymes in one carbon metabolism and the transsulfuration pathway; (1) serine hydroxymethyltransferase (SHMT); (2) glycine decarboxylase of the glycine cleavage system; (3) cystathionine β-synthase (CBS); (4) cystathionine γ-lyase (CSE). Studies in humans and rats have found the PLP-dependent enzymes were impaired in B6 deficiency (125).

The folate cycle converts dietary folate into tetrahydrofolate (THF) form. Folic acid is reduced to THF in a two-step process by dihydrofolate reductase. Throughout the folate cycle, tetrahydrofolate polyglutamate cofactors are the acceptors and donators of one-carbons units differing in the oxidation of N5 or N10 positions (126). SHMT-PLP complex redistributes a one carbon unit from serine to THF to yield glycine

and 5,10-methylene THF. The glycine cleavage system, also PLP-dependent, yields CO_2, NH_3, and a one-carbon unit which is also accepted by THF to form 5,10-methylene THF. 5,10-methyleneTHF can donate a carbon for the synthesis of thymidylate from deoxyuridylate, an initial and rate-limiting step in DNA synthesis. 5,10-methyleneTHF also produces 10-formylTHF in which the formyl group provides two carbons that are incorporated into the purine ring for DNA and RNA synthesis. Methylenetetrahydrofolate reductase partially reduces 5,10-methyleneTHF to 5-methylTHF. This enzyme is disposed to a common genetic polymorphism that reduces its functionality in folate deficiency. 5-methylTHF is the primary transport form in the body. 5-methylTHF is demethylated by methionine synthase using vitamin B12 as a coenzyme to accept the methyl group producing methyl-cobalamin. The methyl group is then transferred to homocysteine producing methionine in the methylation cycle. Methionine is activated by ATP to form S-adenosylmethionine (SAM). SAM is a methyl donor in over 100 methyltransferase reactions such as in DNA methylation, neurotransmitter synthesis, and phospholipid synthesis. SAH is formed when SAM is demethylated. The removal of adenosine from SAH produces homocysteine. Homocysteine has two metabolic fates; it is remethylated by methionine synthase or catabolized in the transsulfuration pathway.

Transsulfuration Pathway

The transsulfuration pathway aides in the regulation of homocysteine and controls the production of cysteine, ultimately yielding glutathione. Homocysteine condenses with serine in a reaction catalyzed by PLP in complex with CBS to produce cystathionine in an irreversible reaction. Cystathionine is then hydrolyzed by CSE to yield cysteine. As seen in the Figure 2-6, the enzymes mentioned can also produce

additional products. H_2S is produced at many points in this pathway. While measuring H_2S content in plasma is difficult, it has been proposed that measuring specific products associated with H_2S production such as homolanthionine and lanthionine can give insight on H_2S production (127).

The transsulfuration pathway has been extensively studied in relation with B6 deficiency due to the dependent enzymes in the pathway. SAM regulates of the transsulfuration pathway by controlling CBS activation such that in restricted conditions, the pathway is driven towards remethylation rather than transsulfuration. Animal studies showed that severe B6 deficiency affected the activity of both enzymes, with CSE being more sensitive (125, 128-132). In human studies, the transsulfuration flux was impaired in B6 deficiency seen by increased homocysteine and cystathionine concentrations (119-122).

Hydrogen Sulfide

H_2S is best known as a toxic pollutant and has been linked to tissue damage and inflammation at high levels. However, recent publications describe H_2S as a gaseous signaling molecule that modulates physiological functions and is regulated (133, 134). Specifically, in the brain H_2S appears to function as a neuromodulator by enhancing N-methyl-D aspartate receptor-mediated responses and facilitating the induction of long term potentiation in the hippocampus (135). H_2S also functions as an endogenous smooth muscle relaxant in vertebrates (136-140). Nitric oxide is a well-known gaseous transmitter with many shared functions *in-vivo*. Nitric oxide has been more extensively studied than H_2S in respect to mechanistic functions. It is not known if H_2S and nitric oxide exert their effects *in-vivo* independently or in tandem, however current research

suggests H_2S is involved in the regulation of nitric oxide mediated signaling events and/or vice versa (141).

Most H_2S is produced endogenously from desulfuration of transsulfuration amino acids catalyzed by CBS and CSE, which are PLP dependent enzymes (142). Mice with the CSE enzyme deleted have reduced levels of H_2S in serum, heart, aorta, and other tissues (143). H_2S concentration is difficult to measure due to rapid oxidation and volatility, and literature shows wide variation in reported H_2S concentrations (144). As H_2S is formed, by-products homolanthionine and lanthionine are also formed. These products have been proposed as biomarkers of hydrogen sulfide production although their quantitative relationship and function of remain unknown (127).

The concentration of endogenously produced H_2S in healthy humans does not approach toxicity. H_2S is metabolized by oxidation in the mitochondria or methylation in the cytosol into sulfate (141). It also can be scavenged by methemoglobin or oxidized glutathione, or consumed by endogenous oxidant species in the vasculature such as nitric oxide (145). The resulting conjugated sulfate is excreted by the kidney (146).

Hypotheses and Specific Aims

Overall Rationale

Previous studies determined the effects of B6 status on specific amino acid concentrations and kinetics. This study aimed to determine the effect of B6 on a complete intracellular amino acid profile in cultured HepG2 cells as well as specific extracellular amino acids. In additional to amino acid patterns, the transsulfuration pathway amino acids were quantified and fractional synthesis rates determined. The focus on the transsulfuration pathway was due to the production of H_2S and its biomarkers, predominantly produced by the CBS and CSE enzymes. These results

provide preliminary evidence regarding the effects of B6 status on H_2S production in an attempt to explain the relationship between B6 deficiency and CVD risk. HepG2 cells were used in this project because previous studies show this cell line simulated physiological conditions under B6 constraints. Hepatic tissue is also the main site for B6 interconversion so by using a human liver cell line, the PL administered will be converted into the enzymatically active form.

Hypotheses

1. The amino acid profile of HepG2 cells will vary according concentration of B6 with some amino acids more directly affected than others.

2. The transsulfuration pathway in B6 deficient cells will be hindered causing an accumulation of two intermediates due to decreased enzyme activity. H_2S concentrations, as well as the biomarkers of H_2S production; lanthionine and homolanthionine, will increase with PLP concentration in HepG2 cells.

3. The flux of amino acids in the transsulfuration pathway will be hindered in PLP deficient cells due to CBS and CSE inhibition.

Specific Aims

- Quantify various amino acids as well as H_2S biomarkers in HepG2 cells adapted to specific concentrations of B6

  a) Modify existing HPLC methods to quantify the intracellular and specific extracellular concentrations of 25 amino acids

  b) Use GC/MS to quantify cystathionine, lanthionine, and homolanthionine intracellular concentrations

- Measure transsulfuration flux and fractional synthesis rates of amino acids associated with the transsulfuration and remethylation pathways in HepG2 cells at various concentrations of cellular B6

Table 2-1. Recommended dietary intakes for B6(mg/day) by age and gender (147).

Age	Male	Female	Pregnancy	Lactation
Birth to 6 months	0.1 mg*	0.1 mg*		
7–12 months	0.3 mg*	0.3 mg*		
1–3 years	0.5 mg	0.5 mg		
4–8 years	0.6 mg	0.6 mg		
9–13 years	1.0 mg	1.0 mg		
14–18 years	1.3 mg	1.2 mg	1.9 mg	2.0 mg
19–50 years	1.3 mg	1.3 mg	1.9 mg	2.0 mg
51+ years	1.7 mg	1.5 mg		

Table 2-2. Tolerable Upper Intake levels for B6(mg/day) by age and gender (147).

Age	Male	Female	Pregnancy	Lactation
Birth to 6 months	NA	NA		
7–12 months	NA	NA		
1–3 years	30 mg	30 mg		
4–8 years	40 mg	40 mg		
9–13 years	60 mg	60 mg		
14–18 years	80 mg	80 mg	80 mg	80 mg
19+ years	100 mg	100 mg	100 mg	100 mg

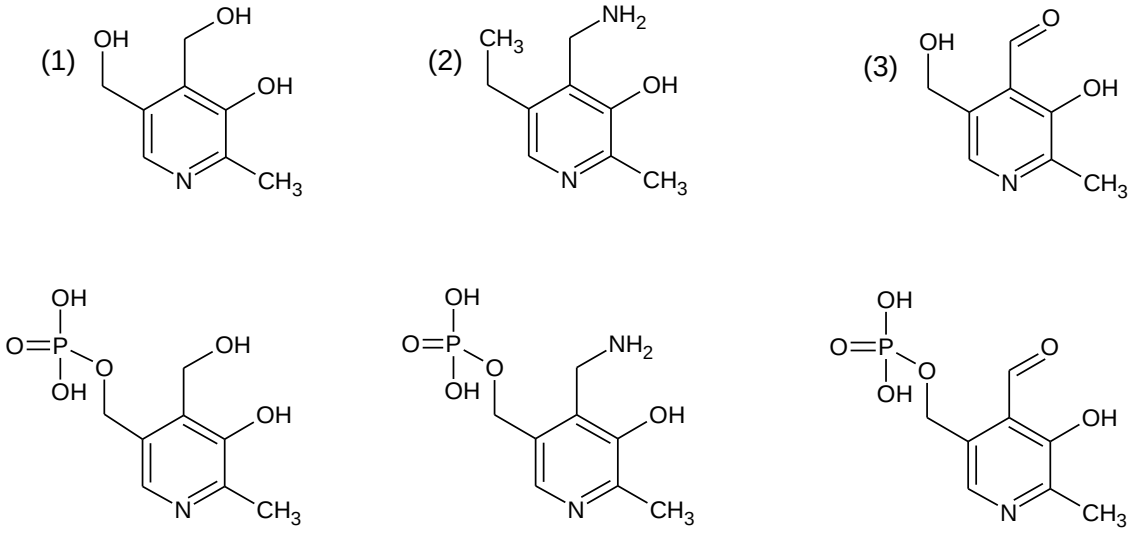

Figure 2-1. (1) pyridoxine, (2) pyridoxamine, (3) pyridoxal chemical structures. Phosphorylated forms are shown below each respective vitamer.

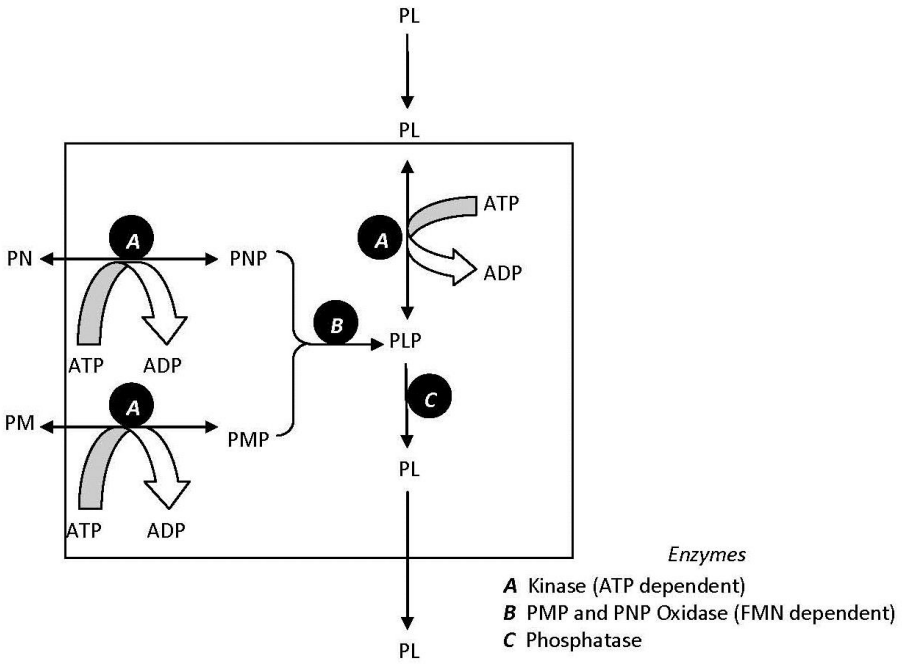

Figure 2-2. This model of vitamin B6 shows cellular trapping, interconversion, and release. The enzymes responsible for each action have also been identified.

