Novel properties of the multifaceted drug phenelzine and its metabolite β-phenylethylidenehydrazine

by

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ABSTRACT

Phenelzine is a monoamine oxidase (MAO) inhibitor that has been used for the treatment of depression and anxiety disorders since the 1960s. In recent years, there has been renewed interest in this drug following reports of its neuroprotective properties in animal models of cerebral ischemia, multiple sclerosis and traumatic brain injury. It has been suggested that phenelzine is metabolized to an active metabolite, namely β-phenylethylidenehydrazine (PEH), by the action of MAO. PEH appears to share some of the neuroprotective properties of phenelzine and may be an interesting new drug in its own right. The work presented in this thesis has investigated the neurochemical effects of phenelzine and PEH and discussed these findings with regard to neuroprotection.

First of all, the metabolism of phenelzine by human MAO-B was examined: this has included confirmation that PEH is a major product of phenelzine oxidation by the enzyme *in vitro*, studies examining the rate of PEH hydrolysis in aqueous media and elucidation of the mechanisms of MAO-B inhibition by phenelzine. Moreover, the effects of phenelzine and geometric isomers of PEH, (*E*)- and (*Z*)-PEH, on rat whole brain levels of amino acids, biogenic amine neurotransmitters and methylamine were compared. Both (*E*)- and (*Z*)-PEH appeared to be equivalent in their neurochemical properties under the conditions used in this study. Phenelzine and the PEH isomers produced marked increases in rat brain levels of γ-aminobutyric acid (GABA) and alanine while reducing levels of glutamine. Phenelzine, but neither PEH isomer, considerably elevated rat brain levels of serotonin, noradrenaline and dopamine. Rat brain levels of methylamine, a substrate for primary amine oxidase (PrAO), were elevated for all three drugs; however, the effect of phenelzine on methylamine was more transient in comparison to both PEH isomers. These findings provide support for the ability of phenelzine and PEH to

inhibit PrAO, an enzyme that catalyzes the formation of toxic aldehydes and whose activity and expression have been reported to be increased in Alzheimer's disease, in the rat brain. In addition, administration of both PEH isomers and phenelzine resulted in dramatic increases in rat whole brain tyrosine levels. It appears that the tyrosine-elevating property of phenelzine is mediated by PEH, as pre-treatment with another MAO inhibitor abolished the effect of phenelzine, but not of PEH, on brain tyrosine levels. Furthermore, phenelzine and PEH were effective at sequestering the toxic reactive aldehydes acrolein, malondialdehyde and methylglyoxal *in vitro*. In mouse cortical neurons, phenelzine and PEH attenuated acrolein-induced toxicity in a dose-dependent manner. However, neither phenelzine nor PEH reduced rat whole brain levels of extractable acrolein; phenelzine, but not PEH, reduced rat whole brain malondialdehyde levels.

The findings presented in this thesis suggest that phenelzine and PEH possess several properties that may be relevant to neuroprotection. As such, they may prove to be useful adjunctive drugs in the treatment of numerous neurological disorders, such as cerebral ischemia, epilepsy, Alzheimer's and Parkinson's diseases. Further investigation of phenelzine and PEH with regard to application in these disorders is warranted.

PREFACE

Chapter 2 of this thesis was done in collaboration with Dr. Andrew Holt, Department of Pharmacology at the University of Alberta. I performed the experiments, data analysis and composition of the chapter. A. Holt was involved with concept formation, data analysis and interpretation, as well as the composition and editing of the chapter. Ines Zuna, a MSc student of A. Holt, contributed to the performance of some of the spectrophotometric experiments.

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A portion of the introduction and discussion of Chapter 5 of this thesis has been published as Matveychuk, D., Dursun, S. M., Wood, P.L. and Baker, G.B. Reactive aldehydes and neurodegenerative disorders. *Bulletin of Clinical Psychopharmacology* 2011; 21(4): 277-288. I was responsible for the literature search and manuscript composition. S.M. Dursun, P.L. Wood and G.B. Baker contributed to the composition and editing of the manuscript.

A section of Chapter 5 regarding acrolein toxicity in mouse cortical neurons was done in collaboration with Dr. Satyabrata Kar, Department of Psychiatry at the University of Alberta. S. Kar was involved with supervision of the experiments. Yanlin Wang, a PhD student of S. Kar, was responsible for obtaining and treating cultured mouse cortical neurons, assessing cell viability and data collection.

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LIST OF ABBREVIATIONS

ε	molar absorptivity coefficient
ADR	adrenaline
ALA	L-alanine
Aβ	amyloid-β
ANOVA	analysis of variance
ARG	L-arginine
AADC	L-aromatic amino acid decarboxylase
ASP	L-aspartate
CNS	central nervous system
CSF	cerebrospinal fluid
DOPAL	3,4-dihydroxyphenylacetaldehyde
L-DOPA	3,4-dihydroxyphenylalanine
DOPEGAL	3,4-dihydroxyphenylglycoaldehyde
DMSO	dimethyl sulfoxide
DA	dopamine
EI	electron ionization
EDTA	ethylenediaminetetraacetic acid
EAE	experimental autoimmune encephalomyelitis
FBS	fetal bovine serum
FAD	flavin adenine dinucleotide
GABA	γ-aminobutyric acid
GC	gas chromatography
GLU	L-glutamate

GLN	L-glutamine
GLY	glycine
HBSS	Hank's balanced salt solution
HPLC	high performance liquid chromatography
HVA	homovanillic acid
H_2O_2	hydrogen peroxide
5-HT	5-hydroxytryptamine, serotonin
HNE	4-hydroxy-2-nonenal
5-HIAA	5-hydroxyindole-3-acetic acid
6-OHDA	6-hydroxydopamine
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPA	hypothalamic-pituitary-adrenal axis
IC_{50}	half-maximal inhibitory concentration
i.p.	intraperitoneal
IBC	N-isobutyryl-L-cysteine
K_i	inhibitor constant
LNAA	large neutral amino acid
LC-MS	liquid chromatography combined with mass spectrometry
MS	mass spectrometry
m/z	mass-to-charge ratio
mRNA	messenger ribonucleic acid
MAO	monoamine oxidase
MAOI	monoamine oxidase inhibitor
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NCI	negative chemical ionization
NAD^+	nicotinamide adenine dinucleotide (oxidized)
NADH	nicotinamide adenine dinucleotide (reduced)
NA	noradrenaline
OD	optical density
OPA	o-phthaldialdehyde
PFBA	pentafluorobenzaldehyde
PFBHA	pentafluorobenzyl hydroxylamine
PFBSC	pentafluorobenzenesulfonyl chloride
PFB	pentafluorobenzyl
PEH	β-phenylethylidenehydrazine
PBS	phosphate-buffered saline
PrAO	primary amine oxidase
PLP	pyridoxal-5'-phosphate
ROS	reactive oxygen species
SSRI	selective serotonin reuptake inhibitor
SSAO	semicarbazide-sensitive amine oxidase
SIM	selective ion monitoring
D-SER	D-serine
L-SER	L-serine
SEM	standard error of the mean
TAUR	taurine
TCP	tranylcypromine
TAT	tyrosine transaminase/aminotransferase

TH	tyrosine hydroxylase
UV	ultraviolet
VAP-1	vascular adhesion protein-1

CHAPTER 1.

General introduction

1.1. FOREWORD

The past several decades have generated extensive research into the potential common mechanisms underlying the neurodegeneration seen in numerous pathological conditions. Neurodegenerative processes are central to chronic neurological disorders such as Alzheimer's and Parkinson's diseases, as well as to acute neurological trauma seen in cerebral ischemia and traumatic brain injury. With current therapies for many of these disorders offering poor clinical efficacy and often severe adverse effects, there is an urgent need for the development of novel neuroprotective agents that may target numerous pathways implicated in neurodegeneration while maintaining an acceptable level of tolerability to patients.

Phenelzine (β-phenylethylhydrazine) is a drug used clinically since the 1960s for the treatment of depression and anxiety disorders. In recent years, phenelzine has received renewed interest as it was shown to be protective in animal models of cerebral ischemia and traumatic brain injury, as well as to increase functional outcomes in an animal model of multiple sclerosis. In addition to inhibiting monoamine oxidase (MAO), phenelzine has numerous other neurochemical effects that may contribute to neuroprotection, including the ability to dramatically elevate brain levels of γ-aminobutyric acid (GABA), inhibit primary amine oxidase and sequester toxic reactive aldehydes. Interestingly, phenelzine is metabolized by MAO to an active metabolite, namely β-phenylethylidenehydrazine (PEH). Findings have shown that PEH shares the aforementioned neuroprotective mechanisms of phenelzine and is also protective in an animal model of cerebral ischemia but, unlike phenelzine, is a poor inhibitor of MAO. The major drawback to clinical use of phenelzine is the potential for tyramine dietary interactions due to irreversible MAO inhibition; PEH should not be associated with this adverse effect and may represent a useful neuroprotective drug in its own right.

The focus of this thesis is to explore the neurochemical properties of phenelzine and PEH and to interpret how these may be of relevance to the neuroprotective effects exhibited by both drugs. Moreover, the mechanisms of MAO-mediated oxidation of phenelzine to PEH will be explored.

1.2 MONOAMINE OXIDASE

Monoamine oxidase (MAO) is a flavin-containing enzyme found in most mammalian tissues and involved in the oxidation of a wide variety of endogenous and exogenous monoamines (Tipton et al. 2004). Inside the cell, MAO is anchored to the outer mitochondrial membrane with its active site exposed to the cytoplasm. The cofactor flavin adenine dinucleotide (FAD) is covalently linked to MAO's active site. During catalysis, the FAD cofactor is reduced and the amine substrates are oxidized to their respective imines, which are subsequently hydrolyzed (non-enzymatically) to an aldehyde and ammonia (for primary amines) or a substituted amine (for secondary amines) (Binda et al. 2002; Tipton et al. 2004; Youdim et al. 2006). To recycle back to the oxidized state, the reduced MAO flavin reacts with oxygen and generates hydrogen peroxide (H_2O_2) as a by-product (Edmondson 2014). In peripheral tissues (including the liver, intestines, lungs and placenta) and cerebral microvessels, MAO is thought to act as a metabolic barrier by facilitating elimination of exogenous amines (Youdim et al. 2006). In the central nervous system (CNS), physiological functions of MAO include protection from foreign amines, cessation of amine neurotransmitter action and regulation of intracellular amine stores (Youdim et al. 2006).

MAO is present in two main isoforms, MAO-A and MAO-B, with 70% sequence homology. The MAO isoforms are distinguished by their sensitivity to pharmacological inhibition, preference for substrates and localization in tissue. Clorgyline is a selective inhibitor of MAO-A, whereas *l*-deprenyl (selegiline) and mofegiline are selective inhibitors of MAO-B. 5-Hydroxytryptamine (5-HT, serotonin) is mostly metabolized by MAO-A, while benzylamine and phenylethylamine are preferentially oxidized by MAO-B. Dopamine, noradrenaline, adrenaline, tryptamine and tyramine are oxidized by both forms of the enzyme (Youdim et al. 2006). In the primate brain, MAO-A is primarily localized to catecholaminergic neurons, while MAO-B is predominantly found in serotonergic neurons and glial cells (Westlund et al. 1985; Riederer et al. 1987; Westlund et al. 1988). Of interest, MAO-B predominates in the human brain, whereas MAO-A is found in greater concentrations in the rat brain (Kennedy et al. 2004). In addition, structural differences appear to exist between the two MAO isoforms: human MAO-A was found to crystallize as a monomer, whereas human MAO-B crystallizes as a dimer (Youdim et al. 2006).

1.3 MONOAMINE OXIDASE INHIBITORS

Iproniazid was the first MAO inhibitor (MAOI) to be discovered in the 1950s. Originally developed as a treatment for tuberculosis, iproniazid was serendipitously found to elevate the mood of patients. This antidepressant effect was attributed to MAO inhibition and consequent increases in brain levels of serotonin and noradrenaline, neurotransmitters that are thought to be functionally deficient in depression (see Baker and Dewhurst [1985] for review). Since then, a variety of MAOIs have been developed. These drugs can be classified according to reversibility of inhibition (reversible and irreversible) and MAO isoform selectivity (non-selective, MAO-A or MAO-B selective).

It appears that inhibition of MAO-A is necessary for increased brain serotonin and noradrenaline levels and the resultant antidepressant effects. Although serotonin is preferentially oxidized by MAO-A, this MAO isoform is present in low levels in serotonergic neurons. Thus, it has been suggested that MAO-A in glial cells surrounding serotonergic neurons may have a vital role in the breakdown of serotonin (Youdim et al. 2006). While noradrenaline can be oxidized by both MAO isoforms, MAO-A is localized to catecholaminergic neurons. Common irreversible non-selective MAOI antidepressants include phenelzine, tranylcypromine and isocarboxazid. However, a major problem associated with clinical use of irreversible MAO-A inhibitors is the potential dietary interaction with tyramine-containing foods such as aged cheeses and meats, overripe fruits and vegetables, as well as fermented beverages. This adverse reaction is commonly referred to as the “cheese effect”. Irreversible inhibition of MAO-A in the small intestine and liver impairs the metabolism of dietary tyramine, a sympathomimetic agent, allowing it to enter the bloodstream and facilitate the release of noradrenaline from ventrolateral medullary adrenergic neurons. Noradrenaline can then stimulate the cardiovascular sympathetic nervous system and result in adverse effects ranging from headache to hypertensive crisis and death (Youdim et al. 2006). To mitigate this dietary interaction, reversible inhibitors of MAO-A, such as moclobemide, have been developed. Moclobemide possesses antidepressant properties but is associated with a reduced risk of the “cheese effect” as high levels of tyramine can out-compete moclobemide for binding to the MAO-A active site.

Selective irreversible MAO-B inhibitors, including selegiline (*l*-deprenyl) and rasagiline, have also been developed. These drugs are poor antidepressants except at high doses when

selectivity for MAO-B is lost and MAO-A is also inhibited. However, there have been reports that administration of transdermal selegiline can produce an antidepressant effect by delivering doses that inhibit MAO-A in the brain without significantly inhibiting MAO-A in the intestine (Frampton and Plosker 2007). Both selegiline and rasagiline are currently approved for treatment of Parkinson's disease. Although these drugs were shown to have some benefit in treatment of early Parkinson's disease (Parkinson Study Group 1993, 2004, 2005; Rascol et al. 2005), they had weaker effects on symptoms than levodopa or dopamine agonists (Caslake et al. 2009). Furthermore, an analysis of clinical trials with MAO-B inhibitors indicated that there is insufficient evidence to suggest that these drugs can delay Parkinson's disease progression (Macleod et al. 2005). Selegiline was also trialled in the treatment of Alzheimer's disease, with largely disappointing results (Birks and Flicker 2003).

1.4 PHENELZINE

Phenelzine (β-phenylethylhydrazine) is a non-selective irreversible MAOI that has been used clinically since the 1960s for the treatment of major depressive disorder and anxiety disorders, including panic disorder, post-traumatic stress disorder and social anxiety disorder (Sheehan et al. 1980; McGrath et al. 1986; Buigues and Vallejo 1987; Davidson et al. 1987; Liebowitz et al. 1988; Zhang and Davidson 2007). Of interest, phenelzine was found to be particularly effective in the treatment of depression with atypical features, such as the presence of mood reactivity (Thase et al. 1995; Stewart and Thase 2007). Despite its clinical efficacy, phenelzine is rarely used as a first-line treatment due to concerns about dietary interactions with tyramine. In addition, phenelzine can produce weight gain, peripheral edema, orthostatic hypotension and sexual dysfunction (Kennedy et al. 2009). There have also been rare instances of hepatotoxicity associated with phenelzine use (Robinson and Kurtz 1987).

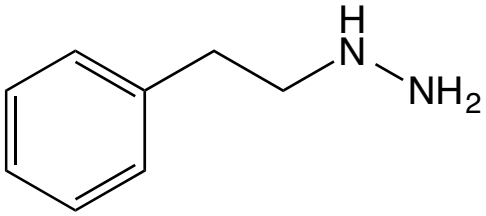
In recent years, there have been numerous reports of neuroprotective properties associated with phenelzine. For example, phenelzine administration reduced neuronal loss in a gerbil model of transient forebrain ischemia (Wood et al. 2006), improved functional outcomes in an experimental autoimmune encephalomyelitis (EAE) mouse model of multiple sclerosis (Musgrave et al. 2011; Benson et al. 2013) and increased cortical tissue sparing in a rat model of traumatic brain injury (Singh et al. 2013). Interestingly, it appears that many of the neuroprotective mechanisms of phenelzine are independent of its ability to inhibit MAO. These

include the ability to significantly elevate brain levels of γ-aminobutyric acid (GABA) due to inhibition of GABA transaminase (Popov and Matthies 1969; Wong et al. 1990; Baker et al. 1991; McManus et al. 1992; Paslawski et al. 1995; Todd and Baker 1995; Todd and Baker 2008), inhibition of primary amine oxidase (PrAO) (Lizcano et al. 1996; MacKenzie 2009) and sequestration of the toxic reactive aldehydes formaldehyde, acrolein and 3-aminopropanal (Wood et al. 2006; Song et al. 2010). These findings will be discussed in further detail in Section 1.6.

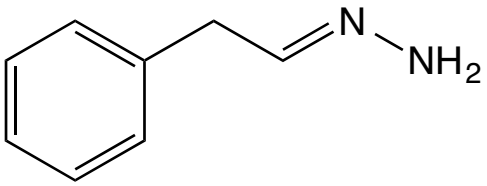
1.5 β-PHENYLETHYLIDENEHYDRAZINE (PEH)

Phenelzine is an interesting MAOI given that in addition to being an inhibitor of MAO, it appears to be a substrate for the enzyme (Clineschmidt and Horita 1969; Tipton 1971) and is oxidized to the imine product β-phenylethylidenehydrazine (PEH) (Tipton and Spires 1972; Patek and Hellerman 1974). The structures of both compounds are shown in **Figure 1-1**. PEH appears to share some neuroprotective properties of phenelzine since it was also found to reduce neuronal loss in a gerbil model of transient global ischemia (Todd et al. 1999). As PEH and phenelzine contain a hydrazine functional group, both compounds were found to be able to sequester the toxic reactive aldehyde formaldehyde and inhibit PrAO (MacKenzie 2009). Furthermore, it has been suggested that PEH is the active metabolite responsible for phenelzine's effects on GABA, given that the phenelzine-induced increase in brain levels of this amino acid can be abolished by pre-treatment with another MAOI (Popov and Matthies 1969; Todd and Baker 1995; MacKenzie 2009). Administration of PEH to rats produces an elevation of brain GABA levels that is comparable to that of phenelzine; however, this effect is not abolished by pre-treatment with another MAOI (MacKenzie 2009).

Although PEH shares many pharmacological properties with phenelzine, it appears to be only a weak inhibitor of MAO-A and MAO-B. While phenelzine was found to inhibit rat whole brain MAO-A and MAO-B by more than 97% and 70%, respectively, for up to 12 hours following administration of the drug, an equal dose of PEH resulted in less than 26% inhibition of either MAO isoform at 1-3 hours and less than 2.5% inhibition at 6-12 hours (Paslawski et al. 2001; MacKenzie et al. 2008a). Furthermore, PEH did not produce any major increases in rat whole brain levels of the MAO substrates noradrenaline, dopamine and serotonin (MacKenzie 2009). Since PEH does not inhibit MAO-A to any appreciable extent, it should not be associated



Phenelzine



PEH

Figure 1-1. Structures of phenelzine (β-phenylethylhydrazine) and PEH (β-phenylethylidenehydrazine).

with tyramine dietary interactions, a major drawback to the clinical use of phenelzine, and may represent a safer alternative to phenelzine.

1.6 THERAPEUTIC MECHANISMS OF PHENELZINE AND/OR PEH

1.6.1 Phenelzine inhibits MAO-A and MAO-B

The ability of phenelzine to exert an antidepressant effect has been attributed to its irreversible and non-selective inhibition of MAO, leading to an elevation of the monoamine neurotransmitters noradrenaline and serotonin in the brain. The mechanism of MAO inhibition by phenelzine is considered to be dependent on the oxidation of phenelzine by the enzyme (Tipton 1971), with some suggesting that either PEH (Tipton 1972) or a diazene intermediate, namely phenylethyldiazene, (Patek and Hellerman 1974; Binda et al. 2008) are responsible for inhibition. Since PEH does not inhibit rat brain MAO-A or MAO-B to any appreciable extent, it is more likely that the mechanism of inhibition proceeds via phenylethyldiazene; this will be explored further using human MAO-B in Chapter 2. The relationship between phenylethyldiazene and PEH is unclear, with speculation that the two products can be formed by separate catalytic pathways (Binda et al. 2008) or by intramolecular rearrangement between phenylethyldiazene and PEH (Patek and Hellerman 1974; Yu and Tipton 1989). The diazene intermediate is very reactive and, in the presence of oxygen, can form an arylalkyl radical that subsequently alkylates the FAD cofactor of MAO, resulting in irreversible inhibition (Binda et al. 2008).

In addition to antidepressant effects, the inhibition of MAO may be associated with some of the neuroprotective properties of phenelzine. The catalytic cycle of MAO results in the production of an imine product and H_2O_2. The imine is then subsequently hydrolyzed to an aldehyde and ammonia (for primary amines) or a substituted amine (for secondary amines). Of interest, high concentrations of ammonia and H_2O_2 have been reported to be neurotoxic (Yang et al. 2003; Wang et al. 2004; Yang et al. 2004; Wood et al. 2006). In addition, MAO-catalyzed oxidation of catecholamines leads to the production of 3,4-dihydroxyphenylacetaldehyde (DOPAL, formed from dopamine) and 3,4-dihydroxyphenylglycolaldehyde (DOPEGAL, formed from norepinephrine and epinephrine), both of which display high toxicity in a variety of *in vitro* and *in vivo* experiments (Eisenhofer et al. 2004; Marchitti et al. 2007). Due to selective targeting

of catecholaminergic neurons, DOPEGAL and DOPAL have been implicated in the etiology of Alzheimer's and Parkinson's diseases (Burke et al. 2003; Burke et al. 2004; Panneton et al. 2010). Taken together, these findings suggest that elevated or sustained MAO activity may lead to deleterious effects on the brain.

In the human brain, the activity of MAO-B, but not MAO-A, was reported to be increased with ageing in a wide variety of studies (Fowler et al. 1980; Sparks et al. 1991; Galva et al. 1995; Fowler et al. 1997; Shemyakov 2001; Volchegorskii et al. 2001; Fowler et al. 2002). In Alzheimer's disease patient brains, activity of MAO-B was increased in comparison to age-matched controls; MAO-A activity was found to be unchanged or increased in certain brain regions (Adolfsson et al. 1980; Oreland and Gottfries 1986; Reinikainen et al. 1988; Jossan et al. 1991; Sparks et al. 1991; Sherif et al. 1992; Saura et al. 1994). Since MAO-B is highly concentrated in glial cells (Riederer et al. 1987), the increase of MAO-B activity in ageing and Alzheimer's disease may be attributed to age- and neurodegeneration-related proliferation of glial cells (Beach et al. 1989; Liu et al. 1996). As such, the use of MAOIs may prevent the excessive production and accumulation of H_2O_2, ammonia and catecholamine-derived aldehydes to toxic levels in ageing and neurodegenerative disorders associated with increased MAO-B and/or MAO-A activity.

1.6.2 Phenelzine and PEH elevate brain levels of GABA

Phenelzine administration has been reported to result in pronounced elevations in rat brain levels of GABA, presumably due to inhibition of GABA transaminase (Popov and Matthies 1969; Baker et al. 1991; McManus et al. 1992; Todd and Baker 1995; Todd and Baker 2008; MacKenzie 2009; Matveychuk et al. 2013). The phenelzine-induced increases in GABA and inhibition of GABA transaminase can be abolished by pre-treatment with another MAOI (Popov and Matthies 1969; Todd and Baker 1995; Todd and Baker 2008; MacKenzie 2009), suggesting that a metabolite of phenelzine formed by MAO is responsible for these effects. PEH appears to be that metabolite, given that administration of PEH also increases brain GABA levels (MacKenzie 2009; Kumpula 2013; Matveychuk et al. 2013) and causes inhibition of brain GABA transaminase (Paslawski et al. 2001; MacKenzie et al. 2008a); these properties of PEH are not affected by prior inhibition of MAO (MacKenzie 2009). GABA transaminase, responsible for breakdown of GABA to succinic semialdehyde in both neurons and astrocytes, is

dependent on pyridoxal-5'-phosphate (PLP) as a cofactor. It has been suggested that the binding of a hydrazine moiety, present on both phenelzine and PEH, to PLP may form a PLP-hydrazone complex that will reduce activity of the enzyme (Yu and Boulton 1991). This is supported by the finding that plasma levels of PLP in 19 patients taking phenelzine were reduced to 54% of a control group (Malcolm et al. 1994). Despite sharing a hydrazine functional group, it is not apparent why PEH, but not phenelzine (without being metabolized to PEH by MAO), is able to inhibit GABA transaminase. Interestingly, administration of phenelzine and PEH to rats was not able to inhibit GABA transaminase activity by more than 50% even at very high doses *ex vivo* (Popov and Matthies 1969; MacKenzie and Baker, unpublished observations) despite the ability of both drugs to almost completely inactivate GABA transaminase *in vitro* (MacKenzie 2009).

The ability of phenelzine and PEH to potentiate GABA levels may have considerable therapeutic relevance. It has been suggested that the anti-anxiety properties of phenelzine are linked to increases in brain GABA levels, as phenelzine produced a clear anxiolytic effect in a rat model of the elevated plus-maze only at doses that increased brain GABA by more than two-fold whereas N^2-acetylphenelzine, a potent inhibitor of MAO but with no GABAergic effects, produced no significant anxiolytic effect (Paslawski et al. 1996). Perturbations in GABA levels may also play a role in affective disorders as patients with depression have been reported to exhibit a deficiency of GABA in cerebrospinal fluid (Gold et al. 1980; Gerner and Hare 1981), plasma (Petty and Sherman 1984; Petty et al. 1990; Petty et al. 1992) and occipital cortex (Sanacora et al. 1999; Kugaya et al. 2003; Sanacora et al. 2004), with electroconvulsive therapy or selective serotonin reuptake inhibitor (SSRI) treatment shown to normalize occipital cortex GABA levels (Sanacora et al. 2002; Sanacora et al. 2003).

Phenelzine and PEH may also be of use in treatment of epilepsy as several established anti-epileptic drugs target GABAergic pathways by augmenting $GABA_A$ receptor activation (benzodiazepines and phenobarbital), inhibiting GABA reuptake (tiagabine) or inhibiting GABA transaminase (vigabatrin) (Howard et al. 2011). Phenelzine is much more potent than vigabatrin at elevating rat whole brain GABA levels (Todd and Baker 2008) and it is expected that the same would be the case with PEH. In addition, PEH, in an *in vitro* rat hippocampal slice model of epilepsy, was able to increase tissue GABA levels and attenuate epileptiform burst frequency (Duffy et al. 2004).

Moreover, the protection of neuronal cells by phenelzine and PEH in a gerbil model of transient global ischemia (Todd et al. 1999; Wood et al. 2006) may be associated with GABA transaminase inhibition. Since GABA is the main inhibitory neurotransmitter in the CNS, increased levels of GABA may counteract pathological increases in excitatory glutamatergic neurotransmission, termed excitotoxicity, by hyperpolarizing the presynaptic membrane (thus preventing glutamate release) and the postsynaptic membrane (thus preventing cell excitation by glutamate) (Schwartz-Bloom and Sah 2001). Excitotoxicity is a central feature of numerous neurological disorders exhibiting a neurodegenerative component, including cerebral ischemia (Choi and Rothman 1990), Alzheimer's disease (Ong et al. 2013; Revett et al. 2013), Parkinson's disease (Olanow and Tatton 1999), amyotrophic lateral sclerosis (Shaw and Ince 1997) and Huntington's disease (Fernandes and Raymond 2009).

1.6.3 Phenelzine and PEH sequester some reactive aldehydes

In recent years, there has been much research dedicated to studying the role of toxic reactive aldehydes in neurodegeneration. These compounds can be produced from a variety of endogenous sources including lipid peroxidation due to oxidative stress, carbohydrate autoxidation and metabolism, cytochrome P450 oxidation of alcohols, myeloperoxidase oxidation of amino acids and catalytic activity of amine oxidases such as MAO, polyamine oxidase and PrAO (O'Brien et al. 2005; Wood 2006). Lipid peroxidation is a source of acrolein, malondialdehyde and 4-hydroxy-2-nonenal (HNE) (Esterbauer et al. 1991; Uchida et al. 1998; Ou et al. 2002); MAO oxidation of catecholamines leads to production of DOPAL and DOPEGAL (Eisenhofer et al. 2004; Marchitti et al. 2007); oxidation of the polyamines spermine, spermidine and 1,3-propanediamine to putrescine by polyamine oxidase, spermine oxidase and diamine oxidase yields acrolein and 3-aminopropanal (Wood et al. 2006); whereas oxidation of methylamine and aminoacetone by PrAO results in production of formaldehyde and methylglyoxal, respectively (Gubisne-Haberle 2004).

Aldehydes are electrophilic and can react rapidly with nucleophilic compounds, especially those containing thiol or amino functional groups. By the mechanism of Michael Addition and/or formation of a Schiff base, aldehydes can form adducts with and cross-link proteins (containing the amino acids histidine, lysine and cysteine), nucleobases of nucleic acids and aminophospholipids (Esterbauer et al. 1991). The formation of these adducts (mostly

irreversible) can lead to multiple deleterious events such as: inhibition of DNA, RNA, and protein synthesis; disruption of protein and cell membrane function; imbalance of calcium homeostasis; and interference with cell respiration and glycolysis pathways (Esterbauer et al. 1991; Lovell et al. 2001; Dang et al. 2010). The aforementioned reactive aldehydes have been shown to be neurotoxic in a wide variety of *in vitro* and *in vivo* experiments (Matveychuk et al. 2011).

There have been numerous reports of increased levels of malondialdehyde and acrolein content in the plasma, serum, erythrocytes and brains of Alzheimer's disease patients (Marcus et al. 1998; Lovell et al. 2001; Casado et al. 2008; Greilberger et al. 2008; Martin-Aragon et al. 2009; Gustaw-Rothenberg et al. 2010; Nam et al. 2010; Padurariu et al. 2010; Sinem et al. 2010). In addition, increased acrolein levels were reported in several brain regions of individuals with early and preclinical Alzheimer's disease (Williams et al. 2006; Bradley et al. 2010), suggesting that accumulation of reactive aldehydes is an early event in the development of the disorder. Furthermore, reactive aldehydes are thought to contribute to the development of several pathological hallmarks of Alzheimer's disease. Malondialdehyde, formaldehyde and methylglyoxal were reported to enhance the rate of amyloid-β (Aβ) oligomer and protofibril formation, as well as to increase the size of the aggregates (Chen et al. 2006). Chronic exposure of rats to acrolein resulted in mild cognitive decline, neuronal loss and astrocyte activation in the hippocampus, up-regulation of β-secretase (BACE-1, responsible for production of Aβ from the amyloid precursor protein) levels in the cortex and down-regulation of α-secretase (ADAM-10, responsible for production of a non-amyloidogenic peptide fragment from the amyloid precursor protein) levels in the hippocampus and cortex (Huang et al. 2013). Acrolein and methylglyoxal were also shown to induce *tau* hyperphosphorylation and accelerate *tau* aggregation into fibrils (Gomez-Ramos et al. 2003; Kuhla et al. 2007; Li et al. 2012).

In Parkinson's disease patients, there are elevated malondialdehyde levels in plasma and cerebrospinal fluid (Ilic et al. 1999; Chen et al. 2009; Serra et al. 2009; Baillet et al. 2010), as well as increased malondialdehyde and acrolein content in the substantia nigra (Dexter et al. 1989; Shamoto-Nagai et al. 2007). As with Alzheimer's disease, it appears that reactive aldehyde accumulation is an early step in Parkinson's disease development as increased levels of malondialdehyde adducts were found in several brain regions of individuals with early stages of

Parkinsonian neuropathology (Dalfo et al. 2005). Acrolein was found to be co-localized with α-synuclein in substantia nigra neurons of Parkinson's disease patients and shown to enhance α-synuclein oligomerization *in vitro* (Shamoto-Nagai et al. 2007). Furthermore, malondialdehyde-modified α-synuclein was discovered in the substantia nigra and frontal cortex of Parkinson's disease patients and individuals with incidental or pre-clinical Parkinson's disease (Dalfo and Ferrer 2008).

Since phenelzine and PEH possess a hydrazine moiety, they are thought to be able to react with aldehydes to form a hydrazone product. Phenelzine and PEH were previously shown to sequester formaldehyde *in vitro* (MacKenzie 2009). In addition, phenelzine was reported to protect neurons and astrocytes against formaldehyde-induced toxicity (Song et al. 2010), exert a protective effect against acrolein and 3-aminopropanal toxicity in rat retinal ganglion cells (Wood et al. 2006) as well as attenuate HNE-induced mitochondrial dysfunction in a rat model of traumatic brain injury (Singh et al. 2013). The ability of PEH and phenelzine to sequester the reactive aldehydes malondialdehyde, acrolein and methylglyoxal *in vitro*, protect against acrolein-induced toxicity in mouse cortical neurons and reduce rat whole brain levels of extractable malondialdehyde and acrolein *ex vivo* were investigated and are reported in Chapter 5 of this thesis.

1.6.4 Phenelzine and PEH inhibit primary amine oxidase (PrAO)

The enzyme PrAO (E.C. 1.4.3.21), formerly called semicarbazide-sensitive amine oxidase (SSAO), is a copper-dependent amine oxidase responsible for the deamination of primary aliphatic and aromatic amines to their corresponding aldehydes, ammonia and H_2O_2 (Precious and Lyles 1988; Lyles 1996). The enzyme is found in two main forms, as a (1) membrane-bound form present in endothelial and vascular smooth muscle cells and adipocytes (Lyles 1996; Jiang et al. 2008), as well as a (2) soluble form circulating in the bloodstream (Abella et al. 2004). It was discovered that PrAO is identical to vascular adhesion protein-1 (VAP-1), an endothelial protein responsible for lymphocyte trafficking and whose expression is induced during inflammation, suggesting that PrAO/VAP-1 may be a dual-function protein that can participate as either an adhesion receptor or an amine oxidase, depending on its location of expression (Smith et al. 1998). Methylamine and aminoacetone have a high affinity for PrAO and are considered to be physiological substrates for the enzyme, undergoing deamination to

their respective aldehydes formaldehyde and methylglyoxal, H_2O_2 and ammonia (Precious and Lyles 1988; Yu 1990; Boor et al. 1992; Lyles and Chalmers 1992; Lyles 1996).

In addition to the presence of copper, PrAO was found to also contain a quinone cofactor (Holt et al. 1998). Compounds containing a hydrazine functional group, including phenelzine and PEH, were suggested to be relatively potent inhibitors of this enzyme due to interaction with a free carbonyl moiety on the quinone cofactor and formation of a quinone-hydrazone complex (Holt et al. 2004). As such, phenelzine has been shown to be a potent inhibitor of bovine lung PrAO *in vitro* (Lizcano et al. 1996), with both PEH and phenelzine subsequently demonstrated to also inhibit human PrAO *in vitro* (MacKenzie 2009). In this thesis, I also address the ability of phenelzine and PEH to increase rat whole brain levels of methylamine, a physiological substrate of PrAO, in Chapter 3.

The activity and/or expression of PrAO has been reported to be increased in diabetes mellitus (Boomsma et al. 1995; Garpenstrand et al. 1999; Meszaros et al. 1999), heart failure (Boomsma et al. 1997; Boomsma et al. 2000) and atherosclerosis (Karadi et al. 2002), as well as hemorrhagic and ischemic stroke (Airas et al. 2008; Hernandez-Guillamon et al. 2012). PrAO may also have a role in the development of Alzheimer's disease, as patients were shown to have increased PrAO activity in blood plasma, overexpression of PrAO in cerebral vasculature and co-localization of PrAO with cerebrovascular Aβ deposits (Ferrer et al. 2002; del Mar Hernandez et al. 2005; Unzeta et al. 2007; Jiang et al. 2008). As methylglyoxal and formaldehyde, products of PrAO oxidation of aminoacetone and methylamine, respectively, have been shown to potentiate Aβ oligomer and protofibril formation (Chen et al. 2006), these aldehydes may contribute for the development of cerebrovascular Aβ deposits seen in Alzheimer's disease patients. Interestingly, the deamination of methylamine by PrAO increased the deposition of Aβ onto blood vessel walls *in vitro* (Jiang et al. 2008). Furthermore, incubation of methylamine with human brain meninges, a tissue with high PrAO content, was shown to produce formaldehyde-protein cross-linkage of bound and soluble proteins (Gubisne-Haberle 2004), an effect that was prevented by prior inhibition of PrAO with a selective inhibitor. Since phenelzine and PEH are inhibitors of PrAO and are also able to sequester the reactive aldehyde products of this enzyme, the use of these drugs may be beneficial in the aforementioned disorders where increased activity and/or expression of PrAO may exacerbate disease pathology.

1.7 THESIS OVERVIEW

The drugs currently used for treating neurodegenerative disorders such as Alzheimer's disease improve the patient quality-of-life for a short period of time but do little to slow the progression of the disease. Moreover, although numerous neuroprotective drug candidates have shown promising results in animal investigations, they are often marred by significant adverse effects and narrow therapeutic windows when introduced into the clinic. As such, there is a pressing need for the development of novel neuroprotective agents that will be well tolerated by patients. Phenelzine, which has been prescribed to patients since the 1960s, appears to be more than just an inhibitor of MAO. As described in this thesis, phenelzine and its active metabolite PEH possess several neuroprotective mechanisms that may be potentially useful in the treatment of neurodegeneration. Furthermore, PEH appears to share numerous neuroprotective properties with phenelzine but is devoid of the potential for adverse dietary interactions with tyramine. Thus, PEH may prove to be an interesting new drug in its own right.

This thesis attempted to address the following questions:

1. What is the mechanism of human MAO-B inhibition by phenelzine? (Chapter 2)
2. Is phenelzine metabolized to PEH by human MAO-B *in vitro*? (Chapter 2)
3. What is the rate of PEH hydrolysis to hydrazine and phenylacetaldehyde *in vitro*? (Chapter 2)
4. What is the effect of phenelzine and geometric isomers of PEH, (*E*)- and (*Z*)-PEH, on rat whole brain levels of amino acids, biogenic amine neurotransmitters and methylamine (a substrate for PrAO)? (Chapter 3)
5. What is the effect of phenelzine, (*E*)- and (*Z*)-PEH on rat whole brain levels of tyrosine? (Chapter 4)
6. Does inhibition of MAO prior to phenelzine or PEH administration abolish the effect of these drugs on rat whole brain tyrosine levels? (Chapter 4)
7. Do phenelzine and/or PEH sequester the reactive aldehydes acrolein, malondialdehyde and methylglyoxal *in vitro*? (Chapter 5)
8. Do phenelzine and/or PEH protect mouse cortical neurons against acrolein toxicity *in vitro*? (Chapter 5)

9. Do phenelzine and/or PEH reduce rat whole brain levels of the extractable reactive aldehydes acrolein and malondialdehyde? (Chapter 5)
10. Is derivatization with pentafluorobenzaldehyde (PFBA) a suitable method for determination of *ex vivo* levels of PEH in rat brain homogenate? (Chapter 6)

1.8 THESIS HYPOTHESES

Based on previous findings in the literature and in preliminary experiments, the following would be expected with regard to the actions of phenelzine and PEH:

Chapter 2: PEH is expected to be a much weaker inhibitor of human MAO-B in comparison to phenelzine. This is based on the rationale that PEH is a weak inhibitor of rat brain MAO-A and MAO-B (Paslawski et al. 2001; MacKenzie et al. 2008a) and does not significantly increase rat whole brain levels of MAO substrates (MacKenzie 2009). Since PEH is a transient inhibitor of rat brain MAO-A and MAO-B, it would be expected to act in the capacity of a reversible inhibitor. In addition, it is unlikely that PEH is the metabolite of phenelzine that is responsible for inhibition of human MAO-B; this effect is likely mediated by production of phenylethyldiazene (Binda et al. 2008).

Chapter 3: It is expected that PEH and phenelzine will increase rat brain levels of the amino acids alanine and GABA, while reducing levels of glutamine and glycine (Popov and Matthies 1969; Wong et al. 1990; Baker et al. 1991; McManus et al. 1992; Paslawski et al. 1995; Todd and Baker 1995; Tanay et al. 2001; Todd and Baker 2008; MacKenzie 2009; Kumpula 2013). Phenelzine, but not PEH, should increase rat whole brain levels of noradrenaline, dopamine and serotonin (MacKenzie 2009). In addition, both phenelzine and PEH have been previously reported to increase rat brain methylamine levels and to inhibit human PrAO *in vitro* (MacKenzie 2009). However, it is not known if geometric isomers of PEH would have different effects with regard to the aforementioned neurochemical properties as previous experiments have only used racemic mixtures of PEH.

Chapter 4: As phenelzine has been previously shown to increase rat brain levels of tyrosine (Dyck and Dewar 1986), administration of both phenelzine and PEH would be expected to produce elevations in brain levels of this amino acid. Since tyrosine is metabolized by tyrosine aminotransferase, a PLP-dependent enzyme, it would be expected that PEH is the active

metabolite responsible for phenelzine-induced increases in tyrosine; pre-treatment with a MAOI should abolish the effects of phenelzine, but not of PEH, on rat brain tyrosine levels. It was previously demonstrated that phenelzine's effects on GABA, alanine and ornithine, also metabolized by enzymes using PLP as a co-factor, are dependent on active MAO and formation of PEH from phenelzine (Popov and Matthies 1969; Todd and Baker 1995; Paslawski et al. 2001; MacKenzie et al. 2008a; MacKenzie et al. 2008b; Todd and Baker 2008; MacKenzie 2009).

Chapter 5: Phenelzine was previously demonstrated to protect rat primary cortical neurons and astrocytes against formaldehyde-induced toxicity (Song et al. 2010), exhibit a protective effect against 3-aminopropanal and acrolein toxicity in rat retinal ganglion cells (Wood et al. 2006) and attenuate 4-hydroxy-2-nonenal (HNE)-induced mitochondrial dysfunction in a rat model of traumatic brain injury (Singh et al. 2013). Furthermore, both phenelzine and PEH were demonstrated to sequester formaldehyde *in vitro* (MacKenzie 2009). Since both phenelzine and PEH possess a hydrazine moiety, it would be expected that both drugs would also be effective at sequestering the reactive aldehydes acrolein, malondialdehyde and methylglyoxal *in vitro*. It would also be expected that phenelzine and PEH would attenuate acrolein-induced toxicity in mouse cortical neurons and lower rat whole brain levels of acrolein and malondialdehyde *ex vivo*.

Chapter 6: In preliminary experiments, it was observed that a major peak for PFBA-derivatized PEH coincides with the peak for PFBA-derivatized hydrazine. As such, it is likely that this assay is actually measuring the hydrazine content of PEH. However, it is unknown whether the hydrazine present is formed from PEH during the derivatization process or is the result of hydrazine present in the PEH stock solution.

1.9 BASIC PRINCIPLES OF ANALYTICAL CHEMISTRY TECHNIQUES USED

Chromatography is an analytical technique used to separate and quantitate compounds of interest (analytes) in a mixture. High performance liquid chromatography (HPLC) and gas chromatography (GC) were used extensively in this thesis. HPLC was combined with fluorimetric, electrochemical or mass spectrometric (MS) detection. GC was combined with MS detection.

1.9.1 Gas chromatography (GC)

A GC system is composed of an inlet, carrier gas, an analytical column that is heated by an oven and a detector. In the inlet, a small volume of sample (usually 1 or 2 μl) is vaporized and injected onto the analytical column. The mobile phase, consisting of an inert carrier gas such as helium, moves the sample through the analytical column. Inside the analytical column, the sample interacts with the stationary phase, a high boiling point liquid, coated onto the walls of the column. Analyte separation occurs due to different affinities of compounds for the stationary phase. Compounds that have greater affinity for the stationary phase will take longer to pass through the analytical column, whereas compounds that have little affinity for the stationary phase will pass through more quickly (Harris 2010). The amount of time required for a sample to travel through the analytical column and enter the detector is called the retention time. If the compounds of interest have similar retention times, changing the temperature parameters of the oven can alter the interactions between the analytes and stationary phase to produce greater separation (Rittenbach and Baker 2007). MS was the detector used for GC analysis in this thesis.

GC is limited to analysis of compounds that are heat-stable and volatile. As aqueous samples can damage the system, compounds of interest must be extracted into organic solvents prior to analysis. In the work-up procedure, compounds of interest are often reacted with other chemicals, a process called derivatization, to form a derivative of greater volatility, stability and/or increased sensitivity of detection (Drozd 1985). For example, methylamine was derivatized with pentafluorobenzenesulfonyl chloride (PFBSC) in Chapter 3, reactive aldehydes were derivatized with pentaflurobenzyl hydroxylamine (PFBHA) in Chapter 5 and PEH and hydrazine were derivatized with pentafluorobenzaldehyde (PFBA) in Chapter 6.

1.9.2 High performance liquid chromatography (HPLC)

The concept behind HPLC is similar to GC, with a few notable differences. The sample is directly injected onto a liquid mobile phase consisting of a mixture of aqueous buffers and organic solvents. A single mobile phase or a gradient of multiple mobile phases can be used throughout the duration of a sample run. Sample separation is achieved by interaction of the analytes with both the mobile phase and the stationary phase coated onto the analytical column (Waters Corporation 2015a).

HPLC offers several advantages over GC. Since the sample is not vaporized, HPLC can be used for compounds that are heat labile or have low volatility (Odontiadis and Rauw 2007). Aqueous samples may be used in the system, preventing the need for the extraction of compounds into an organic solvent. However, a derivatization step is still often required. For example, amino acids were derivatized with N-isobutyryl-L-cysteine (IBC) and o-phthaldialdehyde (OPA) in Chapter 3 and phenelzine, PEH and hydrazine were derivatized with acetone in Chapters 2 and 6.

Several detection methods were used with HPLC in this thesis, including fluorimetric detection, electrochemical detection and MS. Fluorimetric detection, used for the quantitation of amino acids in Chapter 3, relies on a compound's ability to absorb ultraviolet light from the detector and to then emit light of a lower energy back toward the detector (Odontiadis and Rauw 2007). The amount of light fluoresced from the sample is proportional to the concentration of analyte present. The wavelengths of light emitted onto and recorded from the sample can be adjusted depending on the compound of interest. In order to increase the sensitivity of detection, analytes are often derivatized with fluorescent reagents; this was the case with amino acid analysis, where samples were reacted with the fluorescent reagent OPA. In electrochemical detection, used for the quantitation of biogenic amines in Chapter 3 and of tyrosine in Chapter 4, analytes are oxidized on the surface of an electrode inside the detector. The change in electrode current as a result of analyte oxidation is used for quantitation of analyte concentration (Odontiadis and Rauw 2007).

1.9.3 Mass spectrometry (MS)

MS can be used to identify and quantify a compound of interest. Analytes entering the MS detector are either already in the gas phase (as is the case with GC-MS) or need to be converted to the gas phase (as is the case with LC-MS). The molecules are then ionized to gas-phase ions by a variety of techniques, including electrospray ionization, electron ionization and chemical ionization (Waters Corporation 2015b).

Electrospray ionization is generally used when MS is combined with HPLC, as was the case with analysis of phenelzine, PEH and hydrazine in Chapters 2 and 6. Liquid analytes exiting the HPLC are exposed to a high voltage, resulting in formation of ions within an aerosol that can

then enter the MS detector (Waters Corporation 2015b). In electron ionization, used for the detection of methylamine in Chapter 3, reactive aldehydes in Chapter 4 and hydrazine in Chapter 6, analytes in the gas phase exit the GC and are bombarded with a high energy beam of electrons, resulting in formation of ions. In chemical ionization, used for analysis of reactive aldehydes in Chapter 5 and analysis of hydrazine in Chapter 6, a reagent gas (such as methane) is present within the ion source and is ionized by the electron beam. The ionized reagent gas can then interact with the analytes, resulting in formation of analyte ions. Chemical ionization is a lower energy ionization technique in comparison to electron ionization and is therefore regarded as having a higher sensitivity (Waters Corporation 2015b). In all types of ionization techniques, larger analyte ions often fragment into smaller ions of greater stability.

A single quadrupole mass filter, composed of four parallel metal rods, was used in the MS instruments for this thesis. Analyte ions entering the quadrupole are exposed to direct current and radiofrequency fields, causing them to oscillate at amplitudes related to the mass-to-charge ratio (m/z) of the specific ion. By altering the direct current and radio frequency fields, ions of a desired m/z range can be selected for; these ions oscillate at an amplitude that allows them to travel the entire length of the quadrupole and be detected, whereas ions outside of the specified m/z range strike the quadrupole rods and are not detected (Waters Corporation 2015b).

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CHAPTER 2.

Mechanisms of phenelzine metabolism by human monoamine oxidase B

2.1 INTRODUCTION

Monoamine oxidase (MAO) is a flavin-containing enzyme found in most mammalian tissues (Tipton et al. 2004) and involved in the oxidation of a wide variety of endogenous and exogenous monoamines. Inside the cell, MAO is anchored to the outer mitochondrial membrane with its active site exposed to the cytoplasm. The cofactor flavin adenine dinucleotide (FAD) is covalently linked to MAO's active site. During catalysis, the FAD cofactor is reduced and the amine substrates are oxidized to their respective imines, which are subsequently hydrolyzed (non-enzymatically) to an aldehyde and ammonia (for primary amines) or a substituted amine (for secondary amines) (Binda et al. 2002; Tipton et al. 2004; Youdim et al. 2006). To recycle back to the oxidized state, reduced MAO flavin reacts with oxygen and generates hydrogen peroxide (H_2O_2) as a by-product (Edmondson 2014). This reaction scheme is represented for dopamine in **Figure 2-1**. MAO is present in two main isoforms: MAO-A and MAO-B with 70% sequence homology. The MAO isoforms are distinguished by their sensitivity to pharmacological inhibition, preference for substrates and localization in tissue. Clorgyline is a selective inhibitor of MAO-A, whereas *l*-deprenyl (selegiline) and mofegiline are selective inhibitors of MAO-B. 5-Hydroxytryptamine (5-HT, serotonin) is metabolized mostly by MAO-A, while benzylamine and phenylethylamine are preferentially oxidized by MAO-B. Dopamine, noradrenaline, adrenaline, tryptamine and tyramine are oxidized by both forms of the enzyme (Youdim et al. 2006). In the primate brain, MAO-A is primarily localized to catecholaminergic neurons, while MAO-B is predominantly found in serotonergic neurons and glial cells (Westlund et al. 1985; Riederer et al. 1987; Westlund et al. 1988). Of interest, MAO-B predominates in the human brain, whereas MAO-A is found in greater concentrations in the rat brain (Kennedy et al. 2004).

MAO has garnered much interest in psychiatry for the role of its inhibitors in the treatment of depression. The ability of hydrazines to inhibit MAO was first discovered serendipitously with iproniazid in the 1950s (Zeller and Barsky 1952). Originally developed for treatment of tuberculosis, it was found that iproniazid produced mood elevation in patients and had antidepressant properties. The ability of MAO inhibitors to exert an antidepressant effect led to the development of the monoamine hypothesis of depression, stating that depression caused by the result of a functional deficiency of noradrenaline and/or serotonin at specific synapses in the

central nervous system (review: Baker and Dewhurst 1985). Although iproniazid was used as an antidepressant for several years, it was eventually replaced with other hydrazine drugs, such as phenelzine (β-phenylethylhydrazine) and isocarboxazid, due to high incidence of hepatotoxicity.

Phenelzine has been characterized as an irreversible inhibitor of both MAO-A and MAO-B enzyme isoforms. It is an interesting MAO inhibitor in that it also appears to be a substrate for the enzyme (Clineschmidt and Horita 1969a, b; Tipton 1971) and is oxidized by MAO to the imine product β-phenylethylidenehydrazine (PEH) (Tipton and Spires 1972; Patek and Hellerman 1974). The mechanism of MAO inhibition by phenelzine is considered to be dependent on phenelzine oxidation by the enzyme (Tipton 1971), with some suggesting that either PEH (Tipton 1972) or a diazene intermediate (Patek and Hellerman 1974), namely phenylethyldiazene, are responsible for the enzyme inhibition. The relationship between phenylethyldiazene and PEH is unclear, with speculation that the two products can be formed by separate catalytic pathways (Binda et al. 2008) or by intramolecular rearrangement between phenylethyldiazene and PEH (Patek and Hellerman 1974; Yu and Tipton 1989). Binda and colleagues (2008) have suggested that phenelzine can undergo either a C-H bond cleavage to produce PEH or, once every 35-40 catalytic cycles, undergo a N-H bond cleavage to form phenylethyldiazene. This diazene intermediate is very reactive and, in the presence of oxygen, can form an arylalkyl radical that subsequently alkylates the FAD cofactor of MAO, resulting in irreversible inhibition (Binda et al. 2008). An important assumption of this mechanism is that MAO inhibition occurs without the diazene ever leaving the enzyme active site. In the same study, a mass spectral and x-ray structural analysis of MAO-B inhibited by phenelzine determined that the FAD peptide was alkylated with an arylalkyl derivative of phenelzine at the N(5) position of the flavin. The proposed mechanisms of phenelzine inhibition of MAO are summarized in **Figure 2-2**.

In the study reported here, we addressed the stability of PEH as an imine product of MAO-B oxidation of phenelzine and the ability of PEH to inhibit human MAO-B. In addition, we investigated the mechanisms of phenelzine inhibition of MAO-B in relation to the number of phenelzine turnovers by MAO-B per inactivation event and the possibility of phenylethyldiazene leaving the active site prior to alkylation of the FAD cofactor.