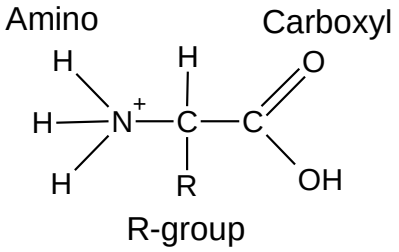

Figure 2-3. The generic structure of α-amino acids.

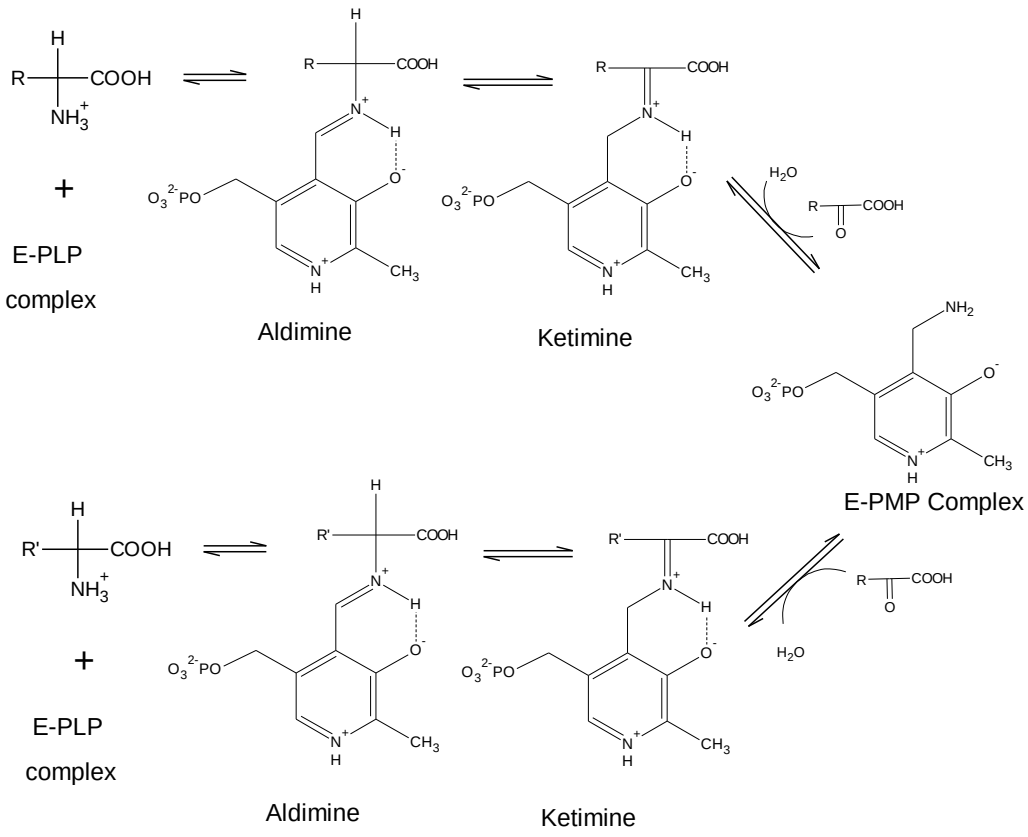

Figure 2-4. The transamination process produces nonessential amino acids using PLP and transaminase enzyme complex.

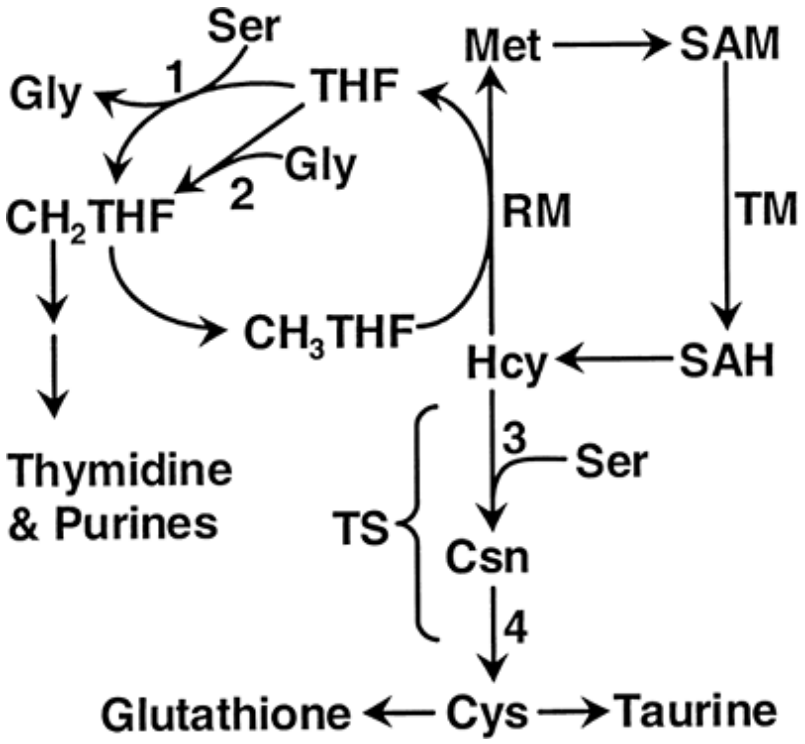

Figure 2-5. One-carbon metabolism and transsulfuration pathway contain four PLP-dependent enzymes: (1) serine hydroxymethyltransferase; (2) glycine decarboxylase of the glycine cleavage system; (3) cystathionine β-synthase; (4) cystathionine γ-lyase (125).

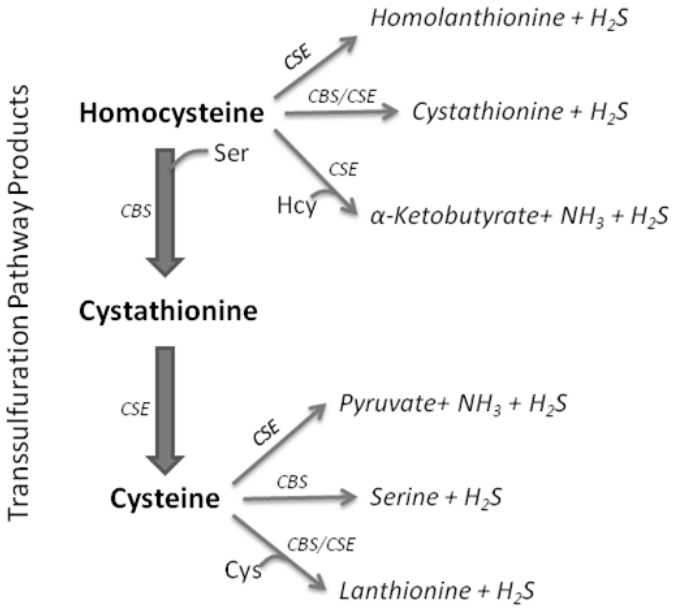

Figure 2-6. The transsulfuration pathway synthesizes H_2S as a by-product catalyzed by CSE and CBS enzymes. Redrawn from (148).

CHAPTER 3
AMINO ACID METABOLISM IN HEPG2 CELLS ARE AFFECTED AT VARIOUS CONCENTRATIONS OF VITAMIN B6

Metabolic perturbations of amino acid concentrations due to altered B6 nutrition demonstrated B6 essentiality in amino acid metabolism. Lanthionine and homolanthionine have been proposed to be biomarkers reflecting the extent of H_2S production. These amino acids are produced by B6 dependent enzymes CSE and CBS during the synthesis of H_2S (127). Some amino acids are more susceptible to B6 inadequacy due to reduced enzyme activity. This experiment used HPLC and GC/MS methods to quantify individual amino acids in HepG2 cells cultivated in media containing 10 nmol/L (severe deficiency), 50 nmol/L (marginal deficiency), 200 nmol/L (adequate), or 2000 nmol/L PL (supraphysiological). By understanding amino acid metabolism in relation to B6 concentration disease risk can be assessed (149).

Materials and Methods

Materials

Human hepatoma cell line (HepG2) was purchased from American Type Culture Collection (Manassas, VA). Cell culture media and all other medium supplements were purchased from HyClone (Logan, UT) or Cellgro (Mannassas, VA). Bradford assay solution was purchased from Bio-Rad (Hercules, CA). All amino acids, used for calibration, were purchased from Sigma Aldrich (St. Louis, MO). All other chemicals and solvents were HPLC grade or above and purchased from Fisher Scientific (Pittsburgh, PA) or Sigma Aldrich (St. Louis, MO).

Cellular Depletion

All media was supplemented with 2 mM L-glutamine, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, antibiotic/antimycotic solution (1x), and 10% fetal

bovine serum. HepG2 cells were grown to confluency in complete media (SH30024.01) for two weeks before transfer into 75.0 cm^2 flasks. Basal media devoid of added B6 (RRC125193) was used to prepare working media with four different concentrations of added pyridoxal; 10 nmol/L PL represented severe deficiency, 50 nmol/L PL represented marginal deficiency, 200 nmol/L represented adequate B6 status, and 2000 nmol/L which was equivalent to the PL concentrations found in commercial media. Cells were passaged every 3-4 days depending on confluency for 6 weeks until intracellular PLP concentration reached a steady state. Cells were passaged by removing the media from each flask and washing with 2 mL of DPBS. Then 1 mL of trypsin was added to each flask and incubated at 37˚C with 5% CO_2 for 4 minutes. Respective media were then added to remove cells from the plate bottom and dilute trypsin. The cells were transferred to new flasks in diluted quantities or collected for analysis. Cells were incubated in 5% CO_2 at 37˚C.

Sample Preparation

Each week, 4 flasks used the above stated cell removal process and were collected in 15mL Eppendorf tubes. The tubes were centrifuged at 50 x g for 10 min and media removed. Cells were then washed three times with DPBS, each followed by centrifugation. The final pellet of cells was diluted into 1.5 mL of cold distilled water and sonicated with a Sonic Dismembrator for 30 seconds in continuous mode, setting 3. The dispersed pellet was immediately aliquoted into separate vials for Bradford Assay, homocysteine assay, amino acid analysis, and PLP assay. Table 3-1 shows the amounts and additions made to each vial before storage at -80˚C.

PLP Analysis

Intracellular PLP concentration was analyzed weekly. Samples collected in the above conditions were sonicated to disrupt cells then 500 µL 10% (w: v) TCA was added immediately for protein precipitation and the mixture was stored until analysis. After samples thawed, the mixture was clarified by centrifugation at 10,600 x g for 10 minutes. A 750 µL portion of the supernatant was transferred to a 15 mL Falcon tube and 50 µL of 0.5 M semicarbazide was added to derivatize PLP and PL into their semicarbazone forms for fluorescence detection. The samples were mixed thoroughly and incubated in a 37˚C water bath for 12-15 minutes. Samples returned to room temperature before repetitive extraction with 3 mL diethylether. A single extraction with 3 mL methylene chloride removed TCA and cellular lipids from the sample. PLP and PL semicarbazone derivatives were measured by HPLC (Thermo, West Palm Beach, FL) with reverse phase Microsorb-MV C18 column (100Å, 3 µm x 4.6 mm x 10 cm, Varian) kept at 42˚C. Post-column alkalinization with 4% NaOH enhanced fluorescence. Peaks were detected with a fluorescence detector at an excitation wavelength of 350 nm and emission wavelength of 478 nm. Isocratic mobile phase at flow rate 1.1 mL/min (0.05M KH_2PO_4 with 3% acetonitrile,2.9 pH) was used to achieve PL and PLP separation (150).