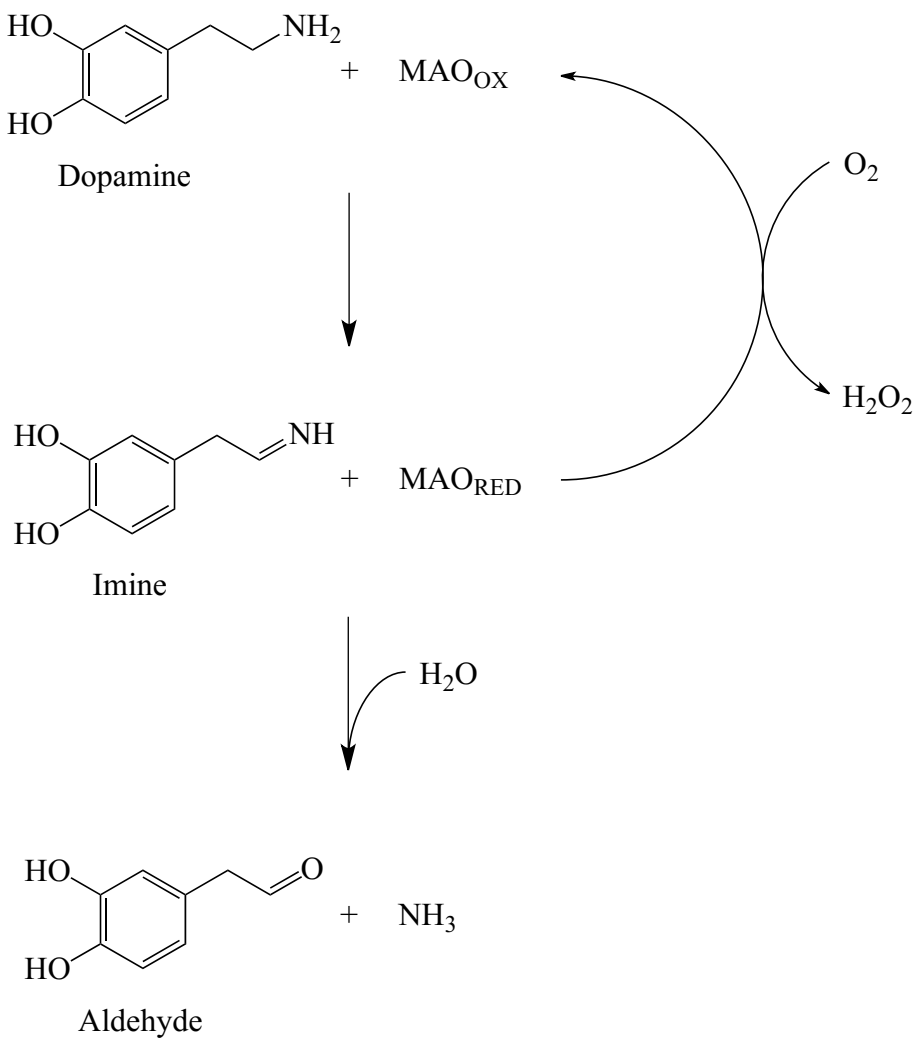


Figure 2-1. Proposed reaction scheme for catalytic turnover of dopamine by MAO.

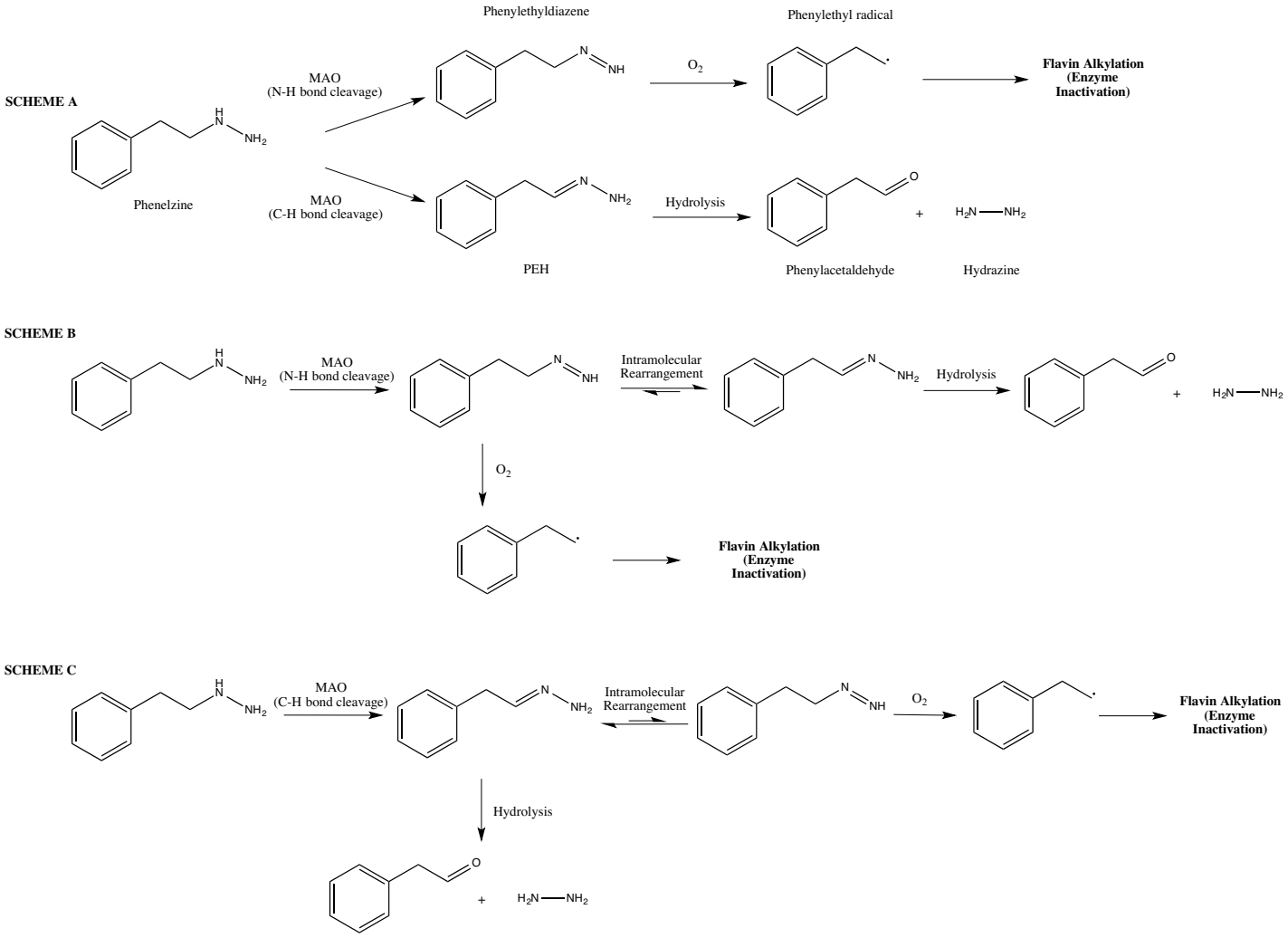


Figure 2-2. Proposed mechanism schemes of phenelzine inactivation of MAO. In **Scheme A**, PEH and phenylethyldiazene are formed by separate catalytic pathways. Alternatively, a single initial product may be formed, either phenylethyldiazene (**Scheme B**) or PEH (**Scheme C**), with subsequent intramolecular rearrangement between phenylethyldiazene and PEH.

2.2 METHODS

2.2.1 Materials

All water used in the experiments was filtered through a Millipore Milli-Q filtration system. Benzylamine hydrochloride, phenelzine sulfate, hydrazine monohydrate, ammonium formate, potassium pyrophosphate, 2-mercaptoethanol, oxidized nicotinamide adenine dinucleotide (NAD^+), peroxidase (type II) from horseradish, catalase from bovine liver and aldehyde dehydrogenase from baker's yeast (*S. cerevisiae*) were purchased from Sigma-Aldrich. Acetone, acetonitrile, methanol, monobasic and dibasic potassium phosphate were obtained from Fisher Scientific. Amplex Red reagent (*N*-acetyl-3,7-dihydroxyphenoxazine) was purchased from Invitrogen. For spectrophotometric experiments, 96-well microplates were obtained from Greiner Bio-One. Full length human MAO-B was expressed in *Pichia pastoris* yeast and purified in the laboratory of Dr. Andrew Holt, Department of Pharmacology at the University of Alberta. PEH was synthesized from phenylacetaldehyde and hydrazine monohydrate in the laboratories of Dr. Velázquez-Martínez in the Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta.

2.2.2 Amplex Red spectrophotometric assay

For measurement of MAO-B activity in some experiments, we employed a modification (McDonald et al. 2010) of a continuous fluorometric assay previously described by Zhou and Panchuk-Voloshina (1997). The concurrent reduction of H_2O_2 (generated as a by-product of the conversion of MAO from the reduced to oxidized state) and oxidation of Amplex Red reagent by horseradish peroxidase stoichiometrically produces the colored compound resorufin (**Figure 2-3**). The production of resorufin can be quantitated by measuring changes in absorbance at a wavelength of 571 nm ($\varepsilon \approx 70{,}600\ M^{-1}\ cm^{-1}$). In 96-well plates, enzyme was incubated ± drugs in physiological buffer, followed by addition of substrate, Amplex Red (20 μM) and horseradish peroxidase enzyme (4 U/ml). A continuous kinetic analysis was carried out Molecular Devices FlexStation 3 microplate reader by measuring production of resorufin via changes in absorbance at 571 nm.

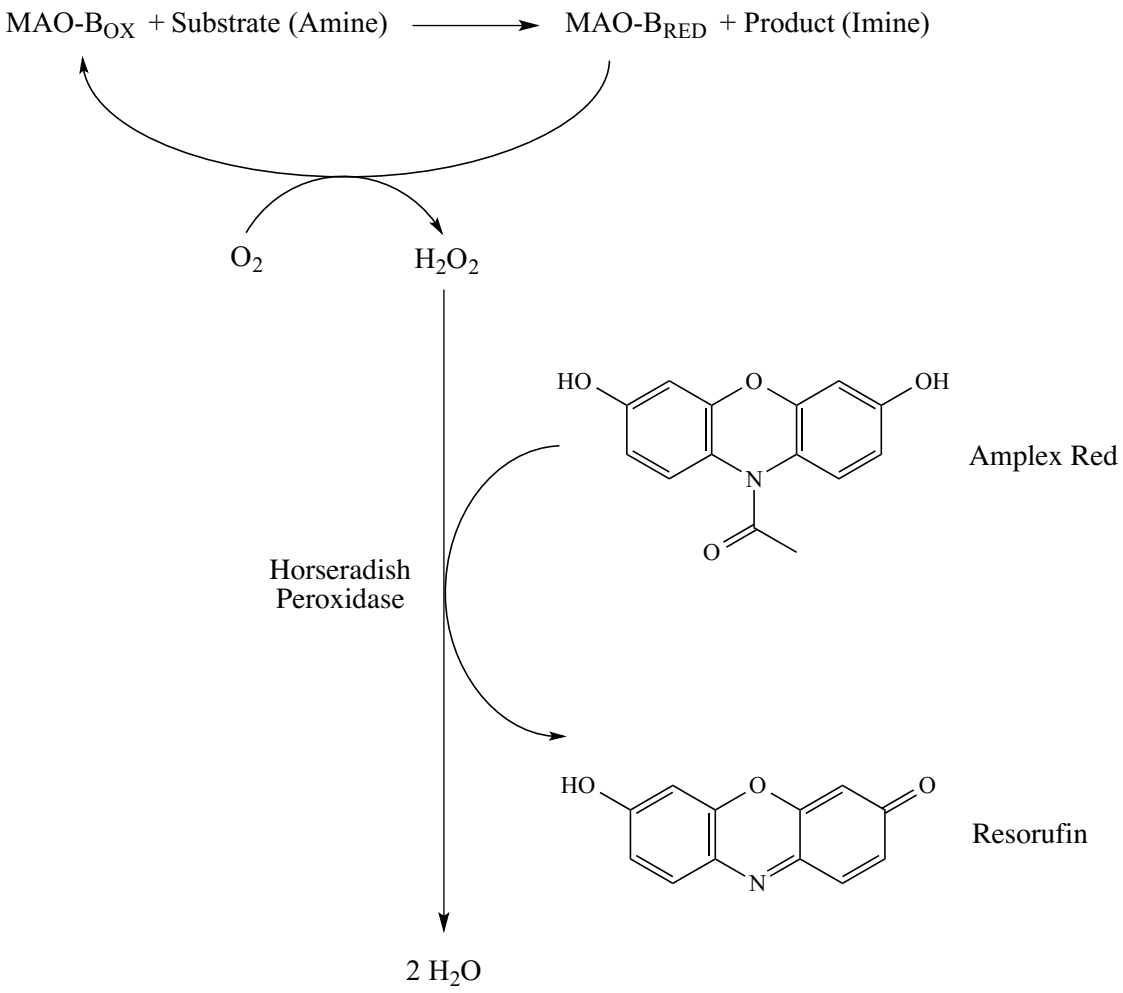


Figure 2-3. Reaction scheme for Amplex Red assay. Production of resorufin is measured spectrophotometrically by monitoring changes in absorbance at a wavelength of 571 nm.

2.2.3 Benzaldehyde spectrophotometric assay

For measurement of MAO-B activity in some experiments, we also employed a spectrophotometric assay for measurement of benzaldehyde production. Benzaldehyde is the hydrolysis product of the imine generated from benzylamine oxidation by MAO-B and has a characteristic absorbance at a wavelength of 254 nm ($\varepsilon \approx 13{,}800\ M^{-1}\ cm^{-1}$). Following incubation of MAO-B ± drugs in a physiological buffer, benzylamine was added and a continuous kinetic analysis was carried out with Molecular Devices FlexStation 3 microplate reader by measuring production of benzaldehyde via changes in absorbance at 254 nm.

2.2.4 Aldehyde dehydrogenase spectrophotometric assay

Hydrolysis of PEH to phenylacetaldehyde was measured using an aldehyde dehydrogenase-coupled spectrophotometric assay. The oxidation of phenylacetaldehyde, produced by the hydrolysis of PEH to phenylacetic acid by aldehyde dehydrogenase is coupled to the reduction of NAD^+ to NADH as demonstrated in **Figure 2-4**. The production of NADH can be quantitated by recording changes in absorbance at a wavelength of 340 nm ($\varepsilon \approx 6{,}220\ M^{-1}\ cm^{-1}$). To an incubation solution in 96-well plates, a mixture of aldehyde dehydrogenase enzyme (0.5 U/ml) in 100 mM potassium pyrophosphate buffer at pH 9.3, the optimal pH for oxidation of aldehydes by this enzyme (Racker 1949), NAD^+ (750 μM) and 2-mercaptoethanol (10 mM) was added to the wells. A continuous kinetic analysis was carried out with Molecular Devices FlexStation 3 microplate reader by measuring production of NADH via changes in absorbance at 340 nm.

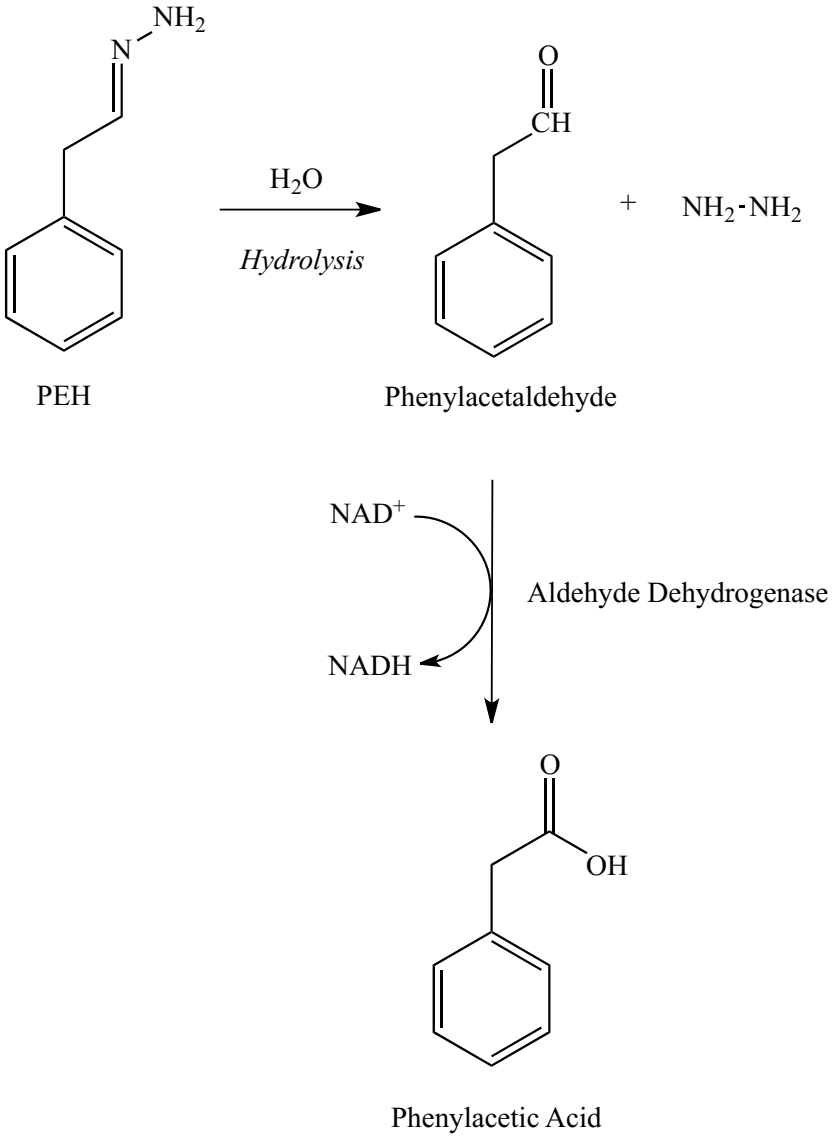


Figure 2-4. Reaction scheme for aldehyde dehydrogenase assay. Production of NADH is measured spectrophotometrically by monitoring changes in absorbance at a wavelength of 340 nm.

2.2.5 Liquid chromatography combined with mass spectrometry (LC-MS)

Derivatization with acetone has previously been reported for determination of hydrazines in a variety of media (Selim and Warner 1978; Holtzclaw et al. 1984; Davis and Li 2008; Sun et al. 2009; Fortin and Chen 2010). We employed this assay for investigation of the kinetics of phenelzine oxidation by MAO-B by means of liquid chromatography combined with mass spectrometry (LC-MS). Derivatization of hydrazines with acetone produces hydrazone compounds, allowing for detection and quantitation of phenelzine, PEH and hydrazine in solution. The proposed derivatization reactions are shown in **Figure 2-5**. In the procedure, 40 μl of sample (in aqueous buffer) were added to 40 μl of acetone and 80 μl of acetonitrile, the mixture vortexed, centrifuged at 10,000 xg for 1 minute and the supernatant (100 μl) transferred to HPLC vials with glass inserts. The analytes were then derivatized in the dark at room temperature for 5 hours. The HPLC instrument was a Waters Alliance 2690 XE with an Atlantis T3 5 μm (3 x 100 mm) analytical column. The mobile phase consisted of 15% 10 mM ammonium formate in water, 25% methanol and 60% acetonitrile with a flow rate of 0.3 ml/minute. The MS instrument was a Waters Micromass ZQ-4000 with the following voltages: capillary = 3.31 kV, cone = 21 V, extractor = 2 V and RF lens = 1 V. The source and desolvation temperatures were 150°C and 350°C, respectively. Nitrogen gas was set at a desolvation flow rate of 350 L/hour and cone flow rate of 90 L/hour. Using positive electrospray ionization mode, selective ion monitoring (SIM) was used to detect the derivatized compounds at the following mass-to-charge ratios (m/z): 176.99 for phenelzine, 174.94 for PEH and 112.92 for hydrazine. The total run time was 7 minutes, with retention times of 2.80 minutes for hydrazine, 3.13 minutes for phenelzine and 3.41 minutes for PEH. Response peak area was used to quantitate the concentration of target compounds based on standard curves generated on the day of the experiment.

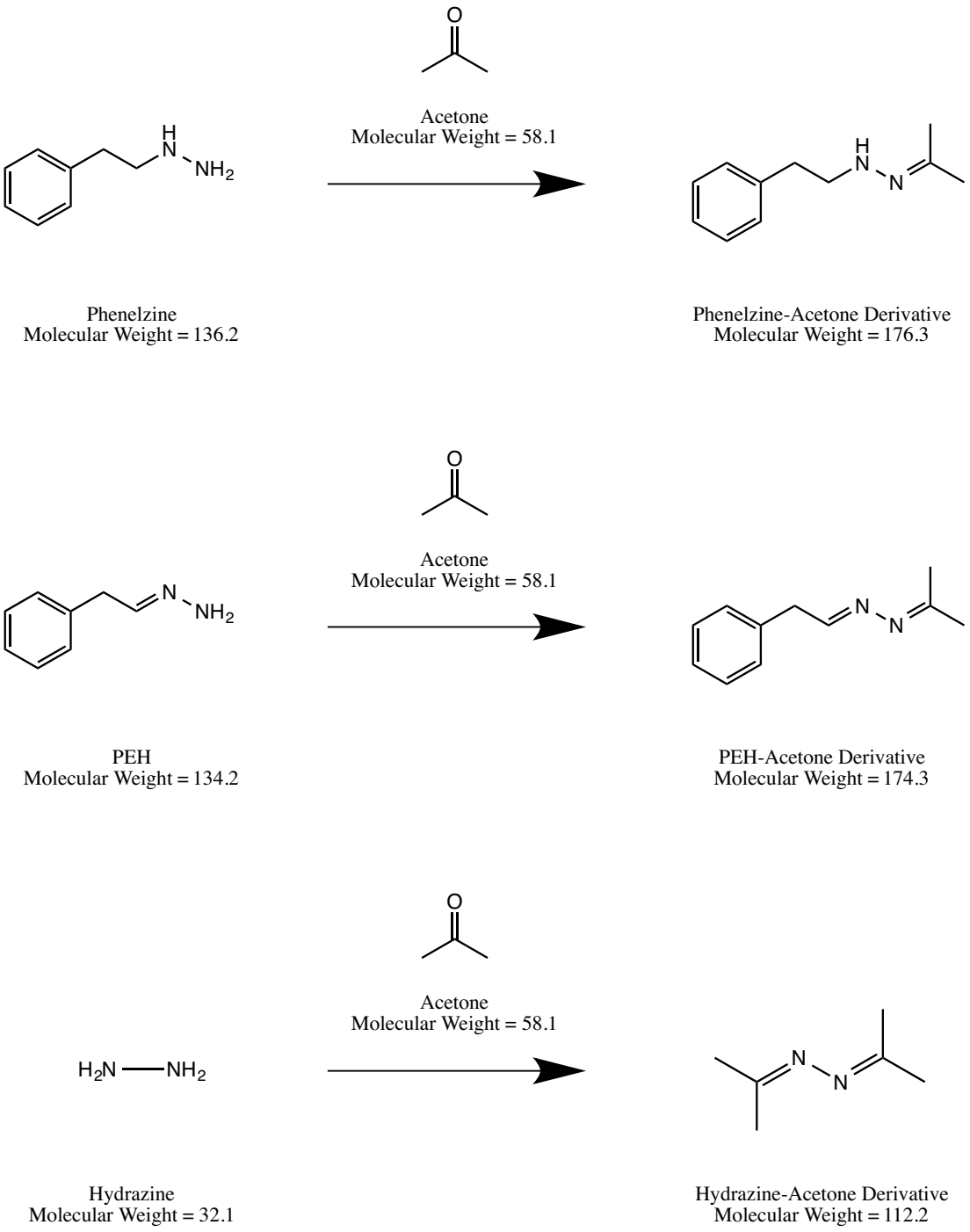


Figure 2-5. Proposed derivatization reaction schemes of acetone with phenelzine, PEH and hydrazine to their respective hydrazone products.

2.3 RESULTS

2.3.1 Determination of MAO-B concentration

The enzyme concentration of purified MAO-B was determined by titration with mofegiline, a selective mechanism-based (irreversible) MAO-B inhibitor, using the Amplex Red assay. In triplicate, a known volume of MAO-B, in 0.1 M potassium phosphate buffer at pH 7.4, was incubated with mofegiline for 1 hour at room temperature, followed by addition of benzylamine as substrate (2 mM final concentration in wells), horseradish peroxidase (4 U/ml) and Amplex Red (20 μM final concentration in wells). Production of resorufin was quantitated by measuring the change in absorbance at 571 nm. The titration is represented in **Figure 2-6**. Concentration of the enzyme was determined by multiplying the x-intercept of the linear portion of the titration by the dilution of the stock enzyme solution. Based on these calculations, the stock solution of MAO-B used in the experiments was 6.75 μM.

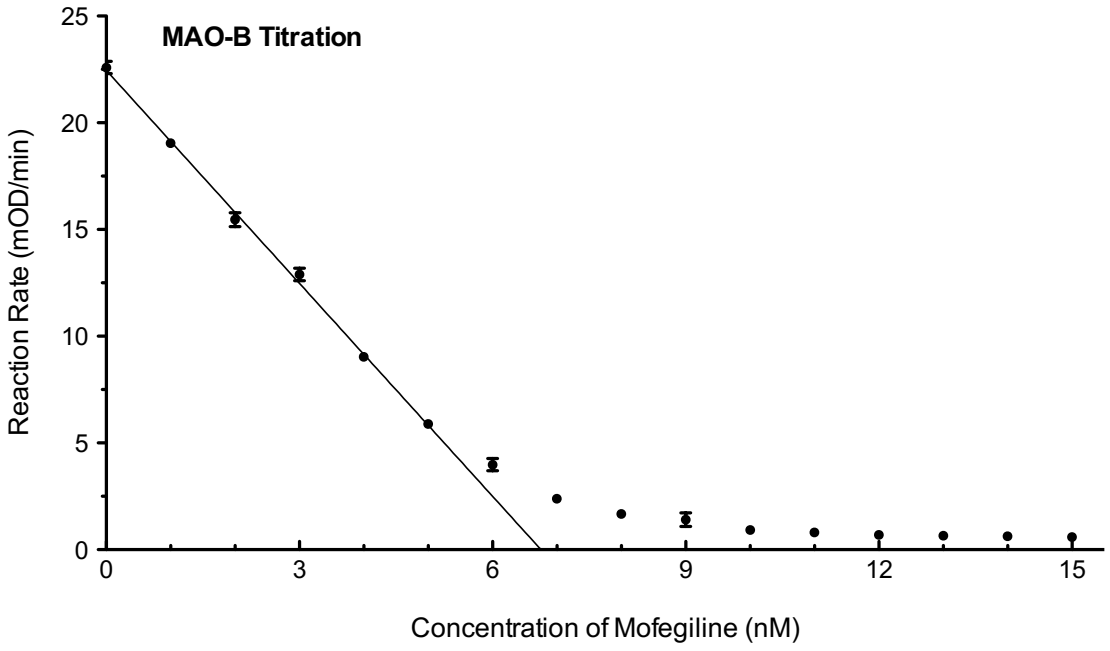


Figure 2-6. Titration of MAO-B with mofegiline to determine stock enzyme concentration. Data are represented as mean ± SEM from 3 replicate determinations.

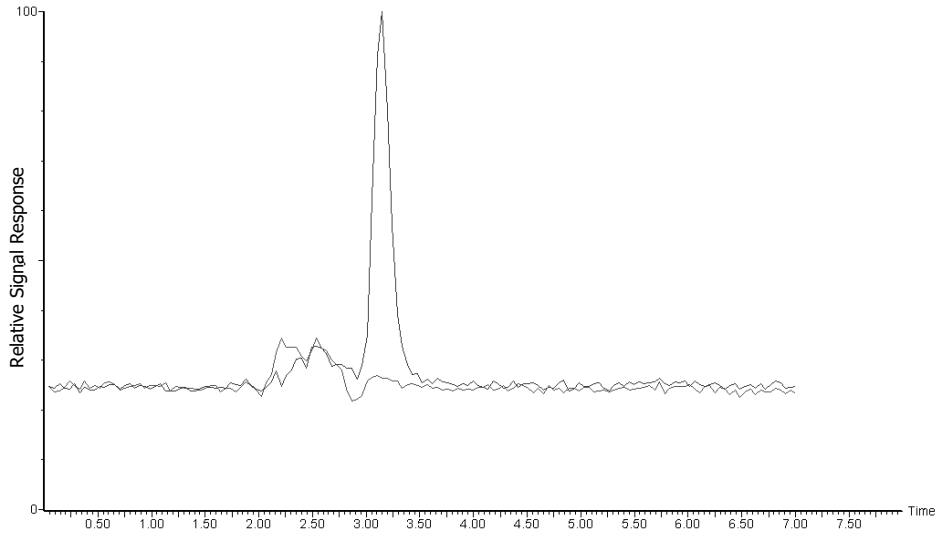
2.3.2 Acetone assay development

Derivatization with acetone has previously been reported for determination of hydrazines in a variety of media (Selim and Warner 1978; Holtzclaw et al. 1984; Davis and Li 2008; Sun et al. 2009; Fortin and Chen 2010). In the method described here, aqueous solutions of phenelzine, PEH and hydrazine were reacted with acetone, followed by dilution with acetonitrile to more closely mimic the mobile phase composition. Representative chromatograms and mass spectra of derivatized phenelzine, PEH and hydrazine are represented in **Figures 2-7** to **2-9**, along with a chromatogram of all three compounds recorded with SIM at m/z of 176.99 for phenelzine, 174.94 for PEH and 112.92 for hydrazine (**Figure 2-10**).

The derivatization time of 5 hours was chosen based on experiments to determine the optimal time required for stability of response for all three compounds. Briefly, 100 μM of phenelzine, PEH or hydrazine (n = 3) in potassium phosphate buffer (pH 7.4) were derivatized with acetone in the presence of acetonitrile, with response peak heights analyzed at time intervals ranging from 0 to 8 hours (**Figure 2-11**). The derivatives were stable for at least 12 hours.

The limits of detection and quantitation for phenelzine, PEH and hydrazine were 50 nM (2 pmol), 250 nM (10 pmol) and 1 μM (40 pmol), respectively. Intra-assay variabilities measured using a 10 μM sample (n = 5) of phenelzine, PEH and hydrazine were 1.9%, 2.8% and 1.1%, respectively. Standard curves for phenelzine, PEH and hydrazine were linear up to 800 μM with r^2 values consistently greater than 0.98. Typical standard curves are represented in **Figure 2-12**. As the sample cone of the mass spectrometer became dirty after prolonged use and peak area of representative standards decreased, standard curves and phenelzine blanks (no MAO-B present) were run daily to ensure accurate quantitation of the compounds of interest in the required concentration ranges. The addition of acetone or acetonitrile was previously reported to abolish the activity of MAO (Woo et al. 1995); this finding was confirmed in our assay for MAO-B (data not shown). Thus, derivatization with acetone is appropriate for determination of the oxidation of phenelzine to PEH by MAO-B at specific time intervals.

A. Chromatogram comparing phenelzine standard with blank



B. Mass spectrum of phenelzine standard peak with retention time of 3.13 min

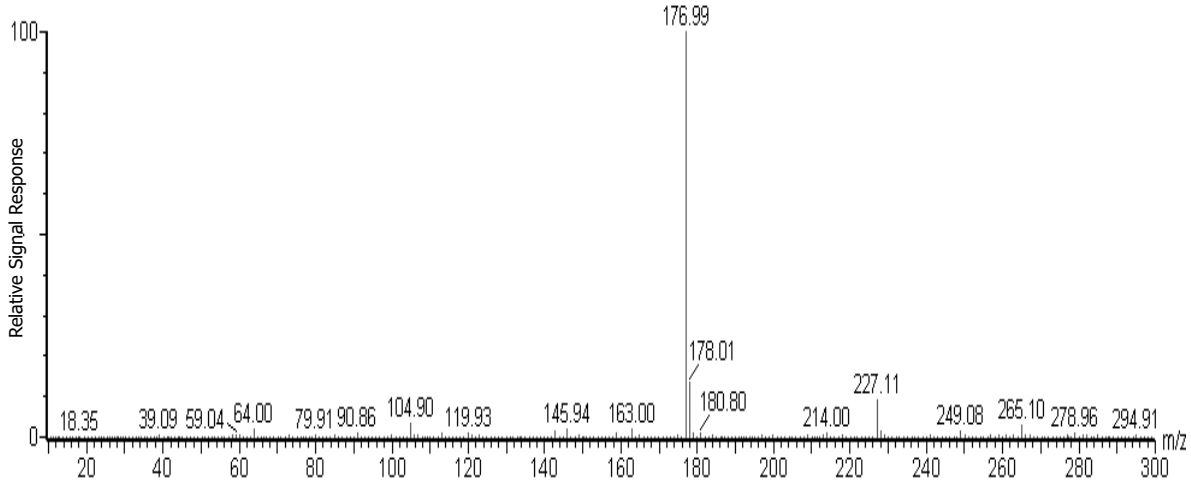
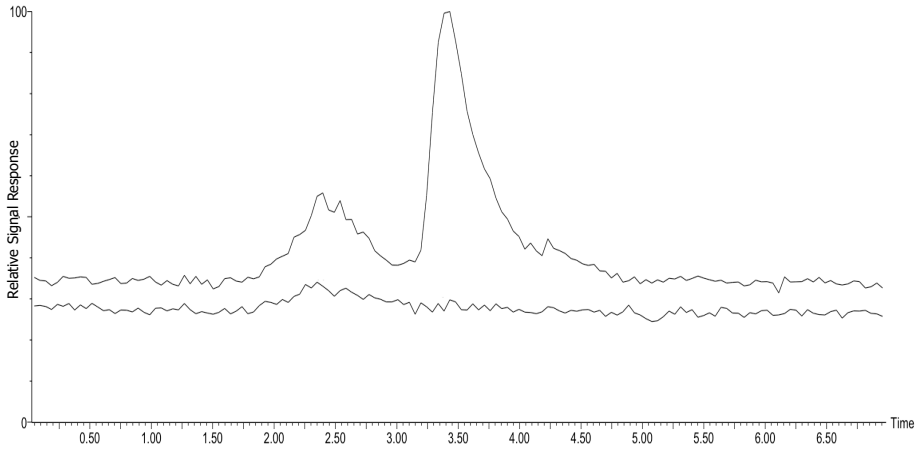


Figure 2-7. Chromatogram of phenelzine standard in comparison to blank, as well as the mass spectrum of the phenelzine peak with a retention time of 3.13 minutes.

A. Chromatogram comparing PEH standard with blank



B. Mass spectrum of PEH standard peak with retention time of 3.41 min

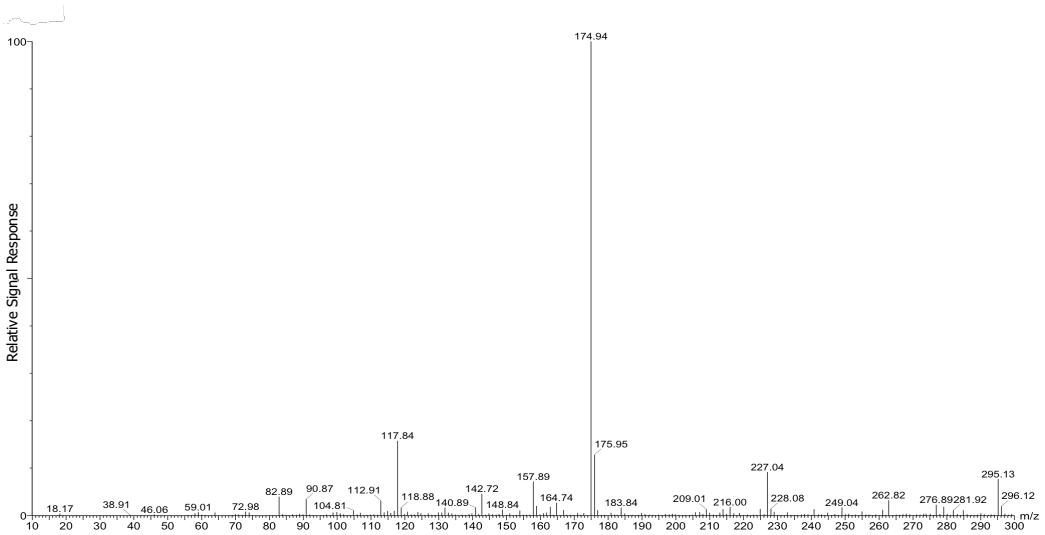
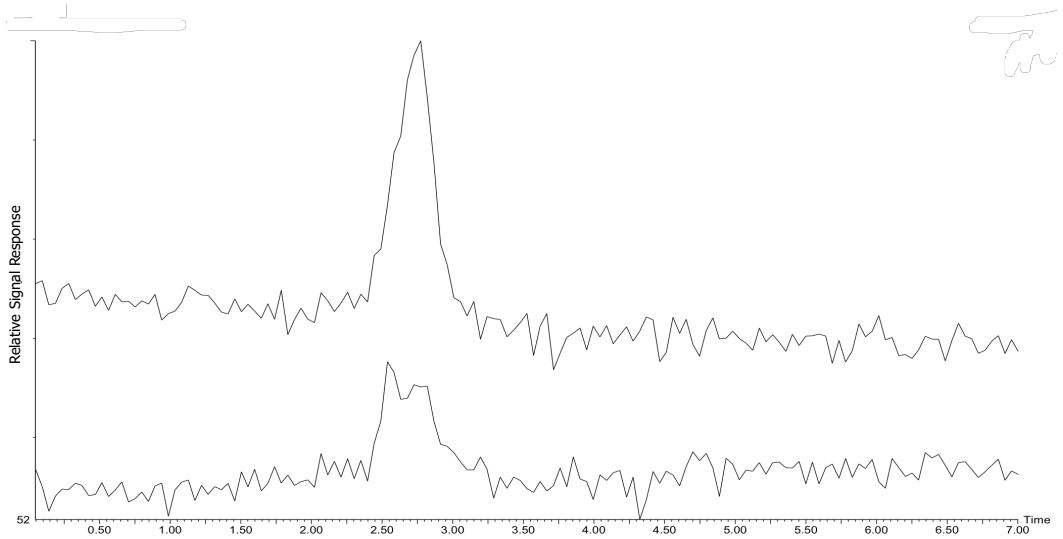


Figure 2-8. Chromatogram of PEH standard in comparison to blank, as well as the mass spectrum of the PEH peak with a retention time of 3.41 minutes.

A. Chromatogram comparing hydrazine standard with blank



B. Mass spectrum of hydrazine standard peak with retention time of 2.80 min

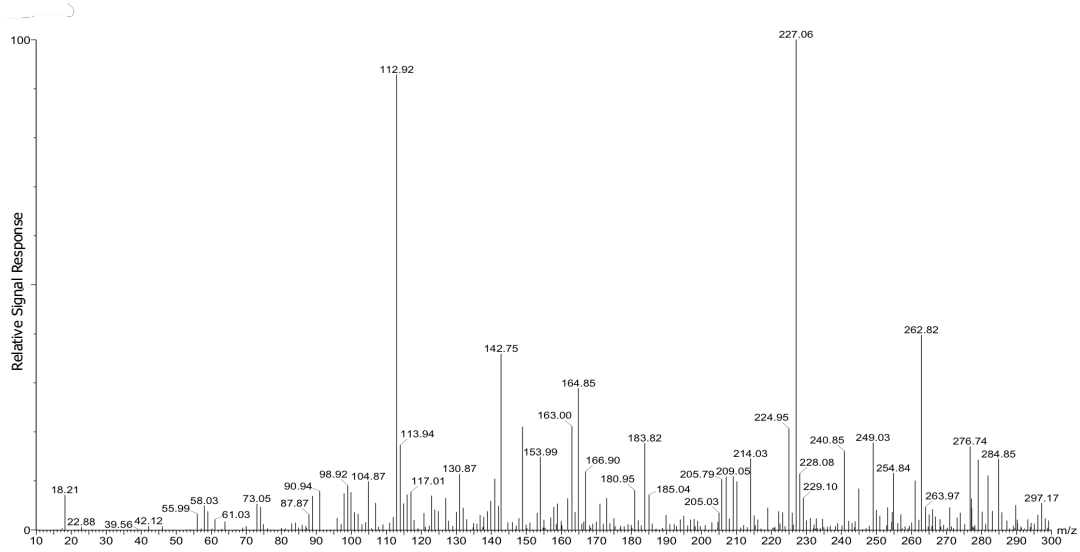


Figure 2-9. Chromatogram of hydrazine standard in comparison to blank, as well as the mass spectrum of the hydrazine peak with a retention time of 2.80 minutes.

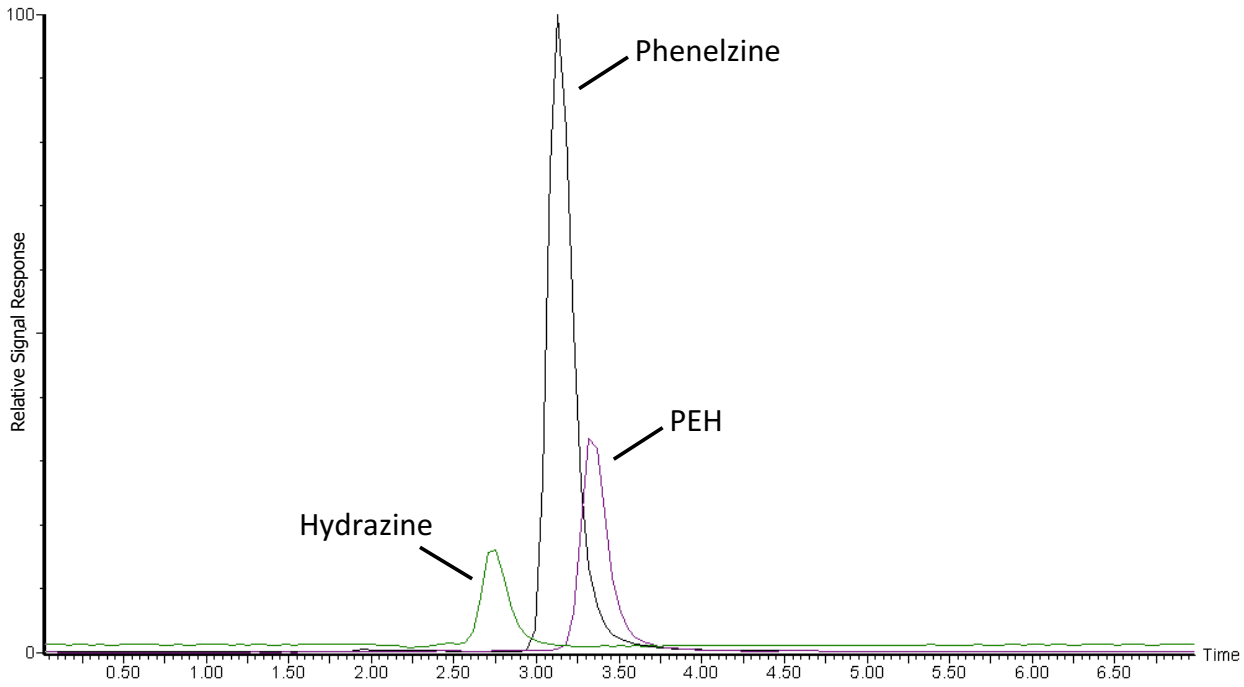


Figure 2-10. Chromatogram of selective ion monitoring (SIM) for hydrazine (m/z of 112.92), phenelzine (m/z of 176.99) and PEH (m/z of 174.99).

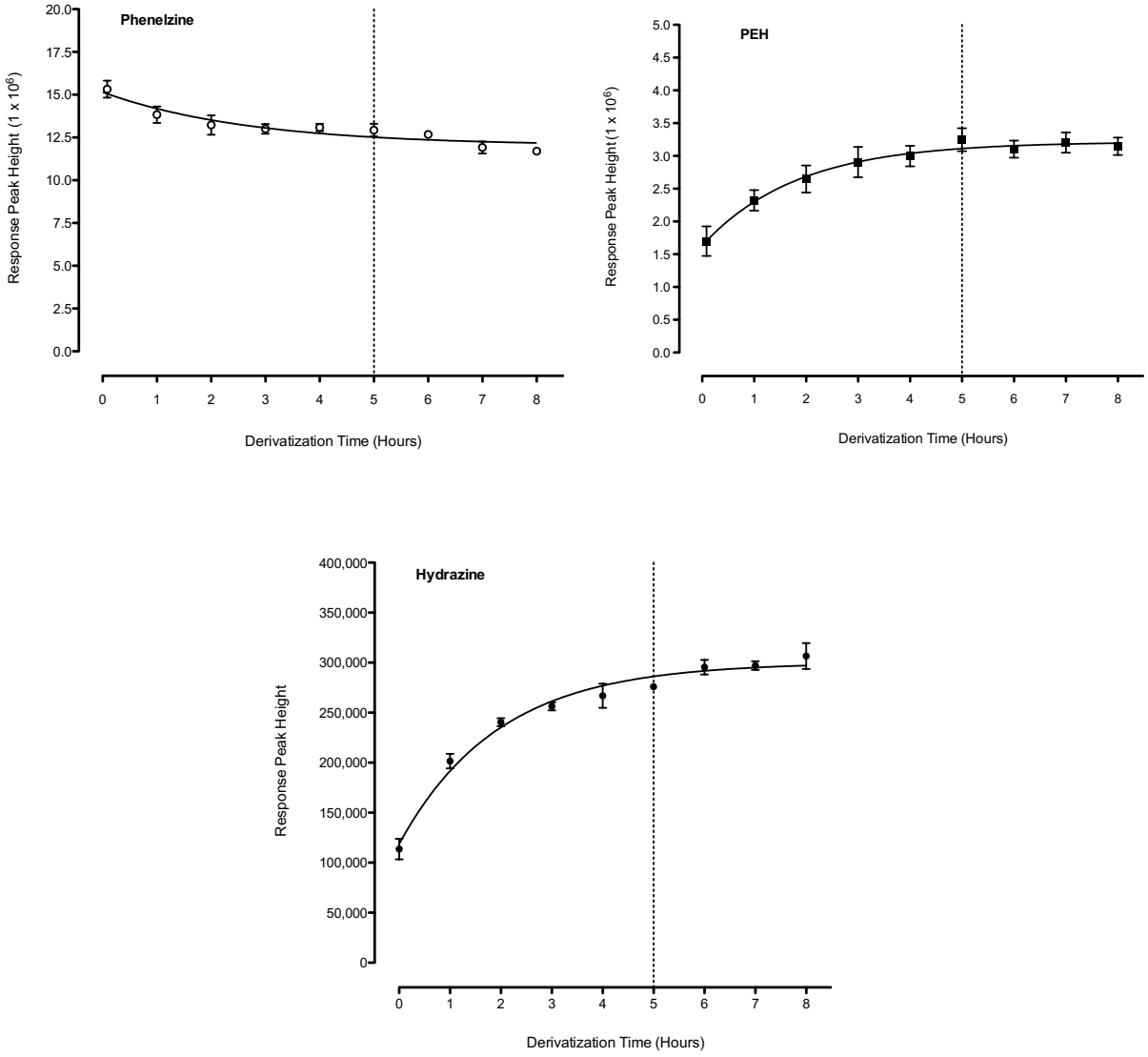


Figure 2-11. Derivatization timeframe of 100 μM phenelzine, PEH and hydrazine with acetone at room temperature. Data (n = 3) are represented as mean ± SEM. Based on this experiment, an optimal derivatization of 5 hours was selected for the assay.

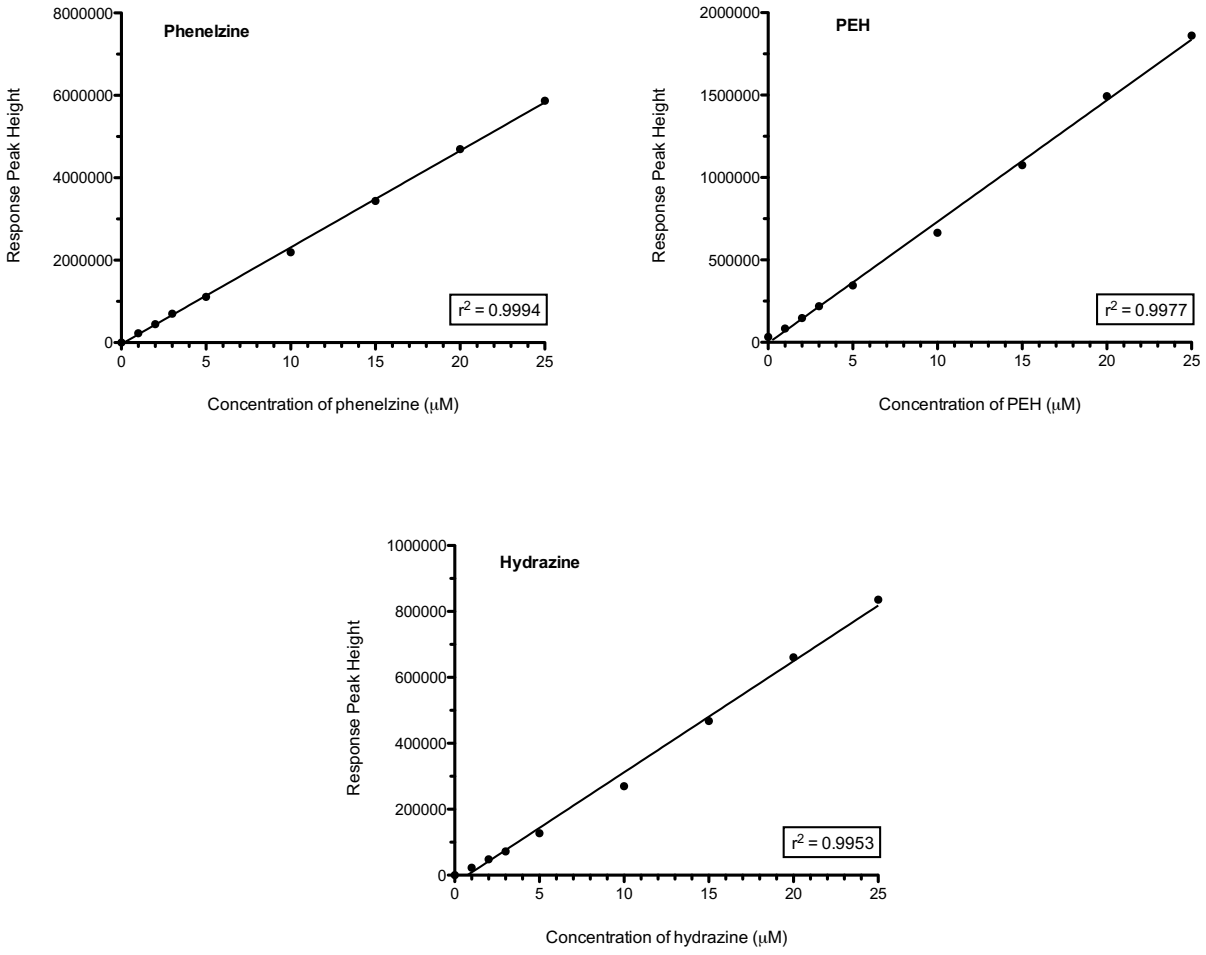


Figure 2-12. Typical standard curves for phenelzine, PEH and hydrazine following derivatization with acetone.

2.3.3 PEH is a relatively stable imine product of MAO-B oxidation of phenelzine

The acetone derivatization assay (LC-MS) was employed to monitor the oxidation of phenelzine by MAO-B by observing the rate of phenelzine disappearance and the rates of PEH and hydrazine formation. MAO-B (30 nM) was incubated with phenelzine (15 μM) for selected time intervals at room temperature. This experiment was carried out in 1 mM potassium phosphate buffer at pH levels of 6.4, 7.4 and 8.4 (n = 3). The results of the experiment are summarized in **Figures 2-13** to **2-15**. At a pH of 7.4, the majority of phenelzine was converted to PEH within the first 30 minutes. This would suggest that PEH is the major metabolite of phenelzine oxidation by MAO-B. The rate of hydrolysis of PEH to hydrazine appears to be pH-dependent, with increasing pH levels corresponding to lower rates of hydrolysis. Over the span of 8 hours, hydrazine concentration reached approximately 3.7 μM, 2.5 μM and 2.0 μM at a buffer pH level of 6.4, 7.4 and 8.4, respectively. This increase in hydrazine was accompanied by comparable reductions in PEH levels. The concentration of hydrazine present in solution appears to plateau by the 8 hour time point, possibly due to an establishment of an equilibrium for the hydrolysis reaction.

As PEH is hydrolyzed to hydrazine and phenylacetaldehyde, we confirmed our findings with hydrazine by quantifying phenylacetaldehyde production from PEH with an aldehyde dehydrogenase assay. The concentration of phenylacetaldehyde was determined using the Beer-Lambert equation ($A = \varepsilon bc$) where A is the absorbance in optical density units (OD), ε is the molar absorptivity coefficient of NADH (6,220 M^{-1} cm^{-1}), b is the path length in cm (0.842 cm) and c is the concentration in M. The results are represented in **Figure 2-16**. The rate of hydrolysis of 15 μM PEH is greatest in water and 5 mM potassium phosphate buffer at pH of 5.8, followed by 5 mM potassium phosphate buffer at pH of 7.4. In comparison, hydrolysis of PEH in 5 mM potassium pyrophosphate buffer at a pH of 9.2 is relatively slow. After a period of 24 hours, 15 μM PEH was converted to approximately 5.4 μM, 3.2 μM and 1.8 μM phenylacetaldehyde at pH levels of 5.8, 7.4 and 9.2, respectively. As with the LC-MS experiments, it appears that not all of the PEH is hydrolyzed over time, suggesting that the imine reaches an equilibrium with its hydrolysis products and is relatively stable in buffered aqueous solutions at a pH of 7.4 or higher.

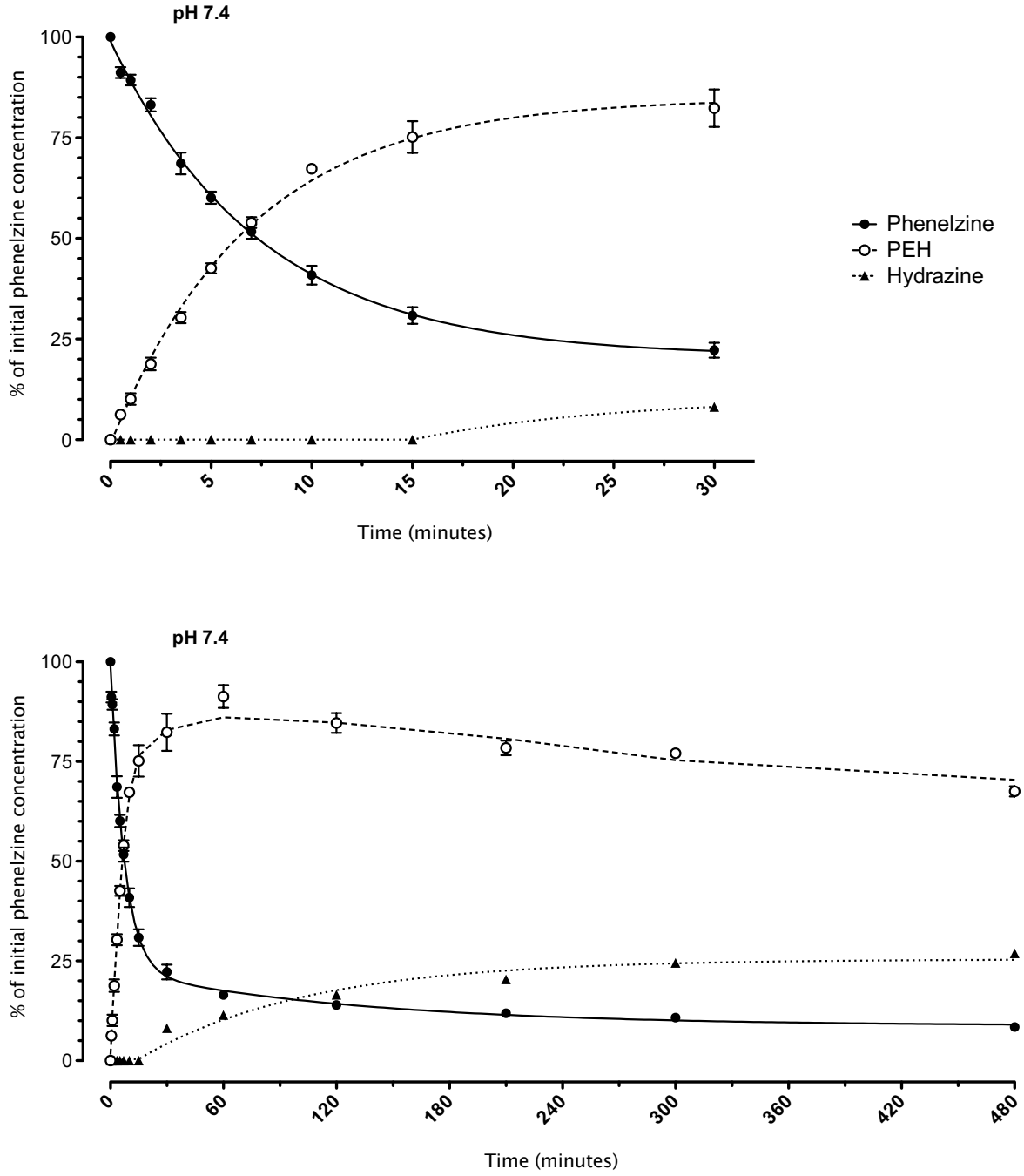


Figure 2-13. Oxidation of 15 μM phenelzine by 30 nM MAO-B over a period of 8 hours in 1 mM potassium phosphate buffer at pH of 7.4. Data (n = 3) are represented as percent of 15 μM initial phenelzine concentration (mean ± SEM).

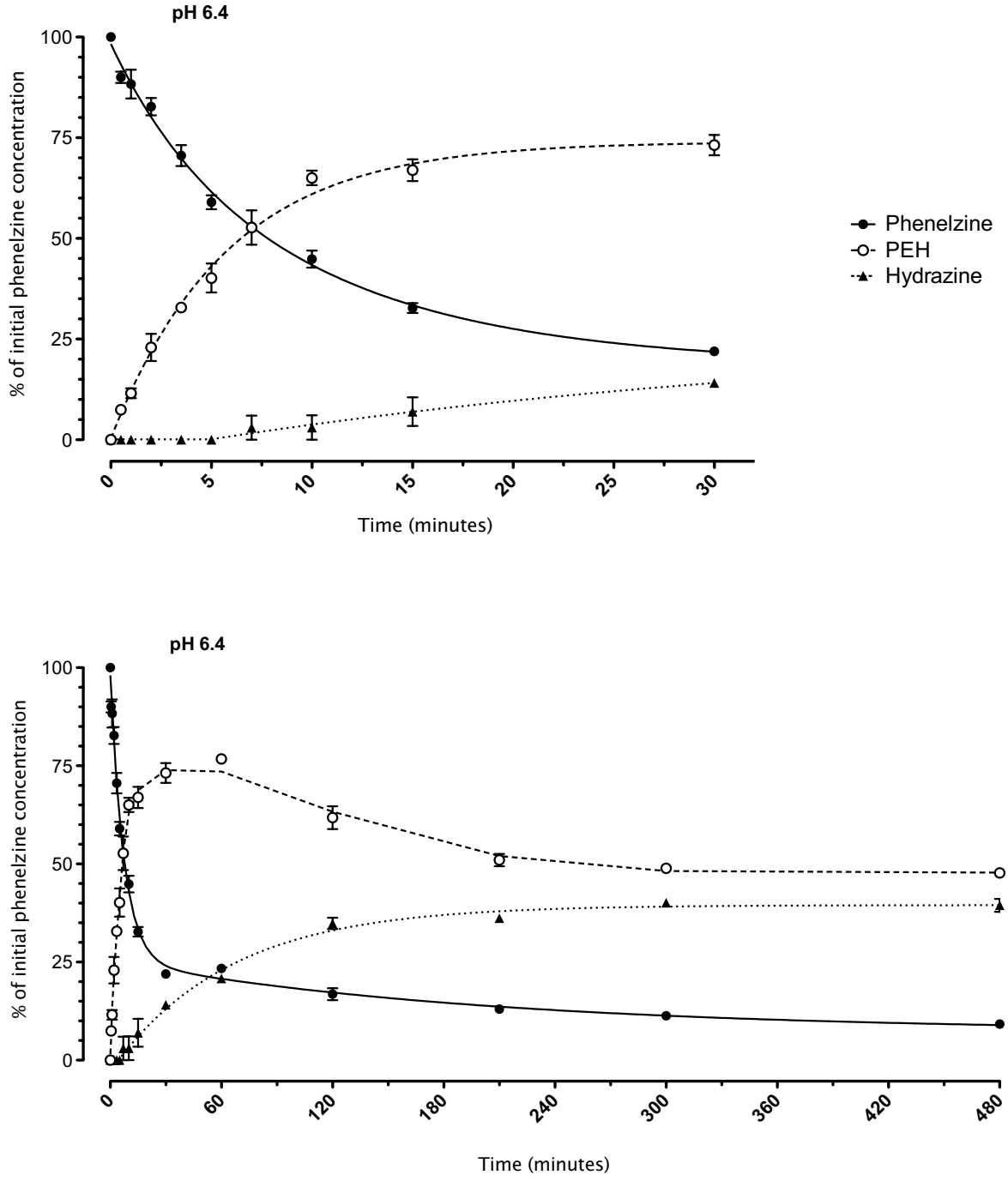


Figure 2-14. Oxidation of 15 μM phenelzine by 30 nM MAO-B over a period of 8 hours in 1 mM potassium phosphate buffer at pH of 6.4. Data (n = 3) are represented as percent of 15 μM initial phenelzine concentration (mean ± SEM).

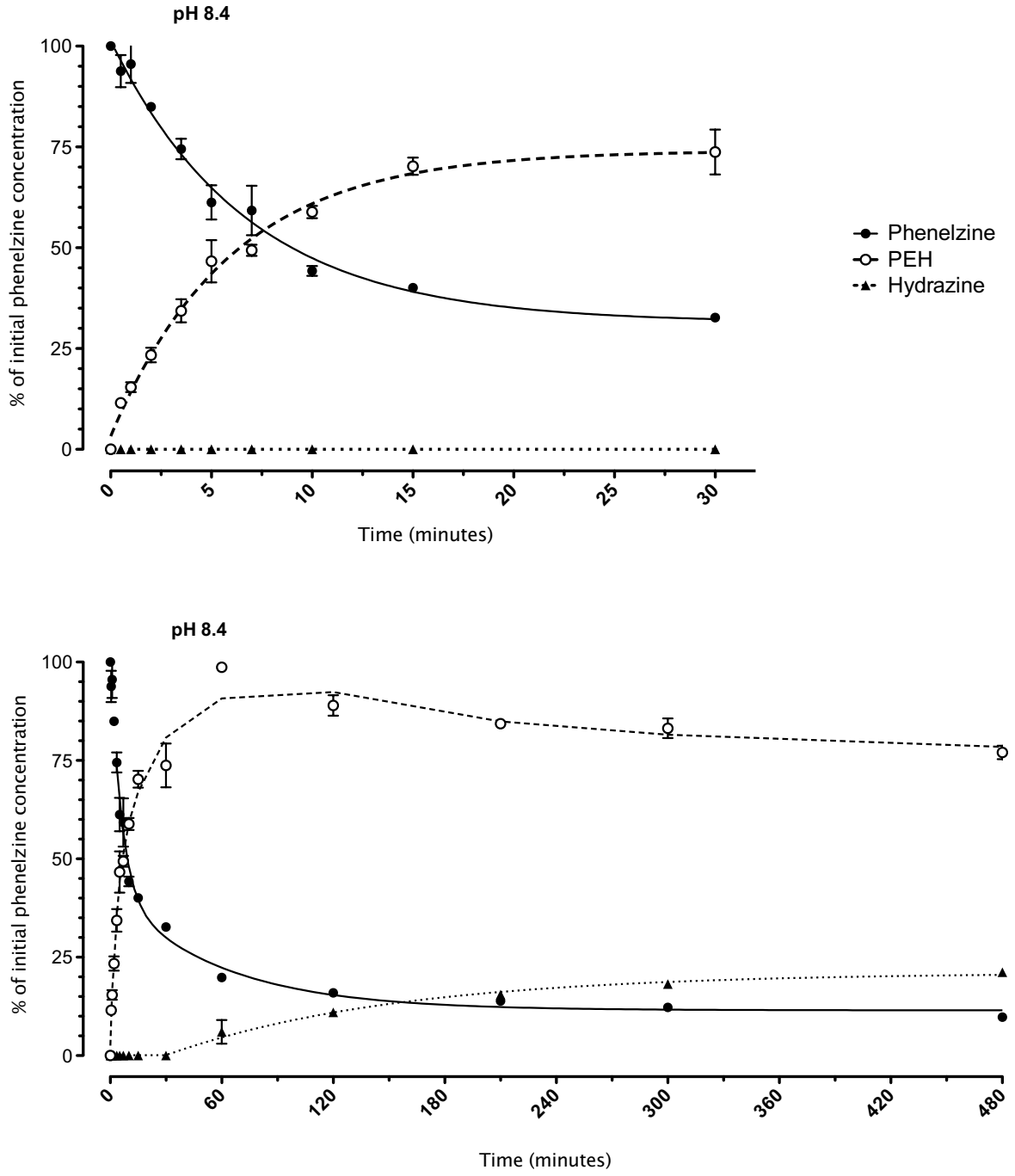


Figure 2-15. Oxidation of 15 μM phenelzine by 30 nM MAO-B over a period of 8 hours in 1 mM potassium phosphate buffer at pH of 8.4. Data (n = 3) are represented as percent of 15 μM initial phenelzine concentration (mean ± SEM).

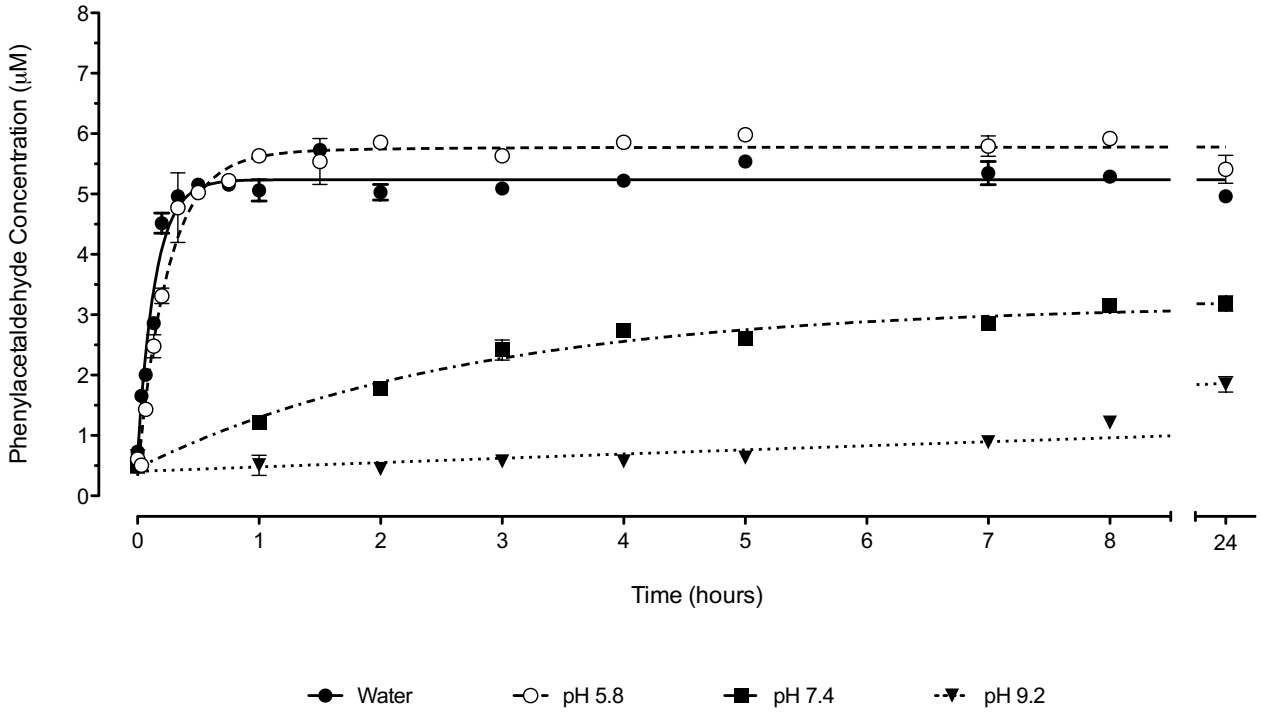


Figure 2-16. Formation of phenylacetaldeyde from hydrolysis of 15 μM PEH in water and 5 mM aqueous buffers at pH levels of 5.8, 7.4 and 9.2 over a span of 24 hours. Data are represented as mean ± SEM from 3 replicate determinations.

2.3.4 PEH is a competitive, reversible inhibitor of MAO-B

Inhibition curves of MAO-B by phenelzine and PEH were determined using the benzaldehyde spectrophotometric assay (the Amplex Red assay could not be employed due to a variety of interferences with reaction components). In triplicate, 30 nM MAO-B was incubated with a range of phenelzine and PEH concentrations for 1 hour at room temperature in 0.1 M potassium phosphate buffer (pH 7.4), followed by addition of 3 mM benzylamine as substrate. The remaining enzyme activity was determined by measuring the rate of benzaldehyde formation at an absorbance wavelength of 254 nm. As demonstrated in **Figure 2-17**, phenelzine was a much more potent inhibitor of MAO-B under these conditions. The calculated IC_{50} values (concentration of inhibitor required for 50% inhibition of enzyme, dependent on substrate) for phenelzine and PEH were 2.4 μM and 330.8 μM, respectively.

To determine if MAO-B inhibition by PEH was of an irreversible nature, the time-dependence of MAO-B inhibition with this drug was investigated since the onset of inhibition by an irreversible inhibitor demonstrates time-dependence. This was carried out using the benzaldehyde spectrophotometric assay, where 30 nM MAO-B was incubated with 500 μM PEH for up to 1 hour at room temperature in 0.1 M potassium phosphate buffer (pH 7.4) in triplicate, followed by addition of 3 mM benzylamine as substrate. The results are shown in **Figure 2-18**. There appears to be a lack of time-dependent inhibition of MAO-B by PEH, suggesting that PEH is acting in the capacity of a reversible inhibitor.

The K_i (the concentration of inhibitor required for 50% inhibition of enzyme, independent of substrate) of PEH for MAO-B was also determined using the benzaldehyde spectrophotometric assay. In triplicate, MAO-B (20 nM) was added to 0, 30, 75 or 180 μM PEH and 30-1500 μM benzylamine in 0.1 M potassium phosphate buffer at pH 7.4 and immediately measured for absorbance at 254 nm. The initial rates of change of absorbance were plotted and analyzed using an equation for competitive inhibition in Graph Pad Prism, with a global r^2 of 0.9952 and K_i of 39.64 ± 1.30 μM (**Figure 2-19).** Fitting of PEH data to an equation for competitive inhibition of oxidized and reduced enzyme (Ramsay et al. 2011) provided K_i values of 83 μM and 72 μM, respectively, with a global r^2 of 0.9964, suggesting that PEH shows no significant preference for either form of MAO-B. In contrast, phenelzine shows much higher

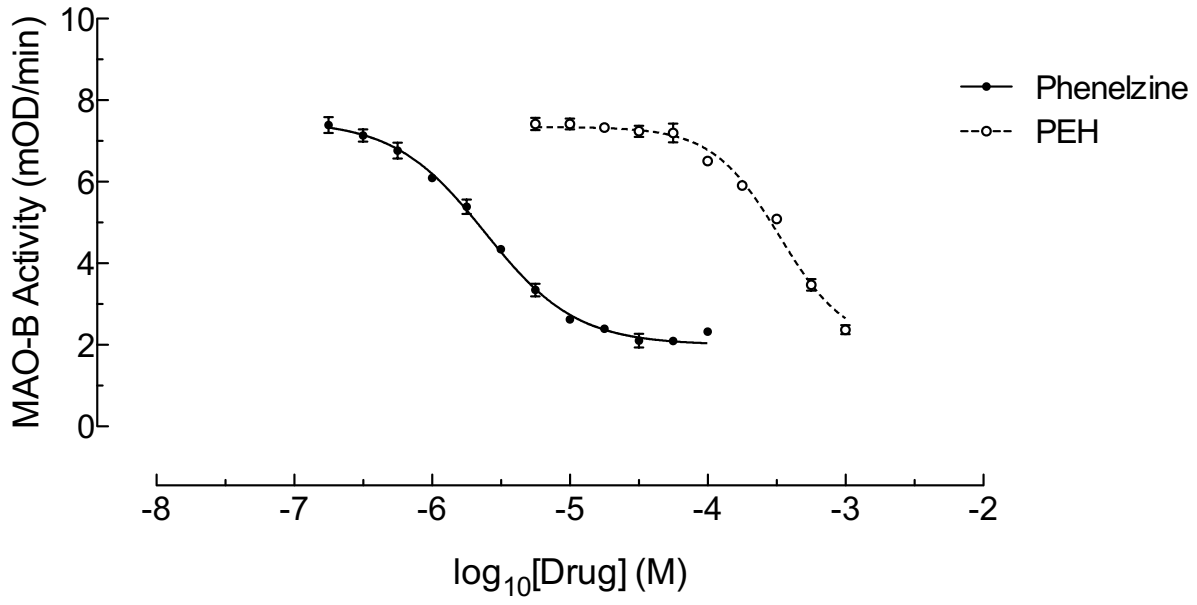


Figure 2-17. Inhibition of MAO-B (30 nM) by phenelzine and PEH following a 1 hour incubation. Remaining enzyme activity was determined with 3 mM benzylamine as substrate using a benzaldehyde spectrophotometric assay. Data are represented as mean ± SEM from 3 replicate determinations.

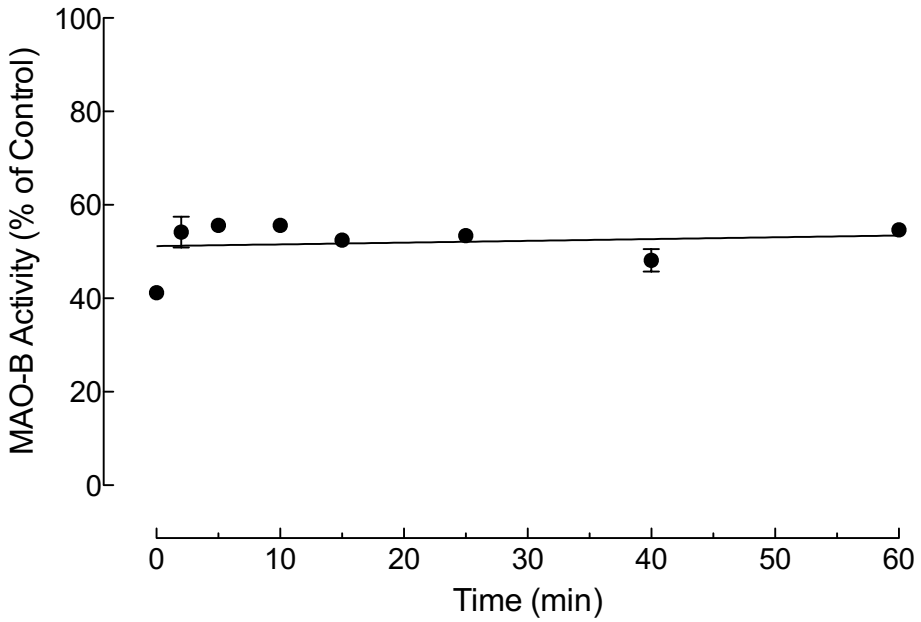


Figure 2-18. Inhibition of 30 nM MAO-B with 500 μM PEH over the span of 1 hour. Remaining enzyme activity was determined with 3 mM benzylamine as substrate using a benzaldehyde spectrophotometric assay. Data are represented as percent of controls (mean ± SEM from 3 replicate determinations) and fitted with a linear regression line.

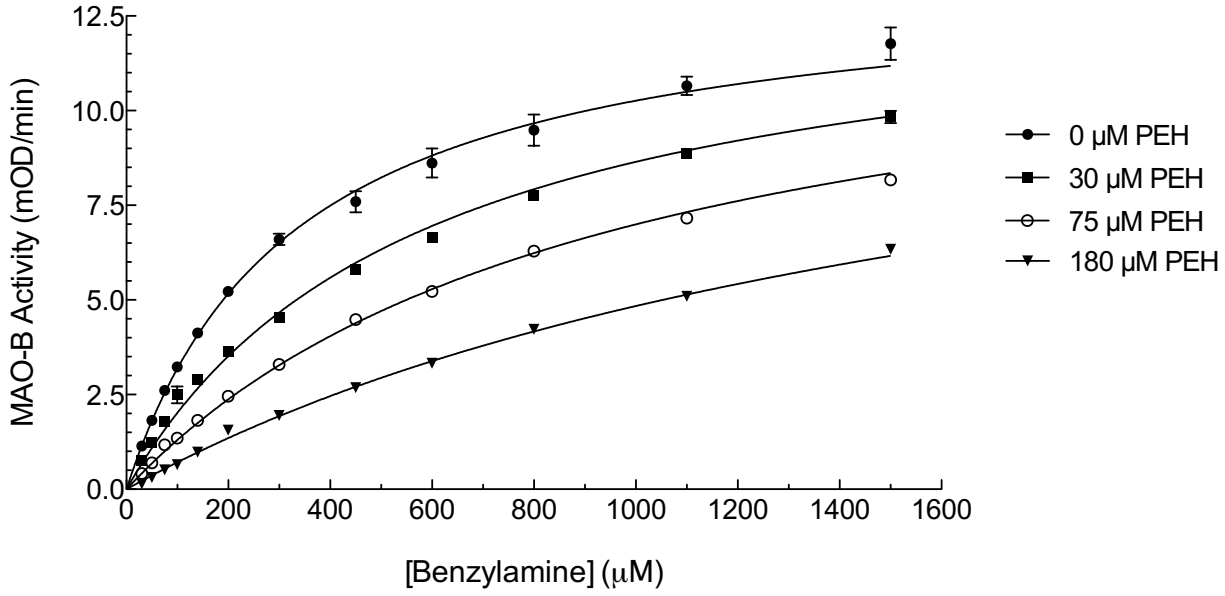


Figure 2-19. Determination of K_i for PEH. Initial rate of benzylamine oxidation was determined using the benzaldehyde spectrophotometric assay for samples containing 20 nM MAO-B, 0-180 μM PEH and 0-1500 μM benzylamine. Data are expressed as mean ± SEM from 3 replicate determinations. Based on fit to an equation for competitive inhibition of oxidized and reduced enzyme (Ramsay et al. 2011), $K_{i\,OX}$ = 83 μM and $K_{i\,RED}$ = 72 μM (global r^2 = 0.9964).

affinity for oxidized MAO-B ($K_{i\,OX}$ = 4.05 ± 0.56 μM) than for reduced enzyme ($K_{i\,RED}$ = 45.1 ± 12.8 μM) (Dr. Andrew Holt, unpublished data).

To evaluate the effect of PEH competitive inhibition on phenelzine oxidation, we employed a benzaldehyde assay to determine remaining MAO-B activity following incubation with a range of phenelzine concentrations. In quadruplicate, MAO-B (120 nM) was incubated with 10 μM, 100 μM and 1 mM phenelzine for a period of 30 minutes at room temperature in 0.1 M potassium phosphate buffer (pH 7.4), followed by addition of 800 μM or 4 mM benzylamine as substrate. To ensure that remaining apparent enzyme activity was not an unaccounted for blank rate, 25 μM mofegiline were added to some wells 10 minutes prior to benzylamine addition. When incubated with up to 1 mM phenelzine, enzyme activity of 120 nM MAO-B is not completely abolished (**Figure 2-20A**). This is confirmed by the observation that 25 μM mofegiline completely inactivated the remaining active enzyme at all of the phenelzine concentrations. When incubated with 1 mM phenelzine, there is evidence of reversible inhibition since the ratio of MAO-B activity in comparison to controls is larger when using a higher concentration of benzylamine (**Figure 2-20 B**); the inhibition with 10 μM and 100 μM phenelzine cannot be overcome by increasing benzylamine concentration, suggesting that there is irreversible inhibition at these phenelzine concentrations. This experiment provides support to the notion that when MAO-B is incubated with high concentrations of phenelzine, the concentration of PEH formed is sufficiently high to cause appreciable competitive inhibition of the enzyme.

2.3.5 Determination of IC_{50} for phenelzine at different concentrations of MAO-B

An Amplex Red spectrophotometric assay was employed to investigate the relationship between concentration of MAO-B and the corresponding IC_{50} values of phenelzine. MAO-B (5-40 nM) was incubated with a range of phenelzine concentrations for 3 hours at room temperature in 0.1 M potassium phosphate buffer at pH 7.4 (n = 3). Remaining activity of the enzyme was assessed by addition of 3 mM benzylamine as substrate, horseradish peroxidase (4 U/ml) and 20 μM Amplex Red, followed by measurement of absorbance at 571 nm. Inhibition curves are shown in **Figure 2-21**, with calculated IC_{50} and phenelzine turnover values demonstrated in **Figure 2-22**. There appears to be a linear but non-stoichiometric relationship between increasing

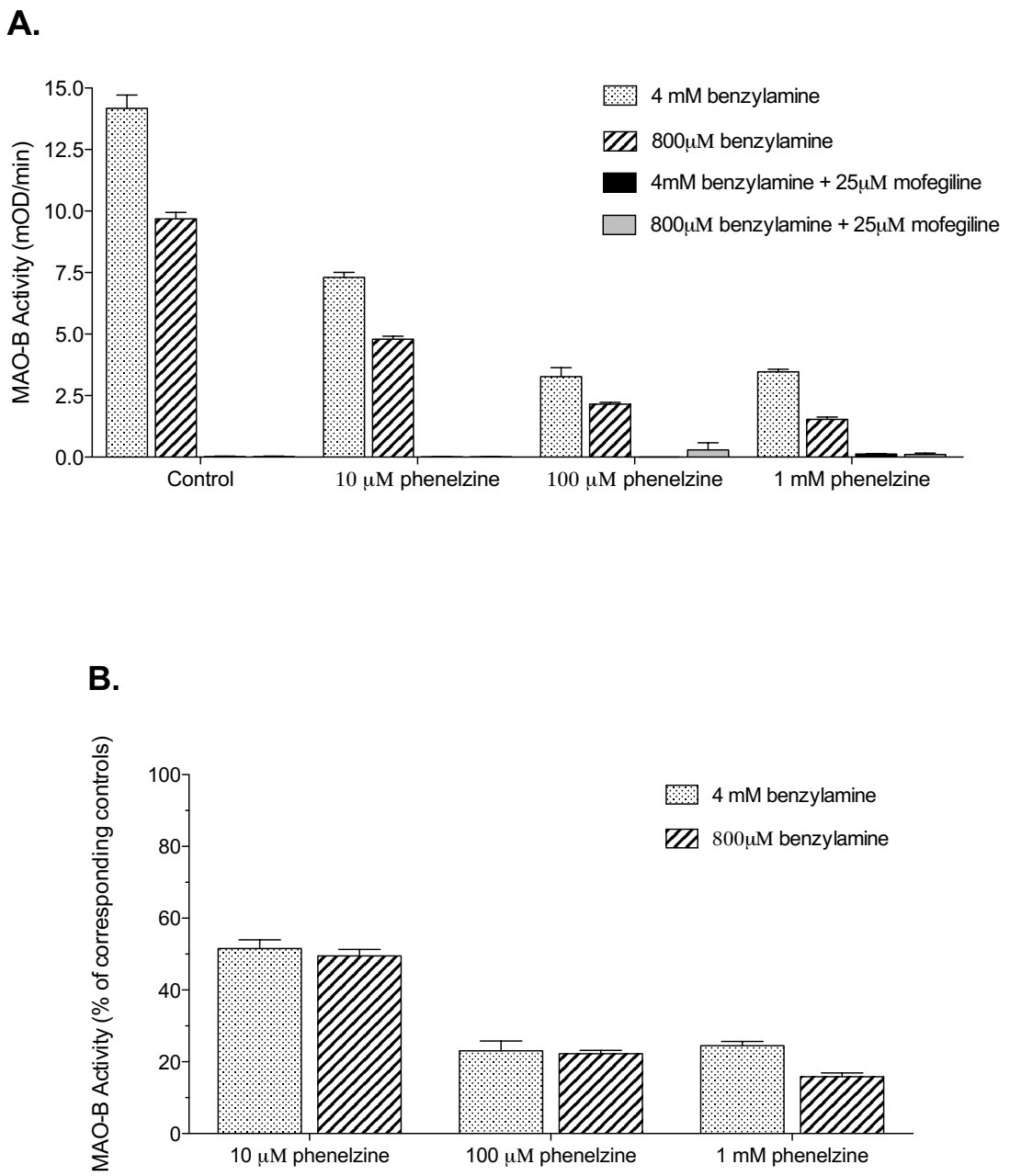


Figure 2-20. Remaining enzyme activity of 120 nM MAO-B following incubation with 10 μM, 100 μM and 1 mM of phenelzine for 30 minutes (**A**). The reversibility of enzyme inhibition was determined by using a high (4 mM) and low (800 μM) concentration of benzylamine as substrate (**B**). Data are expressed as mean ± SEM from 4 replicate determinations.

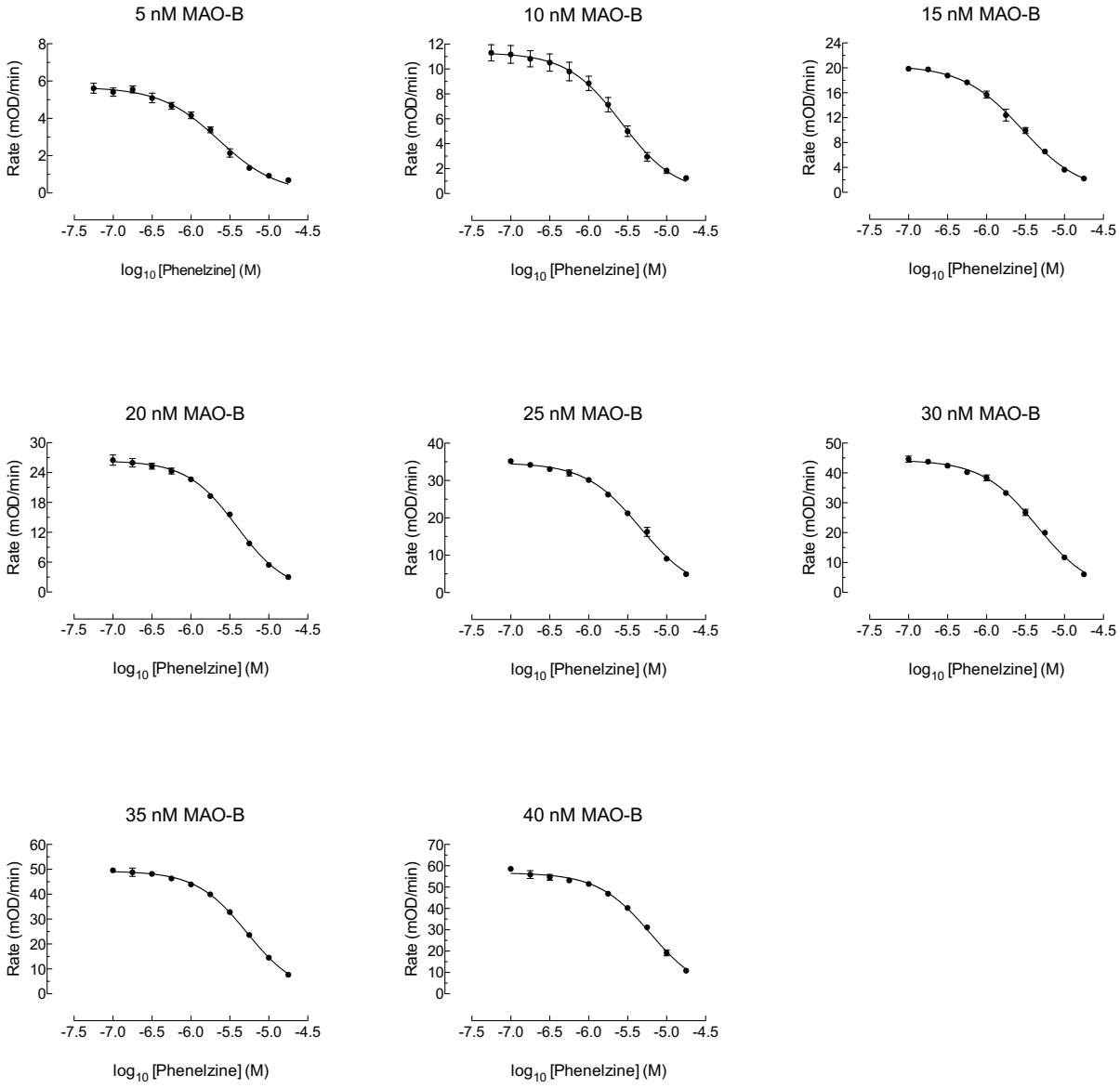


Figure 2-21. Inhibition curves of phenelzine with a range of MAO-B concentrations (5-40 nM) following incubation for 3 hours. Data (n = 3) are expressed as mean ± SEM.

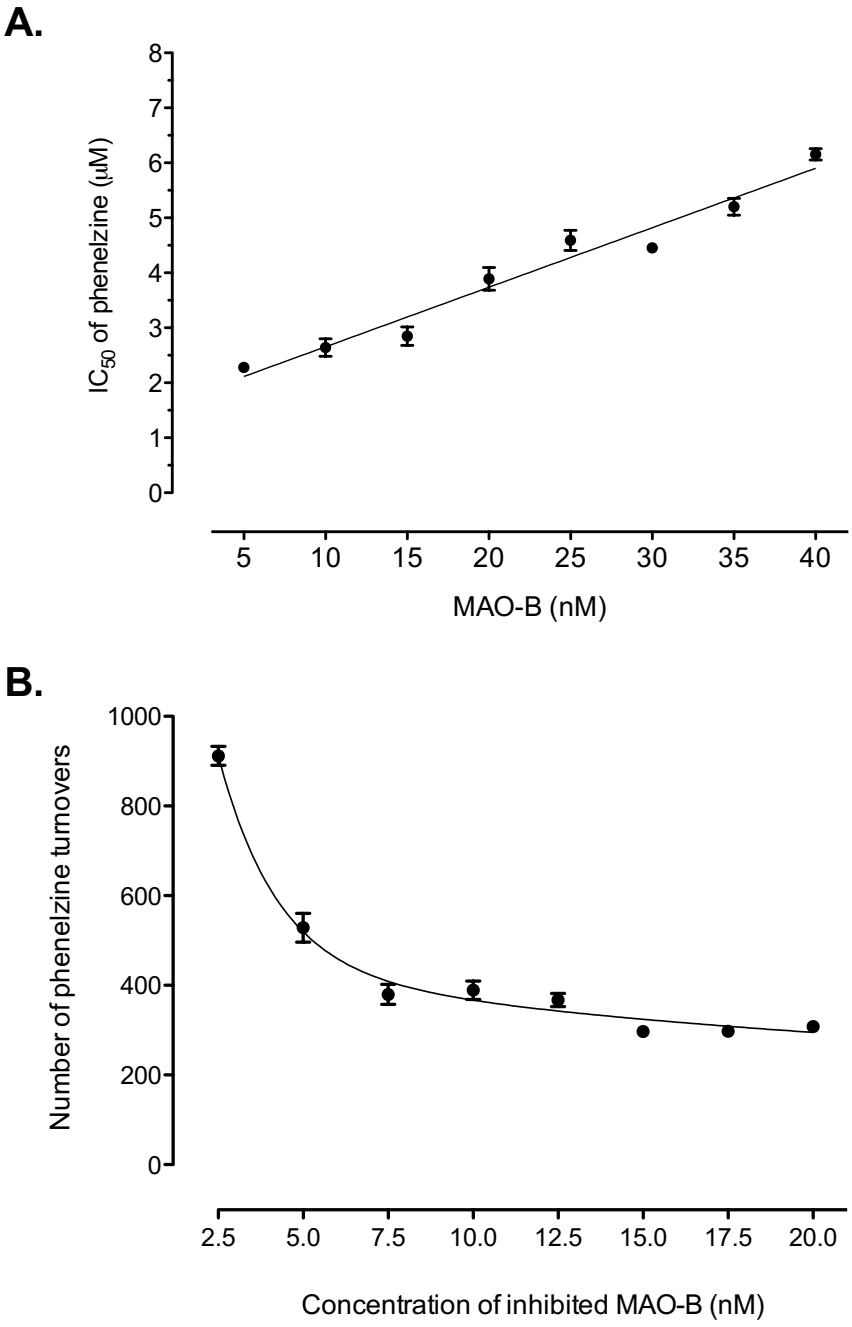


Figure 2-22. Relationship between IC_{50} of phenelzine and MAO-B concentration (**A**) and the turnover number of phenelzine required for inactivation of MAO-B (**B**). Data (n = 3), expressed as mean ± SEM, are fitted with a linear regression line ($r^2 = 0.9363$) (**A**) and a two-phase exponential decay ($r^2 = 0.9721$) (**B**).

MAO-B concentration and the IC_{50} values for phenelzine (r^2 =0.9363). Turnover number of phenelzine required to inhibit specific MAO-B concentrations was calculated by dividing the IC_{50} values of phenelzine by half of the enzyme concentration used in the experiment (since the IC_{50} is the concentration of inhibitor required for 50% inhibition of enzyme). This relationship was fitted to a two-phase exponential decay equation (r^2 = 0.9721).

2.3.6 Kinetics of phenelzine oxidation by MAO-B at a range of phenelzine and MAO-B concentrations

The acetone derivatization assay (LC-MS) was employed to measure the kinetics of phenelzine oxidation by MAO-B. Different concentrations of MAO-B (20 nM and 60 nM) were incubated with a range of phenelzine concentrations (2 μM, 6 μM and 20 μM) in 1 mM potassium phosphate buffer at pH 7.4 (n = 3). Samples were derivatized at different time points, with the change in phenelzine and PEH concentrations quantitated by LC-MS. The kinetics of the reaction were analyzed according to the increase in PEH concentration and reduction in phenelzine concentration over the duration of the oxidation reaction (**Figure 2-23**). The mean values for half times of PEH formation and phenelzine disappearance, as well as the plateau of PEH formation and drop in phenelzine concentration are represented in **Figure 2-24**.

At a constant MAO-B concentration and increasing phenelzine concentrations, the maximum amount of PEH formed and phenelzine oxidized appear to increase linearly (**Figure 2-24 C, D**) whereas the half times for PEH formation and phenelzine disappearance increase exponentially (**Figure 2-24 A, B**). This suggests that while the amount of PEH formed is proportional to the initial phenelzine concentration, it takes longer for MAO-B to be inactivated when there is a higher concentration of phenelzine present at the start of the reaction. Furthermore, the turnover number (calculated as the increase in PEH concentration or drop in phenelzine concentration divided by the concentration of MAO-B) appears to increase with increasing phenelzine concentrations in a linear fashion (**Figure 2-24 E, F)**. These data are consistent with previous findings by Dr. Andrew Holt's laboratory (personal communication, unpublished data) where the turnover number of phenelzine was increased with higher phenelzine concentrations at a constant MAO-B concentration (**Figure 2-25**).

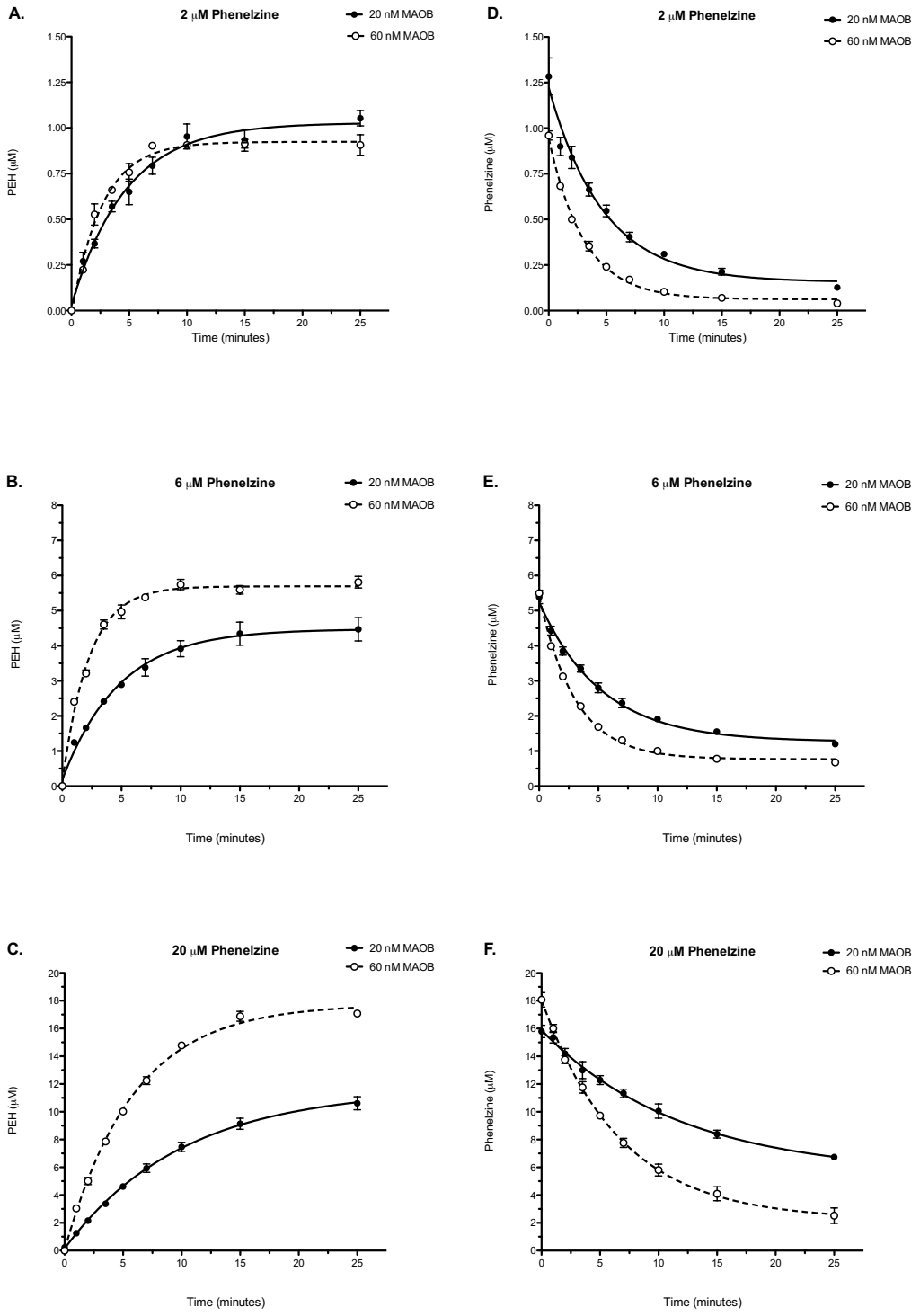


Figure 2-23. Oxidation of phenelzine (2 μM, 6 μM and 20 μM) by MAO-B (20 nM and 60 nM) measured as formation of PEH (**A-C**) and disappearance of phenelzine (**D-F**). Data (n = 3) are expressed as mean ± SEM.

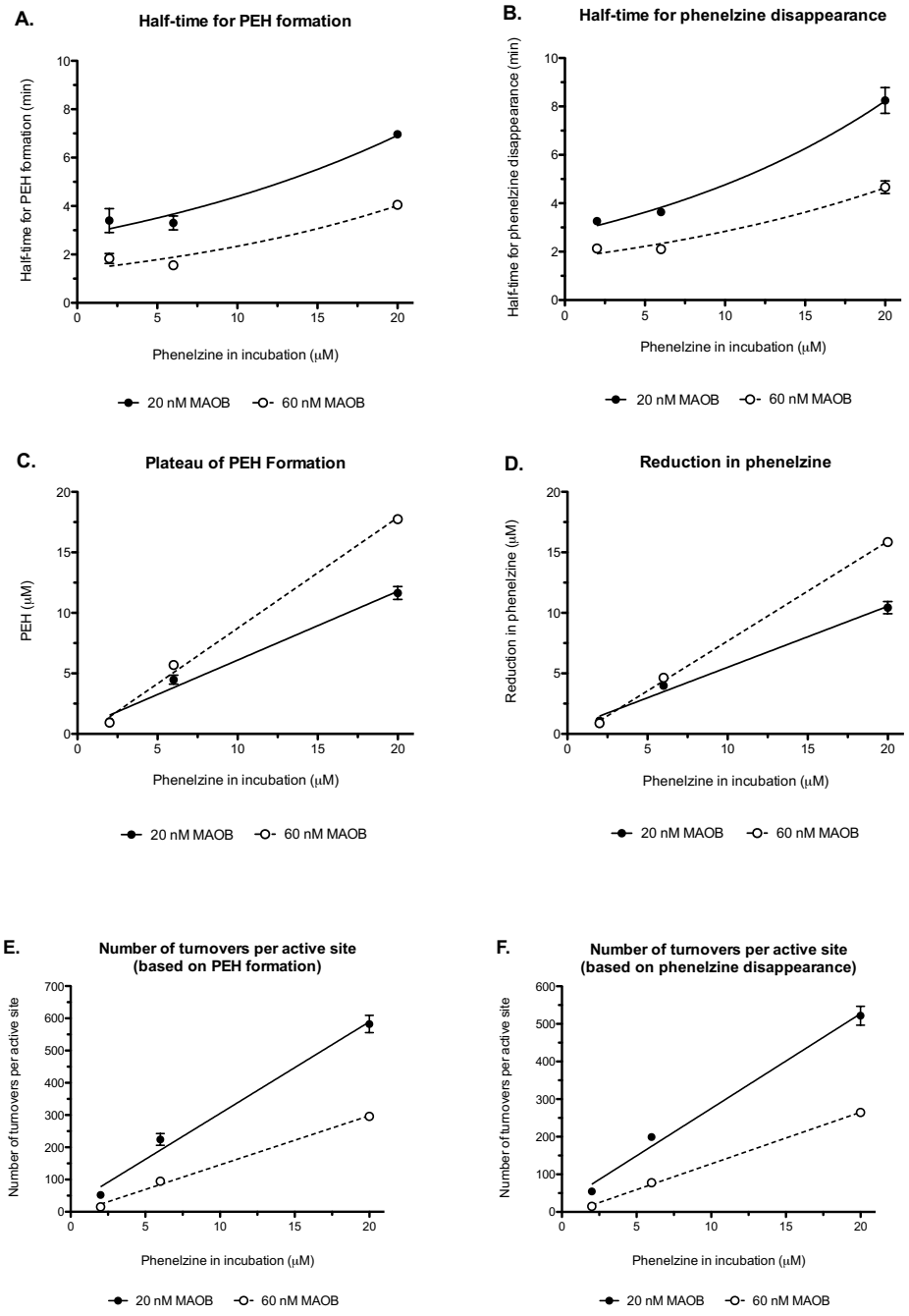


Figure 2-24. Oxidation of phenelzine (2 μM, 6 μM and 20 μM) by MAO-B (20 nM and 60 nM). Data (n = 3) are expressed as mean ± SEM for the half-time of PEH formation (**A**) and phenelzine disappearance (**B**), the plateau of PEH formation (**C**) and reduction in phenelzine concentration (**D**), as well as the turnover number per active site calculated using formation of PEH (**E**) or phenelzine disappearance (**F**).

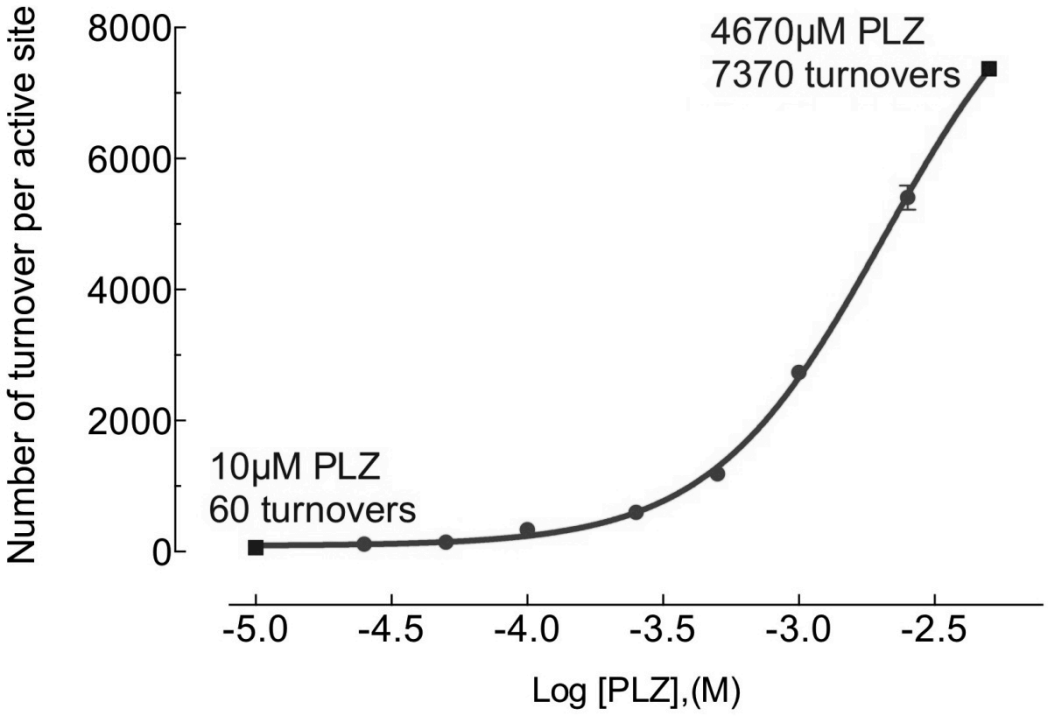


Figure 2-25. Effect of phenelzine concentration on the number of catalytic cycles prior to MAO-B (50 nM) inactivation, measured using the aldehyde dehydrogenase assay. Figure used with permission from Dr. Andrew Holt (Department of Pharmacology, University of Alberta).

When the phenelzine concentration is kept constant and the MAO-B concentration is increased, the half times for PEH formation and phenelzine disappearance are reduced (**Figure 2-24 A, B**). This suggests that the MAO-B may be inhibited at a faster rate when the concentration of the enzyme is increased. This relationship was investigated further in the experiments described in section 2.3.6.

2.3.7 MAO-B oxidation kinetics of phenelzine at a constant initial substrate concentration and increasing enzyme concentrations.

The acetone derivatization (LC-MS) assay was used to investigate the oxidation of 100 μM phenelzine with a range of MAO-B concentrations. MAO-B (1-80 nM) was incubated with 100 μM phenelzine at room temperature (n = 3) in 1 mM potassium phosphate buffer (pH 7.4) containing 0.1 μg/ml catalase (to remove H_2O_2 produced by the MAO-B catalytic cycle). Quantitation of phenelzine concentration in the samples was used to ensure that phenelzine was not depleted during the reaction. The formation of PEH as a function of time is represented in **Figure 2-26**, whereas the plateaus and half-times for PEH formation as a function of MAO-B concentration are shown in **Figure 2-27**. As MAO-B is increased to higher concentrations, the amount of PEH formed appears to head towards a plateau (**Figure 2-27 A**) and the half-life for PEH formation is reduced (**Figure 2-27 B**). These results indicate that at higher concentration of MAO-B, the enzyme is inhibited at a faster rate, allowing less PEH to be formed from phenelzine.