Homocysteine Analysis

Before analysis, each sample was supplemented with saline and an internal standard. Then 10 µL of 10% TCEP was added to reduce disulfide bonds. After 30 minutes of incubation, 100 µL of TCA is added to precipitate proteins. This mixture was then vortexed and centrifuged for 10 minutes at 13,040 x g. A mixture (200 µL) of sodium hydroxide and borate buffer was added to each sample vial along with 50 µL of sample supernatant. The vials were vortexed and incubated for 60 minutes at 60˚C on

a heating block. After returning to room temperature, samples were measured by HPLC using reverse phase Microsorb-MV C8 column (100Å, 5 µm x 4.6mm x 15cm, Varian) maintained at 23˚C. The autosampler temperature was maintained at 4˚C. Isocratic mobile phase (0.05 M acetic acid/acetate buffer, pH 5.2) achieved separation of homocysteine, cysteinylglycine, glutathione, and cysteine by fluorescence detection at an excitation and emission wavelength of 385 nm and 515 nm respectively (151).

Amino Acid Analysis

Cell samples were stored with 500 µL methanol to prevent amino acid degradation. Thawed samples were vortexed then centrifuged for 10 minutes at 3000 x g. The supernatant was removed and dried using a Speed Vac at 35˚C. Samples were reconstituted in 1 mL of 40 mM lithium carbonate buffer (pH 9.5, with HCl). Dansyl chloride dissolved in acetonitrile, 5.56 mM, served as the derivatization reagent. Dns-Cl solution (500 µL) was incubated with the samples for 45 minutes in darkness at room temperature to ensure Dns-amino acid binding. The reaction was quenched by 100 µL of 2% (v:v) enthanolamine. Each sample was filtered (0.45 µm, Fisherbrand) before quantification by HPLC (152). Previously publicized methods did not provide complete separation, so a novel gradient method and column construction was used (152, 153). Mobile phase gradient programs and solvent compositions used are listed in Table 3-2. Luna C18 column (100Å, 5 µm x 4.6mm x 25cm, Phenomenex) and Ultrasphere IP column (5 µm x 4.6mm x 25cm) were used in succession to separate all 21 amino acids at 24˚C.

GC/MS Analysis

A previously validated GC/MS method was modified to quantify lanthionine, homolanthionine and cystathionine. Dowex pipette columns (50WX8-200), packed with

glass wool and containing 0.8 mL of resin (25% (w: v) in 1M NaOH), were equilibrated by acid/base washes. Samples containing 500 µL of the initial cell suspension were acidified with 50 µL of 66% TCA (v:v). The internal standard, norleucine, was added to each sample and standard. This mixture was vortexed, applied to the column and washed with 20 mL of deionized water. The bound amino acids were eluted with 3 mL of NH_4OH into 4 mL reaction vials. Eluates were dried in a Speed-Vac at 35˚C. Once dried, the samples were reconstituted with 10 µL ethanethiol for sulfur containing amino acids and 500 µL of an esterification reagent containing acetyl chloride and 1-propanol. Each sample was capped with nitrogen, vortexed and heated for 40 minutes at 110˚C. After heating, samples were cooled and evaporated with nitrogen at 60˚C. Once the samples dried, they were reconstituted with 100 µL heptafluorobutyric anhydride (HFBA) solution and 10 µL ethanethiol. Samples were capped with nitrogen, vortexed and heated for 40 minutes at 60˚C thereby completing amino acid derivatization. Samples were then dried once more before reconstitution with 200 µL ethyl acetate. Samples were analyzed using GC/MS (154-156).

Homolanthionine Synthesis

To determine the retention time of homolanthionine for HPLC and GC/MS methods, homolanthionine was produced enzymatically. Approximately 7 mM of homocysteine was dissolved 100 mM HEPES buffer, pH 7.4. The sample was preincubated for 5 minutes at 37˚C. Then 40 µL of CSE (2.5 mg/mL in HEPES buffer, provided by Dr. Ruma Banerjee, University of Michigan Medical School) was added and incubated at 37˚C for 45 minutes. The reaction was terminated by the addition of 1 mL of methanol. The sample was centrifuged for 10 minutes at 10,000 x g and the supernatant was transferred into amber vials for storage at -20˚C until analysis (127).

Statistical Analysis

Each concentration of B6 had four replicates. Duplicate injections per sample were performed by HPLC or GC/MS and a complete calibration curve was analyzed after every 10 injections. Amino acid concentrations were identified using Chromeleon software (Thermo Scientific).

Concentrations were statistically analyzed by Dr. Chi at the University of Florida. All data were log transformed to pass the Gaussian assumption. One-way ANOVA with pairwise comparisons was performed for each sample, $P<0.002$. The software used to obtain these results was SAS 9.3.

Results

Vitamin B6 HepG2 Cellular Depletion

Analysis of fetal bovine serum showed that it contained approximately 17 nmol/L PL and negligible PLP, forcing adjustments to the addition of PL. The media targeted to be 10, 50, 200, and 2000 nmol/L PL, respectively, were experimentally determined to be 17.7, 45.3, 239, and 1709 nmol/L PL as shown in Table 3-3.

Four samples per concentration of PL were used to determine weekly PLP status. Concentrations over the 6 week time period are shown as means (Figure 3-1). Amino acid concentration experiments and stable isotope tracer study began after week 6, when a steady PLP concentration of each B6 concentration was maintained for 3 weeks prior. Final PLP concentrations (nmol/mg protein) were 52.5, 105, 123, and 143 for 10, 50, 200, and 200 nmol/L PL respectively at the time of experimentation.

Amino Acid Analysis Method Modifications

Modifications to published amino acid methods allowed 21 amino acids to be separated and determined by HPLC with fluorescence detection. A representative

chromatogram is shown in Figure 3-2. After establishing this method, standard curves were developed for each amino acid, with the exception of homolanthionine. The correlation coefficients for each amino acid are listed in Table 3-4. After the standard curve was deemed adequate, cell and plasma samples were supplemented with approximately 20 µg/mL of specific amino acids to determine the recovery of this assay. The percentage recovery of each amino acid is listed in Table 3-5. After validation of this method, four independent samples of HepG2 cells from each B6 concentration were tested from the week 6 time point.

Homocysteine Analysis Shows Differences between Amino Acid Concentrations in PL Concentration Groups in Cultured Cells and in Extracellular Media

Homocysteine, glutathione (GSH), cysteinylglycine (cysgly), and cysteine were measured in media and cell samples. Each amino acid responded differently to B6 status. Severe (10 nmol/L PL) and marginally (50 nmol/L PL) deficient media contained significantly greater homocysteine concentrations compared to adequate concentrations (200 and 2000 nmol/L PL) of B6. Cellular concentrations of homocysteine did not differ significantly among the various concentrations of PL. GSH concentrations in media also remained approximately constant for all B6 concentrations. In cultured cells, GSH was significantly lower in 10 nmol/L PL (severe B6 deficiency) than in other concentrations of B6. Similar trends were observed for cysgly and cysteine in media; both were significantly greater in 10 nmol/L PL while other B6 concentrations were equivalent. No significant differences were observed for cysgly or cysteine concentrations in cellular analysis. These data for aminothiols are presented in Table 3-6.

Cellular Concentrations of Amino Acids Are Affected by Vitamin B6 Status in HepG2 Cells

The concentration of intracellular amino acids, including lanthionine, cystathionine, and homolanthionine are presented in Table 3-7. GC/MS was used to quantify lanthionine, cystathionine and homolanthionine due to lower detection limits and greater specificity. Homolanthionine was quantified using the cystathionine calibration curve in GC/MS in view of the similarity of these two amino acids and the lack of a commercially available source of pure homolanthionine. Small quantities of homolanthionine were produced spontaneously in the derivatization of homocysteine standards. The amount produced increased in a linear relationship with the concentration of homocysteine. From these amounts, one can conclude endogenous production of homolanthionine was much greater than non-enzymatic production due to derivatization.

Lanthionine, homolanthionine, and cystathionine were three amino acids produced in the transsulfuration pathway by B6 dependent enzymes measured by GC/MS. Homolanthionine and lanthionine concentrations were greater in cells with adequate B6 status. Lanthionine concentration was significantly lower in 10 nmol/L PL and 50 nmol/L PL ($P<0.002$) while homolanthionine trended toward a lower concentration in B6 deficient cells ($P<0.02$). Cystathionine concentration was greater in marginally deficient cells compared to severely deficient cells, $P<0.06$. There was no significant difference in cystathionine concentration between adequate (200 nmol/L PL) and supraphysiological (2000 nmol/L PL) B6 concentrations.

Amino acid analysis by HPLC was used to quantify all other amino acids in Table 3-7. While most amino acids are dependent on B6 for synthesis and/or degradation, not

all showed significant differences in concentration according to B6 restriction. Alanine concentrations were significantly greater in severe (10 nmol/L PL) deficiency compared to marginal (50 nmol/L PL) deficiency and adequate (200 and 2000 nmol/L PL) B6 status. Asparagine, threonine, and valine concentrations were greater in deficient cells compared to other concentrations of B6, ($P<0.002$). Concentrations of leucine ($P<0.01$), lysine ($P<0.008$), and serine ($P<0.03$) trended toward an increase in severe deficiency compared to marginal deficiency. Glycine was significantly greater in severe B6 deficiency compared to marginal deficiency and supraphysiological (2000 nmol/L PL) B6 concentrations. Glutamine concentration was significantly greater in severe and adequate B6 status compared to marginal B6 deficiency and supraphysiological B6 status. Asparagine to aspartate ratios were not statistically different between various concentrations of B6. The ratio of glutamine to glutamate was significantly greater in 10 nmol/L PL compared to 2000 nmol/L PL.

Discussion

This study sought to determine the impact of B6 restriction on metabolism in HepG2 cells by assessing the full profile of amino acids, including the H_2S biomarkers, lanthionine and homolanthionine. Previous research has identified PLP as an essential coenzyme in amino acid metabolism but there has not been a relationship established between B6 status and H_2S production. In addition to quantifying H_2S biomarkers in relation to B6 status, this study also displayed the differences in amino acid concentrations between severe deficiency, marginal deficiency, and adequate B6 status.

In agreement with earlier studies, changes in concentration of amino acids consistent with B6 deficiency were observed. Glycine elevation induced by B6 deficient

media was reported in this study as well as in investigations of both animals and humans, demonstrating a functional change in PLP-dependent metabolism (116). Glycine elevation could be a result of decreased SHMT or glycine decarboxylase activity as both enzymes are sensitive to a loss of activity at varying degrees of B6 (157, 158). Serine elevation in B6 deficient cells may also be explained by impaired SHMT activity although there are many enzymes that are PLP-dependent that catabolize serine. Alanine, asparagine, glutamine, threonine, and valine metabolism were affected by B6 status in this study. While the mechanisms responsible for these significant differences were not studied, one can infer changes were due to the many PLP-dependent enzymes that synthesize or catabolize these amino acids. The ratio of glutamine to glutamate was greater in severely deficient cells (10 nmol/L PL) compared to supraphysiological B6 concentration (2000 nmol/L PL). This is consistent with previous findings in B6 restriction studies in humans (159), although the mechanism is unknown.

Transsulfuration metabolites have been studied extensively in relation to B6 status due to the PLP-dependent enzymes, CBS and CSE. Homocysteine concentration was shown to be inversely associated with PLP concentration in humans (160), but changes are often minor due to tight regulation of one-carbon metabolism. This simple cell model showed a significantly greater concentration of homocysteine in B6 deficient cells, suggesting an imbalance between the transsulfuration pathway and the remethylation cycle. The greater concentration of homocysteine in conjunction with a lower cystathionine concentration ($P<0.06$) could indicate decreased CBS activity in 10 nmol/L PL (120, 124). CBS activity is not affected until severe B6 deficiency

deducing that 10 nmol/L PL was a model of severely deficient cells. Cystathionine elevation is a strong indicator of marginal B6 deficiency ($P<0.09$) (118, 121, 122), which lead to the determination that 50 nmol/L PL was representative of marginal deficiency. Cysteine elevation in media samples in severe B6 deficiency indicated that although CBS and CSE activities decreased, the elevated concentrations of homocysteine forced product formation through the transsulfuration pathway (161). However, the breakdown of cysteine after the transsulfuration pathway also required PLP-dependent enzymes which may have contributed to the elevation in cysteine pools that remained in deficiency. GSH is a final product from the formation of cysteine in the transsulfuration pathway used in many protective roles in the body. Previous research from this laboratory showed contrasting results. Studies by Lima *et al.* and Davis *et al*. showed significant increases in GSH concentrations in human plasma and rat liver when B6 was restricted (124, 162). Lamers *et al*. observed a decrease in GSH synthesis in B6 deficiency and concluded that the effects of marginal deficiency on GSH synthesis were not caused by altered precursor concentrations (123). In the present study, GSH was significantly decreased in severely deficient media. Due to the elevation of cysteine but decrease in GSH concentration, it is suggested that cysteine concentration was not a limiting factor in GSH synthesis.

H_2S and its biomarkers, lanthionine and homolanthionine, are by-products of the transsulfuration pathway. Previous publications have reported a range of values for H_2S concentrations in tissues and biological fluids, which indicates that methods of direct quantification of H_2S may be unreliable. Therefore, the quantification of lanthionine and homolanthionine could be used as indirect measurements for H_2S

production (144). This study demonstrated that lanthionine and homolanthionine were readily measurable by GC/MS in cell extracts. Lanthionine and homolanthionine concentrations were lower in B6 deficient cells, suggesting H_2S production and concentration may have also been lower.

In conclusion, B6 deficiency has been linked to increased disease risk, specifically CVD (149). While there are many indicators of CVD, there is no mechanistic understanding of the relationship between B6 deficiency and CVD (149). This study showed that H_2S biomarker concentrations which may be indicative of H_2S production were lower in B6 deficiency. Mouse model studies have shown CSE depletion decreases H_2S levels in the brain, serum, and heart (143). Because H_2S is a vaso-relaxant and essential in smooth muscle function, it demonstrates a possible explanation of how B6 deficiency increases CVD risk (133, 135, 141, 143). This preliminary study allows for mechanistic effects to be further investigated.

Table 3-1. Final storage conditions for each flask collected for weekly analysis.

Assay	Amount of Cells (µL)	Sample additions
Homocysteine	50	
Bradford	100	300 µL H_2O
PLP	500	500 µL 10% TCA
Amino Acid	500	500 µL Methanol

Table 3-2. Mobile phase gradients programs for separation of Dns-amino acids. Mobile Phases: A. 0.6% acetic acid, 0.08% triethylamine (v:v) in DiH_2O. B. 0.6% acetic acid, 0.08% triethylamine (v:v) in 80/20 acetonitrile/ DiH_2O. Gradient Shapes: 0, no slope; 5, linear.

Time	Acetonitrile (%)	Mobile Phase A	Mobile Phase B	Gradient Shape
0	26.4	67	33	5
33	28.0	65	35	5
37	40.0	50	50	5
50	42.4	47	53	0
54	42.4	47	53	5
72	44.8	44	56	5
86	56.0	30	70	5
97	80.0	0	100	0
102	80.0	0	100	5
104	26.4	67	33	0
114	26.4	67	33	0

Table 3-3. PL concentrations in media determined by HPLC.

Media Concentration (nM)	Actual PL (nM)
10	17.7
50	45.3
200	239
2000	1709

Table 3-4. Correlation coefficients for each amino acid standard curve.

Amino Acid	Correlation Coefficient
Histidine	0.982
Arginine	0.993
Asparagine	0.990
Glutamine	0.987
Serine	0.986
Glutamate	0.979
Hydroxyproline	1.00
Aspartate	0.965
Threonine	0.989
Methionine Sulfone	0.975
Glycine	1.00
Alanine	0.992
Proline	1.00
Methionine	0.998
Valine	0.999
Tryptophan	0.995
Isoleucine	0.988
Phenylalanine	0.965
Leucine	1.00
Lanthionine	0.888
Cystathionine	0.970
Cysteine/Cystine	0.996
Lysine	1.00
Tyrosine	1.00

Data is expressed in 3 points in the standard curve and replicated before each run.

Table 3-5. Recovery analysis for each amino acid in cell analysis.

Amino Acids	Percentage Recovery
Histidine	102
Arginine	116
Asparagine	105
Glutamine	107
Serine	79.5
Hydroxyproline	93.1
Threonine	101
Methionine Sulfone	86.4
Glycine	99.8
Alanine	102
Proline	104
Methionine	100
Valine	99.7
Tryptophan	100
Isoleucine/Phenylalanine	104
Leucine	93.5
Lanthionine	90.8
Cystathionine	91.5
Lysine	93.2

Data expressed as mean, n=3.

Table 3-6. Intracellular and extracellular amino acid concentrations.

Pydridoxal Concentration	10 nM	50 nM	200 nM	2000 nM
		Media (nmol/mg protein)		
Homocysteine	13.2±1.56[a]	9.78±0.432[a]	5.13±0.572[b]	5.93±0.505[b]
GSH	1.02±0.222	1.06±0.249	1.07±0.140	1.17±0.099
CysGly	6.66±0.693[a]	5.34±0.190[b]	5.46±0.341[b]	4.58±0.389[b]
Cysteine	59.1±6.23[a]	35.7±1.99[b]	29.9±3.63[b]	33.6±2.56[b]
		Cell (nmol/mg protein)		
Homocysteine	0.217±0.128	0.209±0.075	0.121±0.036	0.142±0.052
GSH	129±14.1[b]	174±15.8[a]	168±12.1[a]	153±4.60[a,b]
CysGly	1.77±0.039	2.01±0.394	3.51±1.29	1.89±0.110
Cysteine	61.9±3.44	50.3±3.63	55.3±2.92	52.3±2.35

Values are means ±SD, n=4. Data analyzed by ANOVA. Means with unlike superscripts in the same row are different, $p<0.002$.

Table 3-7. Intracellular amino acid concentrations.

Pyridoxal Concentration	10 nM	50 nM	200 nM	2000 nM
		nmol/mg protein		
Alanine	64.9±8.96[a]	29.5±4.54[b]	57.5±2.64[a]	46.2±3.22[a]
Arginine	6.58±0.30	2.87±1.58	7.07±1.2	6.16±0.974
Asparagine	11.9±0.873[a]	8.79±0.730[a]	10.07±1.08[a]	7.71±0.625[b]
Aspartate	39.7±7.08	35.6±16.34	53.1±13.3	40.9±10.71
Glutamate	122±30.1	119±9.80	189±17.1	132±24.3
Glutamine	111±11.3[a]	63.6±6.44[b]	131±3.46[a]	72.8±2.65[b]
Glycine	30.1±0.935[a]	19.8±1.92[b]	25.2±1.12[a]	19.8±0.914[b]
Histidine	18.5±3.56	20.2±5.31	11.8±1.86	10.8±3.28
Leucine	12.8±0.36	8.07±1.92	11.4±0.959	9.02±0.778
Lysine	11.9±1.40	8.85±0.968	9.86±0.454	9.03±0.663
Methionine	2.97±0.392	1.71±0.274	3.35±0.264	1.43±0.269
Proline	30.7±4.34	30.4±1.68	29.0±1.62	28.2±2.28
Serine	13.3±0.867	4.09±2.25	10.7±2.61	8.54±3.22
Threonine	19.5±1.81[a]	6.32±2.33[b]	21.2±3.36[a]	10.4±2.28[a,b]
Tryptophan	2.30±0.342	2.29±0.187	2.31±0.059	2.06±0.125
Tyrosine	4.88±0.846	4.34±0.879	5.04±0.463	4.78±0.647
Valine	9.85±0.386[a]	6.09±0.717[b]	8.76±0.508[a]	6.74±0.448[b]
Cystathionine	4.24±0.215	5.50±0.385	4.71±0.567	4.38±0.793
		pmol/mg protein		
Lanthionine	0.698±1.06[b]	1.38±0.179[b]	1.56±0.181[a]	1.70±0.594[a]
Homolanthionine	93.6±15.8	119±16.8	125±26.2	177±66.0

Values are means ±SD, n=4. Data analyzed by ANOVA. Means with unlike superscripts in the same row are different, $p<0.002$.

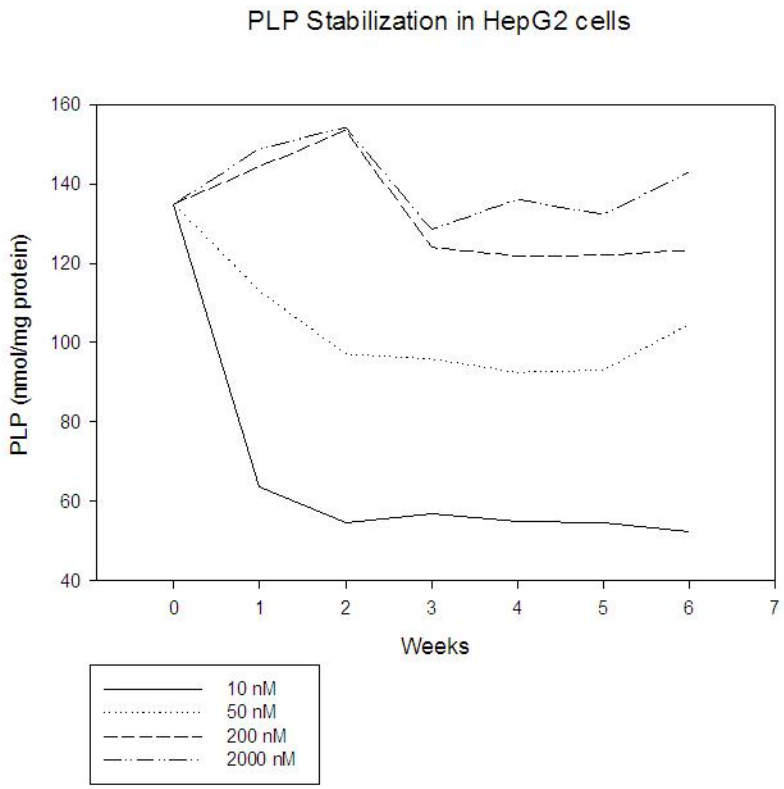

Figure 3-1. Cellular PLP concentration during 6 week stabilization for each concentration of PL.