2.4 DISCUSSION

The work presented here has contributed to the elucidation of the mechanisms of MAO-mediated oxidation of phenelzine to PEH. Employing the acetone assay to directly measure phenelzine and PEH, we were able to show that PEH is the primary metabolite of phenelzine oxidation by human MAO-B *in vitro.* In the literature, a metabolite of phenelzine was reported to be consistent with the spectrophotometric and thin-layer chromatography profiles of PEH in experiments with pig brain and bovine kidney MAO (Tipton and Spires 1972; Patek and Hellerman 1974). However, these methods were either indirect (by measuring phenylacetaldehyde reaction with 2,4-dinitrophenylhydrazine) or not quantitative and lacking

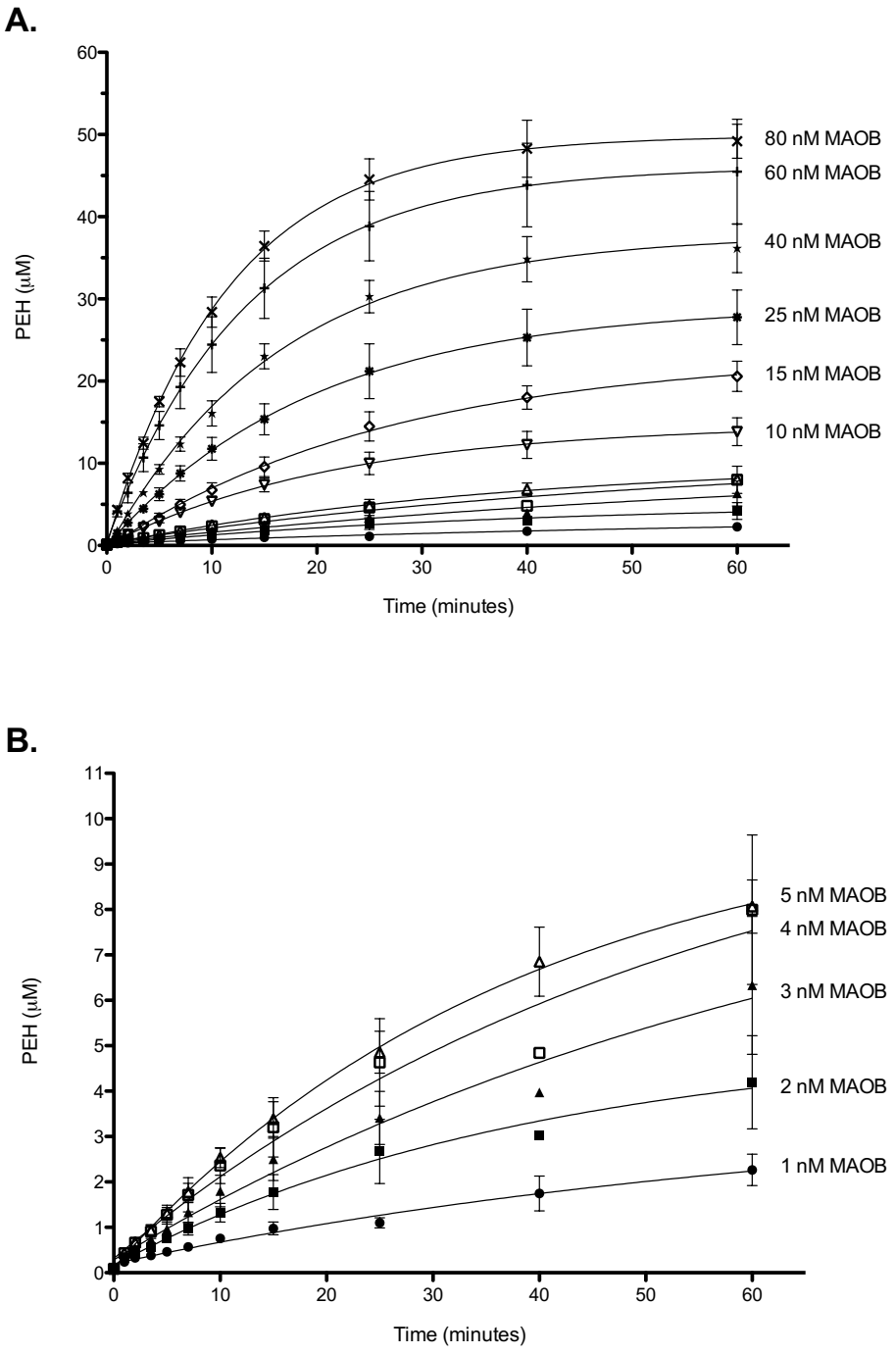


Figure 2-26. Formation of PEH from 100 μM phenelzine and 1-80 nM MAO-B. Data (n = 3) are represented as means ± SEM. Figure (**B**) is a representation of 1-5 nM MAO-B on a smaller scale than Figure (**A**). Data were collected using the acetone assay (LC-MS).

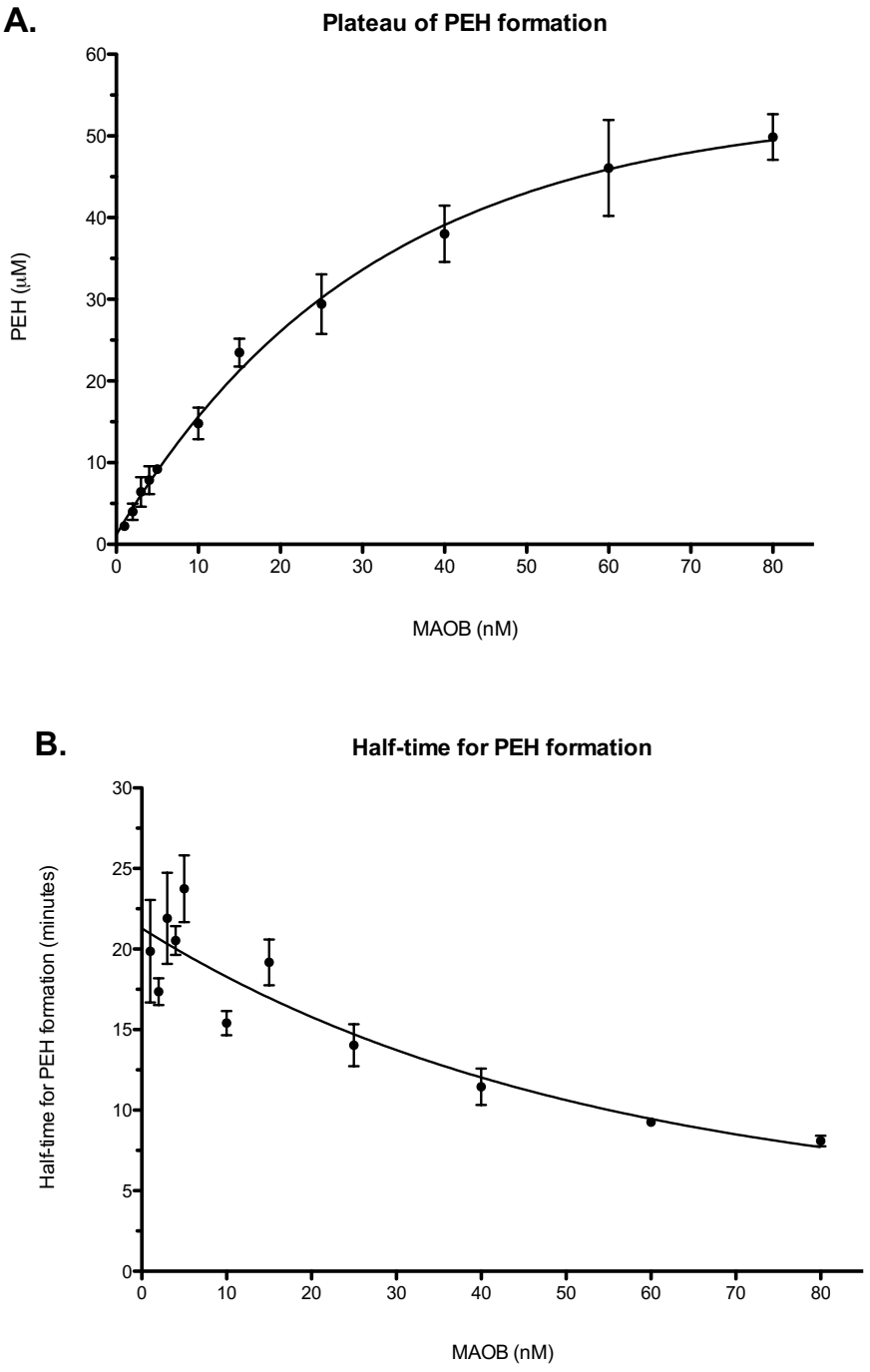


Figure 2-27. Relationship between increasing MAO-B concentration and plateau of PEH formation (**A**) and half-time for PEH formation (**B**) when incubated with 100 μM phenelzine. Data (n = 3) are expressed as means ± SEM.

sensitivity. Moreover, the aforementioned studies were not performed using human enzyme. The *in vivo* production of PEH in the rat brain following phenelzine administration was previously investigated in our laboratories (MacKenzie 2009); however, this assay was determined to be indirectly measuring PEH conversion to hydrazine as described in Chapter 6. With the ability to measure directly both phenelzine disappearance and PEH appearance at specific time intervals of the enzymatic reaction, the results obtained in this investigation provide strong support that phenelzine oxidation by human MAO-B gave almost stoichiometric yields of PEH.

Following oxidation of an amine by MAO-B, it is believed that the imine product dissociates from the oxidized enzyme, leaves the active site and is non-enzymatically hydrolyzed (Edmondson et al. 1993). In general, imines are regarded as unstable compounds that hydrolyze rapidly in water. In an *in vitro* study, phenylacetaldehyde was found to be a major product of phenelzine oxidation by rat liver MAO (Yu and Tipton 1989). Phenylacetic acid, formed from phenylacetaldehyde, was reported to be the major urinary metabolite of phenelzine in rats and humans (Clineschmidt and Horita 1969b; Robinson et al. 1985). These reports suggest that PEH does undergo hydrolysis, although it remained relatively unclear as to how rapidly and to what extent this hydrolysis occurred following phenelzine administration. Our data suggest that PEH is relatively stable in aqueous buffered media at a pH of 7.4, with approximately 20-25% of PEH undergoing hydrolysis to hydrazine and phenylacetaldehyde after 8 hours. The rate of PEH hydrolysis appears to be pH-dependent, with lower pH levels corresponding to increased hydrazine and phenylacetaldehyde formation. In contrast, higher pH levels are protective against PEH hydrolysis. Of interest, PEH hydrolysis is quite rapid in water; as such, PEH should be made up in buffered solution at a pH of 7.4 or higher for future *in vitro* and *in vivo* experiments. Moreover, it appears that PEH and its hydrolysis products reach an equilibrium over time, suggesting that not all of the PEH will be hydrolyzed at a neutral pH level if the hydrolysis products are not removed. The stability of PEH in the present *in vitro* experiments is consistent with observations that PEH administration to rats produces lasting changes (up to 12-24 hours) in brain levels of the amino acids γ-aminobutyric acid (GABA), alanine, tyrosine and ornithine (MacKenzie et al. 2008b; Matveychuk et al. 2013).

The finding that PEH is a reversible, competitive inhibitor of MAO-B with a $K_{i\,OX}$ of 83 μM and $K_{i\,RED}$ of 72 μM is important for understanding the mechanisms of phenelzine inhibition

of MAO. In earlier studies on phenelzine, there was some debate as to the identity of the metabolic product of phenelzine responsible for irreversible MAO inhibition. Tipton (1972) suggested that PEH was a powerful competitive inhibitor which also acted as a time-dependent irreversible inhibitor of pig brain MAO. Our data support the notion that PEH is a competitive inhibitor in human MAO-B, but demonstrates a lack of time-dependent irreversible inhibition. This is in agreement with a study by Patek and Hellerman (1974), who found that treatment of bovine kidney MAO with PEH produced little, if any, irreversible inhibition. Furthermore, administration of PEH to rats did not produce significant changes in activity of brain MAO-A or MAO-B (Paslawski et al. 2001; MacKenzie et al. 2008a) nor increases in brain levels of noradrenaline, serotonin or dopamine (MacKenzie 2009, Chapter 3) at up to 12 hours following injection. Taken together, there is a strong suggestion that PEH is not the inhibitory species of phenelzine oxidation responsible for irreversible inhibition of MAO. However, the ability of PEH to act as a competitive inhibitor does have implications for phenelzine inhibition of MAO-B. When MAO-B is incubated with high concentrations of phenelzine, the half-times for PEH formation and phenelzine disappearance are increased exponentially (**Figure 2-24 A, B**). A possible explanation for this finding is that when relatively high concentrations of PEH are formed from phenelzine, PEH can compete with the remaining phenelzine and with any inhibitor derived from phenelzine for entry to the active site of MAO-B and slow enzyme inactivation. This is supported by the finding that both irreversible and reversible inhibition (caused by phenelzine and PEH, respectively) of MAO-B is present when the enzyme is incubated with a high concentration (1 mM) of phenelzine (**Figure 2-20 B**).

In the literature, there is support for phenylethyldiazene as the irreversible inhibitory species of phenelzine oxidation by MAO-B. It was suggested that phenyldiazene was the inhibitory product of phenylhydrazine oxidation by MAO (Patek and Hellerman 1974). Dideuteration of phenelzine at the carbon-1 position was demonstrated to increase the inhibitory effect on MAO; the authors postulated that dideuteration of carbon-1 interferes with removal of hydrogen from that carbon (to form PEH) and potentiates removal of hydrogen from the neighbouring hydrazine group (to form phenylethyldiazene) (Yu and Tipton 1989). Diazenes are known to be highly reactive compounds and have been shown to react rapidly with oxygen to form an alkyl radical, nitrogen and superoxide anion (Huang and Kosower 1967; Kosower 1971). Of interest, phenelzine was shown to inhibit rat liver cytochrome P-450 by a phenylethyl radical

mechanism (Ortiz de Montellano et al. 1983). In human MAO-B experiments, phenelzine was shown to alkylate the FAD cofactor via an arylalkyl derivative (Binda et al. 2008).

The relationship between PEH and phenylethyldiazene remains relatively unclear. Binda and colleagues (2008) have proposed that PEH and phenylethyldiazene are formed by separate catalytic pathways of MAO: either a C-H bond cleavage to form PEH or a N-H bond cleavage to form phenylethyldiazene. In contrast, others have suggested that phenylethyldiazene can be converted to PEH by intramolecular rearrangement (Patek and Hellerman 1974; Yu and Tipton 1989). These reaction schemes are summarized in **Figure 2-2**. Due to the inability of PEH to exert time-dependent irreversible inhibition of MAO-B (**Figure 2-18)** and given that inhibition of MAO-B caused by PEH if far less extensive than that caused by an equimolar amount of phenelzine (**Figure 2-17)**, it is unlikely that PEH is the initial product of phenelzine oxidation that is subsequently rearranged to phenyethyldiazene (Scheme C of **Figure 2-2**). However, we were unable to differentiate the likelihood of phenelzine oxidation following either Scheme A or B of **Figure 2-2**. This was due, in part, to the instability of phenylethyldiazene and the lack of available assays for detection of diazenes in solution. We were also unsuccessful in our attempts to sequester the phenylethyldiazene using a wide variety of chemical compounds.

It has been proposed that phenylethyldiazene does not leave the MAO catalytic site; rather, it remains within the active site, where it can react with oxygen to form an arylalkyl radical and immediately alkylate the FAD cofactor to inhibit the enzyme (irreversibly) (Binda et al. 2008). However, our data support the theory that phenylethyldiazene exits the MAO-B active site and must then compete with phenelzine and PEH for entry back into the active site. When the initial phenelzine concentration was increased at a constant MAO-B concentration, we observed an increase in the number of phenelzine turnovers per active site prior to enzyme inactivation (**Figure 2-24 E, F** and **Figure 2-25**). The change in the turnover number of phenelzine per inactivation event (ranging from 60 to over 7000) is in conflict with a previous finding of a constant 36-40 turnovers (Binda et al. 2008). The increase in turnover number at higher phenelzine concentrations reflects the increased time taken to inhibit the enzyme, and may be due to increased competition between phenylethyldiazene, phenelzine and PEH for binding to the active site. Since diazenes are highly reactive species, the stability of phenylethyldiazene in solution may be limited. As such, increased competition with phenelzine and/or PEH may keep

phenylethyldiazene in solution long enough for it to decompose and/or alkylate nearby amino acids. At sufficiently high concentrations of phenelzine and/or PEH, the likelihood of the diazene being able to re-access the active site before binding to the FAD cofactor is reduced; thus enzyme is inactivated at a slower rate and consequently more phenelzine can be oxidized to PEH. This observation is further supported by the finding that at constant phenelzine and increasing MAO-B concentrations, the amount of PEH formed appears to reach a plateau (**Figure 2-27 A**) and the half-time for PEH formation is reduced (**Figure 2-27 B**). If phenylethyldiazene did not leave the active site, we would expect that the half-time for PEH formation would increase (due to increased competition between the newly formed PEH and remaining phenelzine, resulting in slower enzyme inactivation) and the relationship between the amount of PEH formed and MAO-B concentration would be linear (if turnover rate of phenelzine per inactivation event is constant, the amount of product formed will be proportional to the MAO-B concentration but it will take longer for the reaction to reach completion). However, our results seem to suggest that the enzyme is actually inactivated more quickly when a higher amount of MAO-B is present. This is also in agreement with the results we obtained from determination of phenelzine IC_{50} for a range of MAO-B concentrations, where higher concentrations of MAO-B required fewer turnovers of phenelzine for enzyme inactivation (**Figure 2-22 B**). We postulate that a higher concentration of enzyme would result in a faster rate of PEH and phenylethyldiazene formation. Since the diazene likely has a much higher affinity for the MAO-B active site in comparison to phenelzine and PEH, a faster rate of diazene formation would correlate to an increased likelihood of diazene binding to the active site and faster enzyme inactivation. If MAO-B is inactivated at a faster rate, the amount of phenelzine that is converted to PEH should decrease with increasing enzyme concentrations.

The reason for the diazene leaving the active site is unclear, but may involve the inability of phenylethyldiazene and/or its arylalkyl radical to bind to the reduced form of FAD. As such, further experiments should be conducted to investigate the affinity of phenylethyldiazene and/or its arylalkyl radical for reduced and oxidized forms of MAO-B. Based on the findings discussed in this chapter, the proposed mechanisms of phenelzine oxidation by human MAO-B are summarized in **Figure 2-28**.

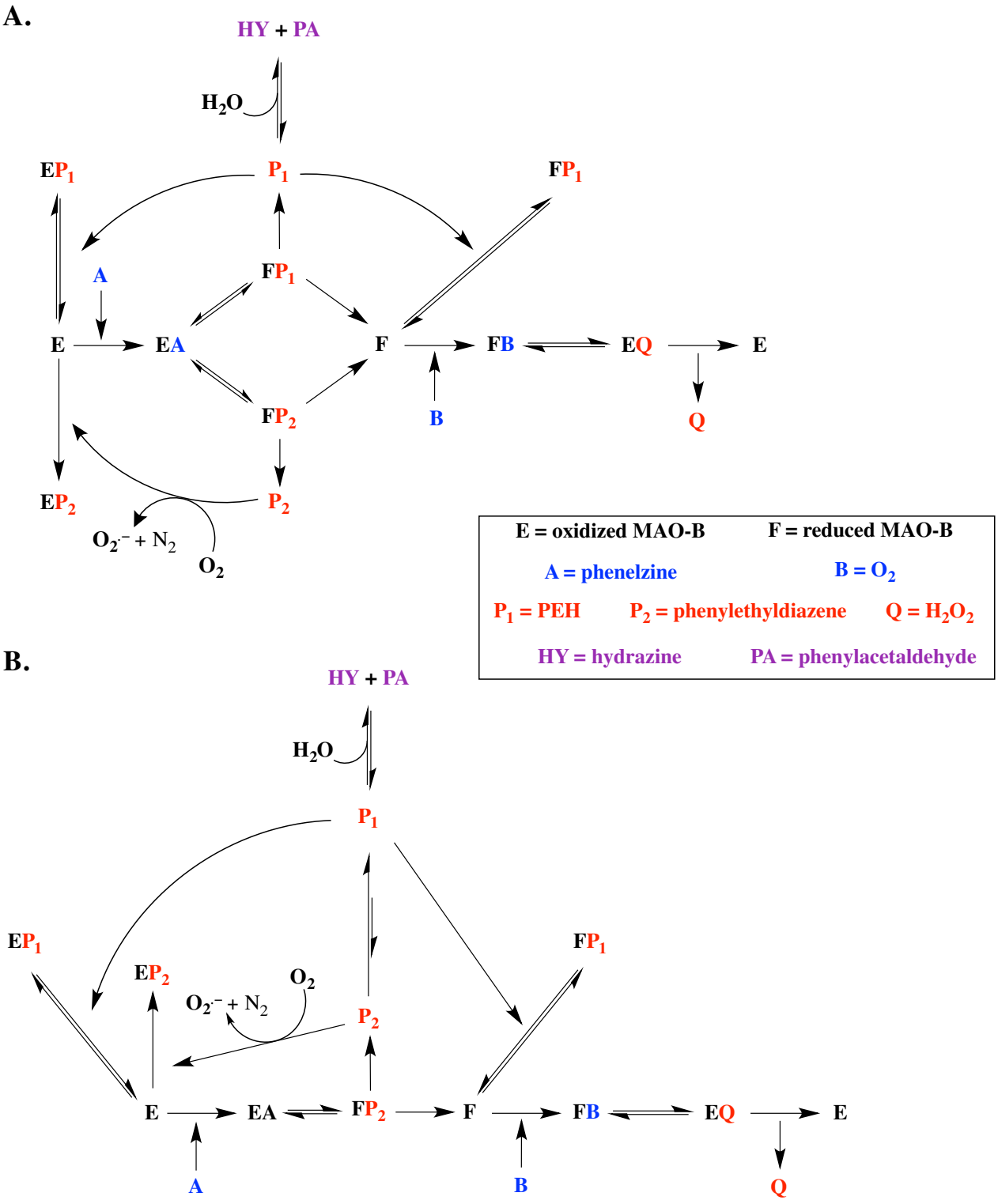


Figure 2-28. Proposed mechanisms for phenelzine oxidation by human MAO-B, with production of PEH and phenylethyldiazene by separate catalytic pathways (**A**) or with phenylethyldiazene being the proximal product (**B**).

2.5 CONCLUSION

The study described here has elucidated several important aspects of phenelzine metabolism by MAO-B. Increasing concentrations of phenelzine may increase the turnover of phenelzine by MAO-B prior to enzyme inhibition, a result attributed to increased competition with high concentrations of PEH acting as a competitive inhibitor. In addition, we have provided support for phenylethyldiazene, the inhibitory species responsible for enzyme inactivation, leaving the MAO-B active site and competing with phenelzine and/or PEH for access to the FAD cofactor. These findings suggest that there may be optimal concentrations of phenelzine for achieving maximum production of PEH or for maximum inhibition of MAO-B. Moreover, the ability of phenylethyldiazene, a very reactive species, to leave the active site and alkylate surrounding proteins may be responsible for some adverse effects of phenelzine, such as rare incidences of hepatotoxicity reported with phenelzine (Robinson and Kurtz 1987). Finally, these experiments should be replicated with human MAO-A as differences in mechanisms of phenelzine oxidation may be present between the two enzyme isoforms.

PEH was found to be relatively stable at a pH of 7.4, with only 20-25% of PEH hydrolyzed to hydrazine and phenylacetaldehyde over a span of 8 hours. It is also a reversible competitive inhibitor of MAO-B with a $K_{i\,OX}$ that is approximately 20-fold higher than that of phenelzine. From previous animal experiments, it appears that PEH is a weak inhibitor of both MAO enzymes. As such, it would be of value to determine if PEH is also a reversible competitive inhibitor of MAO-A in future experiments. If this is the case, PEH should not be associated with potential dietary interactions with tyramine-containing food and beverages, a major drawback to clinical use of phenelzine. Since PEH shares several neuroprotective properties of phenelzine, but should not be associated with irreversible MAO inhibition or phenylethyldiazene production, it may be a useful drug in its own right.

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CHAPTER 3.

Comparison of phenelzine and geometric isomers of β-phenylethylidehydrazine (PEH) on rat brain levels of amino acids, biogenic amine neurotransmitters and methylamine

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3.1 INTRODUCTION

Phenelzine (β-phenylethylhydrazine) is a monoamine oxidase (MAO)-inhibiting antidepressant used for over 50 years in the treatment of not only depression but also several other psychiatric disorders including panic disorder and social anxiety disorder. Phenelzine is an irreversible inhibitor of both MAO-A and MAO-B, and is unique in that it is also a substrate for the MAO enzymes and is metabolized by them to β-phenylethylidenehydrazine (PEH) (Binda et al. 2008; Todd and Baker 2008; MacKenzie 2009). In recent years, there has been a flurry of interest in MAO inhibitors because of their reported neuroprotective properties (Youdim et al. 2006); this interest has included phenelzine and its metabolite PEH as both have been shown to provide protection against neuronal loss in a gerbil model of transient global ischemia (Todd et al. 1999; Wood et al. 2006).

In addition to increasing brain levels of biogenic amines due to MAO inhibition, phenelzine has also been demonstrated to produce a pronounced elevation of brain levels of γ-aminobutyric acid (GABA) and alanine (ALA), presumably due to its inhibition of GABA transaminase and ALA transaminase, respectively (Popov and Matthies 1969; Wong et al. 1990; Baker et al. 1991; McManus et al. 1992; Paslawski et al. 1995; Todd and Baker 1995; Tanay et al. 2001; Todd and Baker 2008). It has been suggested that a metabolite of phenelzine formed by the action of MAO is primarily responsible for the aforementioned effects since phenelzine-induced increases in GABA and ALA can be abolished by pre-treating the animals with another MAO inhibitor (Popov and Matthies 1969; Todd and Baker 1995; MacKenzie 2009); it now appears that PEH is that metabolite. Injection of PEH to rats produces an elevation of brain ALA and GABA levels comparable to that of phenelzine; however, unlike phenelzine, this effect is not abolished by pre-treatment with a MAO inhibitor (MacKenzie, 2009).

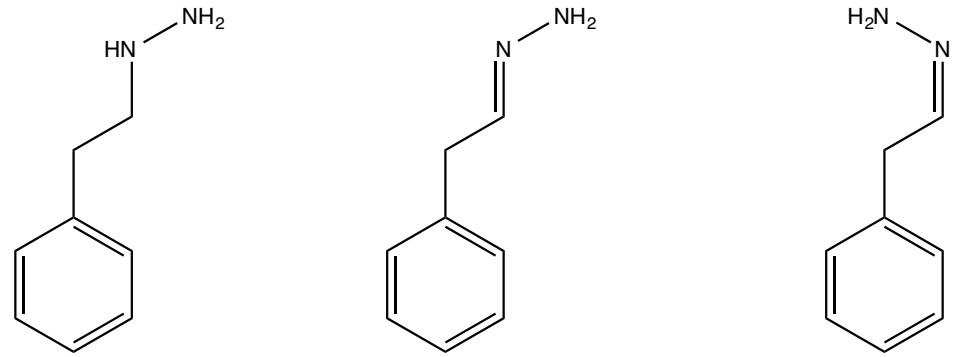
The enzyme primary amine oxidase (PrAO; E.C. 1.4.3.21), formerly called semicarbazide-sensitive amine oxidase (SSAO), is a copper-dependent amine oxidase responsible for the deamination of primary aliphatic and aromatic amines to their corresponding aldehydes, ammonia and hydrogen peroxide (Precious and Lyles 1988; Lyles 1996). The enzyme is found in two main forms, as a (1) membrane-bound form present in endothelial and vascular smooth muscle cells and adipocytes (Lyles 1996; Jiang et al. 2008), as well as a (2) soluble form

circulating in the bloodstream (Abella et al. 2004). It was discovered that PrAO is identical to vascular adhesion protein-1 (VAP-1), an endothelial protein responsible for lymphocyte trafficking and whose expression is induced during inflammation, suggesting that PrAO/VAP-1 may be a dual-function protein that can participate as either an adhesion protein or an amine oxidase, depending on its location of expression (Smith et al. 1998). The activity and/or expression of PrAO has been reported to be increased in diabetes mellitus (Boomsma et al. 1995; Garpenstrand et al. 1999; Meszaros et al. 1999), heart failure (Boomsma et al. 1997; Boomsma et al. 2000), atherosclerosis (Karadi et al. 2002), hemorrhagic and ischemic stroke (Airas et al. 2008; Hernandez-Guillamon et al. 2012), as well as Alzheimer's disease (Ferrer et al. 2002; del Mar Hernandez et al. 2005). Methylamine and aminoacetone have a high affinity for PrAO and are considered to be physiological substrates for the enzyme, undergoing deamination to their respective aldehydes formaldehyde and methylglyoxal (Precious and Lyles 1988; Yu 1990; Boor et al. 1992; Lyles and Chalmers 1992; Lyles 1996). Phenelzine has been shown to be a potent inhibitor of bovine lung PrAO *in vitro* (Lizcano et al. 1996), with both PEH and phenelzine subsequently demonstrated to also inhibit human PrAO *in vitro* (MacKenzie 2009). The use of these drugs may be beneficial in the aforementioned disorders where increased activity and/or expression of PrAO may exacerbate disease pathology.

Although PEH and phenelzine share many pharmacological properties, an important distinction is that PEH is only a weak and transient inhibitor of rat brain MAO-A and MAO-B. While phenelzine was found to inhibit rat whole brain MAO-A and MAO-B by more than 97% and 70%, respectively, for a period of up to 12 hours after administration of a dose of 30 mg/kg, an equal dose of PEH produced less than 26% inhibition of either MAO at 1-3 hours and less than 2.5% inhibition of either MAO at 6-12 hours (Paslawski et al. 2001; MacKenzie et al. 2008). In addition, injection with PEH did not produce any major increase in rat whole brain levels of the biogenic amine neurotransmitters noradrenaline (NA), dopamine (DA) and 5-hydroxytryptamine (5-HT, serotonin) (MacKenzie 2009). Findings presented in Chapter 2 suggest that PEH is a reversible, competitive inhibitor of human MAO-B. A major drawback to clinical use of phenelzine is a potential interaction with tyramine-containing foods such as aged cheeses and meats, overripe fruits and vegetables and fermented beverages. By irreversibly inhibiting MAO-A in the gut, phenelzine prevents the metabolism of dietary tyramine, a sympathomimetic agent, which can then enter the bloodstream and cause unpleasant symptoms

ranging from headache to hypertensive crisis. Since PEH appears to be a poor inhibitor of MAO-A, it should not be associated with this adverse effect and may prove to be an interesting new drug in its own right.

PEH has a double bond and can thus exist as (*E*)- and (*Z*)-isomers (**Figure 3-1**), but to date nobody has compared the isomers with regard to the above-mentioned effects on brain levels of amino acids, biogenic amine neurotransmitters and inhibition of PrAO.
The investigation described here is relevant for potential therapeutic implications of PEH given that the pharmacodynamic and pharmacokinetic properties of isomers can be markedly different, as was the case for stereoisomers of the selective serotonin reuptake inhibitor (SSRI) citalopram (Burke and Kratochvil 2002) and geometric isomers of the tricyclic antidepressant doxepin (Pinder et al. 1977). In the present study, we have measured rat whole brain levels of amino acids, biogenic amine neurotransmitters and their metabolites, as well as methylamine (an endogenous substrate of PrAO) following administration of phenelzine, (*E*)-PEH or (*Z*)-PEH.



phenelzine *(E)-PEH* *(Z)-PEH*

Figure 3-1. Structures of phenelzine (β-phenylethylhydrazine) and the (*E*)- and (*Z*)-isomers of PEH (β-phenylethylidenehydrazine).

3.2. METHODS

3.2.1 Materials

Water used in the experiments was distilled and purified by reverse osmosis using a Millipore Milli-Q filtration system. Methanol, tetrahydrofuran, dimethyl sulfoxide (DMSO), acetonitrile, ethyl acetate and cyclohexane were HPLC-grade and purchased from Fisher Scientific. Perchloric acid (60%), ascorbic acid, EDTA, monobasic and dibasic sodium phosphate, sodium borate and potassium carbonate were also obtained from Fisher Scientific. L-Aspartate (ASP), L-glutamate (GLU), L-serine (L-SER), D-serine (D-SER), L-glutamine (GLN), glycine (GLY), L-arginine (ARG), taurine (TAUR), L-ALA, GABA, NA, DA, 5-hydroxyindoleacetic acid (5-HIAA), homovanillic acid (HVA), 3,4-dihydroxyphenylacetic acid (DOPAC), 5-HT, o-phthaldialdehyde (OPA), benzylamine, pentafluorobenzenesulfonyl chloride (PFBSC), sodium chloride, sodium octyl sulfate and phenelzine sulfate were all obtained from Sigma-Aldrich. N-isobutyryl-L-cysteine (IBC) was purchased from Novabiochem.

Geometric isomers of PEH were synthesized in the laboratory of Dr. Velázquez-Martínez, Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta. By varying the reaction and extraction conditions in preparation of PEH from phenylacetaldehyde and hydrazine monohydrate (**Figure 3-2**), we obtained mixtures of the (*E*)- and (*Z*)-PEH isomers in two different ratios: mixture NU-01 (E/Z = 80%/20%) and NU-02 (E/Z = 9%/91%). These ratios were confirmed using nuclear magnetic resonance spectroscopy. From here on in this chapter, PEH (*E/Z* = 80%/20%) and PEH (*E/Z* = 9%/91%) mixtures will be referred to as (*E*)-PEH and (*Z*)-PEH, respectively.

3.2.2 Animal drug administration

Phenelzine dissolved in H_2O or (*E*)- and (*Z*)-PEH mixtures dissolved in DMSO solution were administered to adult male Sprague-Dawley rats at a concentration of 30 mg/kg by intraperitoneal injection. An injection of H_2O or DMSO alone was used as a control. Groups of rats (n = 4-5 for each treatment condition, with the exception of the H_2O-treated 1 hour group where n = 3) were sacrificed by decapitation at 1, 3, 6 or 12 hours following phenelzine, (*E*)- or (*Z*)-PEH injection, with the brains dissected out and frozen immediately in isopentane on solid carbon dioxide. The brains were then stored at -80°C until the time of analysis. All animal procedures

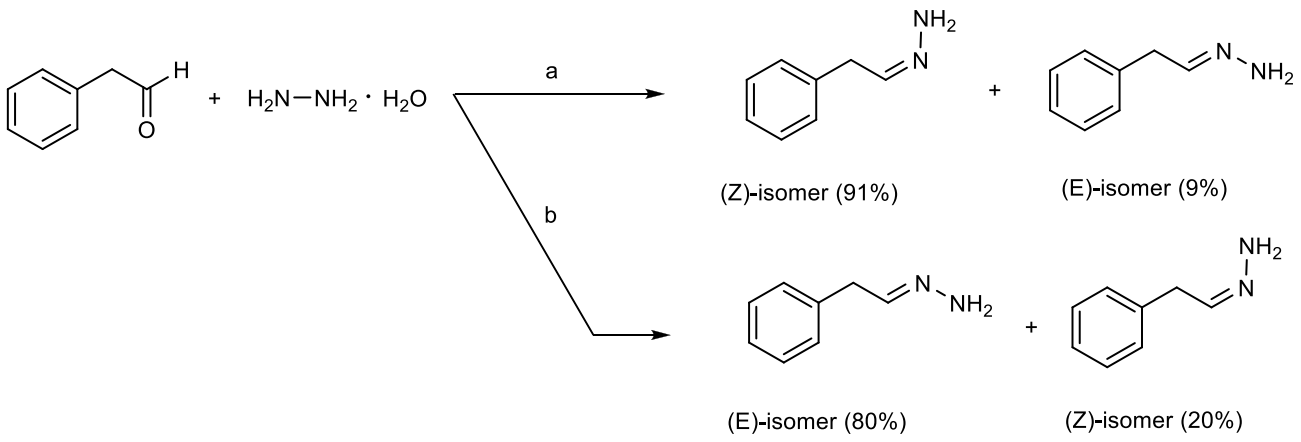


Figure 3-2. Summary of the synthesis of the geometric isomers of PEH from phenylacetaldehyde and hydrazine monohydrate. Reagents and conditions: (**a**) ethanol, reflux, 3 hours; (**b**) no solvent, 110°C, 2 hours.

performed were approved by the University of Alberta Biosciences Animal Care and Use Committee and were in accordance with the guidelines of the Canadian Council on Animal Care.

3.2.3 High performance liquid chromatography (HPLC) with fluorimetric detection

Amino acids (ASP, GLU, L-SER, D-SER, GLN, GLY, ARG, TAUR, ALA and GABA) were analyzed by high performance liquid chromatography (HPLC) with fluorimetric detection after derivatization with IBC and OPA to produce fluorescent derivatives. The procedure is a modification of the one used by Grant et al. (2006). Frozen rat brain was homogenized in 5 volumes of ice-cold methanol and kept on ice for 10 minutes. The homogenate was centrifuged (12,000 xg for 5 minutes at 4°C) and a 50 µl aliquot of supernatant was diluted with 700 µl H_2O. The sample was vortexed and transferred to a HPLC vial. The derivatizing reagent was prepared by dissolving 2 mg OPA and 3 mg IBC in 150 µl methanol followed by dilution with 1350 µl of 0.1 M sodium borate. A sample volume of 5 µl was reacted with a 5 µl mixture of IBC/OPA reagent in the injection loop for 5 minutes prior to injection onto the analytical column. Standard curves were generated for each individual run of samples.

The HPLC system consisted of a Waters Alliance 2695 XE Separations Module equipped with a Waters Symmetry C18 3.5 µm (4.6 x 150 mm) analytical column and a Waters 474 Scanning Fluorescence Detector set at an excitation wavelength of 344 nm and an emission wavelength of 433 nm. Mobile phase "A" was prepared from 85% 0.04 M sodium phosphate buffer and 15% methanol with a pH of 6.2, while mobile phase "B" consisted of 53.4% 0.04 M sodium phosphate buffer, 44.2% methanol and 2.4% tetrahydrofuran, also at a pH of 6.2. The initial mobile phase conditions were be 83% "A" and 17% "B" with a flow rate of 0.5 ml/minute, gradually increasing to 100% "B" and returning back to initial conditions over a span of 60 minutes.

3.2.4 High performance liquid chromatography (HPLC) with electrochemical detection

Biogenic amines and their metabolites (NA, DA, DOPAC, HVA, 5-HT and 5-HIAA) were analyzed by HPLC with electrochemical detection based on the procedure of Parent et al. (2001). Frozen rat brain was homogenized in 5 volumes of ice-cold water, with a 90 µl aliquot transferred to a microfuge tube containing 10 µl of 1N perchloric acid with EDTA (100 mg/dL) and ascorbic

acid (500 μM). The mixture was centrifuged (12,000 xg for 5 minutes at 4°C) and the supernatant transferred to a HPLC vial. Standard curves were generated for each individual run of samples.

The HPLC system consisted of a Waters Alliance 2695 XE Separations Module equipped with a Waters Atlantis dC18 3 μm (3.0 x 100 mm) analytical column and a Waters 2465 Electrochemical Detector set with an applied potential set at 0.64 V. The mobile phase contained 55 mM monosodium phosphate, 850 μM sodium octyl sulfate, 470 μM EDTA, 8% acetonitrile and 2 mM sodium chloride in water with the pH adjusted to 2.9. Sample injection volume was 10 μl with a mobile phase flow rate of 0.6 ml/minute.

3.2.5 Gas chromatography (GC) with mass spectrometry (MS)

Rat whole brain levels of methylamine were analyzed by gas chromatography (GC) combined with mass spectrometry (MS), following derivatization with pentafluorobenzene sulfonyl chloride (PFBSC), based on the procedure of MacKenzie (2009). Frozen rat brain was homogenized in 5 volumes of ice-cold H_2O, with 1.35 ml of homogenate added to 150 μl of 1 N perchloric acid without EDTA or ascorbic acid. The mixture was centrifuged (12,000 xg for 4 minutes) and 1 ml of supernatant was used in the assay. The samples had 200 ng benzylamine added as an internal standard and were then basified with 200 μl of 25% potassium carbonate to a pH of 11-12. A 3 ml volume of ethyl acetate:acetonitrile:PFBSC solution (9:1:0.01 v/v) was added to the samples and the tubes were shaken vigorously for 10 minutes and centrifuged (1,800 xg for 4 minutes). The solvent layer was extracted to a new glass tube and dried under vacuum, reconstituted in cyclohexane and transferred to a GC vial. Standard curves were generated for each individual run of samples.

Analysis was carried out using an Agilent 6890 Series GC System equipped with an Agilent HP-5MS analytical column (length = 30 m, internal diameter = 0.250 mm, film thickness = 0.25 μm) and an Agilent 5973 Mass Selective Detector. Helium was used as the carrier gas. A sample volume of 1 μl was injected into the inlet held at 250°C. The initial column temperature of 80°C was held for 2 minutes, followed by a 15°C/minute increase until a final temperature of 295°C that was held for 10 minutes. The detector was set to selectively monitor ions with a mass-to-charge (m/z) ratio of 337 (benzylamine derivative, internal standard) and 261 (methylamine derivative).

3.2.6 Statistical analysis

Each of the treatment groups had 4-5 animals per time point, with the exception of the 1 hour H_2O-treated group which had 3 animals. The results are expressed as the mean and standard error of the mean (SEM). Differences were statistically evaluated by one-way analysis of variance (ANOVA) followed by the Newman-Keuls multiple comparison test. A P value of < 0.05 was considered to be statistically significant.

3.3 RESULTS

3.3.1 Rat whole brain amino acids

Phenelzine and both PEH isomer mixtures induced significant changes in rat whole brain levels of amino acids in comparison to controls; only TAUR was not significantly affected by (*E*)-PEH, (*Z*)-PEH or phenelzine treatment at any of the time points tested. The calculated levels of amino acids (expressed as μg/g tissue) for vehicle-treated controls (H_2O in the case of phenelzine and DMSO in the case of PEH isomers) across all time points are comparable to the values previously reported in MacKenzie (2009). The alterations in amino acids by drug treatment are represented in **Figures 3-3** to **3-6**, expressed as percent of vehicle-treated controls (H_2O in the case of phenelzine and DMSO in the case of PEH isomers).

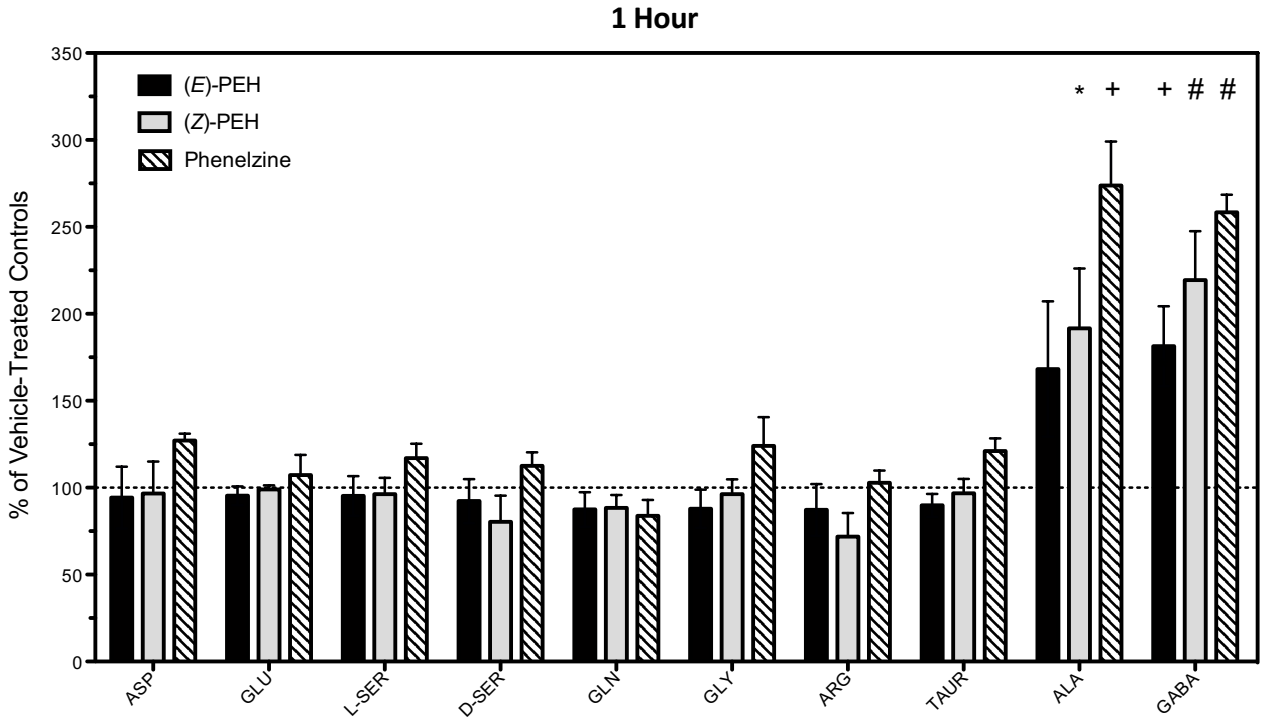


Figure 3-3. Changes in levels of rat whole brain amino acids at 1 hour following treatment with (*E*)-PEH, (*Z*)-PEH or phenelzine (n = 3-5 per treatment group). Data are normalized relative to vehicle-treated controls (DMSO for [*E*]- and [*Z*]-PEH and H_2O for phenelzine). Values are means ± SEM. Symbols show that means are significantly different in comparison to DMSO in the case of (*E*)- and (*Z*)-PEH and H_2O in the case of phenelzine, as assessed by the Newman-Keuls multiple comparison test at $*P < 0.05$, $+ P < 0.01$ and $\# P < 0.001$.

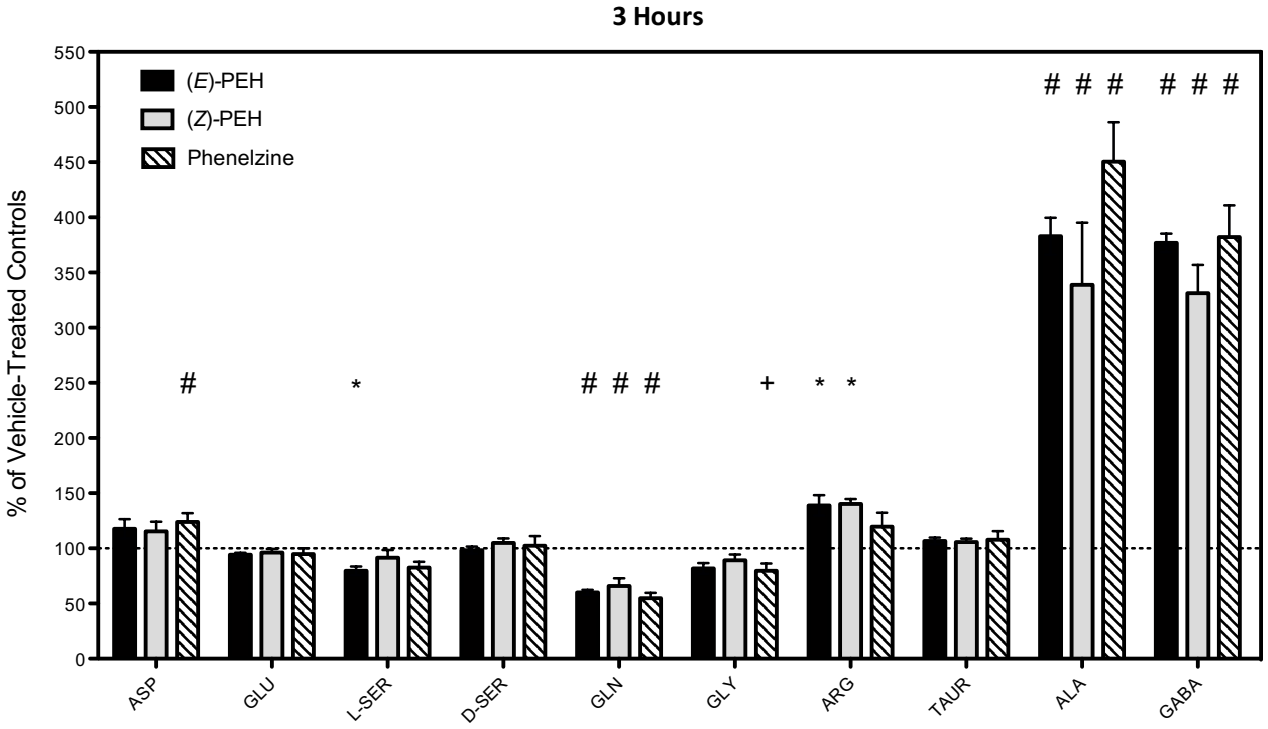


Figure 3-4. Changes in levels of rat whole brain amino acids at 3 hours following treatment with (*E*)-PEH, (*Z*)-PEH or phenelzine (n = 4-5 per treatment group). Data are normalized relative to vehicle-treated controls (DMSO for [*E*]- and [*Z*]-PEH and H_2O for phenelzine). Values are means ± SEM. Symbols show that means are significantly different in comparison to DMSO in the case of (*E*)- and (*Z*)-PEH and H_2O in the case of phenelzine, as assessed by the Newman-Keuls multiple comparison test at $*P < 0.05$, $+ P < 0.01$ and $\# P < 0.001$.

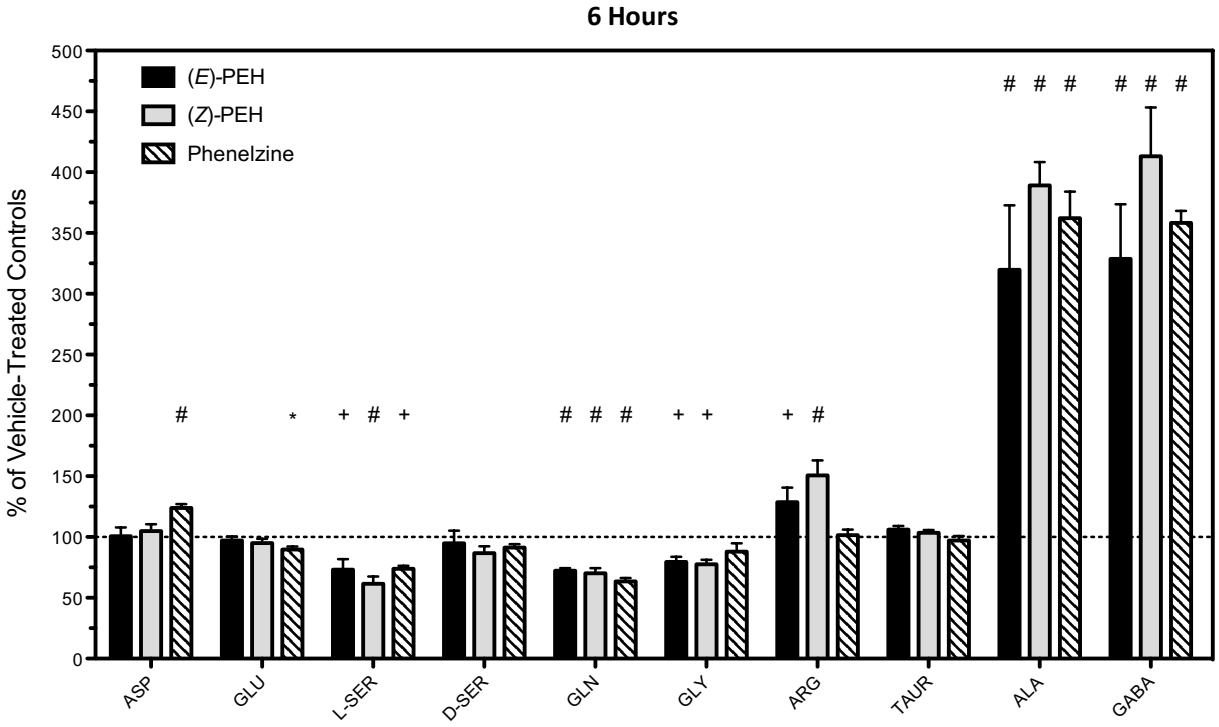


Figure 3-5. Changes in levels of rat whole brain amino acids at 6 hours following treatment with (*E*)-PEH, (*Z*)-PEH or phenelzine (n = 4-5 per treatment group). Data are normalized relative to vehicle-treated controls (DMSO for [*E*]- and [*Z*]-PEH and H_2O for phenelzine). Values are means ± SEM. Symbols show that means are significantly different in comparison to DMSO in the case of (*E*)- and (*Z*)-PEH and H_2O in the case of phenelzine, as assessed by the Newman-Keuls multiple comparison test at $*P < 0.05$, $+ P < 0.01$ and $\# P < 0.001$.

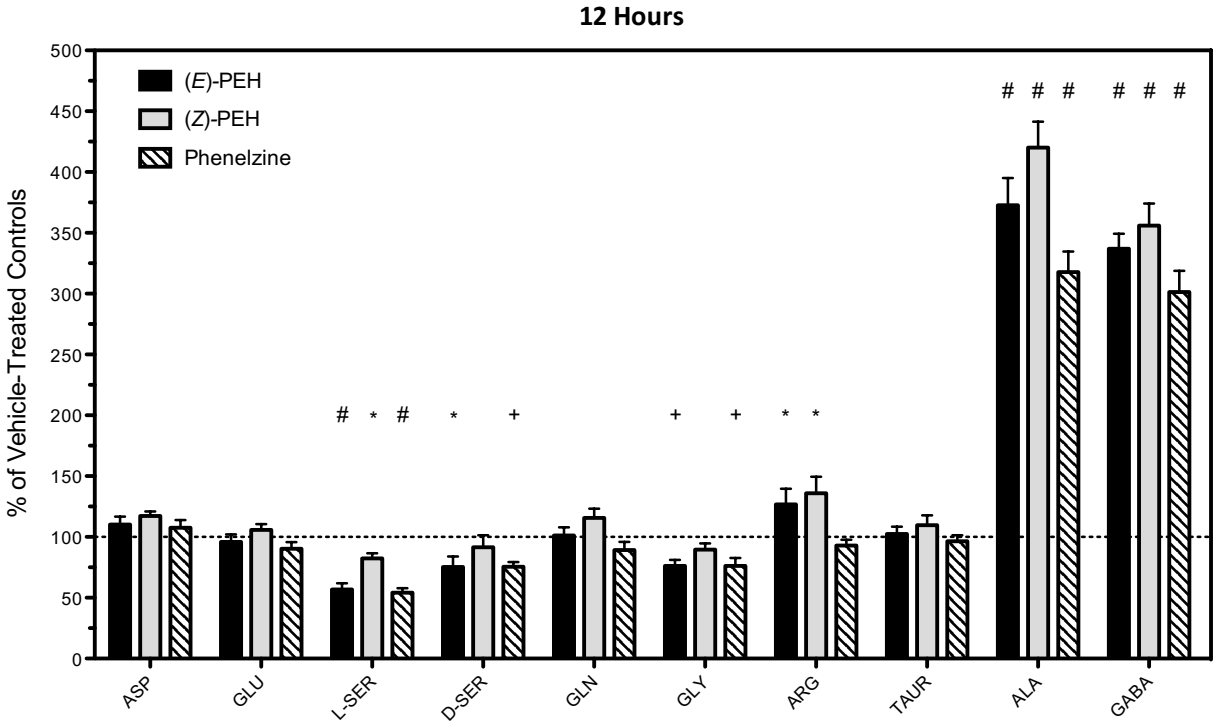


Figure 3-6. Changes in levels of rat whole brain amino acids at 12 hours following treatment with (*E*)-PEH, (*Z*)-PEH or phenelzine (n = 4-5 per treatment group). Data are normalized relative to vehicle-treated controls (DMSO for [*E*]- and [*Z*]-PEH and H_2O for phenelzine). Values are means ± SEM. Symbols show that means are significantly different in comparison to DMSO in the case of (*E*)- and (*Z*)-PEH and H_2O in the case of phenelzine, as assessed by the Newman-Keuls multiple comparison test at **P* < 0.05, + *P* < 0.01 and # *P* < 0.001.

3.3.2 Rat whole brain biogenic amine neurotransmitters

With regard to rat whole brain levels of biogenic amines (NA, DA and 5-HT) and their acid metabolites (DOPAC, HVA and 5-HIAA), there were large differences in the effects of phenelzine and the PEH isomers, as demonstrated in **Figures 3-7** to **3-10**. Phenelzine administration caused pronounced increases in 5-HT levels along with decreases in levels of its acid metabolite 5-HIAA at all time points. In addition, phenelzine also produced large increases in rat whole brain levels of NA and DA at 3 to 12 hours. Levels of DOPAC and HVA, acid metabolites of DA, were either reduced or below the limit of detection at all time points following phenelzine administration. Neither PEH isomer significantly altered levels of NA, DA and 5-HT. However, the PEH isomers produced a modest decrease in rat whole brain levels of DOPAC at 6 and 12 hours. (*E*)-PEH significantly increased brain levels of HVA at 3 hours, but this effect was not observed at any other time point. Both PEH isomers also significantly reduced levels of 5-HIAA at 6 hours, with (*Z*)-PEH significantly increasing levels of 5-HIAA at 12 hours.

3.3.3 Rat whole brain methylamine

Administration of (*E*)- and (*Z*)-PEH caused a substantial accumulation of rat whole brain methylamine concentrations starting at 3 hours (not statistically significant for [*Z*]-PEH) and reaching the highest levels at 12 hours. Phenelzine also produced a robust increase in methylamine levels at 3 hours, although this effect was reduced at 6 and 12 hours. The results are summarized in **Figure 3-11**. The levels of methylamine in vehicle-treated controls across all time points, expressed as mean ± SEM, were 432.0 ± 26.1 and 365 ± 15.4 ng/g tissue in H_2O-treated (n = 16) and DMSO-treated (n = 17) animals, respectively.

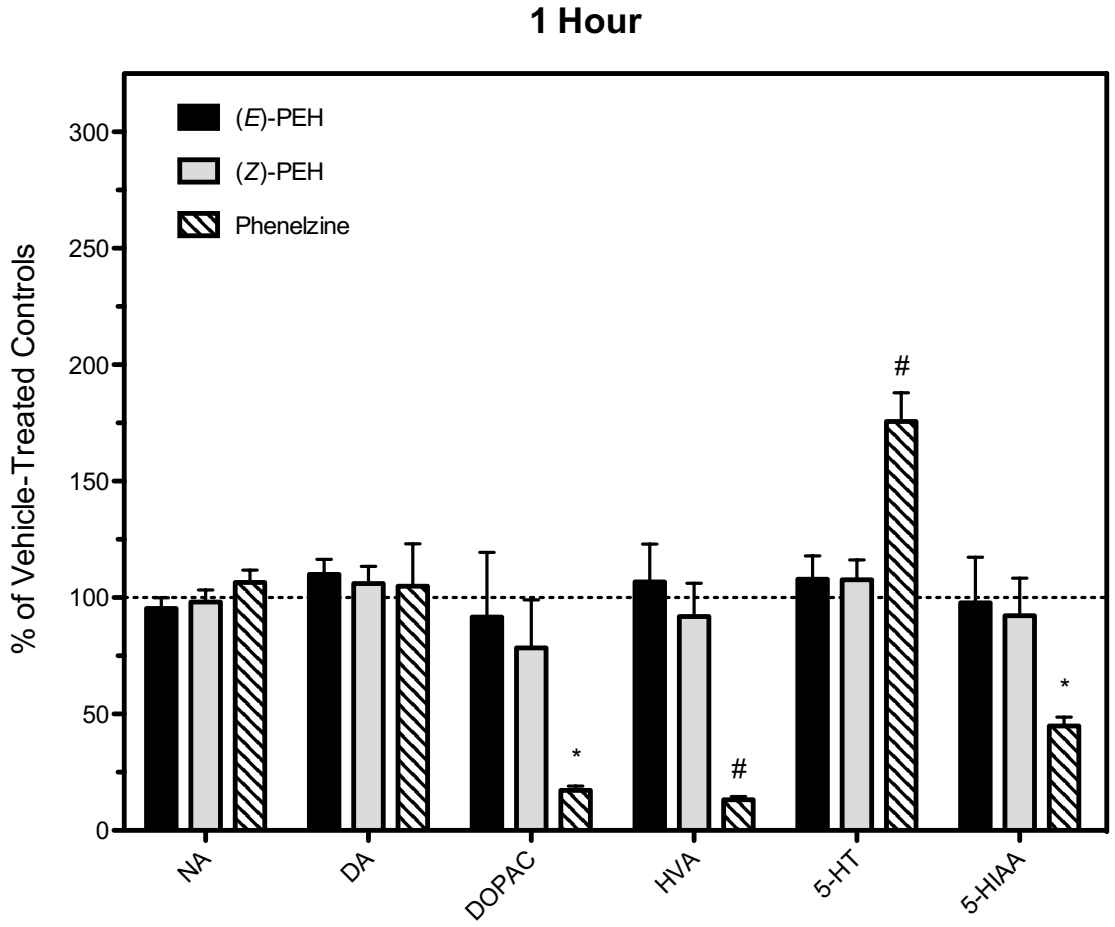


Figure 3-7. Changes in levels of rat whole brain biogenic amines and their acid metabolites at 1 hour following treatment with (*E*)-PEH, (*Z*)-PEH or phenelzine (n = 3-5 per treatment group). Data are normalized relative to vehicle-treated controls (DMSO for [*E*]- and [*Z*]-PEH and H_2O for phenelzine). Values are means ± SEM. Symbols show that means are significantly different in comparison to DMSO in the case of (*E*)- and (*Z*)-PEH and H_2O in the case of phenelzine, as assessed by the Newman-Keuls multiple comparison test at **P* < 0.05, + *P* < 0.01 and # *P* < 0.001.

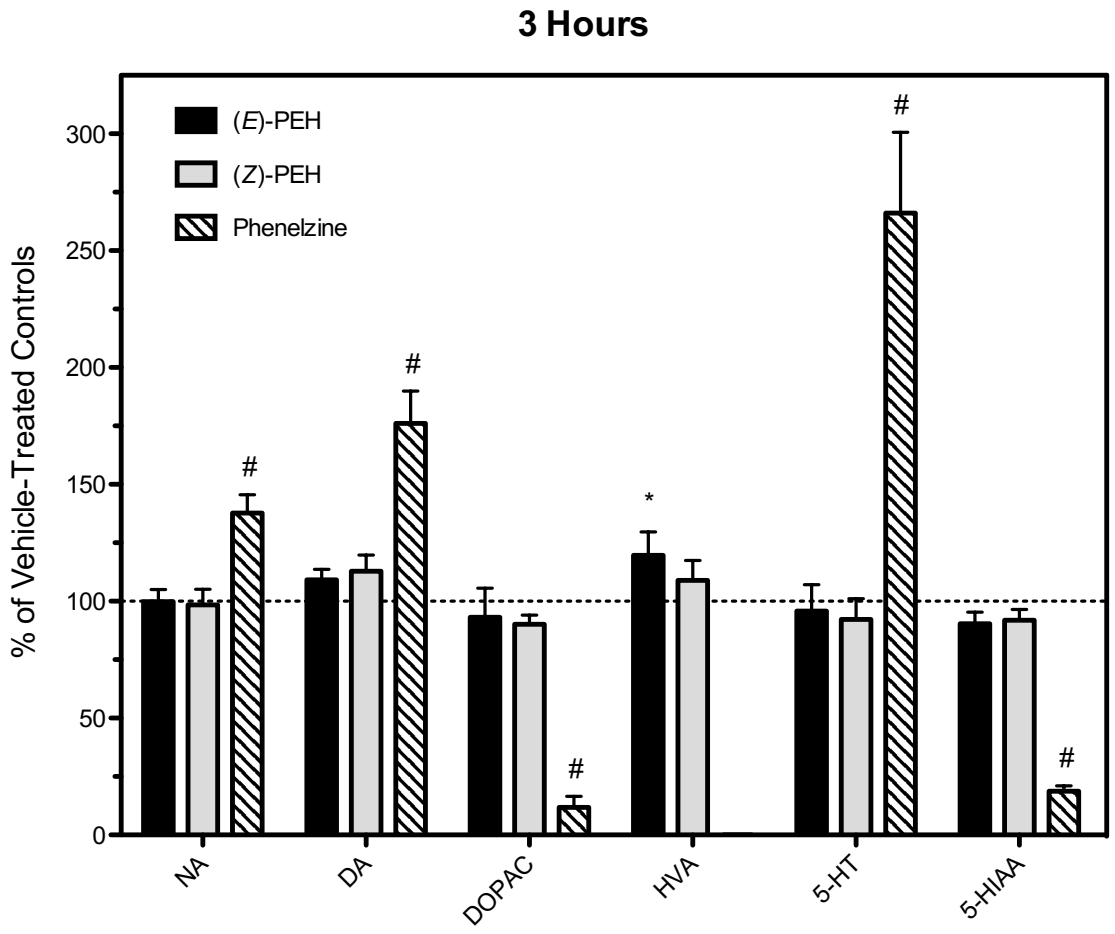


Figure 3-8. Changes in levels of rat whole brain biogenic amines and their acid metabolites at 3 hours following treatment with (*E*)-PEH, (*Z*)-PEH or phenelzine (n = 4-5 per treatment group). Data are normalized relative to vehicle-treated controls (DMSO for [*E*]- and [*Z*]-PEH and H_2O for phenelzine). Values are means ± SEM. Symbols show that means are significantly different in comparison to DMSO in the case of (*E*)- and (*Z*)-PEH and H_2O in the case of phenelzine, as assessed by the Newman-Keuls multiple comparison test at **P* < 0.05, + *P* < 0.01 and # *P* < 0.001.

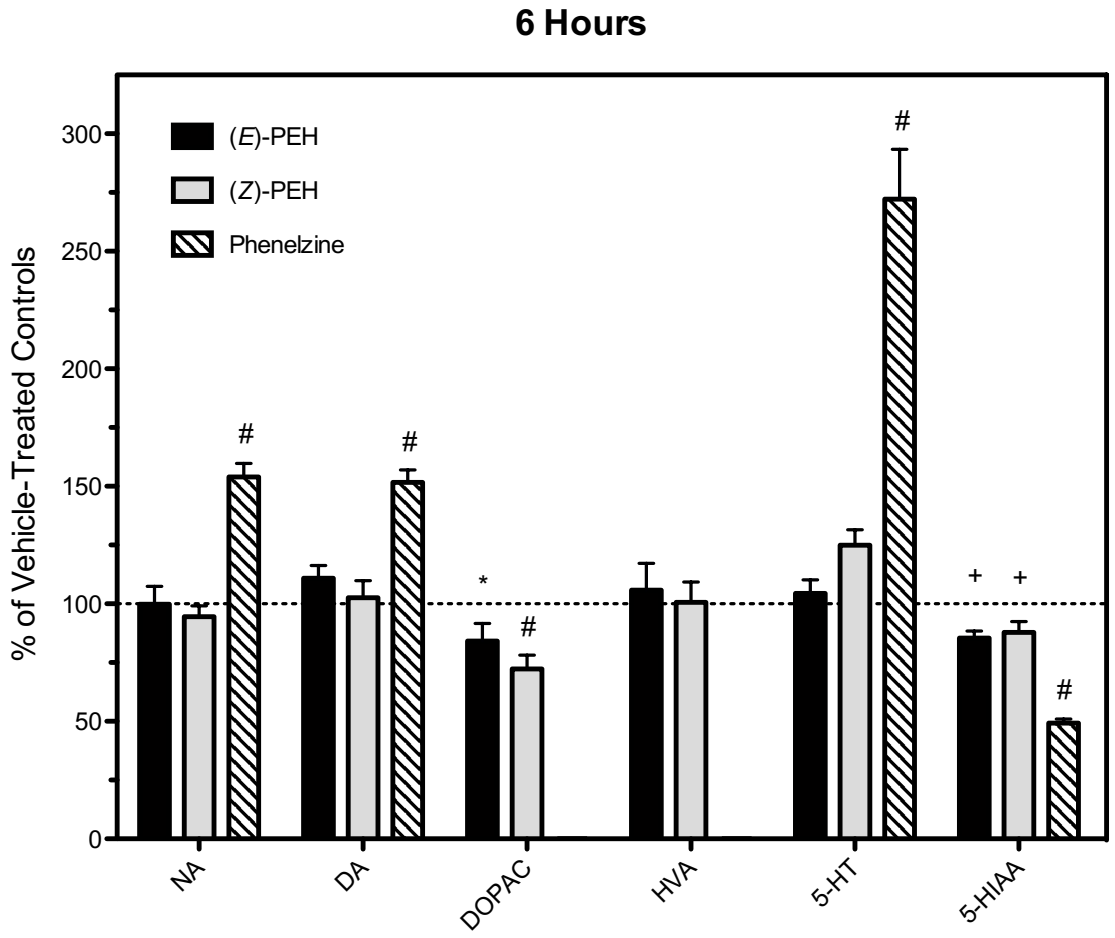


Figure 3-9. Changes in levels of rat whole brain biogenic amines and their acid metabolites at 6 hours following treatment with (*E*)-PEH, (*Z*)-PEH or phenelzine (n = 4-5 per treatment group). Data are normalized relative to vehicle-treated controls (DMSO for [*E*]- and [*Z*]-PEH and H_2O for phenelzine). Values are means ± SEM. Symbols show that means are significantly different in comparison to DMSO in the case of (*E*)- and (*Z*)-PEH and H_2O in the case of phenelzine, as assessed by the Newman-Keuls multiple comparison test at $*P < 0.05$, $+ P < 0.01$ and $\# P < 0.001$.

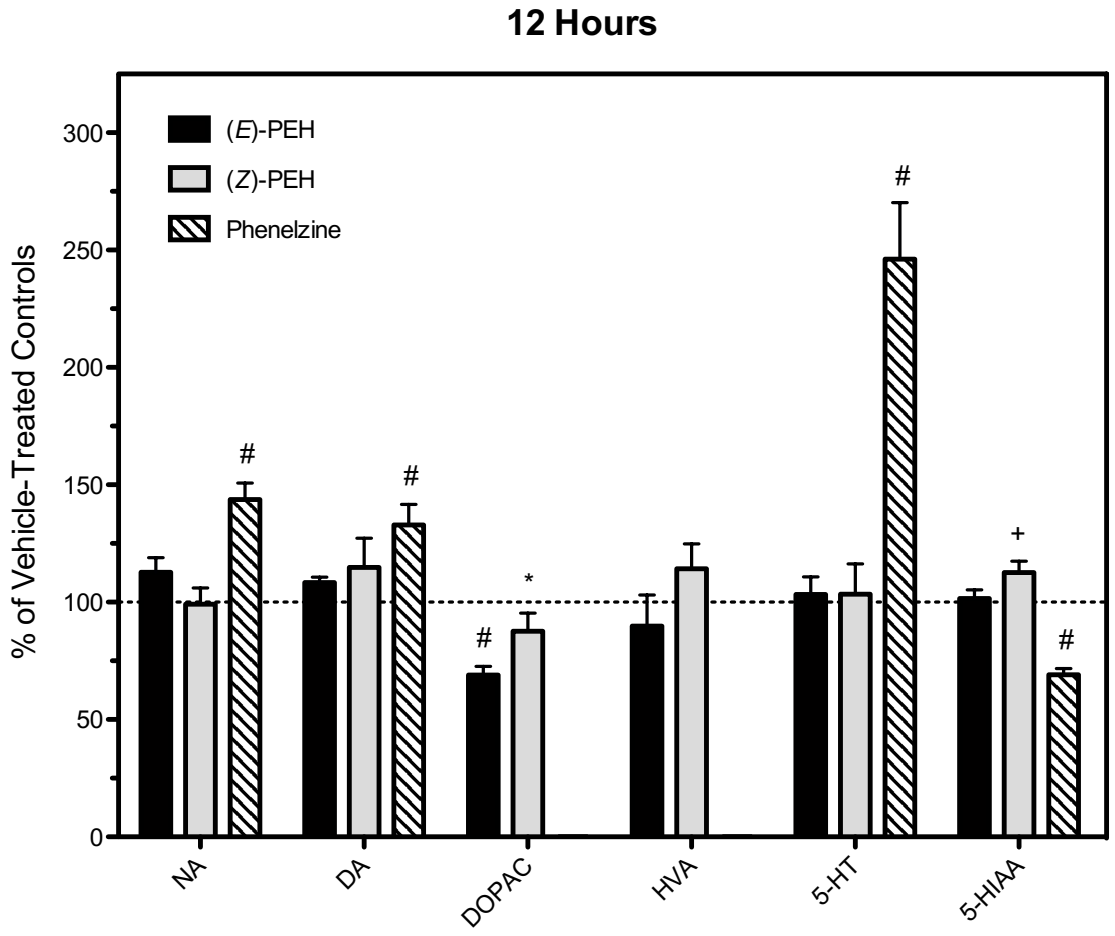


Figure 3-10. Changes in levels of rat whole brain biogenic amines and their acid metabolites at 12 hours following treatment with (*E*)-PEH, (*Z*)-PEH or phenelzine (n = 4-5 per treatment group). Data are normalized relative to vehicle-treated controls (DMSO for [*E*]- and [*Z*]-PEH and H_2O for phenelzine). Values are means ± SEM. Symbols show that means are significantly different in comparison to DMSO in the case of (*E*)- and (*Z*)-PEH and H_2O in the case of phenelzine, as assessed by the Newman-Keuls multiple comparison test at $*P < 0.05$, $+ P < 0.01$ and $\# P < 0.001$.

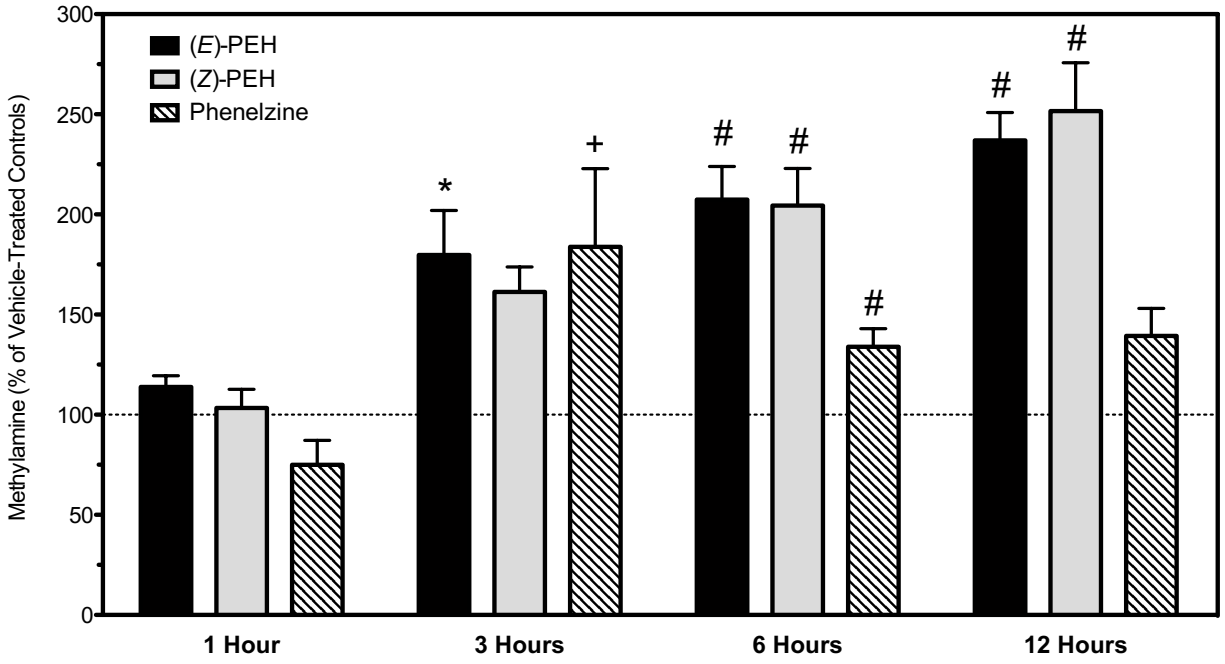


Figure 3-11. Changes in levels of rat whole brain methylamine following treatment with (*E*)-PEH, (*Z*)-PEH or phenelzine (n = 3-5 per treatment group). Data are normalized relative to vehicle-treated controls (DMSO for [*E*]- and [*Z*]-PEH and H_2O for phenelzine). Values are means ± SEM. Symbols show that means are significantly different in comparison to DMSO in the case of (*E*)- and (*Z*)-PEH and H_2O in the case of phenelzine, as assessed by the Newman-Keuls multiple comparison test at $*P < 0.05$, $+ P < 0.01$ and $\# P < 0.001$.

3.4 DISCUSSION

Overall, both (*E*)-PEH and (*Z*)-PEH geometric isomers produced comparable changes to levels of rat whole brain amino acids, biogenic amine neurotransmitters and methylamine. Although a few differences in amino acid and biogenic amine levels did exist between the two PEH isomers, these differences were minor. Our data support the notion that (*E*)-PEH and (*Z*)-PEH are equivalent in their effects on amino acids, biogenic amine neurotransmitters and their metabolites, as well as the PrAO substrate methylamine under the conditions used in this study.

The most substantial effect of (*E*)-PEH, (*Z*)-PEH and phenelzine on the amino acids analyzed was the potentiation of brain GABA and ALA levels starting at 1 hour and still remaining at 12 hours. This is not surprising, as phenelzine was previously shown to inhibit GABA transaminase and ALA transaminase (Popov and Matthies 1969; Wong et al. 1990; Baker et al. 1991; McManus et al. 1992; Paslawski et al. 1995; Todd and Baker 1995; Tanay et al. 2001; Todd and Baker 2008). PEH has also been demonstrated to inhibit GABA transaminase (Paslawski et al. 2001; MacKenzie 2009) and is thought to be the active metabolite of phenelzine in this regard (Popov and Matthies 1969; Todd and Baker 1995; MacKenzie 2009).

The ability of the PEH isomers and phenelzine to potentiate GABA levels may have considerable therapeutic relevance. It has been suggested that the anti-anxiety properties of phenelzine are tied to increases in brain GABA levels, as phenelzine produced a clear anxiolytic effect in a rat model of the elevated plus-maze only at doses that increased brain GABA by more than two-fold whereas N^2-acetylphenelzine, a potent inhibitor of MAO but with no GABAergic effects, produced no significant anxiolytic effect (Paslawski et al. 1996). Perturbations in GABA levels may also play a role in affective disorders as patients with depression have been reported to exhibit a deficiency of GABA in cerebrospinal fluid (Gold et al. 1980; Gerner and Hare 1981), plasma (Petty and Sherman 1984; Petty et al. 1990; Petty et al. 1992) and occipital cortex (Sanacora et al. 1999; Kugaya et al. 2003; Sanacora et al. 2004), with electroconvulsive therapy or SSRI treatment shown to normalize occipital cortex GABA levels (Sanacora et al. 2002; Sanacora et al. 2003). Phenelzine and PEH may also be of use in the treatment of epilepsy as several established anti-epileptic drugs target GABAergic pathways by augmenting $GABA_A$ receptor activation (benzodiazepines and phenobarbital), inhibiting GABA reuptake (tiagabine) or inhibiting GABA transaminase (vigabatrin) (Howard et al. 2011). Phenelzine is much more potent than vigabatrin at

elevating rat whole brain GABA levels (Todd and Baker 2008), and it is expected that the same would be the case with PEH since phenelzine and PEH have similar GABAergic effects as demonstrated in this chapter. In addition, PEH, in an *in vitro* rat hippocampal slice model of epilepsy, was able to increase tissue GABA levels and attenuate epileptiform burst frequency (Duffy et al. 2004). The protection of neuronal cells by phenelzine and PEH in a gerbil model of transient global ischemia (Todd et al. 1999; Wood et al. 2006) may also be associated with GABA transaminase inhibition as increased levels of GABA can counteract excitotoxicity by hyperpolarizing the presynaptic membrane (preventing GLU release) and the postsynaptic membrane (preventing cell excitation by GLU) (Schwartz-Bloom and Sah 2001).

Levels of GLN were decreased by both PEH isomers and phenelzine at 3 and 6 hours. GLN is a nitrogen carrier and serves as a precursor and metabolite of GLU in the GABA shunt metabolic loop. Extracellular GABA is transported into glial cells where it is converted to GLU by GABA transaminase, followed by metabolism of GLU to GLN by GLN synthetase. GLN can then move from the glia to neurons where it is converted back to GLU by glutaminase and then to GABA via glutamic acid decarboxylase. The mechanism for the reduction of GLN levels by phenelzine and PEH is not yet known. Lowered levels of GLN may reflect a functional change in excitatory glutamatergic transmission. It is interesting to note that brain GLU concentrations were not significantly altered (with the exception of a small decrease in GLU with phenelzine at 6 hours) in these experiments, suggesting the possibility of compensatory mechanisms to even out the reduction in the GABA/GLN/GLU flux of the GABA shunt.

Brain levels of GLY were decreased by phenelzine at 3 hours, by both PEH isomers at 6 hours and by (*E*)-PEH and phenelzine at 12 hours. Furthermore, D-SER levels were reduced by (*E*)-PEH and phenelzine at the 12 hour time point; however, the effects on D-SER were not as robust in comparison to GLY. Of interest, GLY and D-SER were recently demonstrated to act as co-agonists at extrasynaptic and synaptic *N*-methyl-D-aspartate (NMDA) receptors, respectively (Papouin et al. 2012). Numerous studies have suggested that extrasynaptic NMDA receptor stimulation mediates cell death, while synaptic NMDA receptor stimulation promotes neuronal survival (Hardingham and Bading 2010). In contrast, the Papouin et al. (2012) paper demonstrated that inhibition of synaptic NMDA receptor stimulation provided neuroprotection against NMDA-induced excitotoxicity, whereas silencing of extrasynaptic NMDA receptors did not produce any protective effects. As such,

the role of extrasynaptic and synaptic NMDA receptor neurotransmission in neurotoxicity still remains a controversial topic and it would be of interest to further explore the role of phenelzine- and PEH-induced reductions in GLY and D-SER in relation to neuroprotection.

Both PEH isomers, but not phenelzine, produced a significant elevation in levels of ARG at 3, 6 and 12 hours. ARG is the immediate precursor to nitric oxide (NO), an important neurotransmitter involved in the pathophysiology of many neurological disorders. The exact role of NO in depression is still uncertain, although it has been reported that plasma levels of NO are decreased in patients with major depression and that the SSRI antidepressant paroxetine reverses this effect (Chrapko et al. 2006).

The lack of change in biogenic amine levels following administration of (*E*)-PEH and (*Z*)-PEH was as expected for a weak MAO inhibitor. Neither PEH isomer affected levels of NA, DA or 5-HT at any time point. However, both PEH isomers decreased levels of DOPAC, a metabolic end-product of DA, at 6 and 12 hours and of 5-HIAA, a metabolic end-product of 5-HT, at 6 hours. In contrast, phenelzine produced robust elevations in NA and DA levels (up to 150-170% of controls) at 3 to 12 hours and even more substantial increases in 5-HT levels (up to 270% of control) at all time points tested. Impaired metabolism of these biogenic amine neurotransmitters is supported by the finding that phenelzine substantially lowered levels of biogenic amine metabolites at all time points: 5-HIAA was significantly decreased whereas levels of DOPAC and HVA, metabolic end-products of DA, were significantly reduced or below the limit of detection. These results support previous observations that PEH is a much weaker inhibitor of rat brain MAO in comparison to phenelzine (Paslawski et al. 2001; MacKenzie et al. 2008).

Methylamine and aminoacetone are considered to be physiological substrates for PrAO that are metabolized to their respective aldehydes, namely formaldehyde and methylglyoxal (Precious and Lyles 1988; Yu 1990; Boor et al. 1992; Lyles and Chalmers 1992; Lyles 1996). Methylamine is formed during metabolism of creatinine and creatine (Yu and Deng 2000; Mitchell and Zhang 2001), adrenaline (Schayer et al. 1952; Yu et al. 1997), choline and lecithin (Zeisel et al. 1983). An investigation of human urinary methylamine levels suggested that the majority of excreted methylamine originates endogenously with no apparent major exogenous dietary sources (Mitchell and Zhang 2001). As inhibition of PrAO activity by a selective inhibitor was shown to substantially increase urinary methylamine in the rat (Lyles and McDougall 1989; Yu and Zuo 1997), we

hypothesized that phenelzine and PEH, both shown to inhibit human PrAO *in vitro* (MacKenzie 2009), would increase brain levels of methylamine in the rat. In the present experiment, (*E*)-PEH and (*Z*)-PEH caused significant elevations in rat whole brain methylamine concentrations that increased over the span of 12 hours. Phenelzine increased rat brain methylamine to a level comparable with PEH isomers at 3 hours; however, this effect on methylamine levels dropped off at 6 and 12 hours. Since methylamine can be formed from MAO-catalyzed metabolism of adrenaline (Schayer et al. 1952; Yu et al. 1997), the attenuation of methylamine accumulation by phenelzine at the longer time points may be due to reduced production of methylamine from this metabolic pathway. Alternatively, the differential effects of phenelzine and PEH on rat brain methylamine levels may be due to pharmacokinetic differences between the drugs. As the activity and/or expression of PrAO has been reported to be increased in diabetes mellitus (Boomsma et al. 1995; Garpenstrand et al. 1999; Meszaros et al. 1999), heart failure (Boomsma et al. 1997; Boomsma et al. 2000), atherosclerosis (Karadi et al. 2002), hemorrhagic and ischaemic stroke (Airas et al. 2008; Hernandez-Guillamon et al. 2012), and Alzheimer's disease (Ferrer et al. 2002; del Mar Hernandez et al. 2005), the use of PrAO inhibitors may be a promising approach in treating certain aspects of these disorders.

Formaldehyde, a product of methylamine metabolism by PrAO, is very reactive and readily interacts with free amine or amide groups to form irreversible methylene bridges, thus enabling cross-linking with proteins and single-stranded DNA (Yu et al. 2003). Incubation of methylamine with human brain meninges, a tissue with high PrAO content, was shown to produce formaldehyde-protein cross-linkage of bound and soluble proteins (Gubisne-Haberle 2004). In the same study, prior inhibition of PrAO with a selective inhibitor completely prevented the formation of formaldehyde-protein cross-linkage. These formaldehyde adducts disrupt normal protein or DNA structure and cause cytotoxicity, as evidenced by formaldehyde-induced dose- and concentration-dependent decreases in cell viability for cultured primary rat cortical neurons and astrocytes *in vitro* (Song et al. 2010) and formaldehyde-induced neuronal apoptosis in rat prefrontal cortex *in vivo* (Zararsiz et al. 2007). There is also evidence for the contribution of formaldehyde in Alzheimer's disease, as it has been demonstrated that vascular deposits of PrAO are co-localized with amyloid-β deposits in post-mortem Alzheimer's disease brains and that the deamination of methylamine by PrAO potentiates the deposition of amyloid-β onto blood vessel walls *in vitro* (Jiang et al. 2008).

Formaldehyde and methylglyoxal have also been shown to increase amyloid-β misfolding, oligomerization and fibrillogenesis (Chen et al. 2006; Chen et al. 2007). It is intriguing that in addition to preventing formaldehyde production by inhibition of PrAO, phenelzine and PEH have both been shown to directly sequester formaldehyde by formation of a hydrazone product (MacKenzie 2009). Phenelzine was also demonstrated to protect neurons and astrocytes from formaldehyde-induced toxicity (Song et al. 2010). Thus, phenelzine and PEH may prove to be useful drugs for the treatment of Alzheimer's disease and a variety of neurological disorders as they not only inhibit generation of formaldehyde by PrAO, but can also sequester whatever formaldehyde is present by a direct chemical reaction. Since PEH does not appear to inhibit MAO to any great extent, it would have an advantage over phenelzine in that the patient diet would not have to be restricted in order to prevent possible hypertensive side effects.

3.5 CONCLUSION

The (*E*)- and (*Z*)- geometric isomers of PEH were equivalent in their effects on rat whole brain (1) changes in levels of amino acids, (2) lack of an increase in biogenic amine neurotransmitter concentrations and (3) accumulation of methylamine. Phenelzine produced comparable changes in levels of amino acids (with the exception of ASP and ARG), but markedly increased levels of biogenic amine neurotransmitters. Although phenelzine and the PEH isomers produced comparable increases in rat brain methylamine levels at 3 hours, the PEH isomer mixtures had a more substantial effect at 6 and 12 hours. Since both phenelzine and PEH have been shown to possess neuroprotective properties in an animal model of stroke, to increase brain GABA levels and to inhibit PrAO, they may prove to be very useful in treating a variety of neurological disorders such as cerebral ischemia, epilepsy and Alzheimer's disease. However, unlike phenelzine, PEH, due to its weak inhibition of MAO-A, should not be associated with tyramine dietary interactions and may represent a safer alternative to phenelzine.

3.6 REFERENCES

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CHAPTER 4.

Elevation of rat brain tyrosine levels by phenelzine is mediated by its active metabolite β-phenylethylidenehydrazine (PEH)

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4.1 INTRODUCTION

Phenelzine (β-phenylethylhydrazine) is a monoamine oxidase (MAO) inhibitor that has been used clinically since the 1960s for the treatment of depression and anxiety disorders, including panic disorder and social anxiety disorder. It is an irreversible inhibitor of both MAO-A and MAO-B, and is unique in that it is also a substrate for these enzymes, with one of the metabolites being β-phenylethylidenehydrazine (PEH) (Chapter 2 of this thesis; Tipton and Spires 1972; Binda et al. 2008; MacKenzie 2009). In recent years, there has been substantial interest in MAO inhibitors, including phenelzine, due to reports of their neuroprotective properties (Youdim et al. 2006; Baker et al. 2012; Song et al. 2013). Phenelzine was recently demonstrated to improve functional outcomes in the experimental autoimmune encephalomyelitis (EAE) mouse model of multiple sclerosis (Musgrave et al. 2011; Benson et al. 2013) and to provide neuroprotection in a rat model of traumatic brain injury (Singh et al. 2013). It appears that PEH shares some of the neuroprotective properties of phenelzine as both drugs were shown to reduce neuronal loss in a gerbil model of transient forebrain ischemia (Todd et al. 1999; Wood et al. 2006).

Acute administration of phenelzine and PEH produces comparable elevations in rat brain levels of γ-aminobutyric acid (GABA), alanine and ornithine, while reducing brain concentrations of glutamine (Popov and Matthies 1969; Wong et al. 1990; Baker et al. 1991; McManus et al. 1992; Todd and Baker 1995; Tanay et al. 2001; MacKenzie et al. 2008b; Todd and Baker 2008; MacKenzie 2009; Kumpula 2013; Matveychuk et al. 2013). These effects of phenelzine appear to be mediated by PEH since pre-treatment of the animals with an irreversible non-selective MAO inhibitor abolishes alterations in the aforementioned amino acids produced by phenelzine, but not by PEH (Popov and Matthies 1969; Todd and Baker 1995; MacKenzie et al. 2008b; Todd and Baker 2008; MacKenzie 2009). As phenelzine has previously been reported to produce acute elevations in rat striatal L-tyrosine (hereafter referred to as tyrosine) (Dyck and Dewar 1986), we wanted to investigate whether PEH also has an effect on levels of this amino acid. A ubiquitous building block for proteins, tyrosine is obtained from exogenous dietary sources or endogenous synthesis from phenylalanine in the liver. It is transported into the brain through a large neutral amino acid (LNAA) transporter and is a precursor for the production of the catecholamine neurotransmitters dopamine (DA), noradrenaline (NA) and adrenaline (ADR) (Fernstrom and Fernstrom 2007). In addition, tyrosine is also a precursor for the production of

melanin in the skin and the brain, as well as for the production of thyroid gland hormones (Cansev and Wurtman 2007).

As PEH can exist as (*E*)- and (*Z*)- geometric isomers, we conducted experiments to determine the time-response effects of acute phenelzine, (*E*)-PEH and (*Z*)-PEH administration on rat whole brain concentrations of tyrosine. The structures of these compounds are shown in **Figure 3-1**. In addition, pre-treatment of the animals with the non-selective irreversible MAO inhibitor tranylcypromine (TCP) was investigated to determine if prior inhibition of MAO had an effect on the tyrosine-elevating properties of phenelzine and PEH.

4.2 METHODS

4.2.1 Materials

Water used in the experiments was distilled and purified by reverse osmosis using a Millipore Milli-Q filtration system. Dimethyl sulfoxide (DMSO), acetonitrile, 2-methylbutane, perchloric acid, ascorbic acid, ethylenediaminetetraacetic acid (EDTA), monobasic sodium phosphate and sodium chloride were purchased from Fisher Scientific. Sodium octyl sulfate, L-tyrosine hydrochloride, phenelzine sulfate and TCP hydrochloride were obtained from Sigma-Aldrich. PEH geometric isomers (in ratios of *E*/*Z* = 80/20 and *E*/*Z* = 9/91, hereafter referred to as [*E*]- and [*Z*]-PEH respectively) and racemic PEH were synthesized in the laboratories of Dr. Velázquez-Martínez in the Faculty of Pharmacy and Pharmaceutical Sciences at the University of Alberta; refer to section 3.2.1 for details on synthesis of PEH.

4.2.2 Animal drug administration

Prior to injection, phenelzine was dissolved in H_2O and PEH in DMSO. Phenelzine or PEH isomer solutions were administered to adult male Sprague-Dawley rats at a concentration of 30 mg/kg (based on free base) by intraperitoneal (i.p.) injection. H_2O or DMSO alone was used as a vehicle control. Groups of rats (n = 4-5 for each treatment condition, with the exception of the H_2O-treated 1 hour group where n = 3) were euthanized by decapitation at 1, 3, 6 or 12 hours following phenelzine, (*E*)-PEH or (*Z*)-PEH injection. The brains were dissected out, immediately flash frozen in 2-methylbutane on solid carbon dioxide, transferred to vials and kept frozen at -80°C until time of analysis.

In the pre-treatment experiments, TCP was administered at 1 mg/kg i.p.; this dose was previously reported to inhibit activities of MAO-A and MAO-B by 80 ± 5% and 95 ± 5%, respectively, at 1 hour following injection (Todd and Baker 1995). At 1 hour after pre-treatment with TCP or vehicle (H_2O), the animals were injected with 30 mg/kg i.p. phenelzine, racemic PEH, H_2O or DMSO. The animals (n = 4-5 per treatment group) were euthanized 3 hours after these injections, with the brains removed and immediately frozen in 2-methylbutane on solid carbon dioxide.

All animal procedures performed were approved by the University of Alberta Biosciences Animal Care and Use Committee and were in accordance with the guidelines of the Canadian Council on Animal Care. As diet is a major source of tyrosine, all animals were maintained on PicoLab rodent diet (23% protein).

4.2.3 High performance liquid chromatography (HPLC) with electrochemical detection

Rat whole brain levels of tyrosine were analyzed by HPLC with electrochemical detection using a modification of the assay reported by Parent et al. (2001). Frozen rat brain was homogenized in 5 volumes of ice-cold H_2O, with a 90 µl aliquot transferred to a microfuge tube containing 10 µl of 1 N perchloric acid with EDTA (100 mg/dL) and ascorbic acid (500 µM). The mixture was centrifuged (12,000 xg for 5 minutes at 4°C) and the supernatant transferred to a HPLC vial. Standard curves were generated for each individual run of samples.

The HPLC system consisted of a Waters Alliance 2695 XE Separations Module equipped with a Waters Atlantis dC18 3 µm (3.0 x 100 mm) analytical column and a Waters 2465 Electrochemical Detector set with an applied potential set at 0.64 V. The mobile phase contained 55 mM monosodium phosphate, 850 µM sodium octyl sulfate, 470 µM EDTA, 8% acetonitrile and 2 mM sodium chloride in water with the pH adjusted to 2.9. Sample injection volume was 10 µl with a mobile phase flow rate of 0.6 ml/minute. Standard curves were run daily and were linear ($r^2 \geq 0.98$).

4.2.4 Statistical analysis

Each of the treatment groups had 4-5 animals, with the exception of the 1 hour H_2O-treated group which had 3 animals. The results are expressed as the mean and standard error of the mean (SEM). Differences between groups were statistically evaluated by one-way ANOVA

followed by the Newman-Keuls multiple comparison test. A P value of < 0.05 was considered to be statistically significant.

4.3 RESULTS

4.3.1 Time-response study on rat brain levels of tyrosine

As shown in **Figure 4-1**, phenelzine and the PEH isomers elevated rat whole brain tyrosine levels compared to vehicle-treated values in the time-response study. (*E*)-PEH and phenelzine significantly elevated tyrosine levels at 1 hour, whereas both PEH isomers and phenelzine produced robust increases in tyrosine at 3 and 6 hours. Only (*E*)-PEH produced a statistically significant effect on tyrosine levels at 12 hours. The effects of the PEH isomers were not statistically different from each other at any of the time points tested. In addition, brain tyrosine concentrations for animals treated with H_2O or DMSO were also not significantly different from each other at any time point. When averaged across all time points, tyrosine concentrations for H_2O- and DMSO-treated animals (12.3 ± 0.7 and 12.6 ± 0.9 μg/g tissue, respectively) were comparable to those previously reported by other researchers (Wurtman et al. 1974; Gibson and Wurtman 1978; Ablett et al. 1984; Dyck and Dewar 1986; Dyck 1987).

4.3.2 Effect of tranylcypromine (TCP) pre-treatment on rat brain tyrosine levels

Since the PEH isomers produced comparable increases in tyrosine levels for the time-response experiment, we used racemic PEH for the TCP pre-treatment experiment. Displayed in **Figure 4-2** are the results from the animals receiving pre-treatment with TCP or vehicle (H_2O) prior to injection with phenelzine, PEH or vehicle (H_2O or DMSO). TCP itself had no effect on rat brain tyrosine levels, as TCP/ H_2O and TCP/DMSO animals had tyrosine levels (12.1 ± 0.6 and 11.4 ± 1.2 μg/g tissue, respectively) comparable to those of H_2O and DMSO (12.3 ± 0.7 and 12.6 ± 0.9 μg/g tissue, respectively) from the time-response study. Pre-treatment with TCP reversed the phenelzine-induced increase in tyrosine levels at 3 hours after phenelzine injection. As assessed by the Newman-Keuls multiple comparison test, both H_2O/Phenelzine and H_2O/PEH were significantly different in comparison to TCP/DMSO, TCP/ H_2O and TCP/Phenelzine at $P < 0.05$. Pre-treatment with TCP had no effect on PEH's elevating action on tyrosine levels, as brain tyrosine concentrations in the TCP/PEH group were also greater than TCP/DMSO, TCP/ H_2O

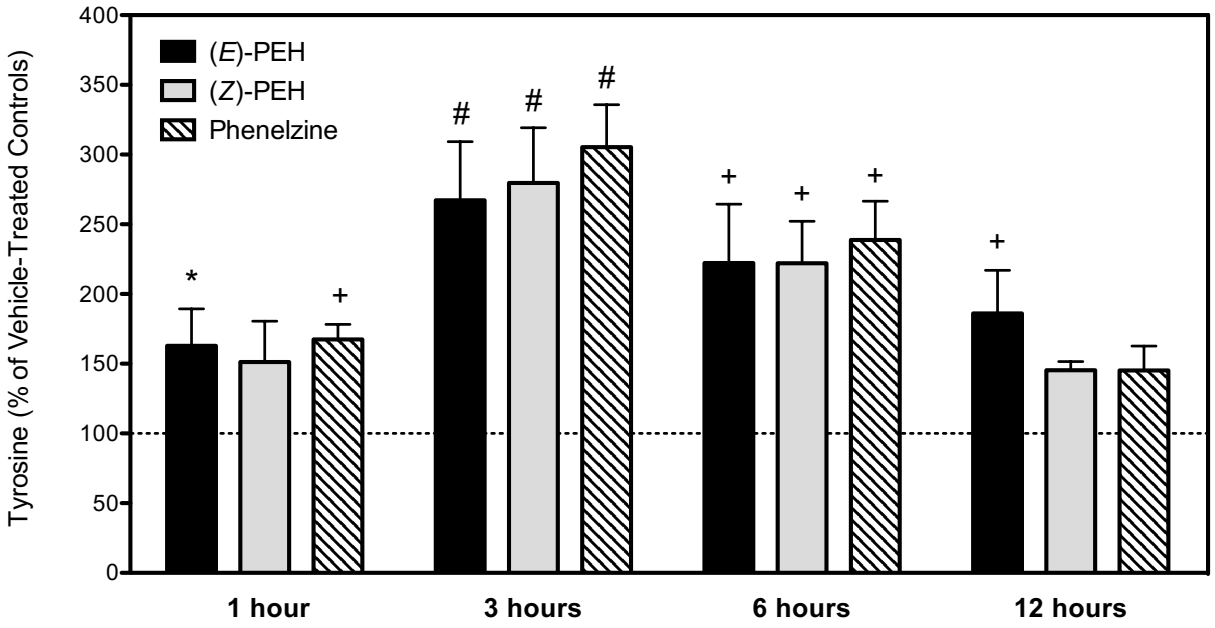

Figure 4-1. Changes in levels of rat whole brain tyrosine following treatment with (*E*)-PEH, (*Z*)-PEH or phenelzine (n = 3-5 per treatment group). Data are normalized relative to vehicle-treated controls (DMSO for [*E*]- and [*Z*]-PEH and H_2O for phenelzine). Values are means ± SEM. Symbols show that means are significantly different in comparison to DMSO in the case of (*E*)- and (*Z*)-PEH and H_2O in the case of phenelzine, as assessed by the Newman-Keuls multiple comparison test at $*P < 0.05$, $+ P < 0.01$ and $\# P < 0.001$.

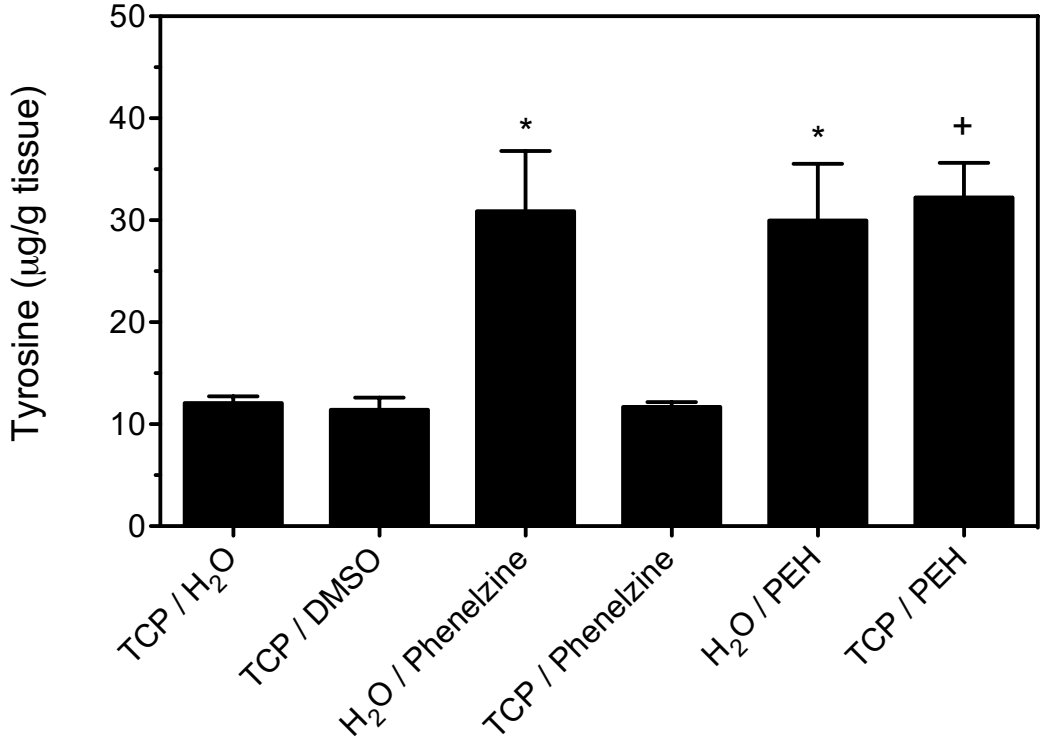

Figure 4-2. Rat whole brain tyrosine levels (means ± SEM) of animals pre-treated with TCP (1 mg/kg i.p.) or H_2O for 1 hour, followed by administration of 30 mg/kg i.p. phenelzine, PEH, H_2O or DMSO. Each treatment group (n = 4-5) was analyzed at 3 hours following the last drug administration. Asterisks show that means are significantly different in comparison to TCP/H_2O in the case of phenelzine and TCP/DMSO in the case of PEH, as assessed by the Newman-Keuls multiple comparison test at $*P < 0.05$ and $+ P < 0.01$.

and TCP/Phenelzine at $P < 0.01$.

4.4 DISCUSSION

The results of the present study demonstrate that a single i.p. injection of the antidepressant/anxiolytic drug phenelzine and the (*E*)- or (*Z*)- isomers of its active metabolite PEH produced comparable time-dependent elevations in rat whole brain tyrosine levels. A similar effect of both PEH isomers on tyrosine is consistent with the previous finding that (*E*)- and (*Z*)-PEH exhibit comparable neurochemical effects to each other on other rat brain amino acids (Chapter 3). The data also support a previous report of increased rat striatal tyrosine following an acute i.p. injection of 100 mg/kg, but not 5 mg/kg, phenelzine (Dyck and Dewar 1986). With the 100 mg/kg dose, striatal tyrosine was significantly elevated at 12 hours (to approximately 300% of controls) and was also increased at 1, 2 and 24 hours following injection but not reaching statistical significance due to large variations in the data. In the current investigation, a 30 mg/kg i.p. injection of phenelzine or PEH isomers produced statistically significant increases in tyrosine within 3 to 6 hours, reaching approximately 220-305% of controls at these time points. This increase was reversed for phenelzine by pre-treatment of the animals with the non-selective irreversible MAO inhibitor TCP. Since functional MAO is required for the conversion of phenelzine to PEH, pre-treatment with TCP should prevent the formation of PEH via this metabolic pathway. TCP pre-treatment did not affect the changes in tyrosine induced by PEH. The data from these pre-treatment experiments support the notion that PEH is responsible for effects of phenelzine on rat brain tyrosine.

In earlier investigations by other researchers, chronic administration of phenelzine via subcutaneous osmotic minipumps for 13 days (0.5 or 2.5 mg/kg/day) or 28 days (10 mg/kg/day) was reported to have no effect on tyrosine in the rat striatum, whole brain or plasma (Dyck et al. 1988; Paetsch and Greenshaw 1991). As a 5 mg/kg acute injection of phenelzine previously failed to evoke an effect on striatal tyrosine (Dyck and Dewar 1986), it can be argued that the investigators used too low a dose in the chronic experiments. Alternatively, long-term administration of phenelzine may inhibit MAO to an extent where only a negligible amount of PEH is formed by the action of the enzyme, resulting in the drop of tyrosine towards control levels. A study examining the effects of chronic administration of PEH on tyrosine is now

warranted**,** as are studies comparing brain levels of phenelzine and PEH with the changes in tyrosine levels observed.