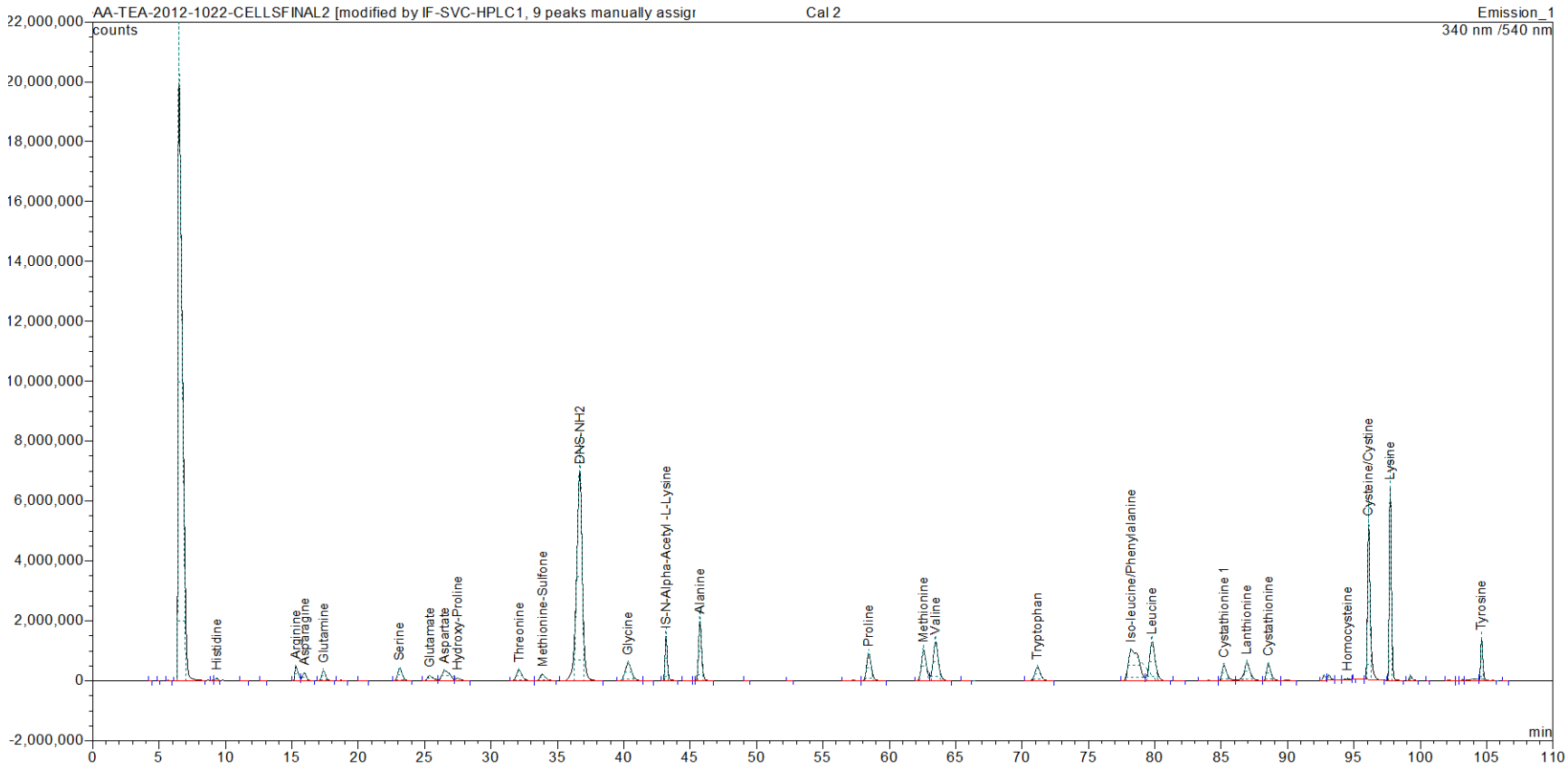

Figure 3-2. A representative chromatogram of amino acid separation.

CHAPTER 4
VITAMIN B6 STATUS IN HEPG2 CELLS AFFECTS THE TRANSSULFURATION AND REMETHYLATION PATHWAYS WHEN ANALYZED BY STABLE ISOTOPE TRACER TIME COURSE

Stable isotope tracer experiments in cell culture and human subjects allow scientists to understand metabolite kinetics over specific time periods. This study focused on quantifying the formation of transsulfuration products. [U-^{13}C] L-methionine and (3,3-D2) L-cysteine were used as precursors of labeled amino acids and H_2S biomarkers in cultured HepG2 cells. Methionine is a precursor of S-adenosylmethionine (SAM) in one carbon metabolism. SAM is the main one carbon donor used in over 100 transmethylation reactions; it is also an allosteric activator of CBS, an enzyme in the transsulfuration pathway. Homocysteine is produced from SAM after which it can either be remethylated for recycling of methionine or shunted into the transsulfuration pathway to form cystathionine and later cysteine. The transsulfuration enzymes catalyze side reactions which produce H_2S and its biomarkers. Condensation of two homocysteine molecules produces homolanthionine while the condensation of two cysteine molecules produces lanthionine. This study was conducted to determine if the labeling of lanthionine and homolanthionine was quantifiable in *in-vivo* stable isotope experiments and also if the kinetics of the transsulfuration pathway were affected in B6 restriction. Previous research in this lab has shown [U-$^{13}C_5$] methionine resulted in [$^{13}C_4$] homocysteine which in turn labeled cystathionine. Fractional synthesis rates were determined and the effect on amino acid metabolism based on B6 status was assessed.

Materials and Methods

Stable Isotope Materials

[U-$^{13}C_5$] L-Methionine, 97-98% purity, and (3,3-D2) L-Cysteine, 98% purity, were purchased from Cambridge Isotopes (Andover, MA). Complete cell culture media was enriched by 20% with [U-$^{13}C_5$] L-Methionine and (3,3-D2) L-Cysteine based upon calculations of methionine and cysteine present in media. Each concentration of B6 (10, 50, 200, and 2000 nmol/L PL) was enriched as stated above. All other chemicals used were HPLC grade of higher and purchased from Fisher Scientific (Pittsburgh, PA) or Sigma Aldrich (St. Louis, MO).

Cellular Preparation

HepG2 cells were maintained as stated previously. After final passage, cells were grown to ~90% confluency and fresh media was added to cell flasks 24 hours prior to experimentation. On the day of the experiment, the media were removed and the cells were washed with DPBS. Enriched media, 15 mL, was added to each 75 cm^2 flask. The time points of this study were 0, 0.5, 1, 2, 4, and 6 hours. At each time point, 500 µL of medium was removed and the cells washed with DPBS. The cells were scraped from the bottoms of the flasks by a rubber spatula and collected in 500 µL DPBS. Cell and medium aliquots (500 µL) were immediately acidified with 50 µL of 66% TCA, vortexed and stored at -80˚C until analysis. The day of analysis, cells were thawed and 100 µL of the internal standard, norleucine, was added. Then samples were homogenized by the ultrasonic homogenizer and both sample sets (cell and media) were centrifuged at 6,100 x g for 15 min. The supernatants were isolated and analysis followed according to GC/MS methods reported previously (154-156).

Kinetic Analysis

[U-$^{13}C_5$] Methionine produced [$^{13}C_4$] homocysteine through the remethylation pathway in one-carbon metabolism. From this point, [$^{13}C_4$] homocysteine could enter the transsulfuration pathway to produce [$^{13}C_4$] cystathionine and α-ketobutyrate or cycle back into [$^{13}C_4$] methionine. Due to homocysteine condensation which produced homolanthionine; the possible products were [$^{13}C_4$] or [$^{13}C_8$] homolanthionine. (3,3-D2) Cysteine produced [D2] or [D4] lanthionine in condensation reactions.

Time points of each amino acid were plotted using SigmaPlot 12.0. Enrichment plateaus (Ep) were determined for each precursor and product that reached steady state within the first 30 minutes. Regression curves from $y=a(1-e^{-bx})$ were applied to each graph to determine Ep and initial rate(I) as represented in Figure 4-8. FSR and ASR were the determined using the following equations. Methionine remethylation and homocysteine production were determined by subsequent equations.

$FSR=I/Ep$ $\quad\quad$ $Remethylation= Ep_{M+4\ methionine}/Ep_{(M+4)+(M+5)\ methionine}$

$ASR=([AA])(FSR)$ $\quad\quad$ $Hcy\ Production= Ep_{M+4\ homocysteine}/Ep_{(M+4)+(M+5)\ methionine}$

Statistical Analysis

All data are presented as mean ± standard deviation. Data were log transformed to pass the Gaussian assumption. One-way ANOVA with pairwise multiple comparisons was used to determine statistical significance between each group of B6 concentrations of FSR and ASR, $P<0.05$. All statistical analyses were performed by SigmaPlot 12.0 software.

Results

The stable isotope tracer experiment produced quantifiable labeled products from [U-$^{13}C_5$] L-methionine and (3,3-D2) L-cysteine. Ratios of labeled to unlabeled amino

acids were plotted versus time (Figures (4-1)-(4-7)). The enrichment plateau for methionine (Table 4-1) in the 10, 50, 200, and 2000 nM PL concentrations indicated ~13% media enrichment. Methionine enrichment plateaus were approximately equivalent in each concentration of B6. The cysteine enrichment was much lower and did not plateau as quickly as methionine (Figure 4-2). The enrichment ratios of cysteine over the six hour time course for 10, 50, 200, and 2000 nmol/L PL were ~1.3% and 50 nmol/L PL was significantly greater than all other enrichment ratios. [$^{13}C_4$] Methionine reached enrichment plateau within 30 minutes, (Figure 4-3) indicating remethylation. While remethylation was significantly different between marginal B6 deficiency (50 nmol/L PL) and adequate B6 status (200 and 2000 nmol/L PL), there did not seem to be a great change in remethylation due to B6 status. [$^{13}C_4$] Homocysteine reached enrichment plateau after 2 hours.

The initial rate of product formation corresponds to the steepest slope in the enrichment curve. The FSR was determined from the enrichment plateau of the precursor directly preceding that amino acid in formation. The apparent ASR was determined by multiplying the FSR with the initial concentration of the amino acid normalized per mg/ protein. The FSR and ASR values for each PL concentration and each labeled product are listed in Table 4-2. FSR of homocysteine was not statistically different between concentrations of B6. FSR of cystathionine was significantly greater in marginal B6 deficiency compared to all other concentrations of B6. [D2] Lanthionine FSR was significantly greater in adequate and supraphysiological concentrations of B6 compared to severe and marginal deficiency. [$^{13}C_4$] homolanthionine FSR was

significantly greater in adequate B6 cells (200 and 2000 nmol/L PL) compared to deficient cells (10 and 50 nmol/L).

Discussion

Stable isotopes are a valuable tool in metabolic kinetic studies. The target enrichment of each precursor in this study was approximately 20%, however [U-$^{13}C_5$] methionine was 13% and [D2] cysteine was 1.3% enriched. The low enrichment of cysteine differed from preliminary experimentation leading to the belief that the stock solution became insoluble or there was human error. The low labeling of cysteine did produce a sufficiently labeled lanthionine product enabling FSR determination. [U-$^{13}C_5$] Methionine reached an enrichment plateau within the first 30 minutes of the 6 hour time course whereas (3,3-D2) cysteine did not, most likely due to its ability to disulfide bond reversibly. Remethylation of methionine determined by [$^{13}C_4$] methionine indicated very small changes due to B6 status.

By labeling amino acids, the fractional synthesis rates of products via the transsulfuration pathway were determined, also determining the synthesis of H_2S biomarkers. Homocysteine FSR as well as homocysteine production rate did not change between B6 concentrations; supporting previous studies (117, 125). Elevations of homocysteine concentration were seen in media samples in deficient cells yet there was no change in FSR. This suggests that the homocysteine pool was greater due to the decreased activity of the transsulfuration enzymes, not because of greater synthesis from the methionine precursor.

While Lamers *et al*. showed an increase in the FSR of cystathionine in B6 restriction in human patients, Davis *et al*. observed no effects of B6 restriction on cystathionine FSR(117, 122). Our study showed severely deficient cells (10 nmol/L PL)

had a lower cystathionine FSR, whereas cells with marginal deficiency (50 nmol/L PL) had a higher cystathionine FSR compared to cells in adequate (200 and 2000 nmol/L PL) B6 concentrations. The concentration of cystathionine in severely deficient cells is also lower than in 50 nmol/L PL concentration. This could be due to decreased CBS activity or dysregulation of SAM since the pool of homocysteine was elevated but the synthesis and concentration of its catabolic product, cystathionine, was depressed. Martinez *et al* showed SAM liver concentration was decreased in rats fed a B6 deficient diet (125). Cystathionine FSR and concentration were greater in marginally deficient cells compared to other B6 concentrations. Cystathionine concentration elevation has been shown to be an indicator of marginal B6 deficiency thereby supporting our conclusion that 50 nmol/L PL represents physiological marginal deficiency (121, 124).