Although the exact mechanism for PEH-induced increases in tyrosine has not yet been confirmed, the inhibition of tyrosine degradation is a likely candidate. There are several pathways for tyrosine metabolism: (1) conversion to 4-hydroxyphenylpyruvate by tyrosine transaminase (TAT, also referred to as tyrosine aminotransferase in the literature); (2) conversion to L-DOPA by tyrosine hydroxylase (TH); and (3) conversion to tyramine by L-aromatic amino acid decarboxylase (AADC). These pathways are summarized in **Figure 4-3**.
PEH-mediated inhibition of TAT, a major metabolic pathway for tyrosine (Fellman et al. 1976), is the most probable explanation. Phenelzine has previously been demonstrated to produce pronounced elevations in rat brain levels of GABA and alanine due to inhibition of GABA transaminase and alanine transaminase, respectively (Popov and Matthies 1969; Wong et al. 1990; Baker et al. 1991; McManus et al. 1992; Todd and Baker 1995; Tanay et al. 2001; Todd and Baker 2008). In addition, phenelzine also increased rat brain ornithine by as much as 6-fold, presumably due to inhibition of ornithine transaminase (MacKenzie et al. 2008b). The phenelzine-induced increases in GABA, alanine and ornithine, as well as the inhibition of GABA transaminase and alanine transaminase can be abolished by pre-treatment with another MAO inhibitor (Popov and Matthies 1969; Todd and Baker 1995; MacKenzie et al. 2008b; Todd and Baker 2008; MacKenzie 2009), suggesting that a metabolite of phenelzine formed by MAO is responsible for these effects. PEH appears to be that metabolite, as administration of PEH also increases brain GABA, alanine and ornithine levels (MacKenzie 2009; Kumpula 2013; Matveychuk et al. 2013) and causes inhibition of brain GABA transaminase (Paslawski et al. 2001; MacKenzie et al. 2008a); these properties of PEH are not affected by prior inhibition of MAO (MacKenzie 2009). The transaminase enzymes for GABA, alanine, ornithine and tyrosine are all dependent on pyridoxal-5'-phosphate (PLP) as a cofactor. It has been suggested that the binding of a hydrazine moiety, present on both phenelzine and PEH, to PLP may form an inactive hydrazone complex that will reduce the activity of associated enzymes and cause an accumulation of substrate (Yu and Boulton 1991). Of interest, plasma levels of PLP in 19 patients taking phenelzine were reduced to 54% of the values in the control group (Malcolm et al. 1994). In fact, phenelzine has been previously reported to inhibit rat liver TAT, an effect that

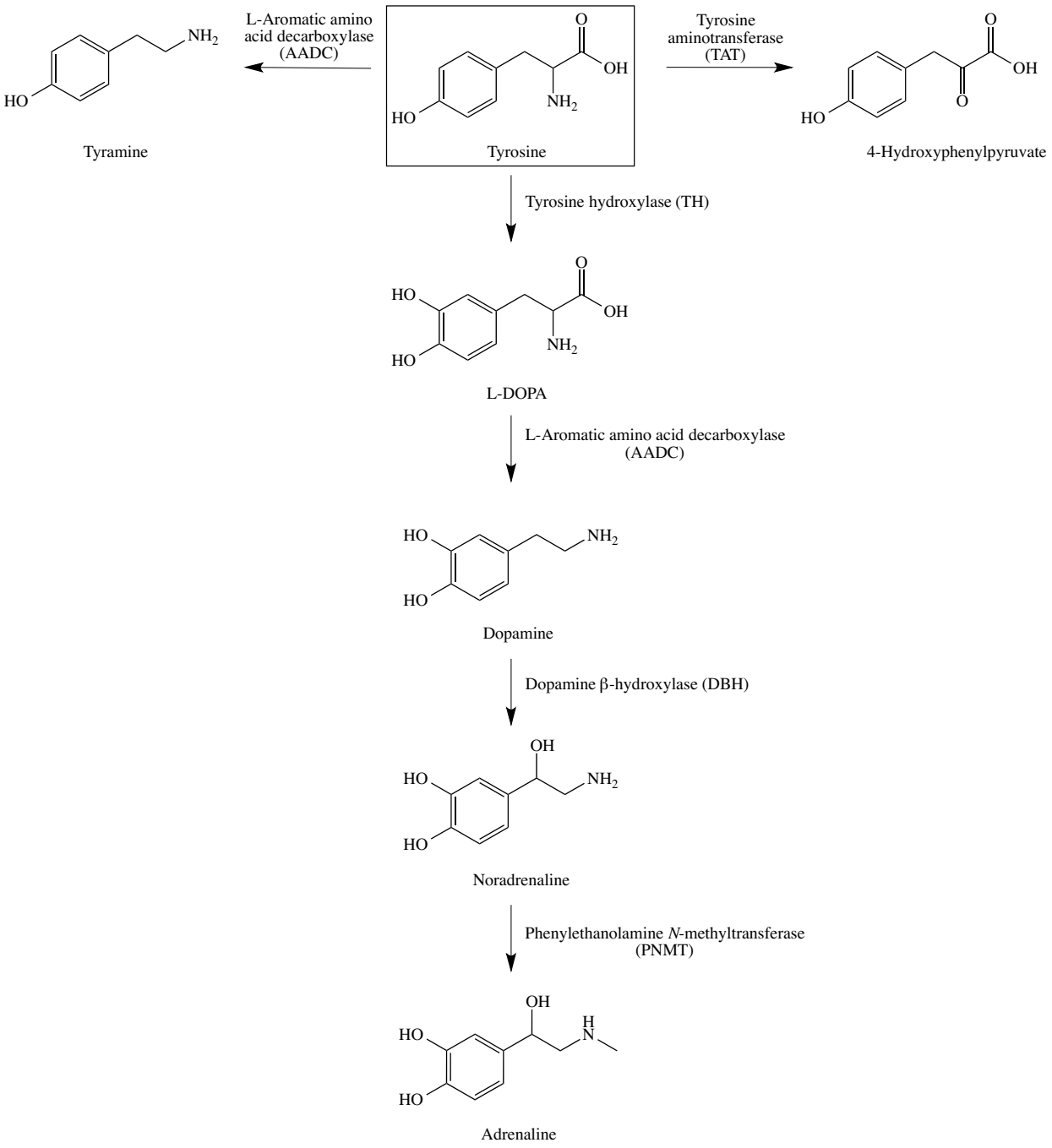

Figure 4-3. Pathways for metabolism of tyrosine.

may increase the amount of tyrosine available for transport into the brain via the LNAA transporter from the periphery (Dyck and Dewar 1986; Dyck 1987). As the assays for TAT function in those studies used liver homogenate (likely containing MAO enzymes), the MAO-mediated metabolism of phenelzine to PEH and subsequent inactivation of TAT by this metabolite may be a viable explanation for the results. A cerebral version of the TAT is also present in the rat brain, with several notable differences from hepatic TAT: it is localized mainly on the inner mitochondrial membrane in comparison to the soluble hepatic enzyme primarily in the cytosol; it contains four versus two PLP molecules per enzyme; and it has different amino acid composition and inhibition characteristics (Mandel and Aunis 2008). Further investigations into whether PEH can inhibit liver and rat brain TAT are needed.

It is unlikely that PEH is an inhibitor of TH, the enzyme responsible for the hydroxylation of tyrosine to L-DOPA (a rate-limiting step in catecholamine synthesis), as rat brain concentrations of DA and NA remain at control levels 1-12 hours following acute administration of PEH (Chapter 3, MacKenzie 2009). It should be noted that the expression of TH may be affected with long-term treatment as a form of a negative feedback mechanism, although there is little experimental consensus on the topic. A study performed with chronic administration of phenelzine (15 mg/kg i.p.) in three replicate experiments showed a 15-20% reduction in rat striatal and hypothalamic TH activity at 3 and 6 weeks in one of the experiments, but failed to reproduce this finding in the other two replicate experiments (Robinson et al. 1979). Furthermore, chronic administration of phenelzine (for 14 days via subcutaneously-implanted minipumps) did not produce a significant reduction in TH mRNA in several rat brain regions (Rovin et al. 2012). However, an additional investigation demonstrated that daily administration of phenelzine (5 mg/kg i.p.) increased TH mRNA levels in the rat locus coeruleus by 70-150% after 2 weeks and by 71-115% after 8 weeks of treatment (Brady et al. 1992).

Another possibility to be considered is PEH-mediated inhibition of AADC, another PLP-dependent enzyme responsible for the conversion of tyrosine to tyramine and the conversion of L-DOPA to DA. However, the decarboxylation of tyrosine by this enzyme is very slow and considered to represent a minor metabolic pathway of tyrosine (Lovenberg et al. 1962; Fellman et al. 1976). In addition, inhibition of AADC should produce a reduction in brain DA and NA levels, an effect not consistent with PEH administration (Chapter 3; MacKenzie 2009). Previous studies have suggested that phenelzine is an inhibitor of rat brain AADC (Dyck and Dewar 1986;

Dyck 1987). However, administration of the drug α-monofluoromethyldopa, a stronger inhibitor of brain AADC but a weaker inhibitor of liver TAT in comparison to phenelzine, did not have an effect on rat whole brain tyrosine levels (Dyck 1987).

Can an increase in brain tyrosine affect production of catecholamines? As the activity of TH is governed by an end-product inhibition mechanism via DA, NA and ADR (Nagatsu et al. 1964), increases in tyrosine levels do not normally have a lasting effect on brain catecholamine concentrations (see Cansev and Wurtman 2007 for review). This is supported by our observation that a single administration of PEH does not significantly increase rat brain DA or NA at 1-12 hours (Chapter 3) despite nearly a 3-fold increase in brain tyrosine at 3 hours. However, experimental evidence suggests that increased tyrosine availability can enhance catecholamine production in monoaminergic neurons under certain conditions, such as increased or sustained neuronal activation (Fernstrom and Fernstrom 2007). This is illustrated by the observation that supplementation with tyrosine in rats potentiated the tyrosine hydroxylation rate in the retina (containing light-sensitive DA neurons) when the animals were exposed to light, but not dark, environments (Fernstrom et al. 1986). There are several possible mechanisms for enhanced catecholamine synthesis from tyrosine upon increased neuronal stimulation: the intraneuronal catecholamine levels are reduced and thereby diminish end-product inhibition of TH (Weiner and Rabadjija 1968), phosphorylation of TH resulting in a more active and precursor-dependent form of the enzyme (Weiner et al. 1978) or increased TH synthesis (Silberstein et al. 1972). Tyrosine-dependent increases in catecholamine production can also be elicited by acceleration of catecholamine turnover through pharmacological interference with catecholaminergic pathways, including administration of an ADCC inhibitor (Wurtman et al. 1974), the DA receptor antagonist haloperidol (Scally et al. 1977), the anesthetic γ-butyrolactone (an activator of TH but also an inhibitor of neuronal firing in nigrostiatal DA neurons) (Sved and Fernstrom 1981), and the combination of a DA antagonist and a DA reuptake inhibitor (Fuller and Snoddy 1982). These findings imply that tyrosine can influence catecholamine synthesis under certain conditions, but can this be of any therapeutic value?

Interest in tyrosine for treatment of mood disorders has been around for several decades. Two of the widely accepted theories of major depression include a central deficiency of NA and/or 5-hydroxytryptamine (5-HT, serotonin) (classical monoamine theory) and the dysfunction of the hypothalamic-pituitary-adrenal (HPA) axis. The rationale for treatment of depression with

tyrosine includes increased production of NA and enhanced resilience against the HPA-mediated stress response. In rats exposed to an acute stressor, pre-treatment with tyrosine prevented NA depletion and behavioural deficits (Reinstein et al. 1984), as well as suppressing the rise in plasma corticosterone levels (Reinstein et al. 1985).

Studies on human volunteers demonstrated that tyrosine supplementation alleviated decrements in working memory and psychomotor tasks, adverse moods and performance impairment following the environmental stress of cold exposure (Banderet and Lieberman 1989; Shurtleff et al. 1994; Mahoney et al. 2007; O'Brien et al. 2007). In addition, tyrosine improved cognitive performance following exposure to acute noise stress (Deijen and Orlebeke 1994) and increased cognitive performance while also reducing blood pressure in cadets following a one-week combat training course (Deijen et al. 1999). In a study comparing tyrosine plasma concentrations of 38 patients with endogenous depression to those of neurotic depressives, schizophrenics and healthy controls, tyrosine concentrations were significantly reduced at 11 AM, but not 8 AM, for the endogenous depression group (Benkert et al. 1971). In a meta-analysis of 8 studies on acute phenylalanine/tyrosine depletion (followed by subsequent reductions in NA and DA) in humans, Ruhé et al. (2007) found that this depletion did not affect mood in healthy controls or previously-depressed patients in remission and not taking antidepressants, but slightly decreased mood in healthy controls with a family history of depression. The most consistent observation was the reduction of mood and relapse into a depressed state associated with phenylalanine/tyrosine depletion in previously-depressed patients in remission who were taking antidepressants (but only when the antidepressant working mechanism targets NA). The authors concluded that depletion of catecholamines does not directly lower mood, but may have such an effect in specific vulnerable populations.

Although initial pilot studies for tyrosine treatment of major depression produced encouraging results (Gelenberg et al. 1980; Gelenberg et al. 1982), a randomized double-blind trial of 65 patients assigned to treatment with tyrosine, imipramine or placebo for 4 weeks failed to show that tyrosine had antidepressant effects (Gelenberg et al. 1990). However, the authors suggest that due to the heterogeneous nature of depression, there may be a subset of depressed patients who would benefit from tyrosine supplementation. One such population may be women suffering from post-partum depression. Levels of estrogens and progesterone reach a peak in the third trimester of pregnancy, but drop sharply within the first few days after delivery (by as much

as 100- to 1000-fold for estradiol and estriol) (Hendrick et al. 1998). There is an inverse relationship between levels of estrogen and MAO-A levels, as demonstrated by a 43% mean increase in an index of MAO-A levels across numerous brain regions (prefrontal cortex, anterior cingulate cortex, anterior temporal cortex, thalamus, dorsal putamen, hippocampus and midbrain) at 4-6 days post-partum (Sacher et al. 2010). Interestingly, increases in MAO-A levels have been reported for the aforementioned brain regions during major depressive episodes (Meyer et al. 2006; Meyer et al. 2009). As MAO-A functions to break down the monoamine neurotransmitters, increases in tyrosine should promote catecholamine synthesis and may allow the brain to keep up with the rapid NA and DA turnover in the post-partum period.

Although PEH is a weak inhibitor of MAO-A and MAO-B and does not significantly elevate NA, DA or serotonin levels under normal conditions (Chapter 3), it can dramatically increase brain GABA levels. In depressed patients, there have been reports of GABA deficiencies in the cerebrospinal fluid (CSF) (Gold et al. 1980; Gerner and Hare 1981) and occipital cortex (Sanacora et al. 1999; Kugaya et al. 2003; Sanacora et al. 2004). Furthermore, occipital cortex GABA levels were reported to be normalized following treatment with electroconvulsive therapy (Sanacora et al. 2003) or selective serotonin reuptake inhibitors (Sanacora et al. 2002). Taken together, PEH may represent an interesting therapeutic for specific subtypes of depressed patients who are responsive to tyrosine-mediated increases in catecholamine production and have deficiencies in GABAergic function.

Patients with neurodegenerative disorders associated with neuronal loss may experience an increase of activation in surviving neurons as an attempt to compensate for functional deficiency, as is observed for Parkinson's disease where there is a substantial loss of nigrostriatal DA neurons (Bernheimer et al. 1973). In rats treated with the catecholaminergic neurotoxin 6-hydroxydopamine (6-OHDA), the basal striatal extracellular DA levels were within the range of control animals until the nigrostriatal DA neuronal content was reduced by more than 80% (Abercrombie et al. 1990). This suggests that certain compensatory presynaptic mechanisms may be activated to increase production of DA, which can include increased activity of surviving DA neurons (Agid et al. 1973; Zigmond et al. 1984) and increased TH activity (Zigmond et al. 1984) or expression (Blanchard et al. 1995). In the rat 6-OHDA model, administration of tyrosine enhanced DA release from the hyperactive nigrostriatal neurons on the lesioned side of the brain but had no effect on the contralateral non-damaged side (Melamed et al. 1980). These

experimental findings were replicated using *in vivo* microdialysis, with exogenous tyrosine increasing striatal DA levels by 139% and 25% on the sides ipsilateral and contralateral to 6-OHDA-induced nigrostriatal lesions, respectively (During et al. 1989). The effect of tyrosine supplementation on CSF levels of tyrosine and homovanillic acid (HVA), a metabolite of DA, was investigated in 23 Parkinson's disease patients (Growdon et al. 1982). In this experiment, all patients were taking 100 mg/kg/day tyrosine for 4-7 days with a subgroup pre-treated with probenecid prior to CSF collection (intended to prevent the transport of HVA across the CSF-blood barrier and facilitate its accumulation in the CSF). CSF levels of tyrosine were increased in all patients regardless of probenecid pre-treatment and levels of CSF HVA were increased in patients taking probenecid, suggesting that tyrosine administration can enhance DA turnover in Parkinson's disease.

In Alzheimer's disease, there is a substantial loss of NA neurons in the locus coeruleus (Tomlinson et al. 1981; Bondareff et al. 1982; Marcyniuk et al. 1986) with compensatory increases in activity of surviving neurons to promote NA production (Raskind et al. 1984; Tohgi et al. 1992), such as increased TH mRNA expression (Szot et al. 2000; Szot et al. 2006). The NA system has been implicated in memory formation and consolidation (Gibbs and Summers 2002), with reductions in brain NA levels demonstrated to coincide with alterations in brain-derived neurotrophic factor (BDNF) expression and to precede memory impairment and behavioural despair in a mouse model of Alzheimer's disease (Francis et al. 2012). The administration of tyrosine to rats prior to an acute stressor (tail shock) prevented NA depletion in the locus coeruleus, hippocampus and hypothalamus, as well as improved the behavioural deficits in the animals (Reinstein et al. 1984). Thus, the elevation of brain tyrosine levels could be investigated as a means of increasing brain NA levels in Alzheimer's disease patients.

The treatment of neurodegenerative disorders with tyrosine may represent an approach to specifically target populations of catecholaminergic neurons that are affected in the disease. Normally-functioning neurons should not be affected by increased tyrosine levels due to presence of negative feedback mechanisms, whereas neurons that have been affected by the disorder (e.g. nigrostriatal DA neurons in Parkinson's disease or locus coeruleus NA neurons in Alzheimer's disease) have increased activity due to compensatory mechanisms and would therefore undergo tyrosine-enhanced synthesis of catecholamine neurotransmitters (Cansev and Wurtman 2007).

4.5 CONCLUSION

Phenelzine and its active metabolite PEH were shown to produce time-dependent elevations in rat whole brain levels of tyrosine. These increases in brain tyrosine concentrations were abolished for phenelzine, but not for PEH, by pre-treatment of the animals with the MAO inhibitor TCP, suggesting that the tyrosine-elevating properties of phenelzine are MAO-mediated and are likely the result of PEH formation. As indicated by Matveychuk et al. (2013) and Baker et al. (2012), PEH may be a useful adjunctive drug for a number of neurological and psychiatric disorders because of its ability to elevate brain GABA levels, inhibit primary amine oxidase and sequester toxic aldehydes. The results from the present study suggest that elevation of tyrosine levels could also be a beneficial effect of PEH in the treatment of such disorders.

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CHAPTER 5.

Sequestration of toxic reactive aldehydes by phenelzine and its metabolite β-phenylethylidenehydrazine (PEH)

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5.1 INTRODUCTION

The concepts of excitotoxicity, mitochondrial dysfunction and oxidative stress are intimately linked and have garnered much attention in the study of neurological disorders. Oxidative stress, an imbalance between the production of reactive oxygen species and the ability to detoxify these reactive intermediates, has been linked to acute trauma seen in cerebral ischemia (Love 1999) and traumatic brain injury (Rodriguez-Rodriguez et al. 2014), as well as to neurodegenerative disorders such as Alzheimer's disease and Parkinson's disease (Lin and Beal 2006). Unfortunately, treatment of these conditions with antioxidant agents in human clinical studies has been largely disappointing (Gilgun-Sherki et al. 2002; Slemmer et al. 2008; Snow et al. 2010; Bains and Hall 2012; Mecocci and Polidori 2012). However, there is still optimism surrounding development of novel antioxidant agents that are more permeable to the blood-brain barrier and the use of multiple drugs to target different components of the oxidative stress pathway and achieve a synergistic effect. One of the consequences of oxidative stress is an increase in the generation of neurotoxic reactive aldehydes from lipid peroxidation, with suggestions that a possible link may exist between the excess production of these aldehydes and the selective neuronal and/or glial cell loss seen in numerous neurological disorders. Thus, investigation of the toxic aldehyde pathways is warranted for development of potential future interventions.

As mentioned above, a major source of endogenous aldehydes is lipid peroxidation. This process involves damage of membrane polyunsaturated fatty acids by reactive oxygen species (hydrogen peroxide [H_2O_2], hydroxyl radicals [$OH\bullet$] and superoxide radicals [$O_2^{-}\bullet$]), producing α,β-unsaturated aldehyde compounds such as 4-hydroxy-2-nonenal (HNE), acrolein and malondialdehyde (Esterbauer et al. 1991; Uchida et al. 1998; Ou et al. 2002). However, sources of endogenous reactive aldehydes are not limited to lipid peroxidation and also include carbohydrate autoxidation and metabolism, cytochrome P450 oxidation of alcohols, myeloperoxidase oxidation of amino acids and amine oxidase reactions (including monoamine oxidase, polyamine oxidase and primary amine oxidase) (O'Brien et al. 2005; Wood et al. 2006). Monoamine oxidase-catalyzed oxidation of catecholamines leads to the production of 3,4-dihydroxyphenylacetaldehyde (DOPAL, formed from dopamine) and 3,4-dihydroxyphenylglycolaldehyde (DOPEGAL, formed from noreprinephrine and epinephrine),

both of which display high toxicity in a variety of *in vitro* and *in vivo* experiments (Eisenhofer et al. 2004; Marchitti et al. 2007). Due to selective targeting of catecholaminergic neurons, DOPEGAL and DOPAL have been implicated in the etiology of Alzheimer's and Parkinson's diseases (Burke et al. 2003; Burke et al. 2004; Panneton et al. 2010). Oxidation of the polyamines spermine, spermidine and 1,3-propanediamine to putrescine by polyamine oxidase, spermine oxidase and diamine oxidase yields the reactive aldehydes 3-aminopropanal and acrolein as by-products (Wood et al. 2006). In addition, formaldehyde and methylglyoxal are produced from the precursors methylamine and aminoacetone, respectively, by primary amine oxidase (Gubisne-Haberle 2004). The reactive aldehydes are known to be metabolized by three major pathways: conjugation with glutathione by glutathione-S-transferases and glyoxalases, oxidation to a carboxylic acid by aldehyde dehydrogenase, and reduction to an alcohol by aldo-keto reductases (Kuhla et al. 2005; O'Brien et al. 2005). The enzymes that are involved with aldehyde detoxification are distributed in the central nervous system in a species-, region-, cell- and organelle-specific manner (Picklo 2006).

The structures of aldehydes relevant to this chapter are represented in **Figure 5-1**. Acrolein, HNE and malondialdehyde are classified as α,β-unsaturated aldehydes, whereas methylglyoxal is an α-dicarbonyl aldehyde. In comparison to free radicals, reactive aldehydes have a relatively long life span and can disperse to a wide range of intracellular and/or extracellular targets from their source of synthesis (Esterbauer et al. 1991). Aldehydes are electrophilic and can react rapidly with nucleophilic compounds, especially those containing thiol or amino functional groups. Through the mechanism of Michael Addition and/or formation of a Schiff base, aldehydes can form adducts with and cross-link proteins (containing the amino acids histidine, lysine and cysteine), nucleobases of nucleic acids and aminophospholipids (Esterbauer et al. 1991). The formation of a cross-link between two amino acids with acrolein is demonstrated in **Figure 5-2**. Of interest, acrolein is very strongly electrophilic and is considered to be the most reactive of the α,β-unsaturated aldehydes (Esterbauer et al. 1991). The formation of these adducts (mostly irreversible) can lead to multiple deleterious events such as: inhibition of DNA, RNA, and protein synthesis; disruption of protein and cell membrane function; imbalance of calcium homeostasis; and interference with cell respiration and glycolysis pathways (Esterbauer et al. 1991; Lovell et al. 2001; Dang et al. 2010). The role of lipid peroxidation aldehydes (acrolein, HNE and malondialdehyde) in cytotoxicity has been closely

tied to the mitochondria, as this organelle is the site of free radical formation and its membrane is a significant source of arachidonic and linoleic acids for the production of α,β-unsaturated aldehydes. Acrolein, HNE and malondialdehyde have been shown to inhibit brain mitochondrial respiration (Picklo et al. 1999; Picklo and Montine 2001; Pocernich and Butterfield 2003; Long et al. 2008; Vaishnav et al. 2010). In cultured primary rat hippocampal neurons, both HNE and acrolein produced time- and concentration-dependent cell death, with evidence of disrupted cell membrane polarization and increasing intracellular calcium concentrations (Mark et al. 1997; Lovell et al. 2001). In the Lovell et al. (2001) study, acrolein was more toxic than HNE at equal concentrations. In human neuroblastoma SH-SY5Y cells, short-term methylglyoxal exposure induced plasma membrane depolarization, generation of reactive oxygen species and glutamate release, whereas long-term exposure (24 hours) caused a reduction in cell viability and intracellular ATP levels (de Arriba et al. 2006). Moreover, it appears that accumulation of reactive aldehydes can potentiate further oxidative stress and production of more aldehydes. For example, rats exposed to sub-chronic and chronic doses of acrolein experienced an increase in brain malondialdehyde levels (Huang et al. 2013; Rashedinia et al. 2013).

Levels of malondialdehyde were reported to be increased in the plasma (Martin-Aragon et al. 2009; Gustaw-Rothenberg et al. 2010), serum (Greilberger et al. 2008; Sinem et al. 2010), erythrocytes (Casado et al. 2008) and temporal cortex (Marcus et al. 1998) of Alzheimer's disease patients, as well as in the serum of individuals with mild cognitive impairment (Padurariu et al. 2010). Alzheimer's disease brains have been reported to exhibit two-fold higher levels of nuclear DNA acrolein-guanosine adducts in the hippocampus (Nam et al. 2010) and increased levels of extractable acrolein in the amygdala and hippocampus/parahippocampal gyrus (Lovell et al. 2001). Elevated acrolein content was also reported in the superior and middle temporal gyrus of subjects with mild cognitive impairment and the hippocampus/parahippocampal gyrus, superior and middle temporal gyrus and cerebellum of subjects with early Alzheimer's disease (Williams et al. 2006). Furthermore, extractable acrolein was increased in the hippocampus/parahippocampal gyrus but reduced in the cerebellum of individuals with preclinical Alzheimer's disease pathology at autopsy but normal ante-mortem performance on neuropsychological tests (Bradley et al. 2010). These studies suggest that accumulation of reactive aldehydes is an event that occurs early in the development of the disease and prior to the onset of significant clinical symptoms. In addition to increased

production of reactive aldehydes in Alzheimer's disease, the accumulation of these toxic compounds may be amplified by their impaired metabolism as patients were reported to have decreased activity and levels of the enzyme glutathione-S-transferase in the ventricular cerebrospinal fluid (CSF), amygdala, hippocampus and parahippocampal gyrus (Lovell et al. 1998).

In Parkinson's disease patients, there are reports of elevated malondialdehyde levels in plasma and CSF (Ilic et al. 1999; Chen et al. 2009; Serra et al. 2009; Baillet et al. 2010). In the Chen et al. (2009) study, the authors noted that plasma malondialdehyde levels peaked at early disease stages and were correlated with Parkinson's disease severity. In addition, malondialdehyde and acrolein content were elevated in the substantia nigra of individuals with Parkinson's disease (Dexter et al. 1989; Shamoto-Nagai et al. 2007). Furthermore, the concentration of malondialdehyde-lysine adducts were significantly increased in the frontal cortex, amygdala and substantia nigra of patients with early stages of Parkinsonian neuropathology (Dalfo et al. 2005), suggesting that protein modification by reactive aldehydes may be an early step in the development of this disorder.

Although there is uncertainty regarding whether reactive aldehydes play a central role in neurodegenerative disease initiation or are simply the result of other underlying neuropathological changes, the literature suggests there is a strong contribution of aldehyde toxicity to disease exacerbation. One pharmacological approach to reducing the aldehyde load is by directly sequestering the aldehydes. Since phenelzine and β-phenylethylidenehydrazine (PEH) possess a hydrazine moiety, they are thought to be able to react with aldehydes to form a hydrazone product (**Figure 5-3**). Both drugs were previously shown to sequester formaldehyde *in vitro* (MacKenzie 2009). In addition, phenelzine was protective against formaldehyde, 3-aminopropanal and acrolein toxicity in rat primary cortical neurons and astrocytes and in rat retinal ganglion cells (Wood et al. 2006; Song et al. 2010). A recent investigation also reported that phenelzine attenuated HNE-induced mitochondrial dysfunction and improved cortical tissue sparing in a rat model of traumatic brain injury (Singh et al. 2013). In the present study, we evaluated the ability of phenelzine and PEH to sequester the reactive aldehydes malondialdehyde, acrolein and methylglyoxal *in vitro*, as well as the ability of both drugs to attenuate acrolein-induced toxicity in a mouse cortical neuron culture. Furthermore, we investigated the effect of

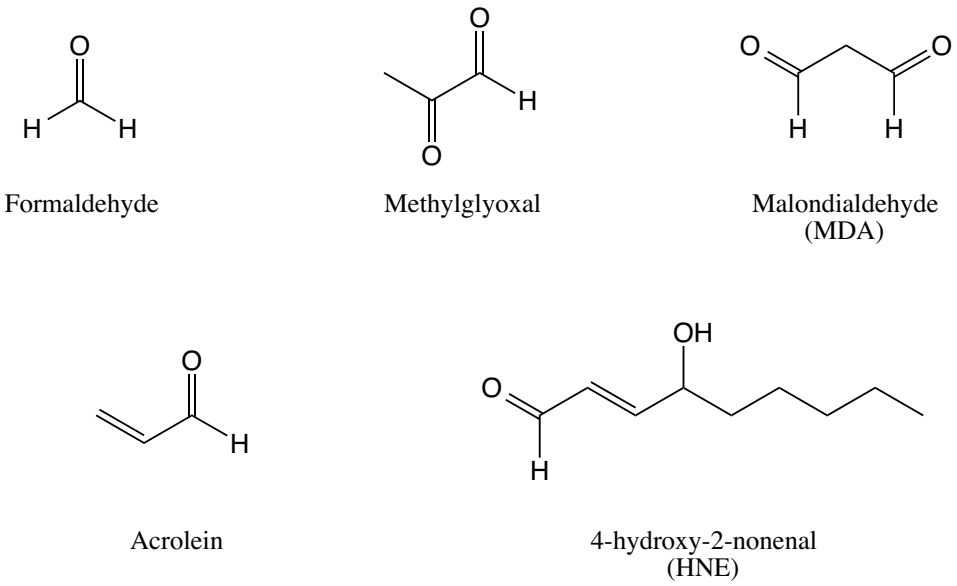


Figure 5-1. Structures of relevant reactive aldehydes.

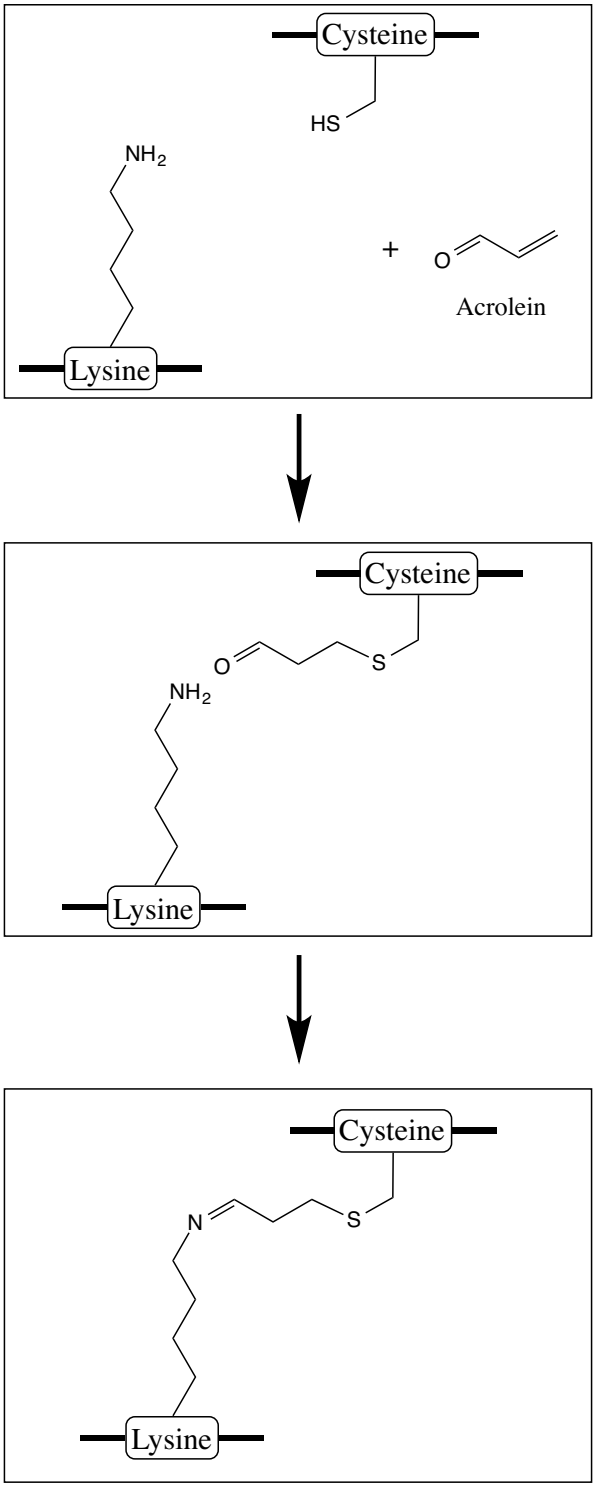


Figure 5-2. Proposed reaction scheme for the formation of a cross-link between two amino acids with acrolein.

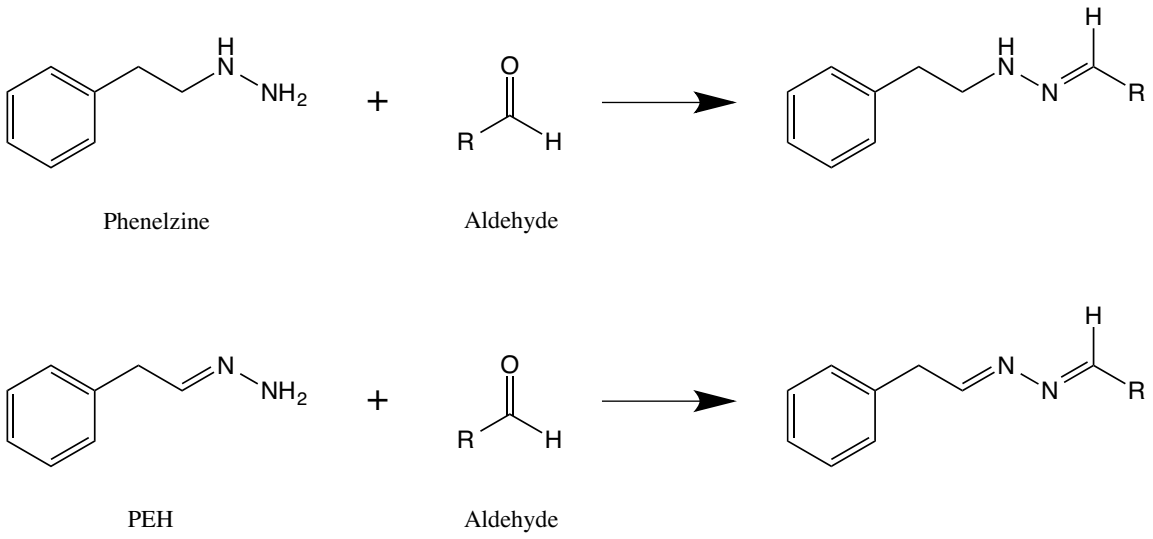


Figure 5-3. Proposed reaction scheme for sequestration of aldehydes by phenelzine and PEH to form a hydrazone product.

acute administration of phenelzine and PEH on rat whole brain levels of extractable malondialdehyde and acrolein *ex vivo*.

5.2 METHODS

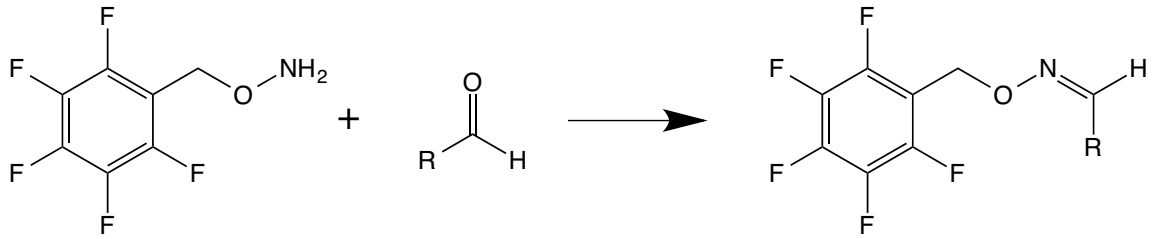
5.2.1 Materials

Water used in the experiments was distilled and purified by reverse osmosis using a Millipore Milli-Q filtration system. Dimethyl sulfoxide (DMSO), 2-methylbutane, hexane, sulfuric acid, ethylenediaminetetraacetic acid (EDTA), sodium sulfate and ethanol were purchased from Fisher Scientific. O-(2,3,4,5,6-Pentafluorobenzyl)-hydroxylamine hydrochloride (PFBHA), phenelzine sulfate, potassium phthalate monobasic, butylated hydroxytoluene, malondialdehyde tetrabutylammonium salt, methylglyoxal and $^{13}C_3$-acrolein were obtained from Sigma-Aldrich. Acrolein was purchased from Sigma-Aldrich and Ultra Scientific. Benzaldehyde-2,3,4,5,6-d_5 was obtained from CDN Isotopes. PEH was synthesized in the laboratory of Dr. Velázquez-Martínez in the Faculty of Pharmacy and Pharmaceutical Sciences at the University of Alberta.

5.2.2 *In vitro* sequestration of reactive aldehydes by phenelzine and PEH

The sequestration of reactive aldehydes by phenelzine and PEH was measured *in vitro* using a modification of the assay procedure described by Munch et al. (1998). Acrolein, malondialdehyde and methylglyoxal were incubated with phenelzine or PEH to allow for the formation of hydrazone products (**Figure 5-3**). The remaining free aldehydes in the solution were derivatized with PFBHA to produce pentafluorobenzyl (PFB) oximes (**Figure 5-4**). The PFBHA-derivatized aldehydes were measured using gas chromatography combined with mass spectrometry (GC-MS) in electron ionization (EI) mode.

The aldehydes and phenelzine or PEH (n = 5 per group) were prepared in phosphate-buffered saline (PBS, pH = 7.4). A volume of 100 μl aldehyde was reacted with 100 μl phenelzine or PEH in a glass extraction tube and incubated at room temperature for a period of 30 minutes or 12 hours. Following incubation, 100 μl of 0.250 M potassium phthalate monobasic was added to adjust the pH to 4 (the optimal pH for derivatization) followed by addition of 50 μl of PFBHA (20 mM for acrolein and methylglyoxal experiments, 40 mM for malondialdehyde



PFBHA Aldehyde

Figure 5-4. Proposed reaction scheme for derivatization of unsequestered aldehydes by PFBHA to produce a PFB oxime compound.

experiments) dissolved in H_2O. The derivatization reaction was allowed to proceed for 60 minutes at room temperature. To each tube, several drops of concentrated sulfuric acid were added to prevent the extraction of excess derivatizing reagent, phenelzine or PEH. Derivatized aldehydes were extracted by addition of 300 μl hexane, vortexing for 5 minutes, transferring of the mixture to microfuge tubes and centrifugation for 2.5 minutes at 6,000 xg. A portion of the top solvent layer (130 μl) was transferred to GC vials.

Analysis was carried out using an Agilent 6890 Series GC System equipped with an Agilent HP-5MS analytical column (30 m length, 250 mm internal diameter and 0.25 μm film thickness) and an Agilent 5983 Mass Selective Detector in EI mode. The oven settings varied for each aldehyde: for acrolein, the initial oven temperature of 50°C was held for 1 minute, increased by 4°C/minute to 130°C, followed by an increase of 30°C/minute to 290°C; for malondialdehyde and methylglyoxal, the initial oven temperature of 50°C was held for 1 minute, increased by 10°C/minute to 200°C and 2°C/minute to 220°C, followed by an increase of 30°C/minute to 290°C. The injector temperature was maintained at 220°C in splitless mode and the GC to MS transfer line at 295°C. For analysis, 1 μl of sample was injected onto the column with helium as the carrier gas (1 ml/minute). The MS source and quad temperatures were set at 230°C and 150°C, respectively.

The chromatograms of the PFBHA-oximes for each aldehyde, along with mass spectra of each peak captured using scan mode are shown in **Figures 5-5** to **5-7**. Chromatograms of derivatized acrolein produced two separate peaks, presumably due to the formation of (*E*)- and (*Z*)-isomers at the oxime double bond (**Figure 5-8**). The number of peaks increased to three for the dicarbonyl compounds malondialdehyde and methylglyoxal since oxime formation can occur with both carbonyl groups, thus increasing the number of possible isomers. For quantitation purposes, the response signal was measured as the sum of the multiple peaks heights for each compound. All PFB-oximes universally exhibit a fragment ion at a mass-to-charge ratio (m/z) of 181, whereas the acrolein-derived PFB-oxime also exhibits a molecular ion at m/z of 251 (Beranek et al. 2010), the malondialdehyde-derived PFB-oxime has a fragment ion at m/z of 250 (Spiteller et al. 1999; Kawai et al. 2007; Berdyshev 2011) and the methylglyoxal-derived FPB-oxime has a molecular ion at m/z of 462 and a fragment ion at m/z of 265 (De Revel and Bertrand 1993; Lapolla et al. 2003). The experiments used for data collection were monitored

using selective ion monitoring (SIM) at m/z of 251 for acrolein, 250 for malondialdehyde and 462 for methylglyoxal. Response peak height was used to quantitate the concentration of the target compounds based on standard curves generated on the day of the experiment.

5.2.3 Effect of phenelzine and PEH on acrolein toxicity in mouse cortical neurons

These experiments were performed in collaboration with Dr. Satyabrata Kar's laboratory, Department of Psychiatry at the University of Alberta. Timed-pregnant BALB/c mice purchased from Charles River (St. Constant, QC, Canada) were maintained according to the Animal Care and Use Committee of the University of Alberta and the Canadian Council for Animal Care Committee guidelines. Primary cortical neuronal cultures were prepared from 17-day old embryos of timed-pregnant BALB/c mice as described previously (Amritraj et al. 2009; Amritraj et al. 2013). In brief, the pregnant mice were anaesthetized with halothane and decapitated. The cortices from pup brains were dissected in Hank's balanced salt solution (HBSS) supplemented with 15 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 10 U/ml penicillin, 10 μg/ml streptomycin and digested with TrypLE Express enzyme. The cell suspension was filtered through a cell strainer and then plated on 96-well plates (5×10^4 cells/well for cell viability assay). The cultures were maintained in Neurobasal medium supplemented with B27, 50 μM glutamine, 15 mM HEPES, 10 U/ml penicillin, 10 μg/ml streptomycin and 1% fetal bovine serum (FBS) at 37˚C with 5% CO_2/95% air. The medium was replaced 1 day later without FBS and all experiments were performed on day 7 after plating.

Neurons were treated with either phenelzine or PEH (25, 50 and 100 μM) with or without acrolein (25 and 50 μM) for a period of 24 hours (n = 3). Viability of neurons was determined using the colorimetric 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay described previously (Song et al. 2008; Wei et al. 2008). In brief, neurons were incubated with MTT at 0.5 mg/ml in culture medium for 4 hours at 37˚C with 5% CO_2/95% air. The medium was removed after incubation and the formazan was dissolved in DMSO. Absorbance was measured at a wavelength of 570 nm with a microplate reader.

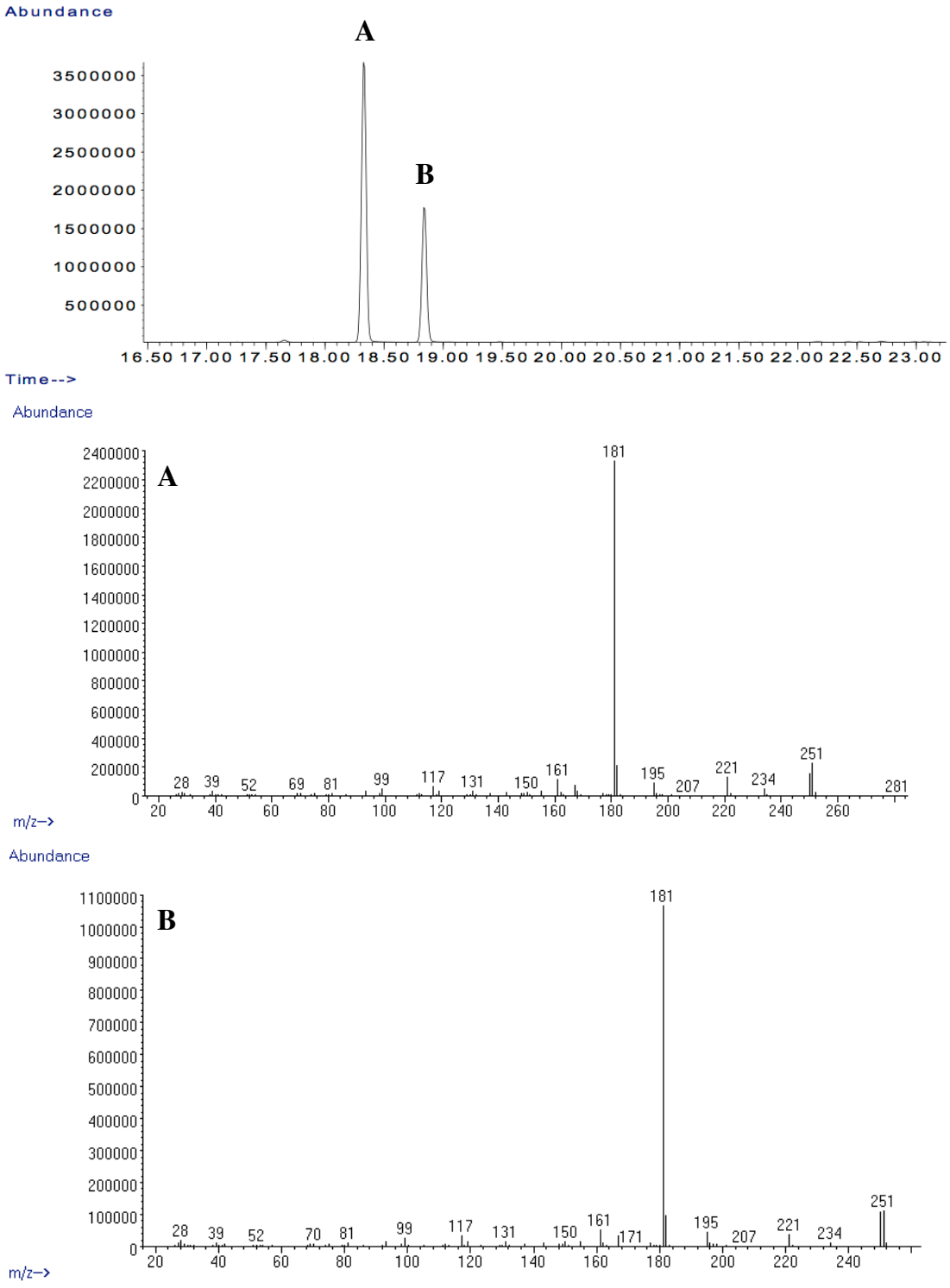


Figure 5-5. Representative chromatogram and mass spectra of acrolein derivatized with PFBHA (GC-MS in EI mode).

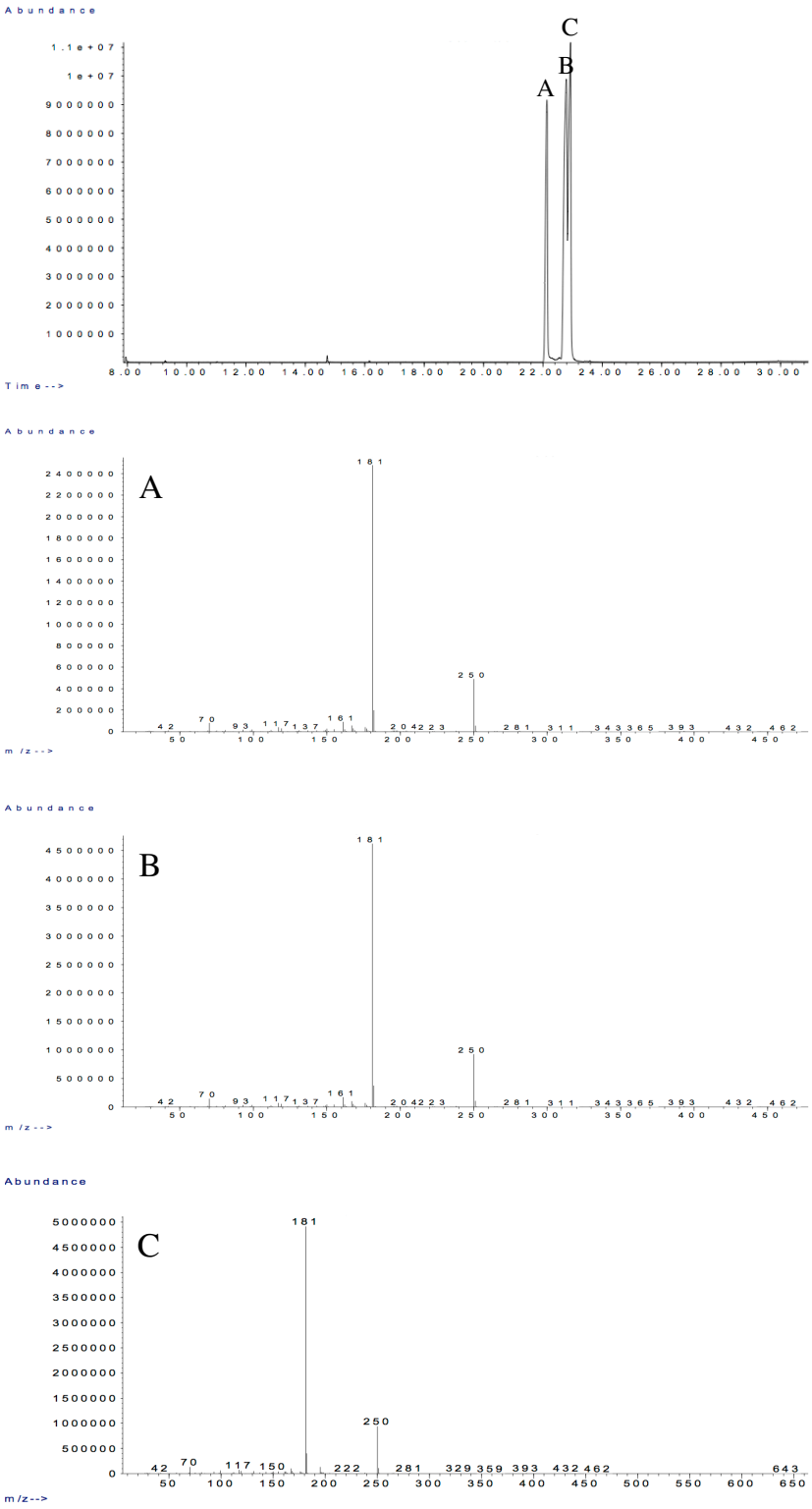


Figure 5-6. Representative chromatogram and mass spectra of malondialdehyde derivatized with PFBHA (GC-MS in EI mode).

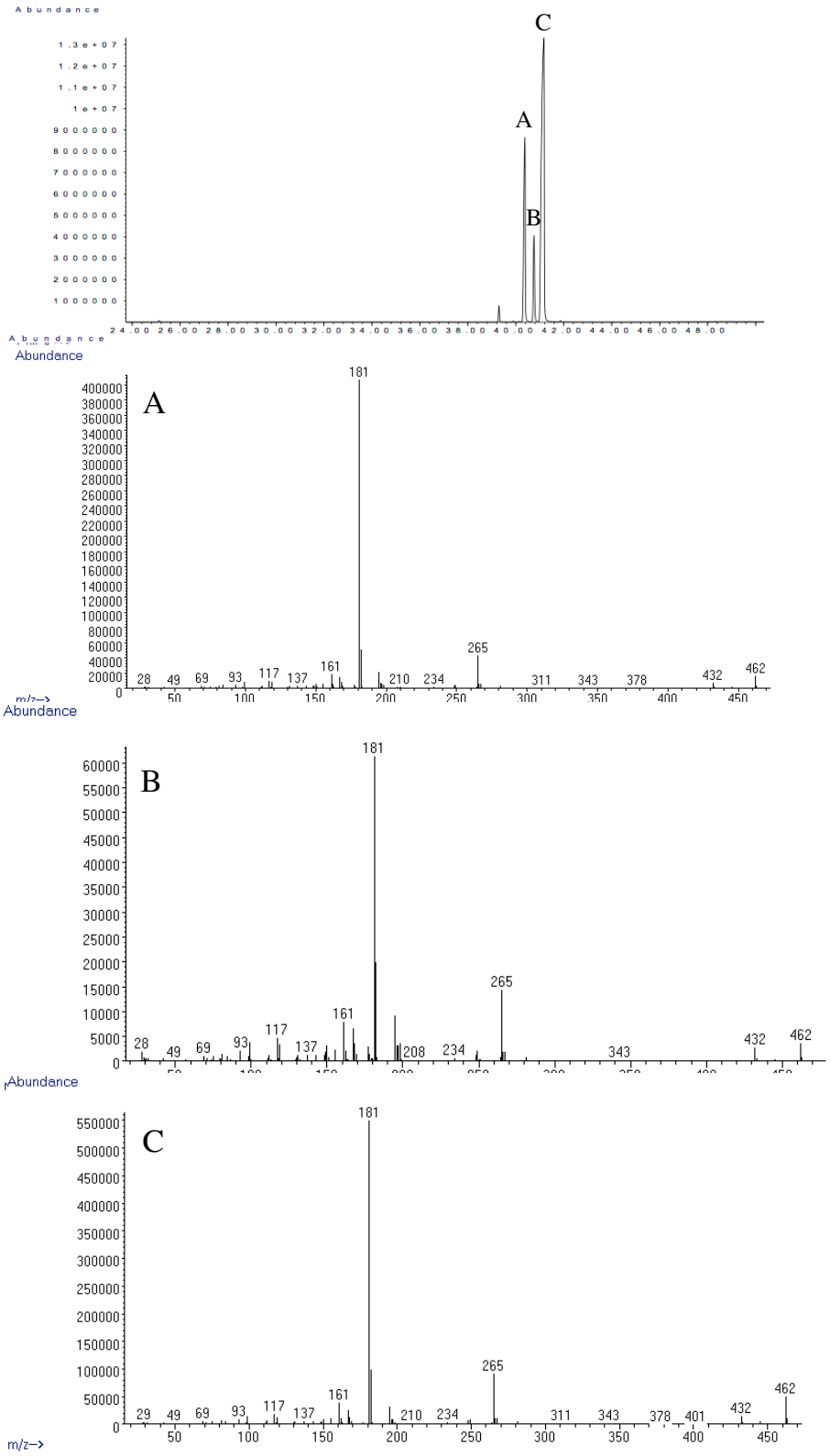
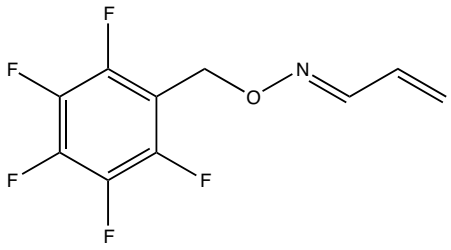
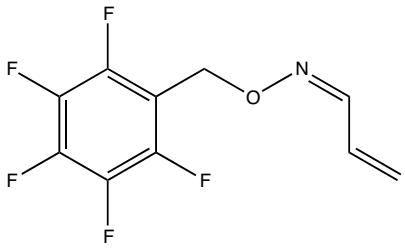


Figure 5-7. Representative chromatogram and mass spectra of methylglyoxal derivatized with PFBHA (GC-MS in EI mode).