In addition to the traditional products of the transsulfuration pathway (homocysteine, cystathionine, and cysteine), lanthionine and homolanthionine were also measured for enrichment and FSR. [$^{13}C_4$] Homolanthionine and lanthionine FSRs were significantly greater in cells of adequate (200 and 200 nmol/L PL) B6 status compared to cells deficient (10 and 50 nmol/L PL) in B6. Greater synthesis of homolanthionine and lanthionine in B6 adequacy suggests the production of H_2S was also greater in cells of adequate B6 status (127). Dually labeled homolanthionine and lanthionine were visibly present in the spectrum but too low to reliably quantify for this experiment. There was, however, a rise in presence of these products throughout the time course.

In conjunction with the conclusion that greater H_2S biomarker production was associated with higher B6 concentrations, data from Appendix showed the H_2S production capacity was greater in cells of adequate B6 status compared to deficient

cells. Drs. Ruma Banerjee and Omer Kabil, University of Michigan Medical School, quantified by GC the capacity at which cells in each concentration of B6 could produce H_2S. Cell-lysates placed in buffers containing high concentrations of cysteine and homocysteine (>>K_m) allowed CSE and CBS to produce H_2S at V_{max}. These *in-vitro* conditions allowed assessment of the influence of cellular B6 availability on the capacity to produce H_2S. The ability to produce H_2S and the increased production of H_2S biomarkers observed in this study showed that H_2S production was affected by B6 status in HepG2 cells. Figure 4-9 and 4-10 displayed the relationship between lanthionine and homolanthionine concentration in accordance to H_2S production capacity.

Table 4-1. Enrichment plateaus and methylation cycle kinetics.

	10 nM	50 nM	200 nM	2000 nM
		Ep		
[U-$^{13}C_5$]Methionine	0.130±0.00253	0.131±0.000523	0.128±0.00143	0.137±0.00771
[D2]Cysteine	0.0113±0.00103[b]	0.0192±0.000676[a]	0.0122±0.00238[b]	0.0101±0.000830[b]
[$^{13}C_4$]Methionine	0.0187±.000483[a]	0.0107±0.000231[b]	0.0198±0.00138[a]	0.0213±0.00253[a]
Methionine Remethylation	0.162±0.00342[a, b]	0.145±0.00156[b]	0.174±0.0110[a]	0.177±0.0160[a]
Homocysteine Production	0.972±0.0608	1.03±0.0248	0.964±0.0393	0.980±0.0827

Values are means ±SD, n=4. Data analyzed by ANOVA. Symbols indicate statistically difference $p<0.05$.

Table 4-2. FSR and ASR of amino acids in various concentrations of B6.

	10 nM		50 nM		200 nM		2000 nM	
	FSR (hr^{-1})	ASR (pmol/mg protein/hr)	FSR (hr^{-1})	ASR (pmol/mg protein/hr)	FSR (hr^{-1})	ASR (pmol/mg protein/hr)	FSR (hr^{-1})	ASR (nmol/mg protein/hr)
[$^{13}C_4$] Cystathionine	0.135 ±0.0134^{c}	354±35.1^{a}	0.243 ±0.00488^{a}	1100±222^{b}	0.145 ±0.00552^{c}	756±28.8^{c}	0.173 ±.00719^{b}	1310±54.7^{d}
[$^{13}C_4$] Homocysteine	0.941 ±0.137	204±29.6^{a}	0.816 ±0.173	170±36.2^{a}	0.733 ±0.0195	88.7±2.36^{b}	0.708 ±0.0816	100±11.6^{b}
[D2] Lanthionine	0.245 ±0.0788^{b}	0.171±0.0551^{b}	0.106 ±0.0140^{b}	0.148±0.0195^{b}	0.298 ±0.119^{a,b}	0.477±0.191^{b}	0.697 ±0.307^{a}	1.19±0.522^{a}
[$^{13}C_4$] Homolanthionine	0.00662 ±0.00221^{b}	0.620±0.206^{b}	0.00487 ±0.000971^{b}	0.579±0.116^{b}	0.00999 ±0.00364^{a}	1.25±0.455^{a,b}	0.0287 ±0.0224^{a}	5.09±3.97^{a}

Values are means ±SD, n=4. Data analyzed by ANOVA. Symbols indicate statistically difference $p<0.05$.

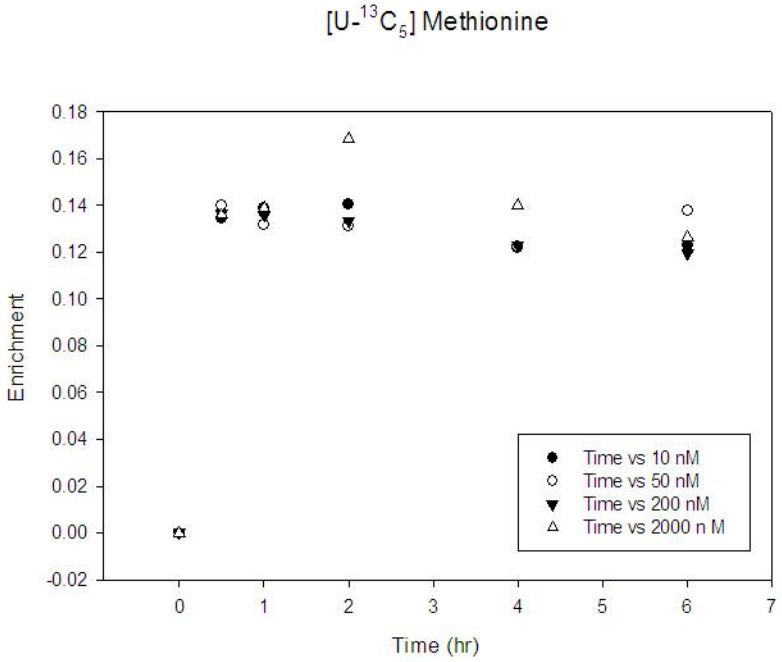

Figure 4-1. Enrichment time course of precursor [U-^{13}C] methionine.

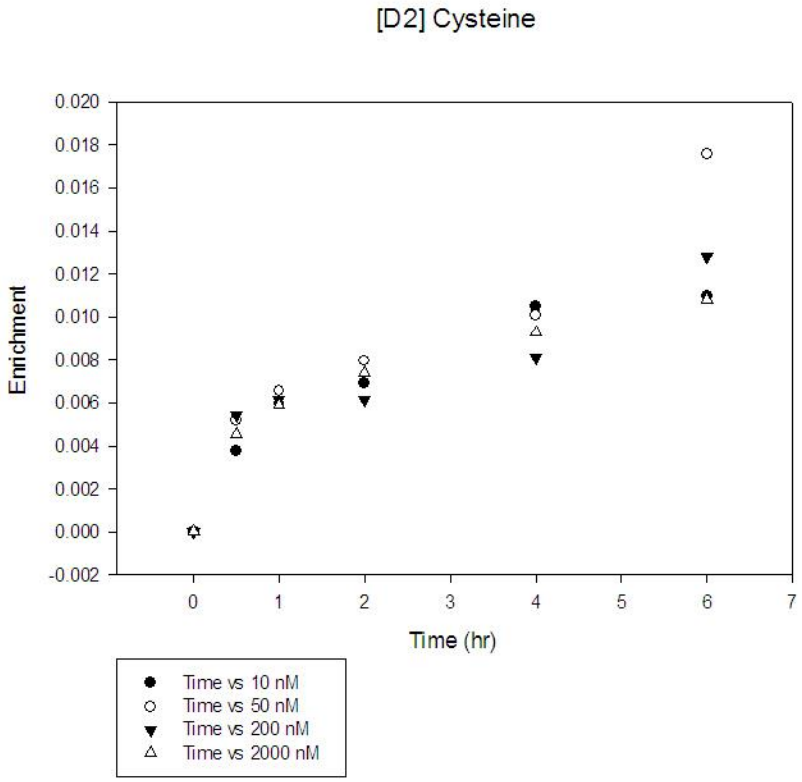

Figure 4-2. Enrichment time course of precursor [D2] cysteine.

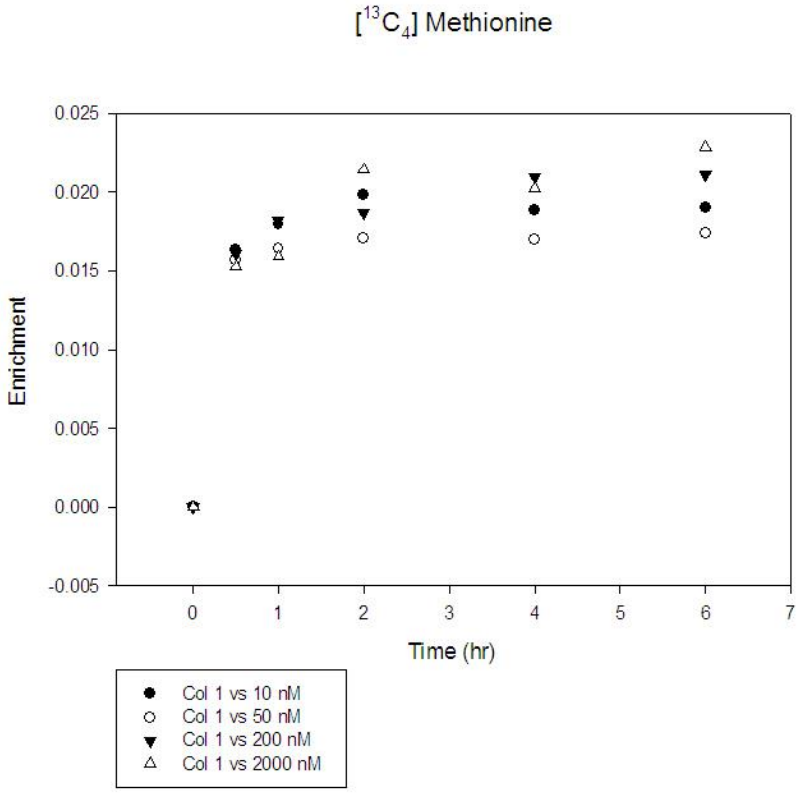

Figure 4-3. Enrichment time course of product [$^{13}C_4$] methionine.

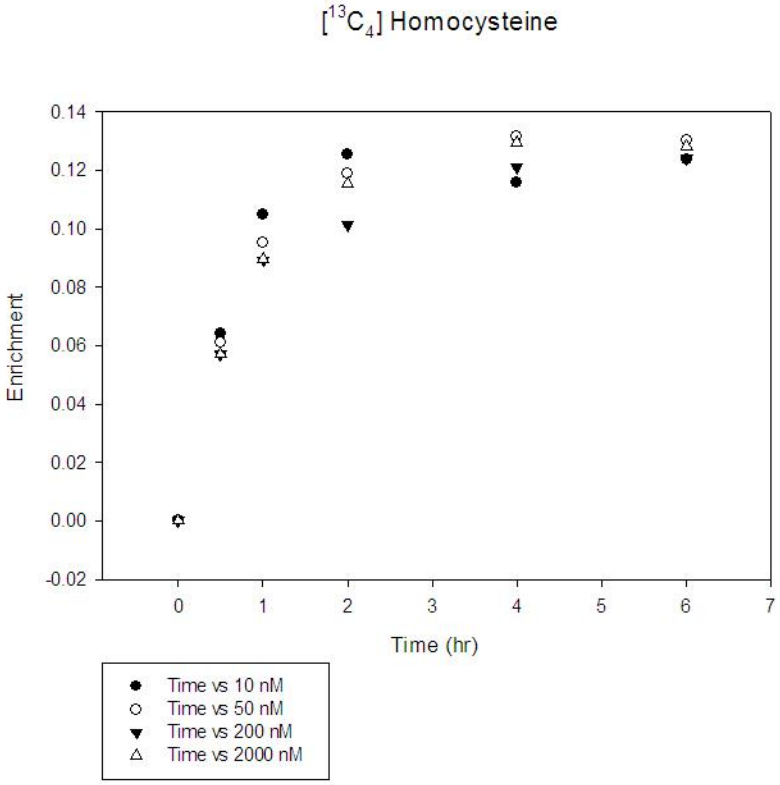

Figure 4-4. Enrichment time course of product [$^{13}C_4$] homocysteine.

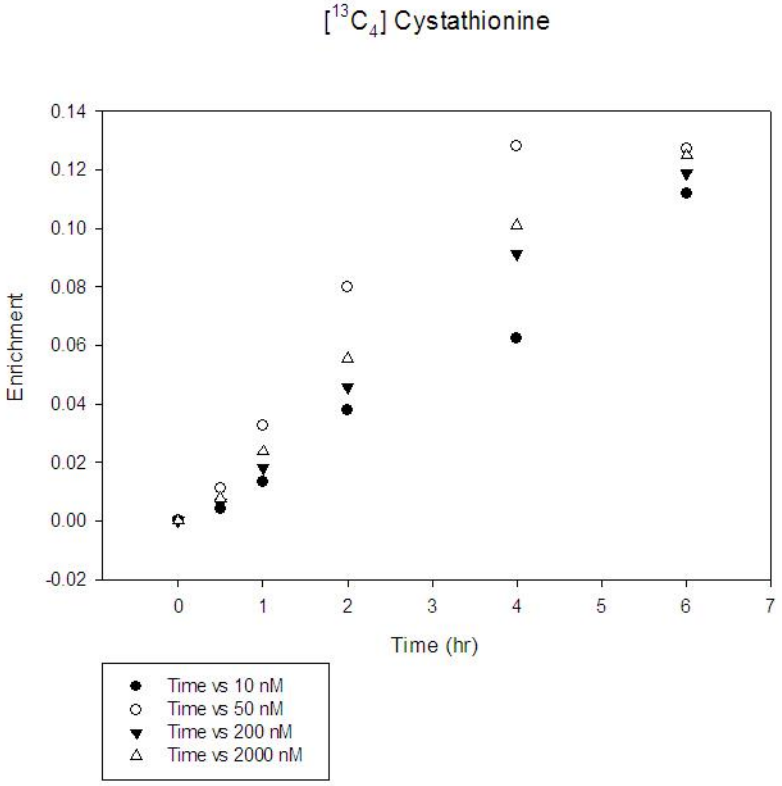

Figure 4-5. Enrichment time course of product [$^{13}C_4$] cystathionine.

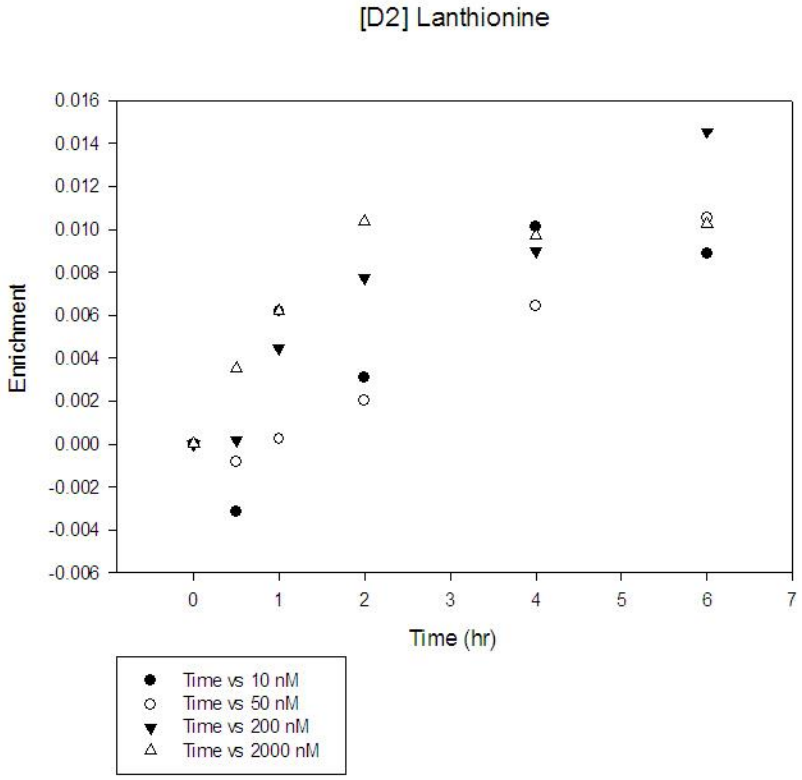

Figure 4-6. Enrichment time course of product [D2] lanthionine.

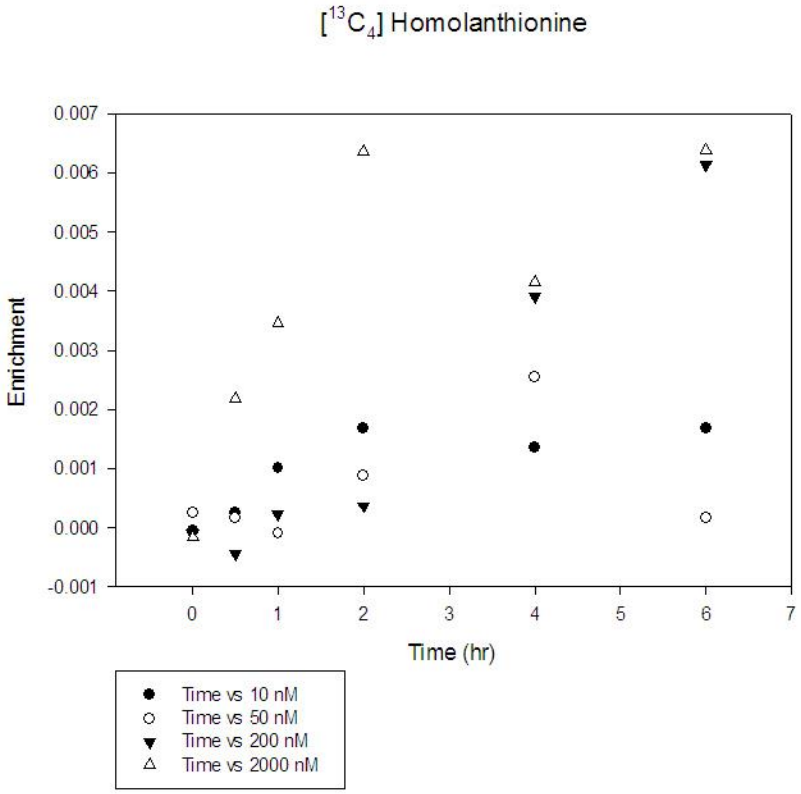

Figure 4-7. Enrichment time course of product [$^{13}C_4$] homolanthionine.

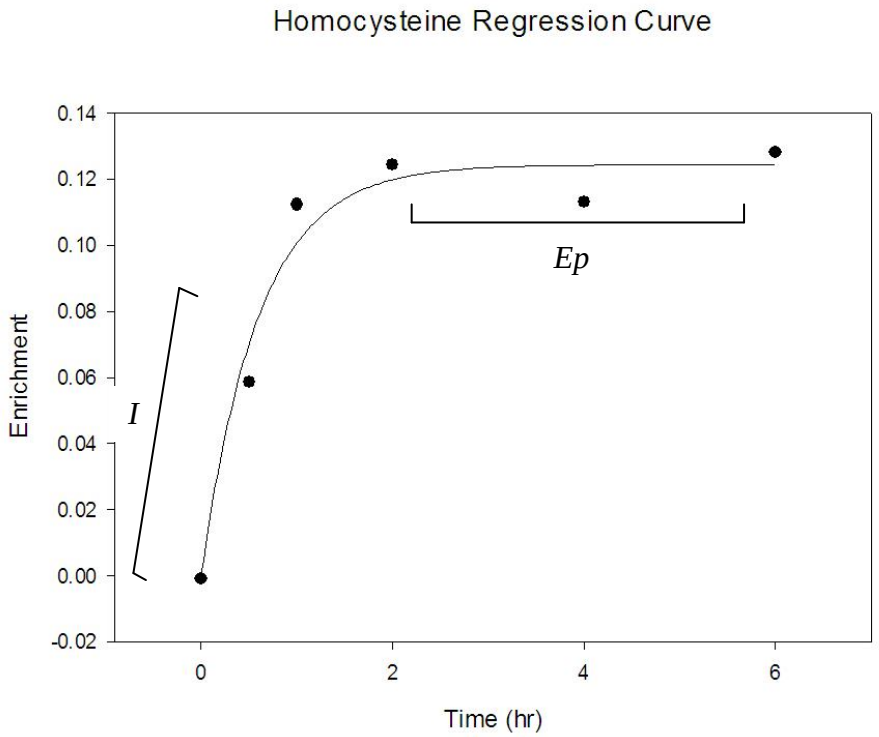

Figure 4-8. Regression curve analysis in SignmaPlot 12.0 determined initial rate (I) and enrichment plateau (Ep) for each amino acid in each sample.

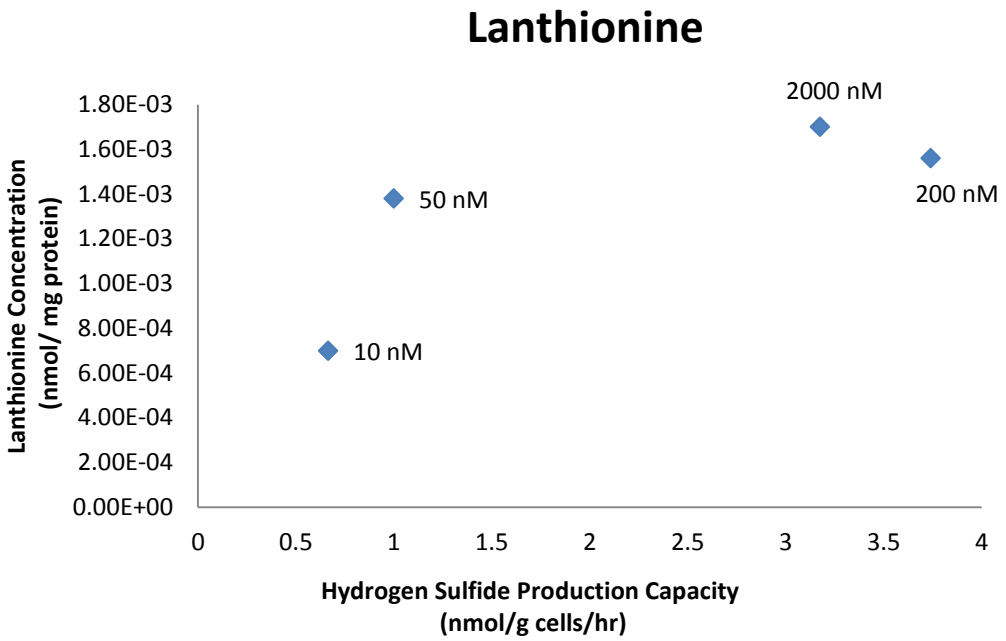

Figure 4-9. Hydrogen sulfide production capacity plotted versus lanthionine concentration in HepG2 cells.