(E)-PFB oxime



(Z)-PFB oxime

Figure 5-8. Structures of (*E*)- and (*Z*)-PFB oxime isomers formed from the derivatization of acrolein with PFBHA.

5.2.4 *Ex vivo* determination of rat brain extractable reactive aldehydes

Phenelzine (dissolved in H_2O) or PEH (dissolved in DMSO) was administered to adult male Sprague-Dawley rats at a concentration of 30 mg/kg by intraperitoneal (i.p.) injection. H_2O or DMSO alone was used as a vehicle control. Groups of rats were euthanized by decapitation at 3, 6 or 12 hours following phenelzine or PEH injection (n = 4-5 for each treatment group at 3 and 6 hours; n = 8 for each treatment group at 12 hours). The brains were dissected out, immediately flash frozen in 2-methylbutane on solid carbon dioxide, transferred to vials and kept frozen at -80°C until time of analysis. All animal procedures performed were approved by the University of Alberta Biosciences Animal Care and Use Committee and were in accordance with the guidelines of the Canadian Council on Animal Care.

Brain tissue levels of extractable aldehydes were analyzed by GC-MS with negative chemical ionization (NCI) using a modification of previously described methods (Luo et al. 1995; Fitzmaurice et al. 2006; Bradley et al. 2010). Frozen rat brain tissue was homogenized in 11 volumes of H_2O with 400 μM EDTA and 20 μM butylated hydroxytoluene to attenuate artifactual oxidation during the derivatization process. To 1 ml of homogenate, 10 nmol of $^{13}C_3$-acrolein and 1 nmol of d_5-benzaldehyde were added as internal standards. Homogenate was then derivatized with 200 μl of 50 mM PFBHA at room temperature for 60 minutes. Following derivatization, proteins were precipitated with addition of 750 μl ethanol and centrifugation at 13,000 xg for 10 minutes. Derivatized aldehydes were extracted by addition of 2 ml hexane, vortexing for 2 minutes, centrifugation at 3,000 RPM for 5 minutes and transfer of the hexane layer to clean glass tubes. The hexane extraction was repeated and the hexane layers combined. Residual H_2O was removed by addition of 0.5 g of sodium sulfate and centrifugation at 3,000 RPM for 5 minutes. The samples were then taken to dryness under vacuum at ambient temperature in a SPD SpeedVac (Thermo Electron Corporation), reconstituted in 100 μl hexane and transferred to GC vials with glass inserts.

Analysis was carried out using an Agilent 6890 Series GC System equipped with an Agilent HP-5MS analytical column (30 m length, 0.250 mm internal diameter and 0.25 μm film thickness) and an Agilent 5973 Mass Selective Detector. The initial oven temperature was held at 50°C for 1 minute, increased at a rate of 10°C/minute until 260°C, followed by an increase of

20°C/minute until reaching a final temperature of 295°C, which was held for 5 minutes. The injector temperature was maintained at 250°C in pulsed splitless mode and the GC to MS transfer line at 295°C. For analysis, 1 µl of sample was injected onto the column with helium as the carrier gas (1 ml/minute). The detector was set to NCI mode with methane as the reagent gas and MS source and quad temperatures set to 150°C. SIM was used to detect aldehydes with m/z of 231 for acrolein, 234 for $^{13}C_3$-acrolein, 286 for d_5-benzaldehyde and 204 for malondialdehyde (Fitzmaurice et al. 2006; Bradley et al. 2010).

Representative chromatograms and mass spectra for the derivatized aldehydes are shown in **Figures 5-9** to **5-13**. Since several peaks were present for each aldehyde, the response was measured as the sum of the multiple peak areas. The ratio of response peak area of unlabeled aldehyde (acrolein and malondialdehyde) to internal standard ($^{13}C_3$-acrolein for acrolein and d_5-benzaldehyde for malondialdehyde) was used for quantitation based on standard curves in naïve homogenate generated on the day of the experiment.

5.2.5 Statistical analysis

For *in vitro* sequestration experiments, statistical analysis was performed using one-way analysis of variance (ANOVA) followed by the Newman-Keuls multiple comparison test. For the acrolein experiments in cultured mouse cortical neurons, differences between groups were evaluated by two-way ANOVA followed by analysis of simple main effects with a Bonferroni correction for multiple comparisons. Differences in rat whole brain extractable aldehyde levels were evaluated by two-tailed unpaired t-test. A P value of < 0.05 was considered to be statistically significant.

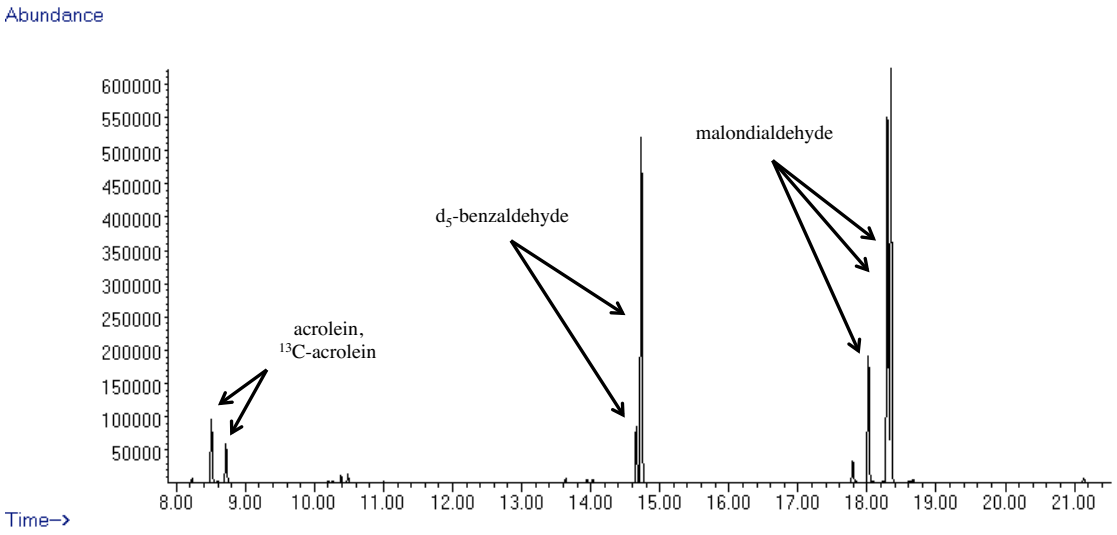


Figure 5-9. Representative chromatogram of aldehydes derivatized with PFBHA (GC-MS in NCI mode). Acrolein (m/z of 231) and ^{13}C-acrolein (m/z of 234) were at retention times of 8.52 and 8.73 minutes; d_5-benzaldehyde (m/z of 286) was at retention times of 14.65 and 14.73 minutes; and malondialdehyde (m/z of 204) was at retention times of 18.01, 18.29 and 18.34 minutes.

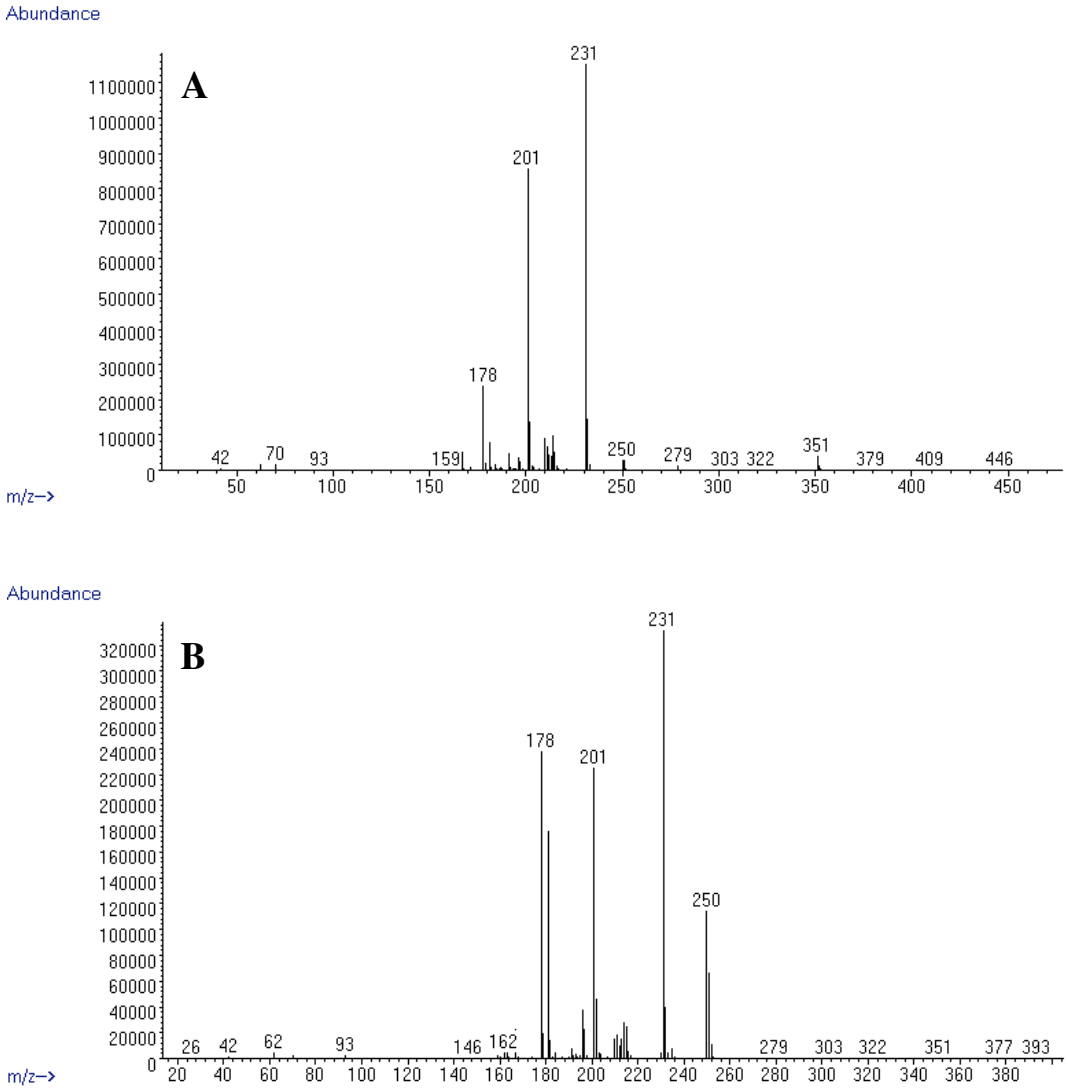


Figure 5-10. Representative mass spectra of acrolein derivatized with PFBHA (GC-MS in NCI mode) at retention times of (**A**) 8.52 and (**B**) 8.73 minutes.

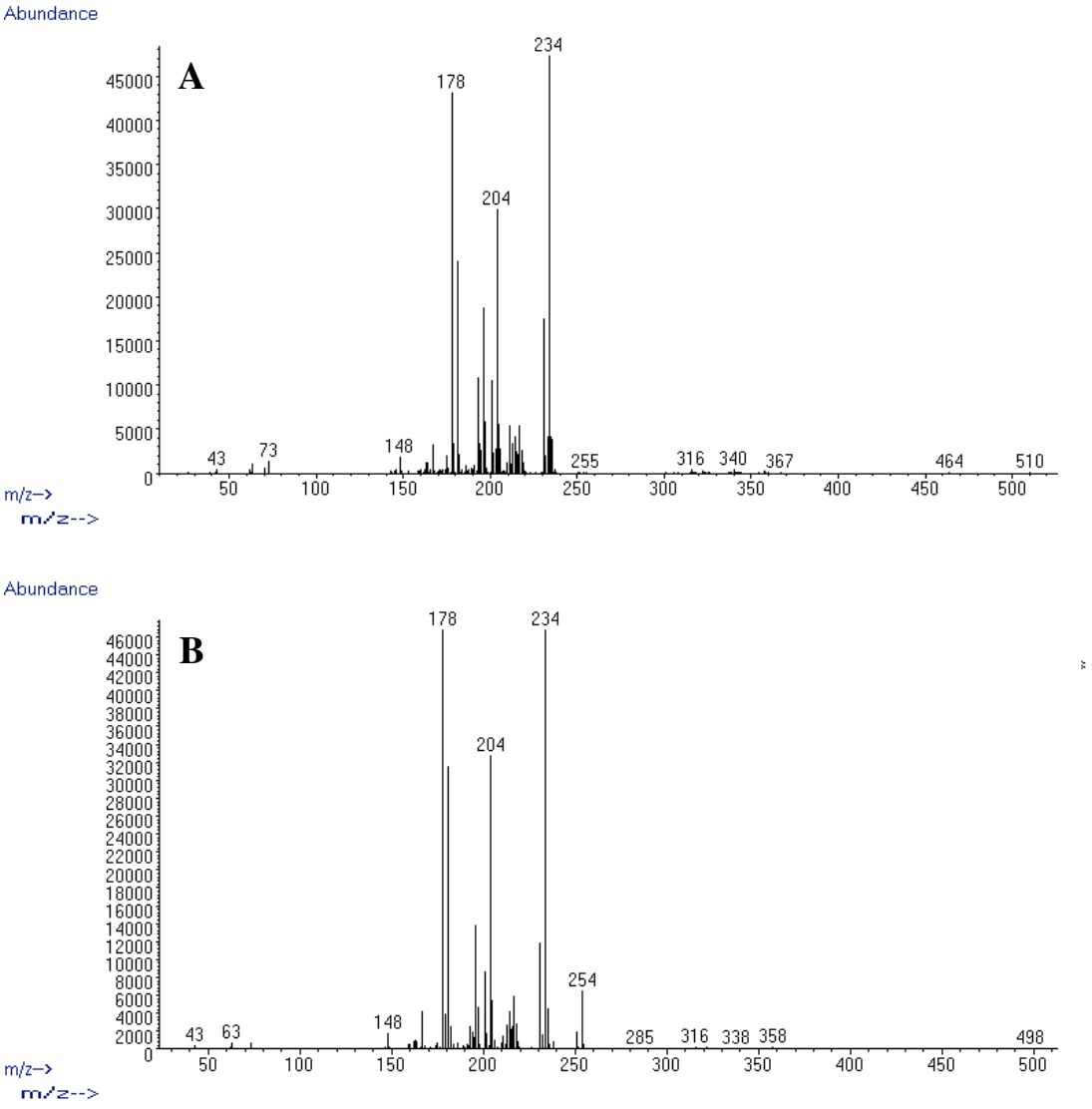


Figure 5-11. Representative mass spectra of ^{13}C-acrolein derivatized with PFBHA (GC-MS in NCI mode) at retention times of (**A**) 8.52 and (**B**) 8.73 minutes.

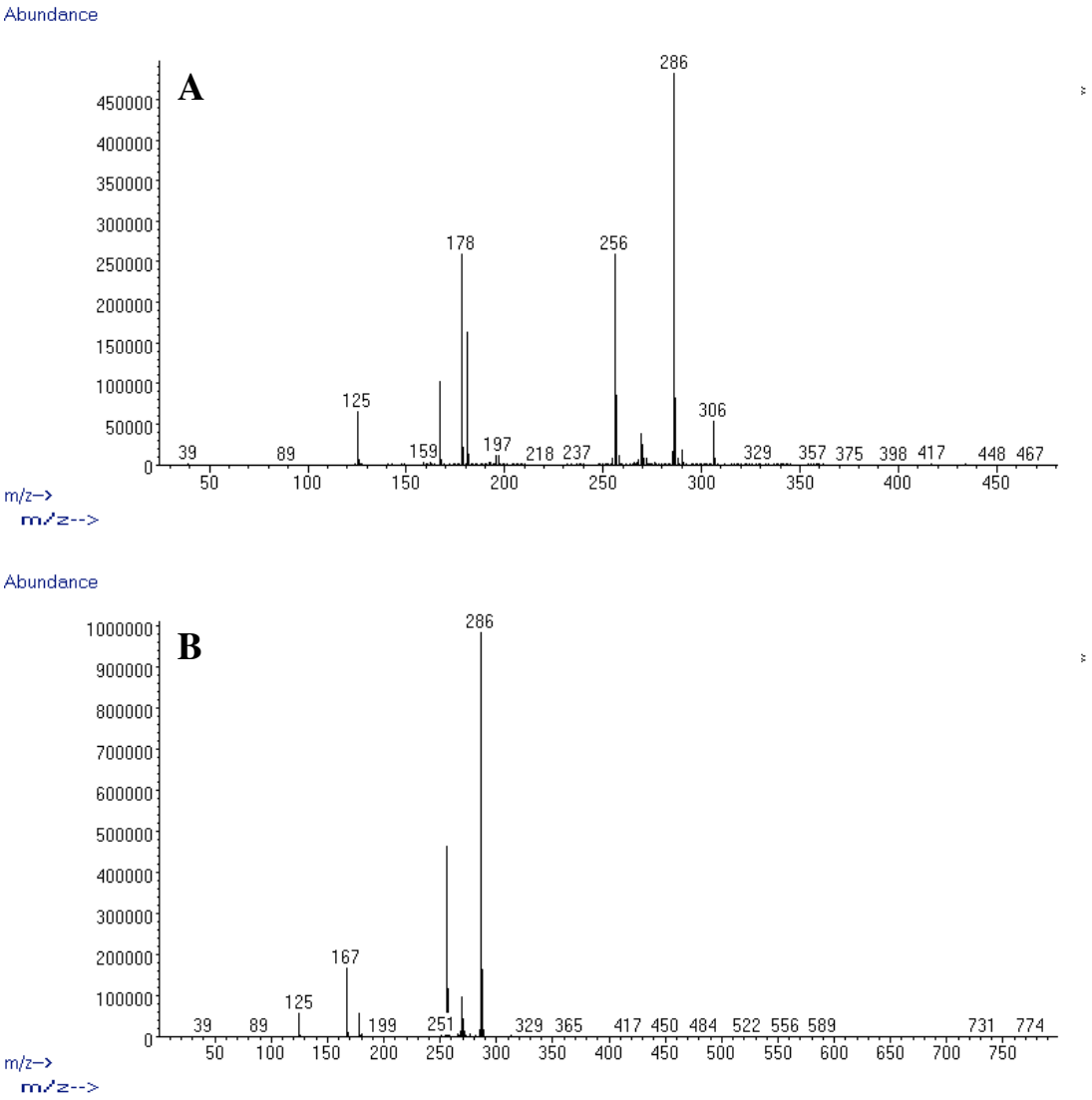


Figure 5-12. Representative mass spectra of d_5-benzaldehyde derivatized with PFBHA (GC-MS in NCI mode) at retention times of (**A**) 14.65 and (**B**) 14.73 minutes.

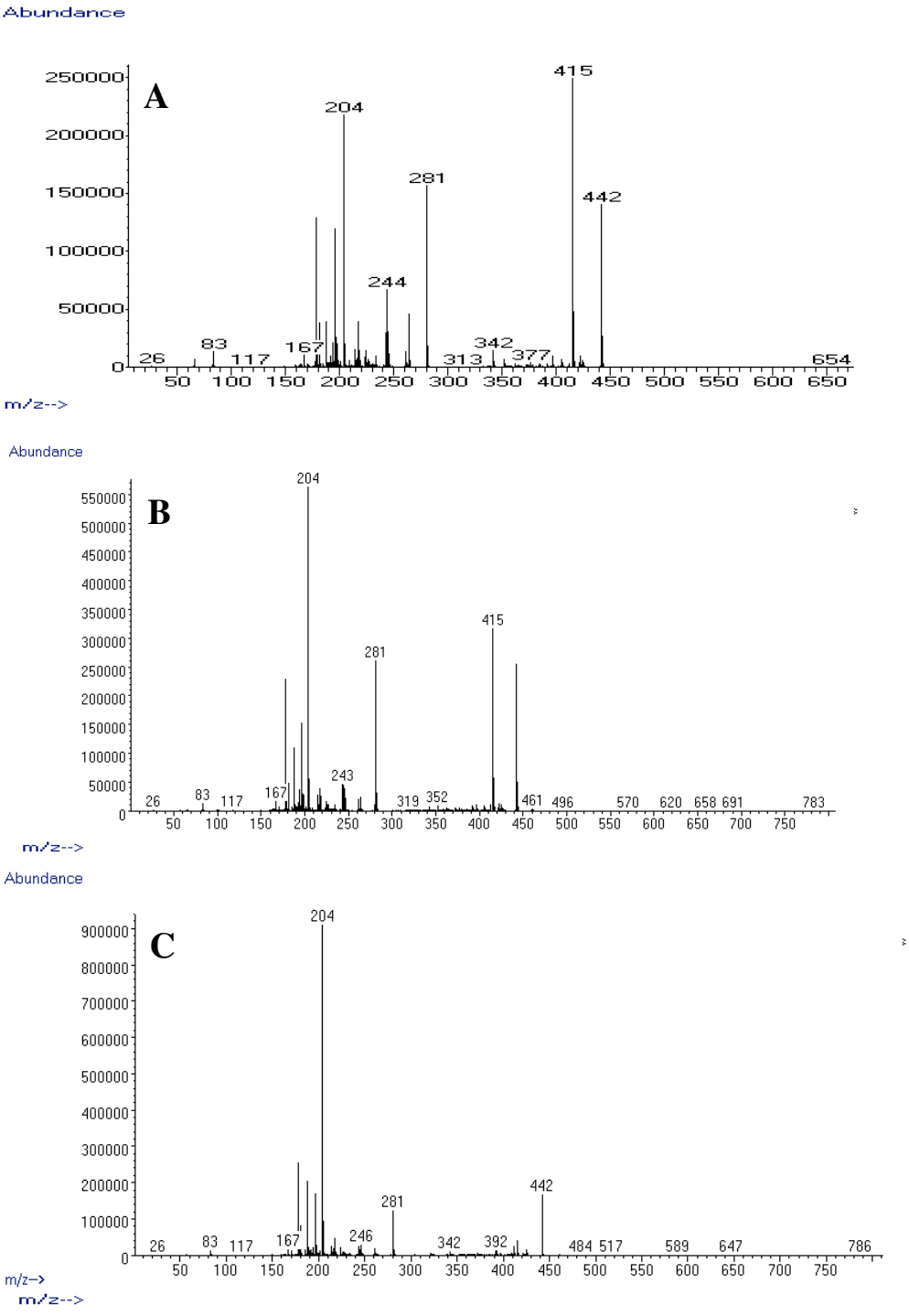


Figure 5-13. Representative mass spectra of malondialdehyde derivatized with PFBHA (GC-MS in NCI mode) at retention times of (**A**) 18.01, (**B**) 18.29 and (**C**) 18.34 minutes.

5.3 RESULTS

5.3.1 Phenelzine and PEH are effective at sequestering reactive aldehydes *in vitro*

The sequestration of aldehydes by phenelzine or PEH was assessed by comparing the amount of aldehyde remaining in solution following incubation with each drug to that of aldehyde incubated with PBS alone. Incubation periods with each drug were 30 minutes (for all aldehydes) and 12 hours (for malondialdehyde and methylglyoxal). Standard curves for each aldehyde were consistently linear with an $r^2 \geq 0.98$ (**Figures 5-14** to **5-16)**.

Both drugs were most effective at sequestering acrolein (**Figure 5-17)**. Following a 30 minute incubation with 300 nmol acrolein, 150 nmol phenelzine and PEH reduced acrolein content to slightly less than half of controls, whereas 300 nmol phenelzine and PEH reduced acrolein content to less than 20% of controls. Both drugs appear to be equally effective at sequestration of acrolein.

Sequestration of malondialdehyde and methylglyoxal by phenelzine and PEH was both dose- and time-dependent (**Figures 5-18** and **5-19**). Phenelzine and PEH were more effective at sequestering these aldehydes when incubated for 12 hours in comparison to 30 minutes. Although both drugs appear to have similar effects in sequestration of malondialdehyde, PEH was more effective at reducing methylglyoxal levels in the samples.

5.3.2 Attenuation of acrolein toxicity by phenelzine and PEH in mouse cortical neurons

The effect of phenelzine and PEH treatment alone on viability of cultured mouse cortical neurons is demonstrated in **Figure 5-20**. When phenelzine was applied to the neurons, there was a slight reduction in cell viability at the highest dose (100 μM) but this effect was not statistically significant. PEH treatment at the highest dose (100 μM) significantly reduced cell viability to approximately 77% of controls.

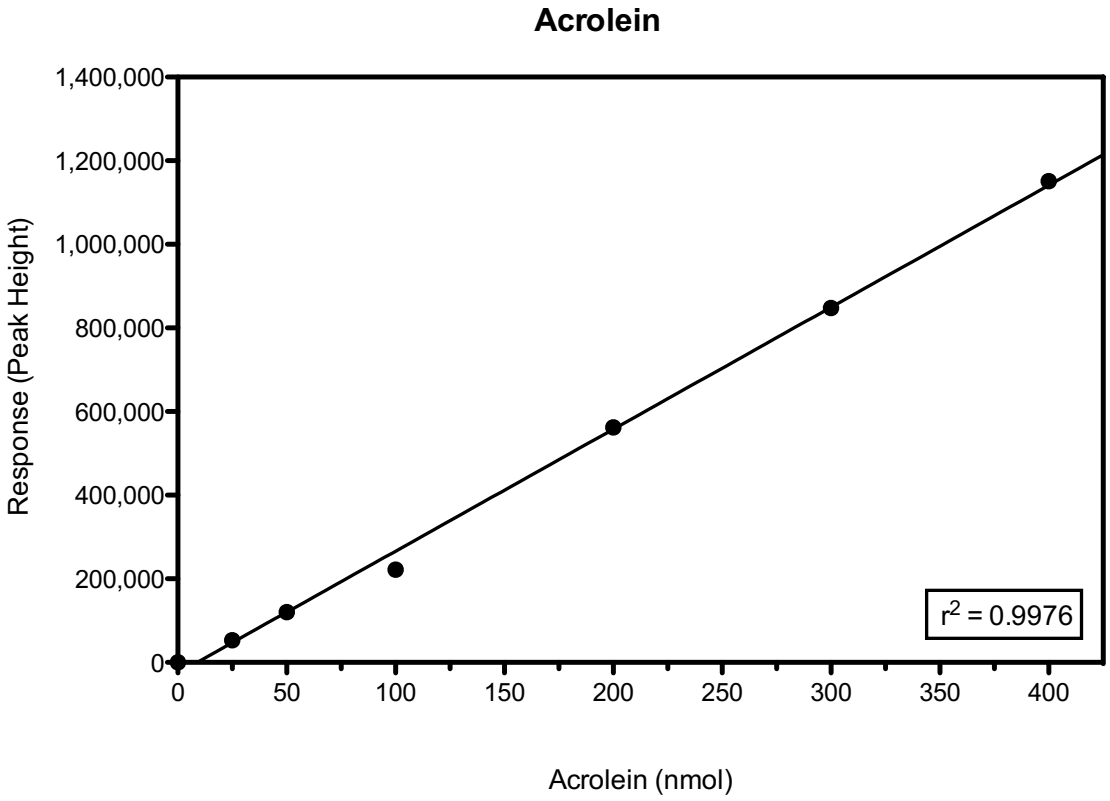


Figure 5-14. Standard curve of acrolein following derivatization with PFBHA. Acrolein was quantified using SIM at m/z of 251 by GC-MS in EI mode.

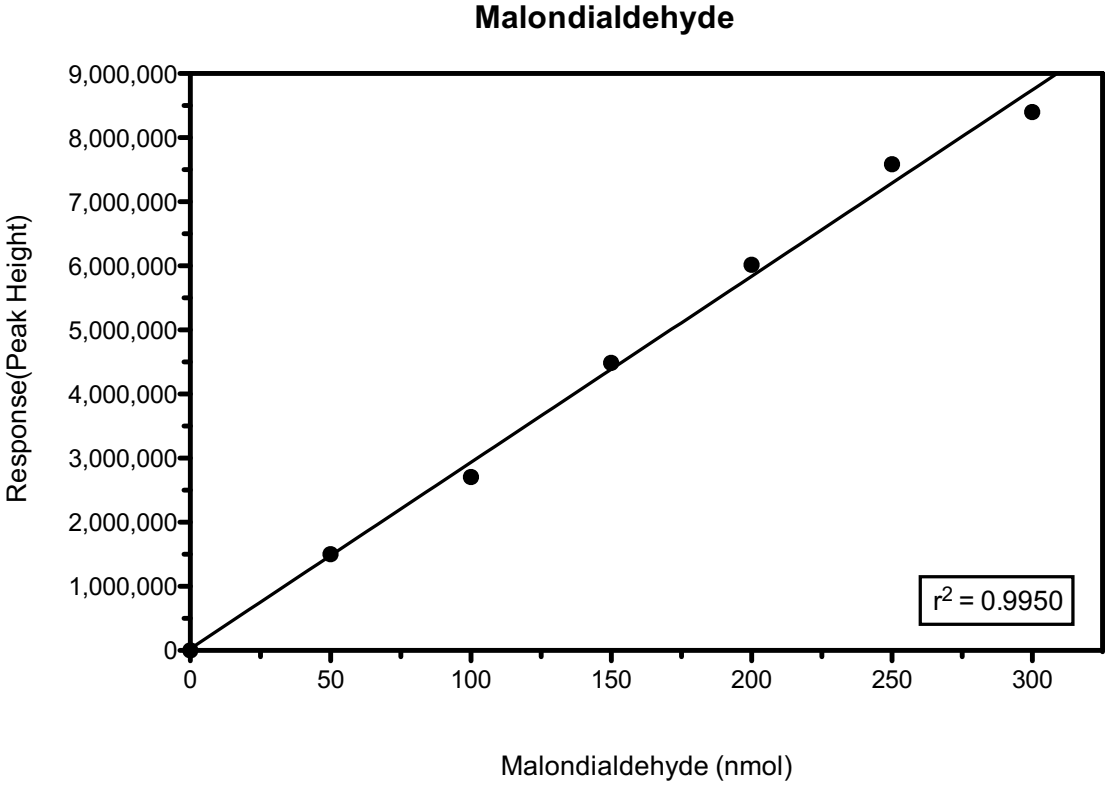


Figure 5-15. Standard curve of malondialdehyde following derivatization with PFBHA. Malondialdehyde was quantified using SIM at m/z of 250 by GC-MS in EI mode.

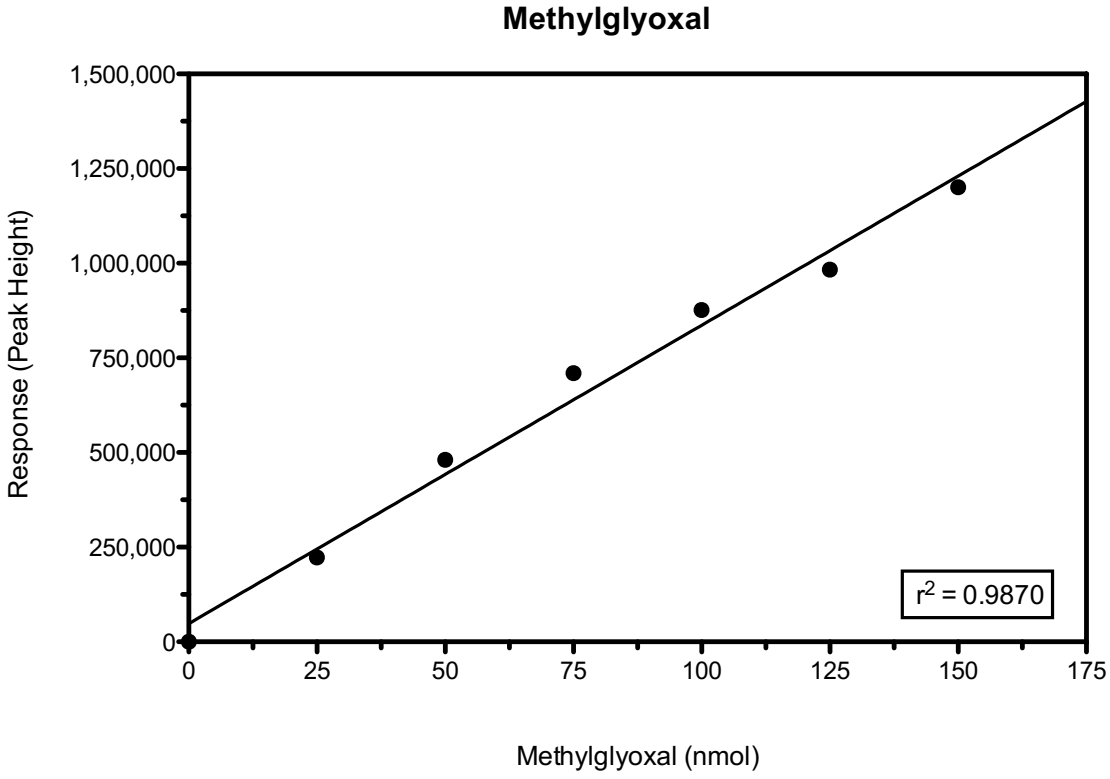


Figure 5-16. Standard curve of methylglyoxal following derivatization with PFBHA. Methylglyoxal was quantified using SIM at m/z of 462 by GC-MS in EI mode.

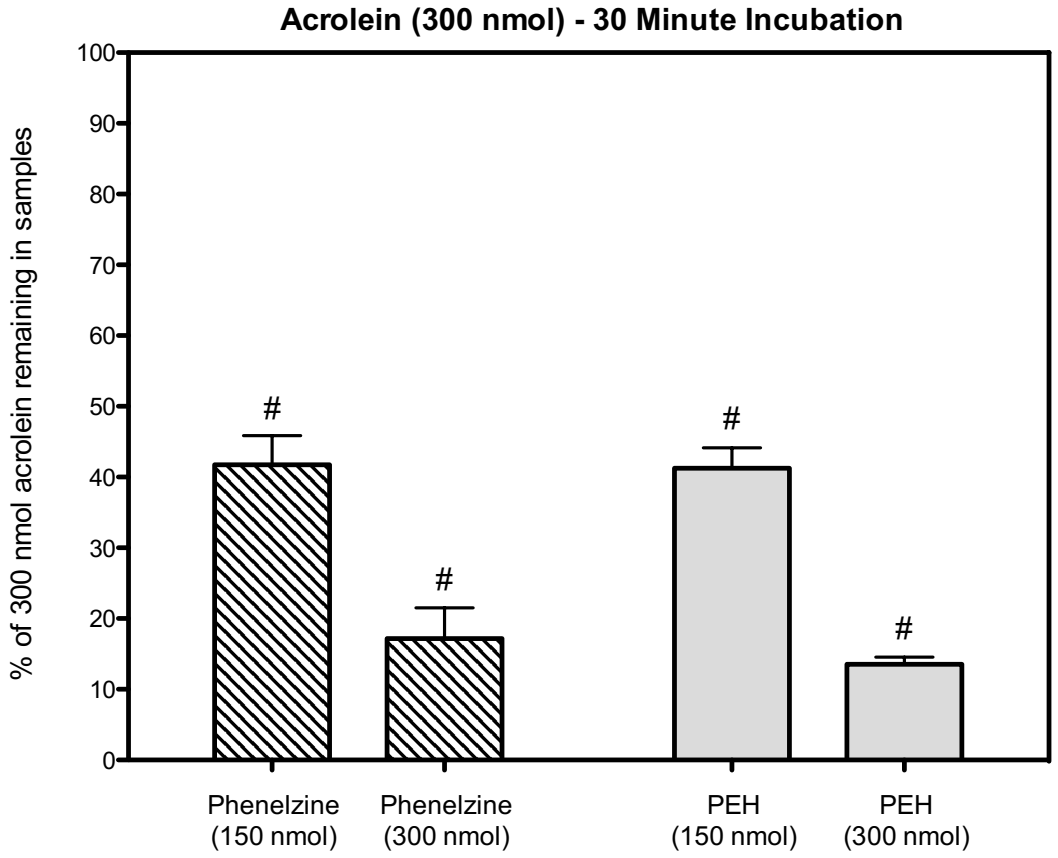


Figure 5-17. Sequestration of 300 nmol acrolein by 150 and 300 nmol phenelzine or PEH (n = 5 per treatment group) over a span of 30 minutes. Data are normalized relative to controls. Values are means ± SEM. Symbols show that means are significantly different in comparison to controls, as assessed by the Newman-Keuls multiple comparison test, # $P < 0.001$.

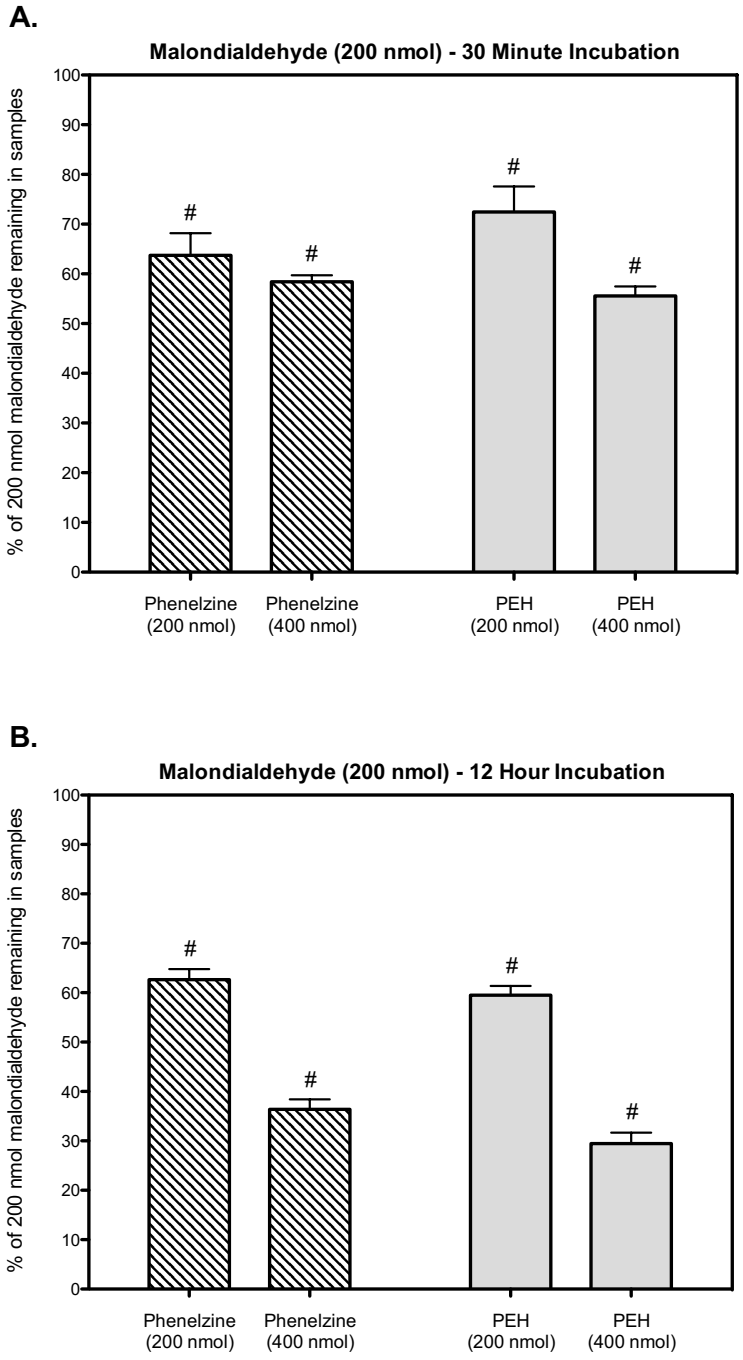


Figure 5-18. Sequestration of 200 nmol malondialdehyde by 200 and 400 nmol phenelzine or PEH (n = 5 per treatment group) over a span of 30 minutes (**A**) and 12 hours (**B**). Data are normalized relative to controls. Values are means ± SEM. Symbols show that means are significantly different in comparison to controls, as assessed by the Newman-Keuls multiple comparison test, # $P < 0.001$.

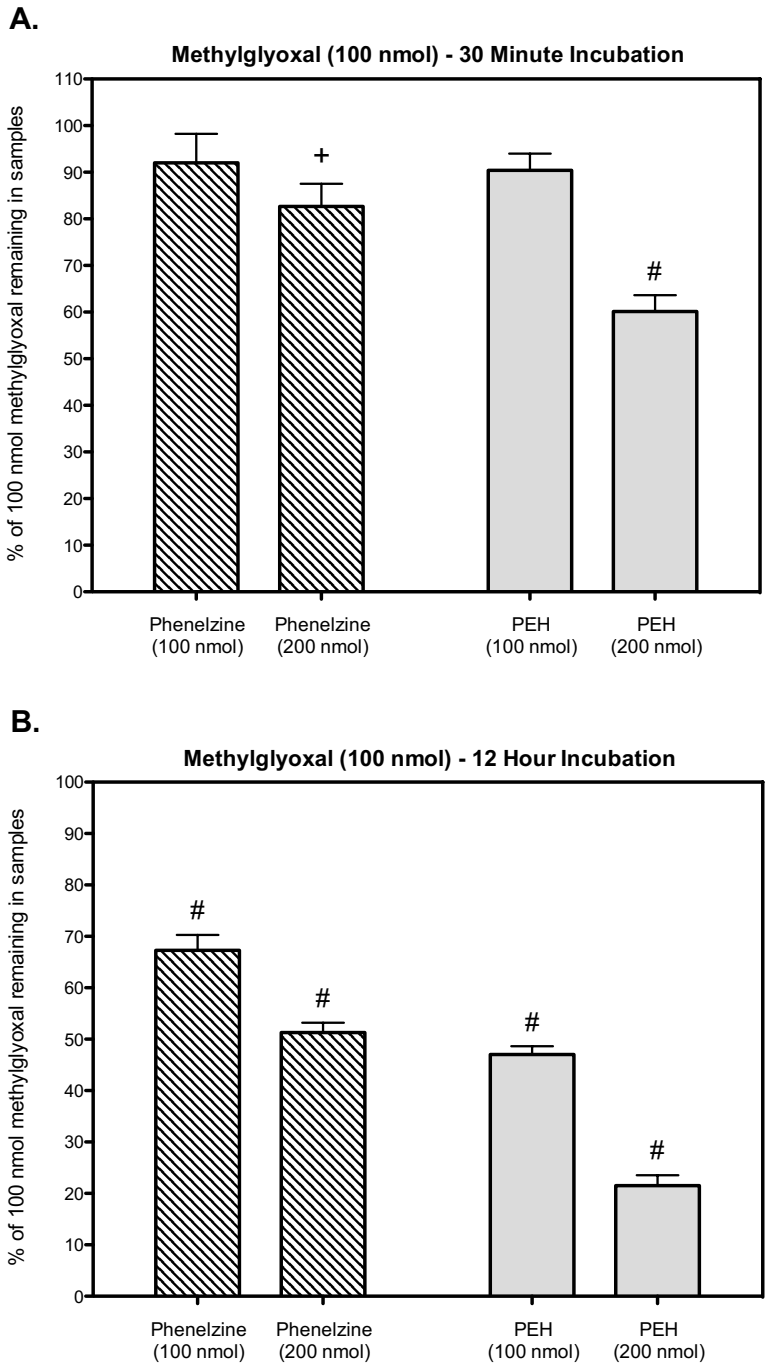


Figure 5-19. Sequestration of 100 nmol methylglyoxal by 100 and 200 nmol phenelzine or PEH (n = 5 per treatment group) over a span of 30 minutes (**A**) and 12 hours (**B**). Data are normalized relative to controls. Values are means ± SEM. Symbols show that means are significantly different in comparison to controls, as assessed by the Newman-Keuls multiple comparison test, + $P < 0.01$ and # $P < 0.001$.

Acrolein was toxic to cultured mouse cortical neurons, reducing cell viability to approximately 70% and 20% of controls at 25 and 50 μM, respectively. Cell viability was significantly increased when 25 μM acrolein was co-treated with 25 or 50 μM phenelzine (**Figure 5-21**). When 50 μM acrolein was applied to the neurons, cell viability was significantly increased when co-treated with 25-100 μM phenelzine or 50-100 μM PEH (**Figure 5-22**). The increase in cell viability for both drugs appears to be dose-dependent, with phenelzine being more effective at protecting mouse cortical neurons from acrolein toxicity at the 25 and 50 μM concentrations of acrolein in comparison to PEH.

5.3.3 Effect of phenelzine and PEH on rat brain levels of extractable acrolein and malondialdehyde

We were able to detect and quantify levels of extractable acrolein and malondialdehyde (but not methylglyoxal, formaldehyde nor HNE) in rat whole brain homogenate. Standard curves for both aldehydes in homogenate were consistently linear with an $r^2 \geq 0.98$ (**Figures 5-23** and **5-24**). The intra-assay coefficient of variation for acrolein and malondialdehyde in rat whole brain homogenate (n = 6) was 6.1% and 8.5%, respectively. Across all time points, rat whole brain acrolein levels were 434 ± 19 and 481 ± 36 nmol per gram tissue (mean ± SEM) and rat whole brain malondialdehyde levels were 3.56 ± 0.36 and 3.49 ± 0.38 nmol per gram tissue (mean ± SEM) for H_2O- and DMSO-treated animals, respectively.

Rat whole brain levels of extractable acrolein and malondialdehyde following treatment with phenelzine and PEH are demonstrated in **Figures 5-25** and **5-26**. Neither phenelzine nor PEH treatment had a significant effect on rat whole brain extractable acrolein levels at any time point. Phenelzine significantly reduced extractable malondialdehyde levels to approximately 80% of controls at the 6 and 12 hour time points. However, there was no significant effect of PEH on rat whole brain malondialdehyde levels.

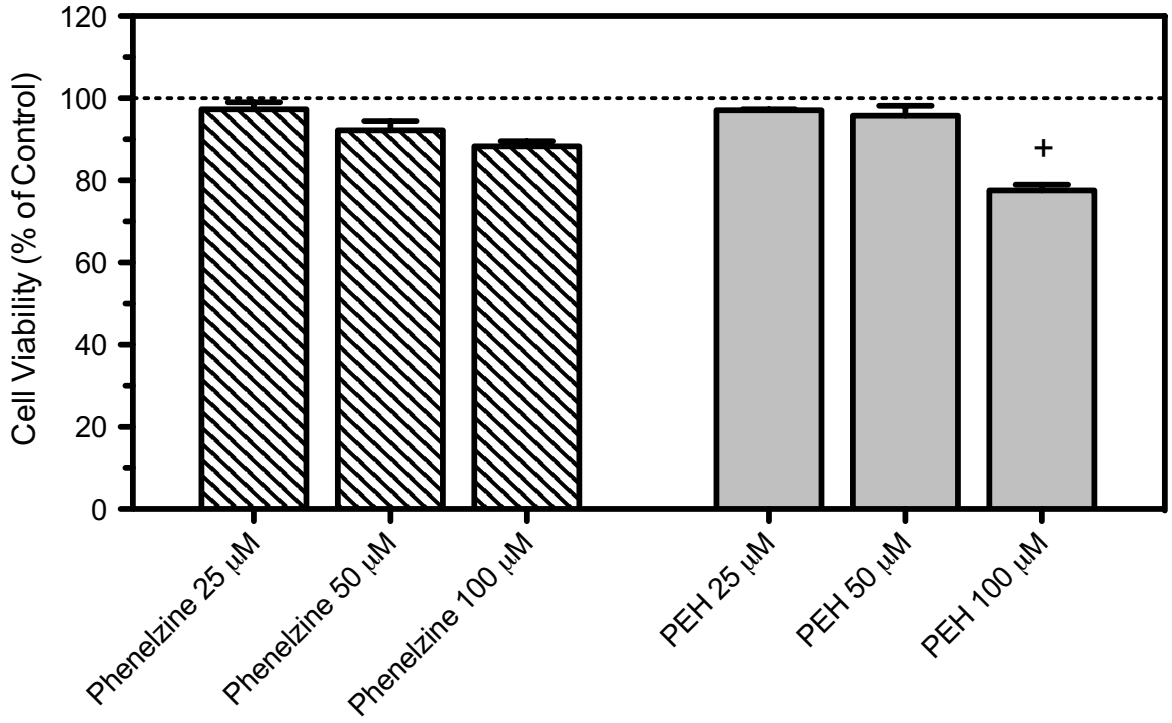


Figure 5-20. Viability of cultured mouse cortical neurons following treatment with 25-100 μM phenelzine or PEH (n = 3). Data are normalized relative to controls. Values are expressed as means ± SEM. Symbols show that means are significantly different in comparison to controls, as assessed by analysis of simple main effects with a Bonferroni correction for multiple comparisons, + $P < 0.01$.

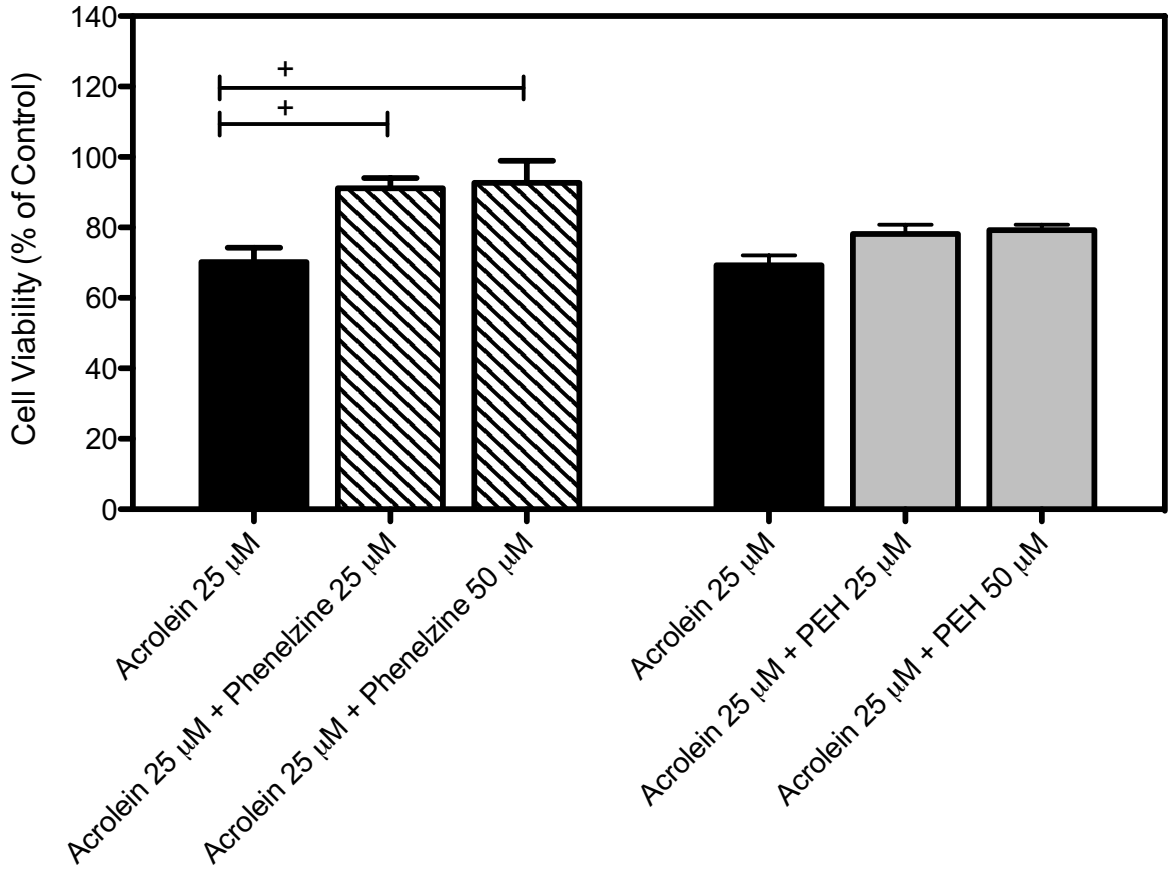


Figure 5-21. Viability of cultured mouse cortical neurons following treatment with 25 μM acrolein ± varying concentrations of phenelzine or PEH (n = 3). Data are normalized relative to controls. Values are expressed as means ± SEM. Symbols show that means are significantly different in comparison to controls, as assessed by analysis of simple main effects with a Bonferroni correction for multiple comparisons, + $P < 0.01$.