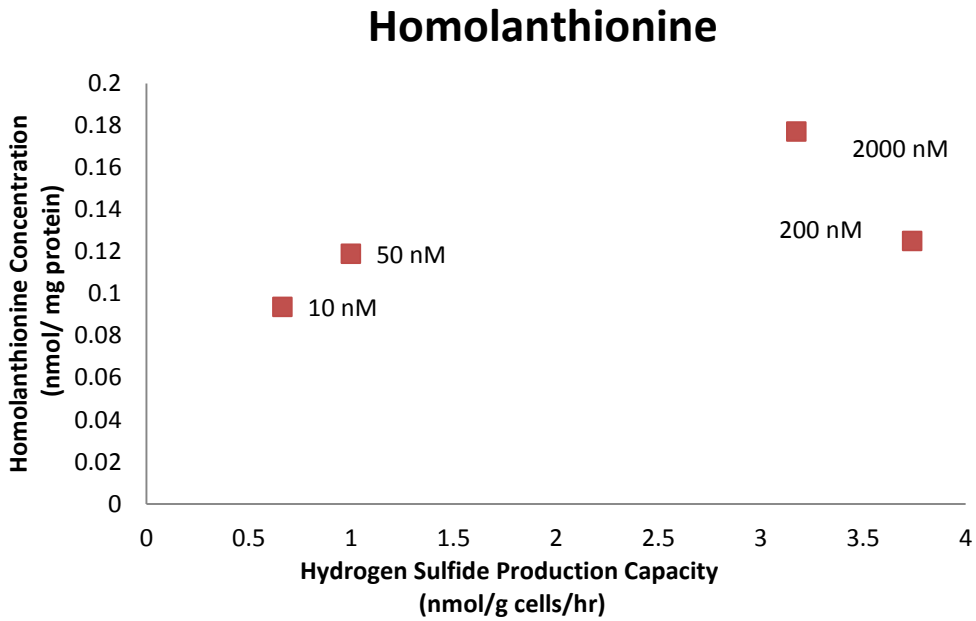

Figure 4-10. Hydrogen sulfide production capacity plotted versus homolanthionine concentration in HepG2 cells.

CHAPTER 5
CONCLUSIONS

The vast number of reactions that require B6 demonstrates its essentiality in bodily functions. From neurotransmitter synthesis, to amino acid metabolism, to disease, B6 is involved in at least one mechanism needed for each process. While most of these mechanisms are well-understood, the mechanism in which B6 relates to CVD is unknown. Several hypotheses have been developed with experimental support but more possibilities exist. This research project sought to determine the relationship between B6 status H_2S production to provide preliminary data for another such mechanism to relate B6 and CVD.

H_2S, once thought to be solely a toxic chemical, has now been shown to be produced and regulated in the body. Endogenously produced H_2S acts as a gas signaling molecule as well as a physiologic vasodilator and regulator of blood pressure (143). CSE and CBS are the main producers of H_2S, both requiring PLP as a coenzyme. Therefore, H_2S biogenesis is PLP-dependent. Since PLP could possibly regulate H_2S production and that production affects vasodilation and blood pressure, both impact factors for CVD, one can theoretically define a relationship between B6 and H_2S modulating CVD risk.

The initial experiment quantified amino acid concentrations in cells of various concentrations of B6 ranging from severely deficient to adequate. Biomarkers of H_2S production, lanthionine and homolanthionine, could indirectly quantify the production of H_2S in the cells since direct quantification was unavailable and most likely unreliable. Data from this experiment showed that severe deficiency (10 nmol/L PL) and marginal deficiency (50 nmol/L PL) impaired the metabolism of amino acids: glycine, alanine,

asparagine, glutamate, leucine, and valine. The ratio of glutamine to glutamate was also affected by B6 status although the mechanism of this result is unknown. The transsulfuration products; homocysteine, cystathionine, glutathione, and cysteine were also affected in deficiency due to decreased activity of CSE and CBS. The concentration of lanthionine and homolanthionine decreased in B6 deficient cells. Most of these data were in agreement with previous studies in humans, cells, or rats on the effects of B6 status on amino acid concentration. This novel determination of H_2S biomarker concentrations gives an initial view into connection between H_2S concentration and B6 status.

The second experiment sought to determine the kinetics of the transsulfuration pathway as well as the methionine cycle by the use of isotopic labeling techniques. H_2S biomarkers production were determined and quantified, indicating endogenous production was substantial. Lanthionine and homolanthionine FSRs were lower in B6 deficiency compared to adequate B6 status. The methylation cycle was not affected by B6 restriction but the activity of the transsulfuration enzymes were, causing significant differences in synthesis rates of transsulfuration amino acids. The inhibition of CBS and CSE as well as the lower FSRs of lanthionine and homolanthionine suggest that H_2S production was inhibited by B6 restriction.

Collaboration with Dr. Ruma Banerjee, University of Michigan Medical School, reinforced the association of H_2S with B6 concentration. *In-vitro* experiments of HepG2 cell-lysates placed in buffers with high concentrations of homocysteine and cysteine induced H_2S production at maximum rate. Thus the limiting factor was the concentration of B6 which influences the activities of CBS and CSE. This experiment

studied the production capacity of H_2S in each concentration of B6. A correlation between B6 concentration and H_2S production capacity was seen among replicates, showing production capacity was decreased in B6 deficiency. These data also showed lanthionine and homolanthionine were accurate biomarkers of H_2S production because their concentrations in cells generally paralleled the production capacity. However, homolanthionine is likely to be a better biomarker due to its resistance to degradation (163).

HepG2 cells responded to B6 deficiency similar to previously published data in rat and human studies. Therefore, HepG2 cells can be used to model physiological results of B6 restriction in humans. Since this is a reliable model, the effect of B6 concentration on lanthionine and homolanthionine concentration should be seen in other studies. These experiments also related the production and concentration of H_2S biomarkers to the production capacity of H_2S. Therefore, by quantifying the concentration of lanthionine and homolanthionine one can infer the production of H_2S is directly related, concluding that B6 status affected H_2S production in HepG2 cells. This project provides preliminary data to support another mechanism in which B6 status is related to CVD.

Presently, H_2S is known as a neuromodulator and vaso-relaxant in many species and its regulation is necessary to maintain normal cardiovascular function. There is a link between B6 deficiency and CVD although the mechanism is uncertain. Future studies in rats and humans could determine if this correlation remains. Furthermore, studies in humans with CVD may identify if these mechanistic predictions

exist in diseased states as well as in the excretory pathways. Continuing research in H_2S production could allow for a reliable method of determining CVD risk.

APPENDIX A
GENERAL METHODS

Protein Concentration Measurement

The Bradford assay was used to determine protein concentration in cells in order to normalize data. BioRad Protein Assay Solution was diluted 1:5 in HPLC grade water. The dye solution was then filtered through Whatman #1 filter paper. Bovine serum albumin (BSA) diluted in to 1, 0.8, 0.4, 0.2, 0.1 mg/mL by serial dilutions to act as a calibration curve. 50 µL of each sample of standard was added to 2.5 mL working dye solution. Samples were vortexed and allowed to sit for 5 minutes before analysis. Samples were read at 595 nm absorbance using a spectrophotometer (Beckman DU 640)(164).

Absorbance was recorded for all samples. A graph of standard concentrations versus absorbance allowed for the concentration of samples to be determined. A representative graph from standards is shown below.

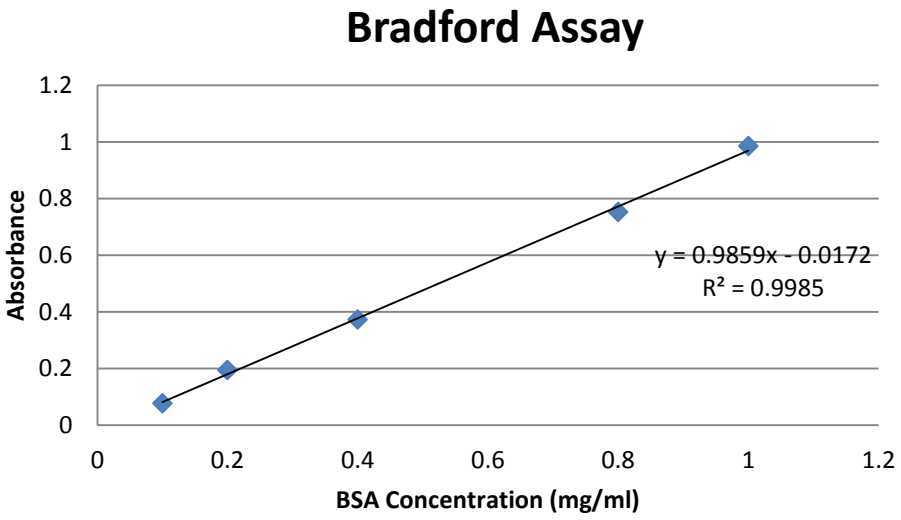

Figure A-1. Absorbance of BSA standards versus concentration of standards provides the linear equation needed to quantify total protein concentration in cell samples.

Hydrogen Sulfide Measurement

Collaboration with Dr. Ruma Banerjee and Dr. Omer Kabil at the University of Michigan resulted in the quantification of H_2S production capacity in the four concentrations of B6. *In-vitro* experiments with cell-lysates of each concentration of PL were analyzed by GC for H_2S concentration. Cell-lysates were added to a buffer containing 10 mM cysteine and 10 mM homocysteine (>> K_m). Concentration of substrates greater than K_m enabled the enzymes producing hydrogen sulfide to operate at V_{max} with B6 concentration acting as the limiting factor. These experiments were replicated 5 times on separate days. Figure A-2 shows H_2S production capacity was decreased in cells under B6 restriction. This data supplements results found from quantifying H_2S biomarkers as indicators of H_2S production.

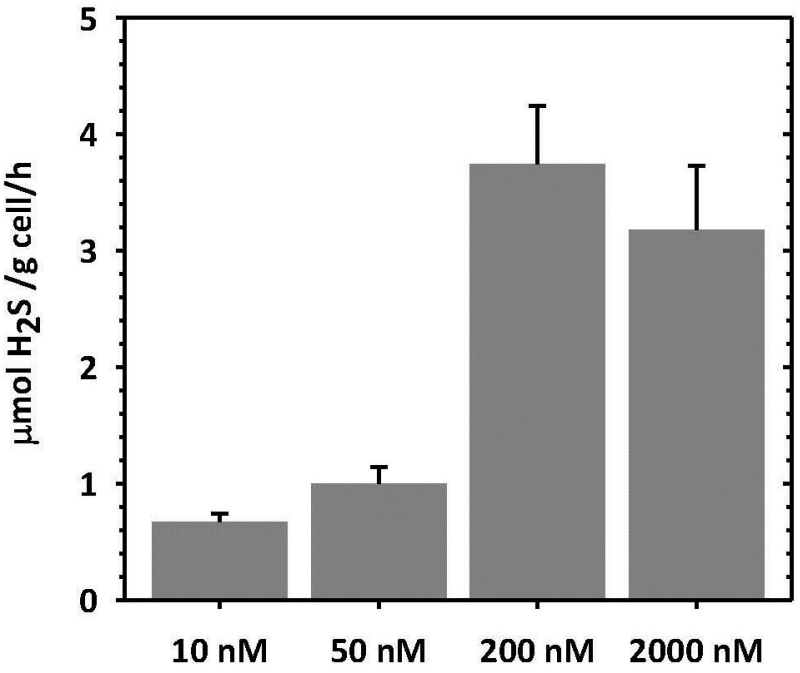

Figure A-2. Hydrogen sulfide production capacity differs under varying concentrations of B6 (10, 50, 200, and 2000 nmol/L PL), displayed as mean ± standard deviation.

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BIOGRAPHICAL SKETCH

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