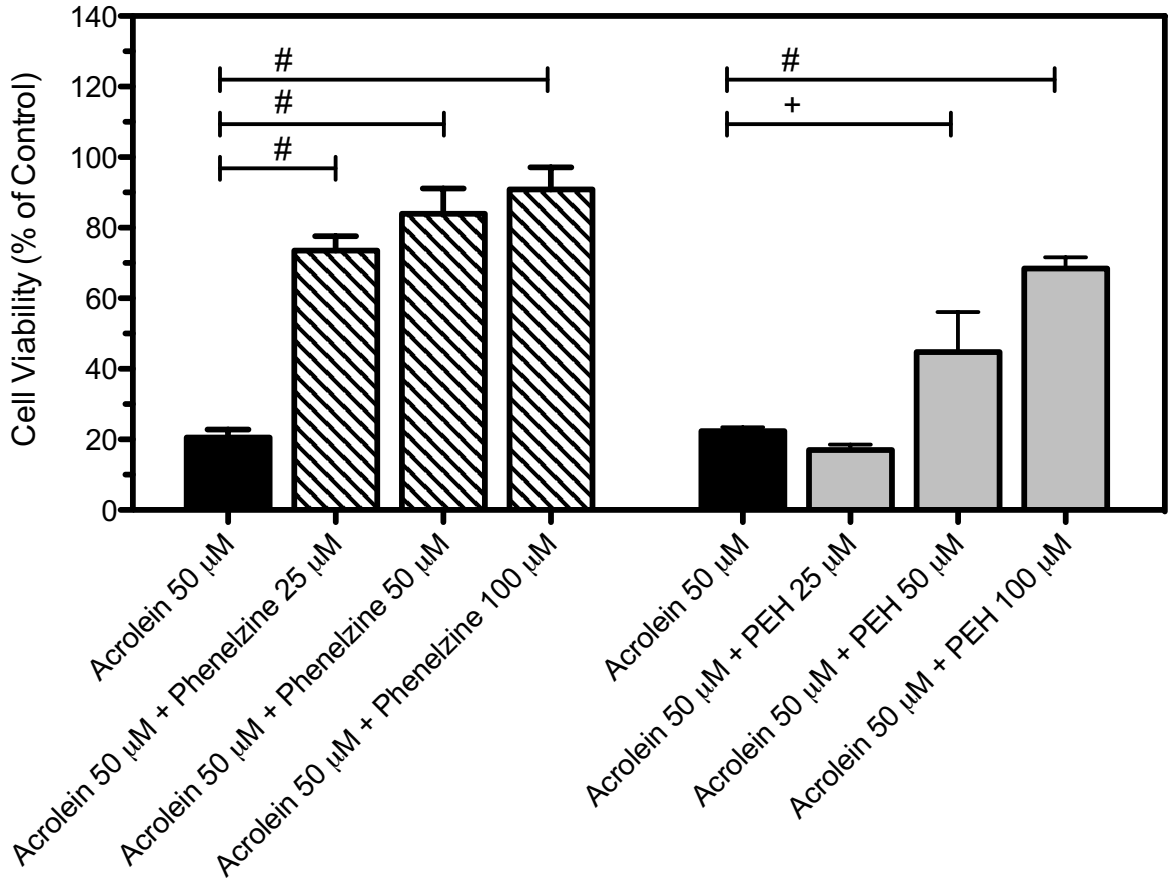


Figure 5-22. Viability of cultured mouse cortical neurons following treatment with 50 μM acrolein ± varying concentrations of phenelzine or PEH (n = 3). Data are normalized relative to controls. Values are expressed as means ± SEM. Symbols show that means are significantly different in comparison to controls, as assessed by analysis of simple main effects with a Bonferroni correction for multiple comparisons, + $P < 0.01$ and # $P < 0.001$.

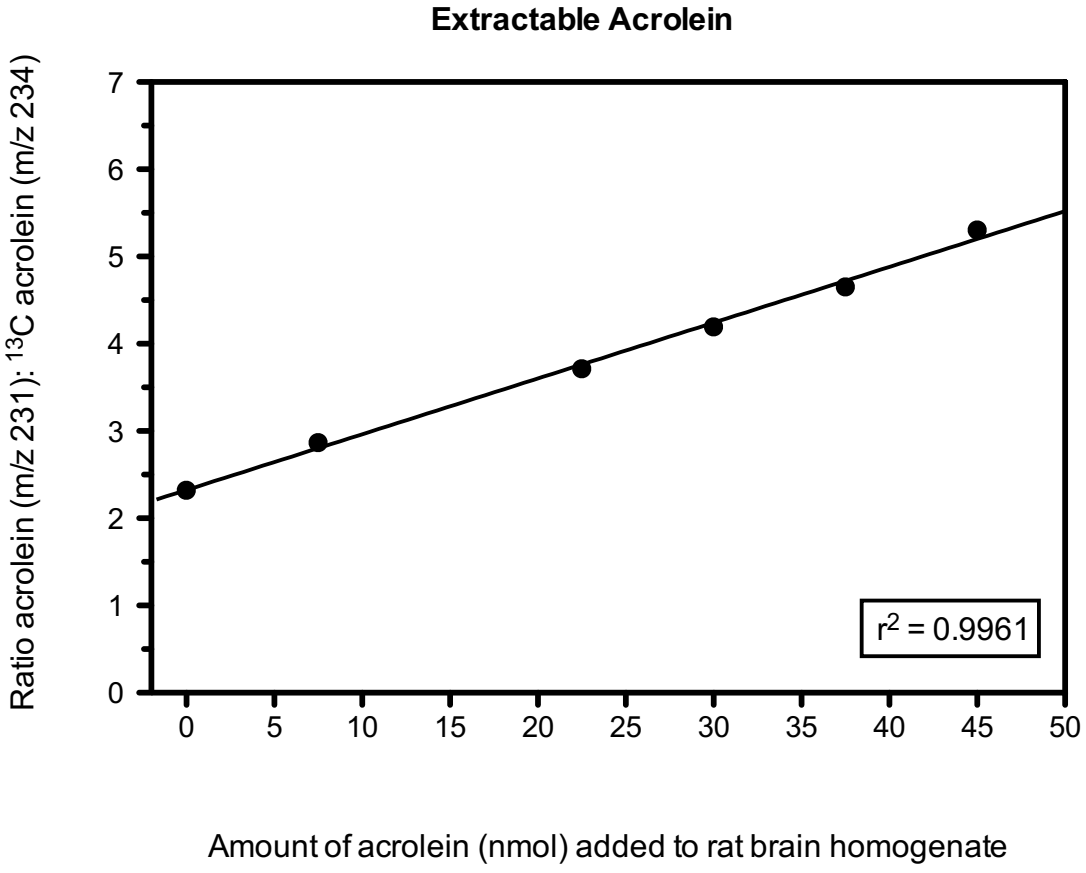


Figure 5-23. Standard curve of acrolein in rat whole brain homogenate (quantified by GC-MS in NCI mode).

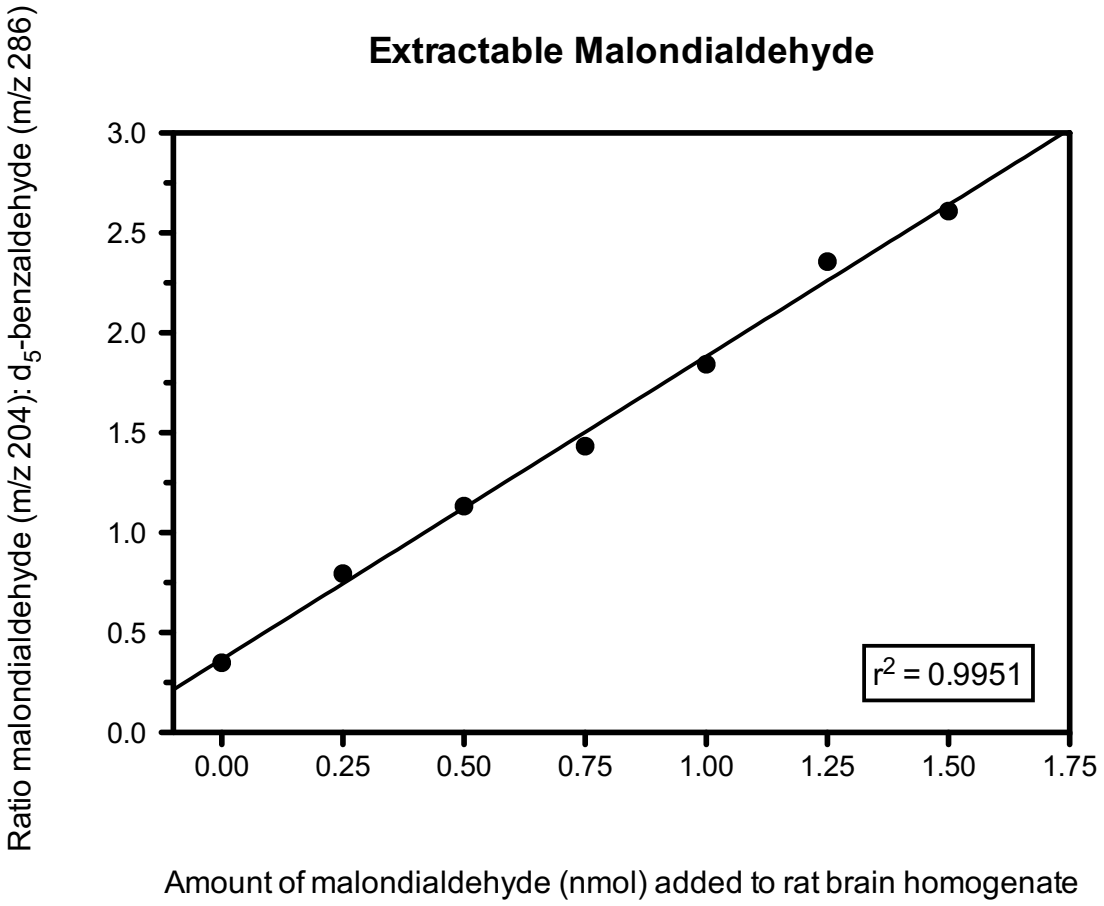


Figure 5-24. Standard curve of malondialdehyde in rat whole brain homogenate (quantified by GC-MS in NCI mode).

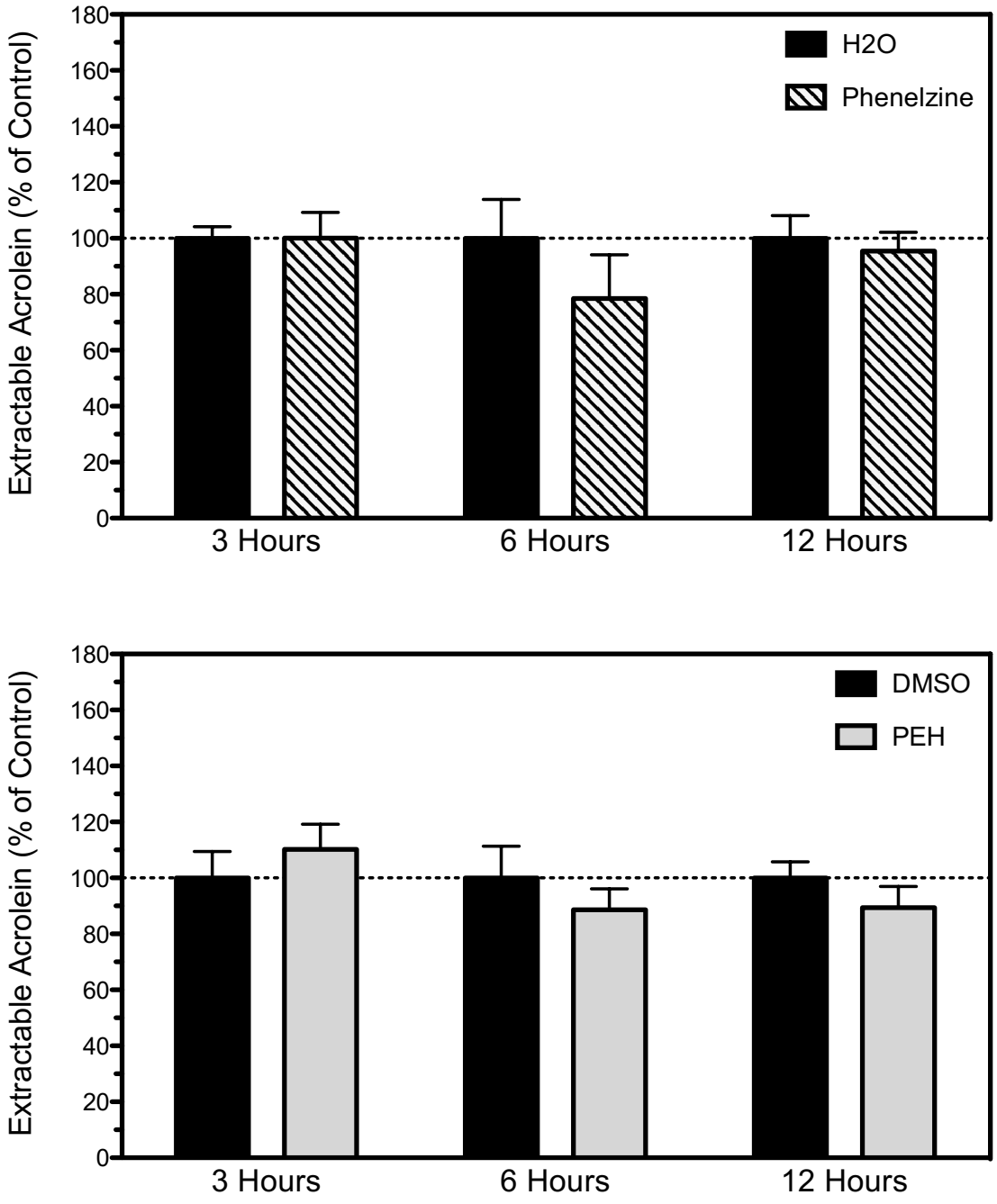


Figure 5-25. Changes in rat whole brain extractable acrolein levels following treatment with phenelzine or PEH (n = 4-8 per treatment group). Data are normalized relative to vehicle-treated controls (H_2O for phenelzine and DMSO for PEH). Values are means ± SEM.

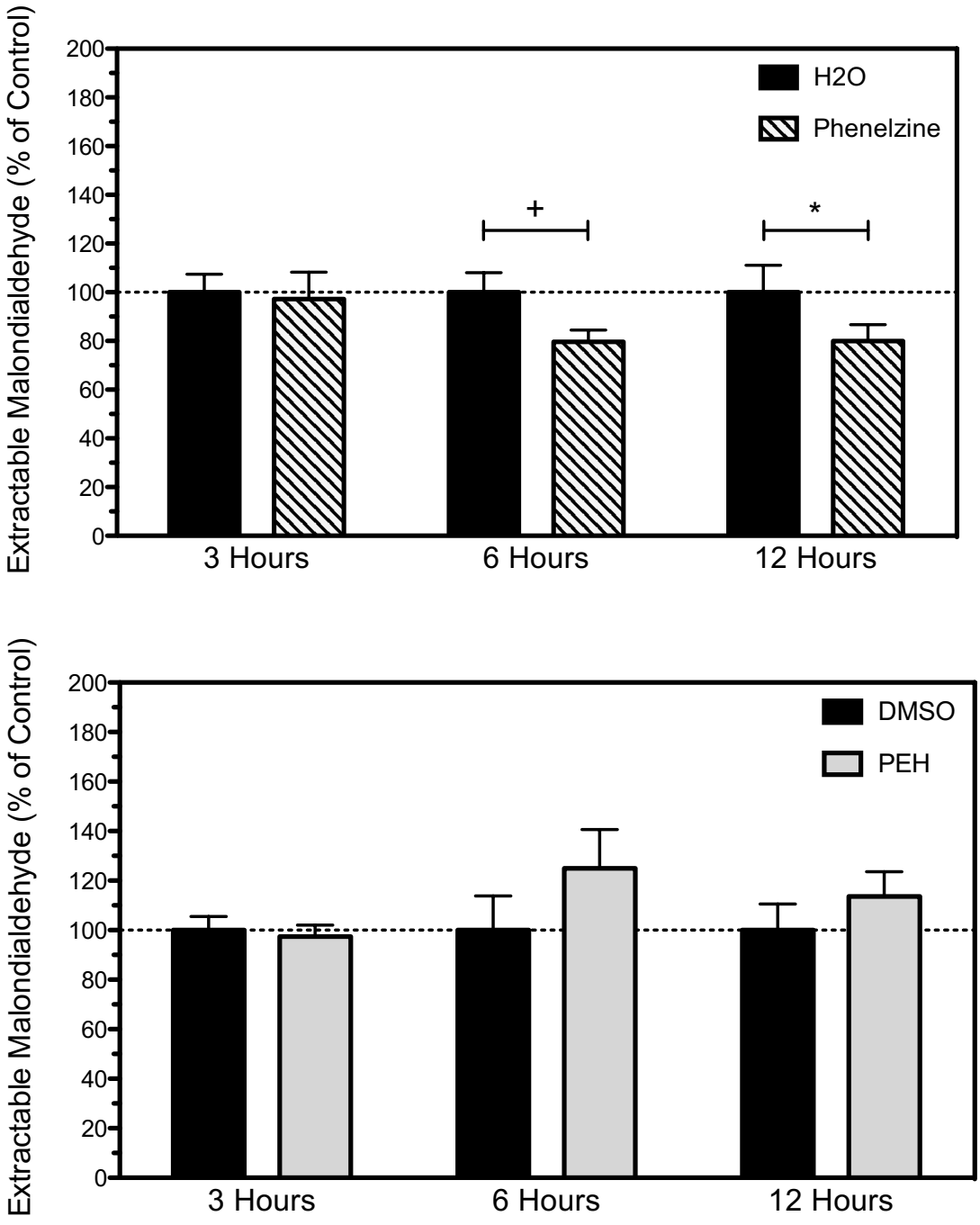


Figure 5-26. Changes in rat whole brain extractable malondialdehyde levels following treatment with phenelzine or PEH (n = 4-8 per treatment group). Data are normalized relative to vehicle-treated controls (H_2O for phenelzine and DMSO for PEH). Values are means ± SEM. Symbols show that means are significantly different in comparison to vehicle-treated controls, as assessed by unpaired two-tailed t-test at $*P < 0.05$, $+ P < 0.01$.

5.4 DISCUSSION

It appears that both phenelzine and PEH are able to reduce free acrolein, malondialdehyde and methylglyoxal levels *in vitro*. Out of the aldehydes investigated, the sequestration of acrolein was the most rapid and significant. This is not surprising, as acrolein is considered to be very strongly electrophilic and the most reactive of the aldehydes (Esterbauer et al. 1991). While both phenelzine and PEH appeared to be equally effective at sequestering acrolein and malondialdehyde, PEH had a greater effect on methylglyoxal.

In mouse cultured cortical neurons, acrolein produced a dose-dependent reduction in cell viability. The concentrations of acrolein used in the experiments (25 and 50 μM) are physiologically relevant as the concentration of acrolein in human serum was reported to range as high as 50 μM (Satoh et al. 1999). In comparison to PEH, phenelzine was more effective at attenuating acrolein toxicity. When co-treated with 50 μM acrolein, 25 and 100 μM phenelzine were able to increase cell viability from 20% to 74% and 91%, respectively. This is consistent with a previous study employing rat retinal ganglion cells, where 100 μM phenelzine provided greater than 95% protection against 50 μM acrolein (Wood et al. 2006). In the current experiment, PEH also protected cortical neurons from 50 μM, but not 25 μM, acrolein toxicity but was required in higher concentrations (50 or 100 μM) to produce this effect. Of interest, when co-treated with 50 μM acrolein, 25 μM phenelzine increased cell viability to a similar extent as 100 μM PEH. At this time, it is not apparent why phenelzine produced a greater protective effect than PEH under the experimental conditions used. It is possible that phenelzine may exert other protective actions in addition to acrolein sequestration; for example, phenelzine was shown to reverse formaldehyde-induced alterations in glutamate uptake and levels of the second messengers AKT and p38 in rat primary cortical neurons and astrocytes (Song et al. 2010).

In our study, rat whole brain levels of extractable acrolein were 434 ± 19 and 481 ± 36 nmol per gram tissue (mean ± SEM) for the H_2O- and DMSO-treated control animals, respectively. Considering that protein content of the rat brain is estimated to be 10-13% of wet tissue weight (Banay-Schwartz et al. 1992), these values are comparable to previously reported values of 2-5 nmol acrolein per mg protein in human brain tissue (Bradley et al. 2010). Rat

whole brain malondialdehyde levels were 3.56 ± 0.36 and 3.49 ± 0.38 nmol per gram tissue (mean ± SEM) for H_2O- and DMSO-treated control animals, respectively. These levels of malondialdehyde were much lower than the previously reported values of 0.4 – 0.9 nmol malondialdehyde per mg protein in human brain tissue (Fitzmaurice et al. 2006); this discrepancy may be due to species- and/or age-related differences. In addition, previous studies have examined individual brain regions whereas the present experiment investigated whole brain levels of aldehydes.

Although phenelzine and PEH were equally effective at reducing free malondialdehyde content in the *in vitro* experiments, only phenelzine significantly reduced rat whole brain levels of extractable malondialdehyde *ex vivo* at the 6 and 12 hour time points. Neither phenelzine nor PEH treatment resulted in a significant reduction in rat whole brain extractable acrolein levels. These results indicate that both drugs may not be quite as effective at reducing aldehyde levels *ex vivo*. However, it should be noted that there are several limitations to this study. First of all, levels of protein-bound aldehydes should also be investigated. Secondly, it may be necessary to further examine separate brain regions as opposed to the whole brain since region-specific differences in aldehyde distribution may exist. For example, it was previously reported that extractable levels of acrolein were increased in the hippocampus/parahippocampal gyrus but were decreased in the cerebellum of individuals with preclinical Alzheimer's disease (Bradley et al. 2010). Thirdly, it is worth investigating the effect of chronic treatment with phenelzine or PEH on extractable aldehyde levels. As the concentration of rat whole brain extractable acrolein was much higher than that of malondialdehyde (approximately 435-480 in comparison to 3.5 nmol per gram tissue), it is possible that prolonged treatment with phenelzine or PEH is required to detect a notable reduction in acrolein levels. Finally, as the current study was conducted on healthy adult rats, the ability of phenelzine and PEH to reduce aldehyde levels may have been attenuated by endogenous clearance mechanisms for aldehydes. As such, it would be more clinically relevant to determine the effect of both drugs on extractable aldehyde levels in an animal model of oxidative stress that produces an imbalance of aldehyde accumulation and the ability of the brain to detoxify these aldehydes. Taking this into account, further investigation should be conducted prior to determining whether phenelzine or PEH are able to sequester rat brain aldehydes.

If phenelzine and PEH are indeed able to sequester reactive aldehydes in the brain, this may have important implications for treatment of a number of neurological disorders. As summarized in the introduction of this chapter, there are numerous reports of increased reactive aldehyde content in the bloodstream and several relevant brain regions of patients with Alzheimer's disease and mild cognitive impairment. There are considered to be four pathological hallmarks of Alzheimer's disease, including (1) neuronal cell loss, the presence of (2) extracellular neuritic/senile plaques composed of with amyloid-β (Aβ) protein and (3) intracellular neurofibrillary tangles containing hyperphosphorylated *tau* protein, as well as (4) cerebrovascular Aβ deposition (Selkoe 1991); reactive aldehydes may contribute to the development of all four of these disease characteristics. As summarized in Section 5.1 and confirmed by our results with acrolein in mouse cortical neurons, reactive aldehydes can cause neuronal cell death. There is also an indication that reactive aldehydes may have a relationship with Aβ toxicity. An increase in rat brain malondialdehyde levels was observed following intra-ventricular and intra-hippocampal injections of Aβ (Bagheri et al. 2011; Briyal et al. 2011), while malondialdehyde, formaldehyde and methylglyoxal were reported to dose-dependently enhance the rate of Aβ oligomer and protofibril formation as well as increase the size of the aggregates (Chen et al. 2006). Chronic oral exposure of rats to acrolein (2.5 mg/kg/day for 8 weeks) caused mild cognitive declination, loss of neurons and astroglial activation in the hippocampus, as well as up-regulation of β-secretase (BACE-1) protein levels in the cortex and down-regulation of α-secretase (ADAM-10) protein levels in the hippocampus and cortex (Huang et al. 2013). Since the amyloid precursor protein can be processed by two alternate pathways, either cleavage by α-secretase to produce a non-amyloidogenic peptide fragment or cleavage by β-secretase and γ-secretase to produce the Aβ peptide, the aforementioned alterations in α-secretase and β-secretase protein expression suggest that reactive aldehydes may cause alterations in Aβ metabolism that promote formation of the Aβ peptide. Furthermore, adducts of acrolein and malondialdehyde were determined to be concentrated in the neurofibrillary tangles of Alzheimer's disease patients (Yan et al. 1994; Calingasan et al. 1999). Acrolein and methylglyoxal were shown to induce *tau* hyperphosphorylation and accelerate *tau* aggregation into fibrils in a variety of *in vitro* experiments (Gomez-Ramos et al. 2003; Kuhla et al. 2007; Li et al. 2012). Primary amine oxidase, an enzyme that is present in cerebral blood vessels and blood plasma, is responsible for oxidation of methylamine and aminoacetone to their

respective aldehydes, namely formaldehyde and methylglyoxal (Precious and Lyles 1988; Yu 1990; Boor et al. 1992; Lyles and Chalmers 1992; Lyles 1996). In Alzheimer's disease patients, there have been reports of increased primary amine oxidase activity in blood plasma, overexpression of primary amine oxidase in cerebral blood vessels and co-localization of this enzyme with cerebrovascular Aβ deposits (Ferrer et al. 2002; del Mar Hernandez et al. 2005; Unzeta et al. 2007; Jiang et al. 2008). Given that formaldehyde and methylglyoxal potentiated Aβ oligomer and protofibril formation (Chen et al. 2006) and that the deamination of methylamine by primary amine oxidase increased the deposition of Aβ onto blood vessel walls *in vitro* (Jiang et al. 2008), formaldehyde and methylglyoxal may play a role in the formation of cerebrovascular Aβ deposition seen in Alzheimer's disease. As reactive aldehydes appear to contribute to all four pathological hallmarks of Alzheimer's disease, the development of drugs able to sequester these aldehydes should be explored further. According to the current study, phenelzine and, to a lesser extent, PEH may be promising therapeutics in this regard. Furthermore, in addition to sequestering formaldehyde (MacKenzie 2009) and methylglyoxal *in vitro*, both phenelzine and PEH were shown to inhibit human primary amine oxidase *in vitro* (MacKenzie 2009) and increase rat whole brain levels of methylamine, an endogenous substrate for primary amine oxidase (Chapter 3**)**.

In relation to Parkinson's disease, there are numerous reports of increased reactive aldehyde content in plasma, CSF and numerous brain regions (including the substantia nigra) of patients (please refer to Section 5.1 for a more detailed summary). The pathological hallmark of Parkinson's disease is the presence of neuronal cytoplasmic Lewy bodies primarily composed of aggregated and fibrillized α-synuclein protein (Galvin 2006). In Parkinson's disease neuromelanin-containing substantia nigra neurons, there was an 8-fold increase of acrolein co-localization with cytoplasmic α-synuclein when compared to controls, as well as evidence of acrolein-modified α-synuclein (Shamoto-Nagai et al. 2007). The authors also demonstrated that acrolein can enhance α-synuclein oligomerization *in vitro*. In addition, the presence of malondialdehyde-modified α-synuclein was found in the substantia nigra and frontal cortex of Parkinson's disease patients and individuals with incidental or pre-clinical Parkinson's disease (Dalfo and Ferrer 2008). Thus, the sequestration of reactive aldehydes may prove to be a novel approach to attenuating certain features of neurodegeneration present in this disorder.

The ability to pharmacologically reduce reactive aldehyde load may also be relevant to other neurological disorders. Phenelzine was shown to attenuate HNE-induced mitochondrial dysfunction and improved cortical tissue sparing in a rat model of traumatic brain injury (Singh et al. 2013). Furthermore, increased malondialdehyde content was found to be present in the CSF of multiple sclerosis patients (Hunter et al. 1985; Calabrese et al. 1994; Ghabaee et al. 2010). In experimental autoimmune encephalomyelitis (EAE) mice, an animal model of multiple sclerosis, treatment with hydralazine (a drug that shares a hydrazine moiety with phenelzine and PEH) delayed onset of symptoms, reduced severity of paralysis and decreased spinal cord demyelination (Leung et al. 2011). The authors noted that acrolein adducts were significantly increased in the spinal cords of EAE mice and that hydralazine treatment reduced levels of these adducts, although this effect was not statistically significant. Of interest, phenelzine was shown to improve behavioural and functional outcomes in the EAE mouse model (Musgrave et al. 2011; Benson et al. 2013). As such, it would be important to determine if the aldehyde-scavenging property of phenelzine contributed to this improvement in clinical outcomes of the EAE animal model.

5.5 CONCLUSION

Phenelzine and its active metabolite PEH were shown to produce dose-dependent sequestration of acrolein, malondialdehyde and methylglyoxal *in vitro*. Both drugs were also protective against acrolein toxicity in a mouse cortical neuron culture. Following acute administration of phenelzine, but not PEH, rat whole brain extractable malondialdehyde levels were significantly reduced at 6 and 12 hours following injection. Neither phenelzine nor PEH had an effect on rat whole brain extractable acrolein levels. As such, further investigation should be conducted on whether phenelzine and PEH are able to sequester reactive aldehydes *in vivo.* The present results suggest that there may be potential for both drugs as useful adjunctive treatments in neurological diseases with increased reactive aldehyde loads, such as Alzheimer's and Parkinson's diseases.

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CHAPTER 6.

Formation of hydrazine from β-phenylethylidenehydrazine (PEH) by reaction with pentafluorobenzaldehyde

6.1 INTRODUCTION

The fluorinated reagent pentafluorobenzaldehyde (PFBA) has been used to derivatize hydrazines for analysis by gas chromatography (Liu et al. 1974; Jindal et al. 1980; Mozayani et al. 1987; Preece et al. 1992; Riggs et al. 2008) and was employed in our laboratory in an attempt to analyze brain levels of β-phenylethylidenehydrazine (PEH), a metabolite of the antidepressant drug phenelzine (β-phenylethylhydrazine). The structures of both compounds are shown in **Figure 6-1**. PEH is of interest as a drug in its own right because it has been reported to have neuroprotective effects in an animal model of stroke (Todd et al. 1999). It would thus be useful to study brain levels of PEH when administered on its own or following administration of phenelzine, which has also been reported to have neuroprotective properties (Wood et al. 2006; Musgrave et al. 2011; Benson et al. 2013; Singh et al. 2013).

However, during our investigation of phenelzine- and PEH-treated brain tissue using PFBA as a derivatizing agent, we found a major peak corresponding to derivatized hydrazine **(Figure 6-2)**. This finding suggested that our PEH sample was being broken down to hydrazine in the presence of PFBA. To investigate this matter further, we have analyzed chromatograms and mass spectra of PEH or hydrazine spiked into rat brain tissue methanolic homogenates or methanol, followed by PFBA derivatization. This was carried out using gas chromatography-mass spectrometry (GC-MS) in electron ionization (EI) and negative chemical ionization (NCI) modes. In addition, we employed an assay using liquid chromatography-mass spectrometry (LC-MS) following derivatization with acetone to determine whether the hydrazine signal present in the PEH samples is due to the residual hydrazine content of PEH stocks, since hydrazine is one of the reactants used in the synthesis of PEH, or due to conversion of PEH to hydrazine during the PFBA derivatization process.

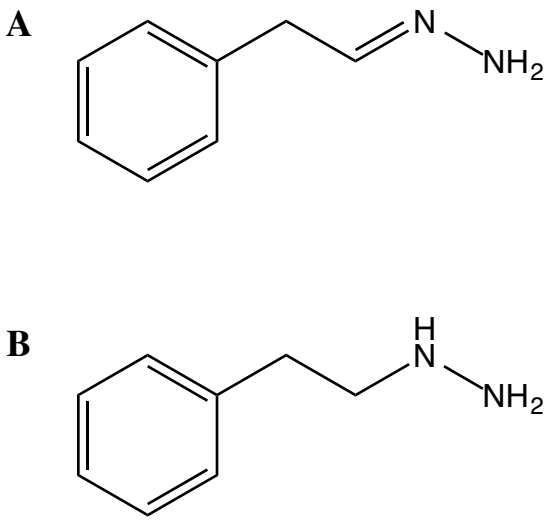


Figure 6-1. Structures of (**A**) PEH (β-phenylethylidenehydrazine) and (**B**) phenelzine (β-phenylethylhydrazine).

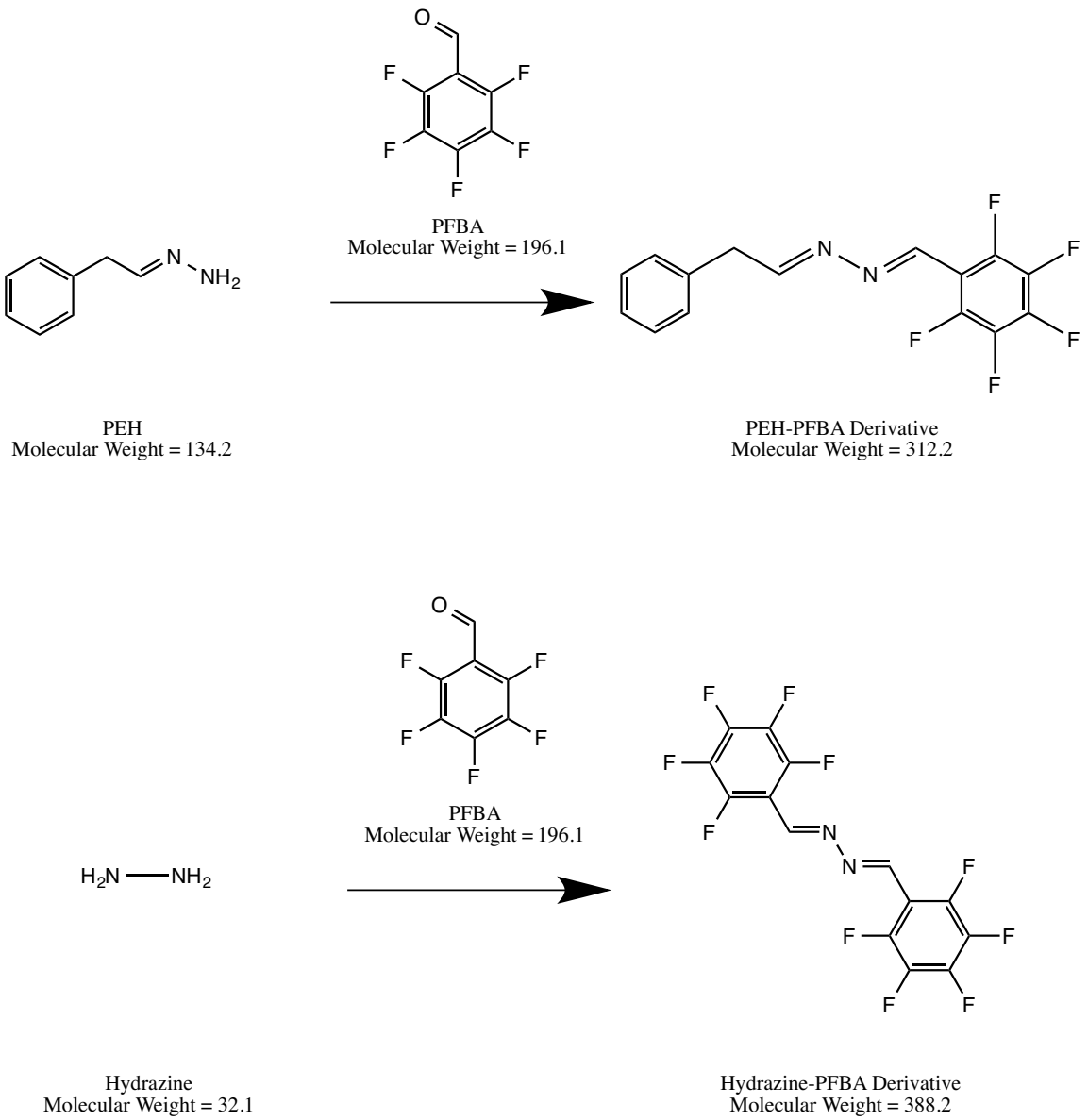


Figure 6-2. Proposed derivatization reaction schemes of PEH and hydrazine with pentafluorobenzaldehyde (PFBA).

6.2 METHODS

6.2.1 Materials

Methanol, acetone, acetonitrile, cyclohexane and 2-methylbutane were HPLC-grade and purchased from Fisher Scientific. Hydrazine monohydrate, ammonium formate and 2,3,4,5,6-pentafluorobenzaldehyde (PFBA) were obtained from Sigma-Aldrich. PEH was synthesized from phenylacetaldehyde and hydrazine monohydrate in the laboratory of Dr. Velázquez-Martínez in the Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta.

6.2.2 Animals

Adult male Sprague-Dawley rat brains were dissected out and flash frozen immediately in 2-methylbutane on solid carbon dioxide. The brains were kept frozen at -80°C until time of analysis. All animal procedures performed were approved by the University of Alberta Biosciences Animal Care and Use Committee and were in accordance with the guidelines of the Canadian Council on Animal Care.

6.2.3 Derivatization with pentafluorobenzaldehyde (PFBA)

Rat brain tissue was homogenized in 8 volumes of ice-cold methanol and an aliquot of 1 ml was transferred to a microfuge tube and spiked with 2 μg of PEH or hydrazine (prepared in methanol), followed by centrifugation at 13,000 xg for 10 minutes at 4°C. A 500 μl volume of the resultant supernatant was then transferred into a glass tube with subsequent addition of 10 μl PFBA and derivatization conducted in the dark at room temperature for 30 minutes. The samples were then taken to dryness under vacuum at ambient temperature in a SPD SpeedVac (Thermo Electron Corporation), reconstituted in 200 μl cyclohexane and centrifuged for 5 minutes at 13,000 xg. The supernatant was then transferred to GC vials with glass inserts. For samples prepared in methanol, 400 μl portions of methanol with known concentrations of PEH or hydrazine were derivatized with 10 μl of PFBA and the same procedure followed as outlined above. Analysis was carried out using an Agilent 6890 Series GC System equipped with an Agilent HP-5MS analytical column (30 m length, 0.250 mm internal diameter and 0.25 μm film thickness) and an Agilent 5973 Mass Selective Detector. The initial oven temperature was set to 80°C and increased at a rate of 15°C/minute until reaching a final temperature of 295°C. The

oven was held at 295°C for 15 minutes prior to returning to initial conditions. The injector temperature was maintained at 250°C in splitless mode and the GC to MS transfer line at 295°C. For analysis, 2 μl of sample was injected onto the column with helium as the carrier gas (1 ml/minute). The detector was set to either EI or NCI mode. For EI, the MS source and quad temperatures were set at 230°C and 150°C, respectively. NCI employed methane as the reagent gas, with MS source and quad temperatures of 150°C. The MS detector was set to scan for mass-to-charge ratios (m/z) between 25 and 500 to obtain a complete mass spectrum of blanks and samples spiked with PEH or hydrazine, followed by selective ion monitoring (SIM) at a m/z of 388 for quantitation of hydrazine content. Response peak area was used to quantitate the concentration of target compounds based on standard curves generated on the day of the experiment.

6.2.4 Derivatization with acetone

Derivatization with acetone has previously been reported for determination of hydrazines in a variety of media (Selim and Warner 1978; Holtzclaw et al. 1984; Davis and Li 2008; Sun et al. 2009; Fortin and Chen 2010). We have employed this derivatization technique with LC-MS for *in vitro* studies on metabolism of phenelzine by human monoamine oxidase B in Chapter 2. The proposed derivatization reaction schemes of acetone with hydrazine and PEH are demonstrated in **Figure 6-3.** In the protocol, 40 μl of sample (in methanol) were added to 40 μl of acetone and 80 μl of acetonitrile, the mixture vortexed, centrifuged at 10,000 xg for 1 minute and 100 μl transferred to HPLC vials with glass inserts. The analytes were then derivatized in the dark at room temperature for 5 hours. The HPLC instrument was a Waters Alliance 2690 XE with an Atlantis T3 5 μm (3 x 100 mm) analytical column. The mobile phase consisted of 15% 10 mM ammonium formate in water, 25% methanol and 60% acetonitrile with a flow rate of 0.3 ml/minute. The MS instrument was a Waters Micromass ZQ-4000 with the following voltages: capillary = 3.31 kV, cone = 21 V, extractor = 2 V and RF lens = 1 V. The source and desolvation temperatures were 150°C and 350°C, respectively. Nitrogen gas was set at a desolvation flow rate of 350 L/hour and cone flow rate of 90 L/hour. Using positive electrospray ionization mode, SIM was used to detect the derivatized compounds at the following m/z: 174.94 for PEH and 112.92 for hydrazine. The total run time was 7 minutes, with retention times of 2.80 minutes for hydrazine and 3.41 minutes for PEH. Response peak area was used to quantitate the

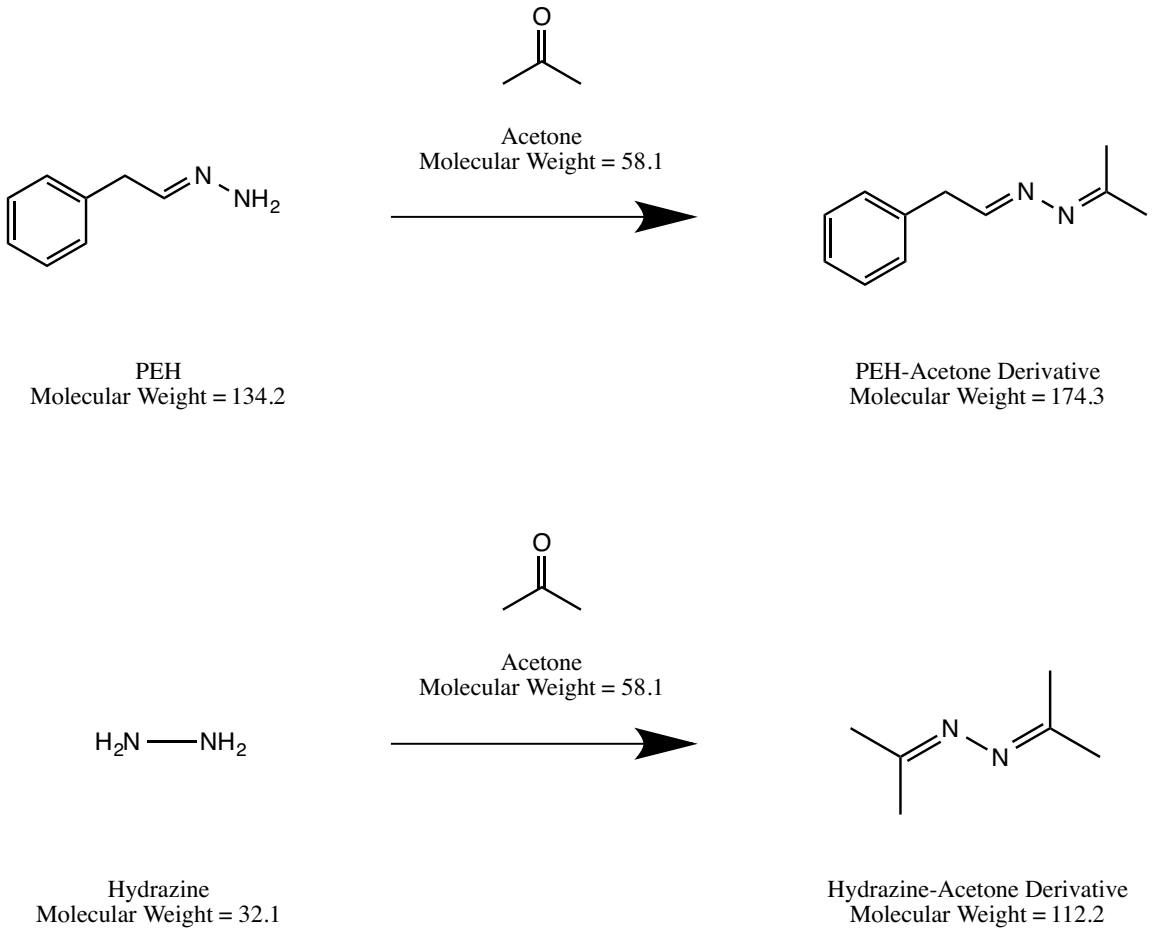


Figure 6-3. Proposed derivatization reaction schemes of PEH and hydrazine with acetone.

concentration of target compounds based on standard curves generated daily.

6.3 RESULTS

6.3.1 PFBA derivatization: PEH or hydrazine spiked into methanol and rat brain tissue homogenate (GC-MS in EI mode)

Methanol was spiked with 2 μg of PEH (14.9 nmol) or hydrazine (62.4 nmol), derivatized with PFBA and analyzed by GC-MS in EI mode. Representative chromatogram and mass spectra are shown in **Figure 6-4**. The peak with a retention time of 11.62 minutes and base peak ion of m/z 388 was present in both the PEH and hydrazine samples. This signal was much greater in the hydrazine sample, as would be expected due to a larger spiked amount of hydrazine (62.4 nmol) in comparison to PEH (14.9 nmol). Furthermore, the PEH sample had an additional peak with a retention time of 11.19 minutes and base peak ion with m/z of 298. To rule out the possibility that this variation could be due to differences in batches of synthesized PEH, we analyzed six older stocks of PEH and found similar chromatographic and mass spectrometric profiles.

When rat brain tissue homogenized in methanol was spiked with either 2 μg of PEH (14.9 nmol) or hydrazine (62.4 nmol), a prominent peak with a retention time of 11.62 minutes and a base peak ion with m/z of 388, corresponding to PFBA-derivatized hydrazine, was observed (**Figure 6-5**). In addition, the PEH-spiked brain sample contained a minor peak at a retention time of 11.19 minutes and base peak ion of m/z 298.

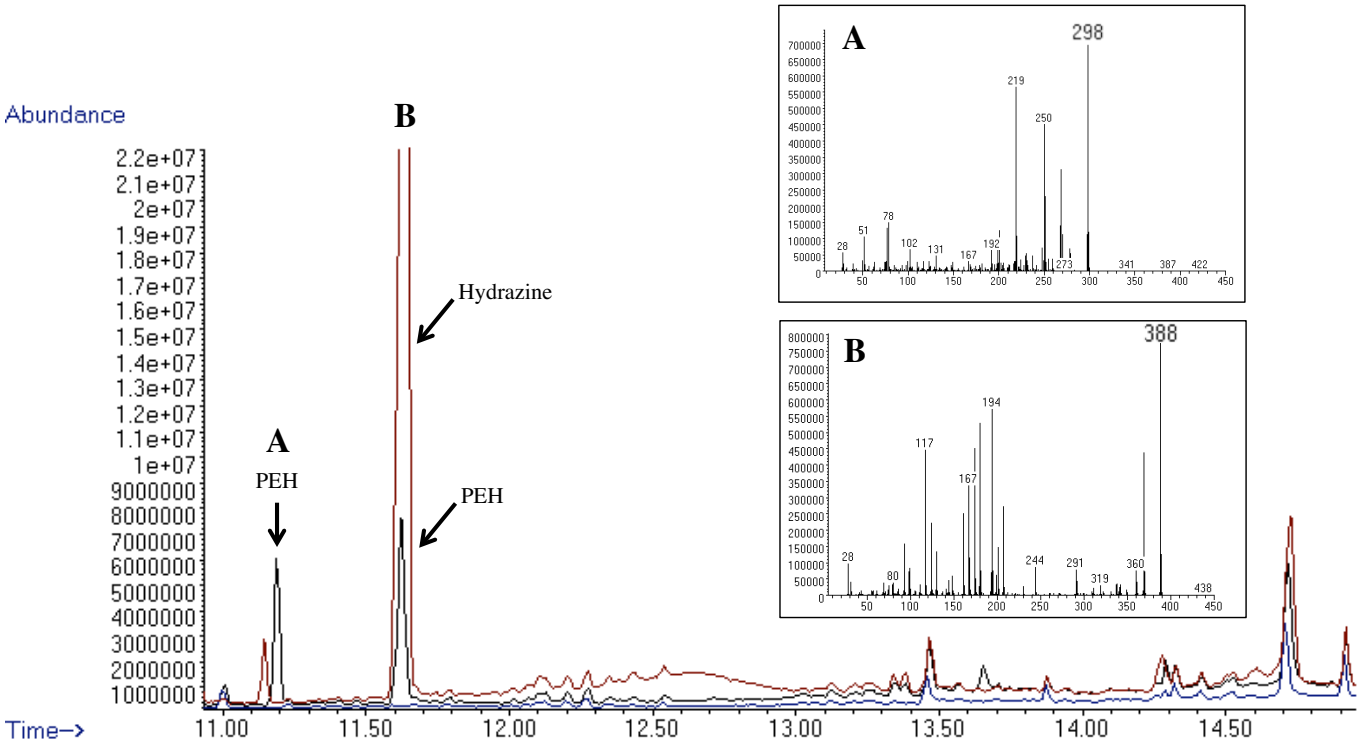


Figure 6-4. Methanol spiked with 2 μg of PEH (14.9 nmol) or hydrazine (62.4 nmol), derivatized with PFBA and analyzed by GC-MS in EI mode. The chromatogram represents an overlay of a methanol blank with PEH- and hydrazine-spiked methanol.

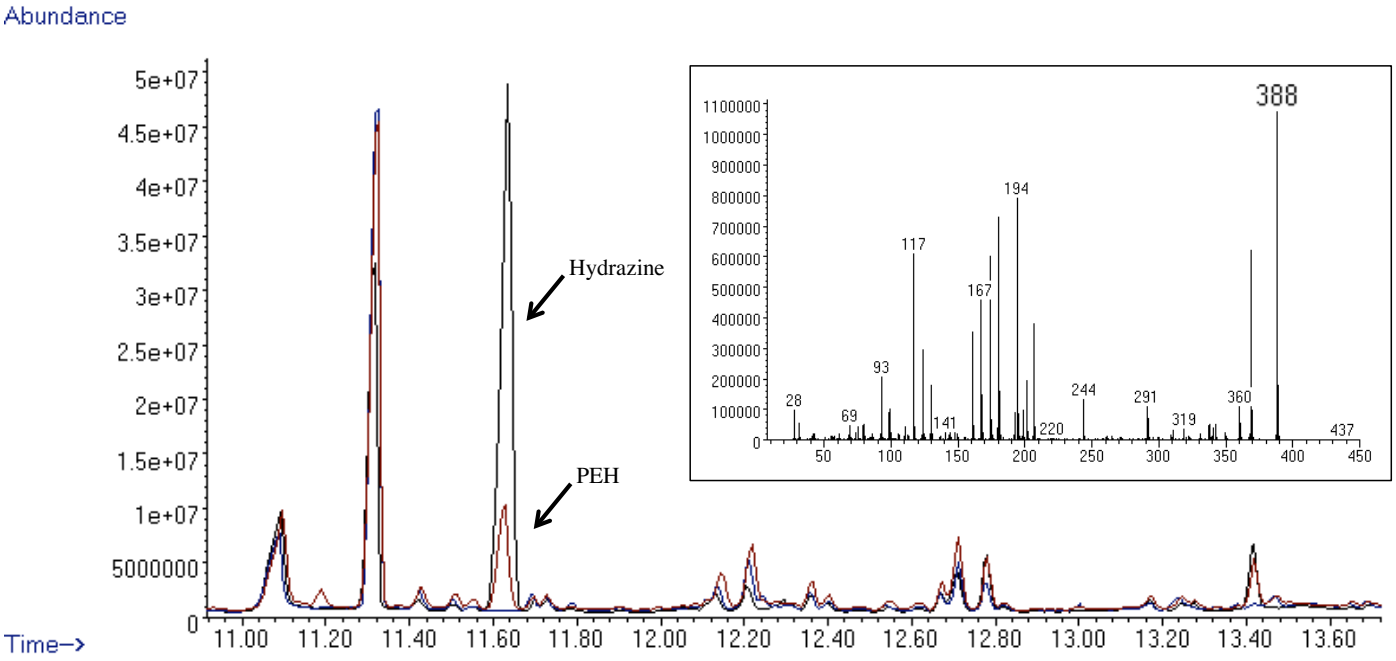


Figure 6-5. Rat whole brain homogenate spiked with 2 μg of PEH (14.9 nmol) or hydrazine (62.4 nmol), derivatized with PFBA and analyzed using GC-MS in EI mode. The chromatogram represents an overlay of naïve rat brain homogenate with PEH- and hydrazine-spiked rat brain homogenate.

6.3.2 PFBA derivatization: hydrazine standard curve in methanol (GC-MS in NCI mode)

To increase the sensitivity of the assay, we investigated PEH and hydrazine spiked into methanol using NCI mode. Furthermore, NCI mode was used for quantitation of hydrazine content in PEH samples. When a high standard of PEH (6 nmol) was analyzed, two peaks were observed when compared with a blank. The chromatogram and mass spectra are shown in **Figure 6-6**. The peak with a retention time of 10.18 minutes and base peak ion with m/z of 388 was prominent in both the PEH and hydrazine standards but completely absent in the blank. In addition, the PEH standard produced a minor peak at a retention time of 9.67 minutes with a base peak ion of m/z 298. Overall, the chromatography profiles of PEH and hydrazine samples in NCI mode are similar to those of EI mode. A standard curve of hydrazine was constructed by SIM at a m/z of 388. The standard curve was linear ($r^2 > 0.98$) and is represented in **Figure 6-7**.

6.3.3 Acetone derivatization: PEH and hydrazine standard curves in methanol (LC-MS)

This assay allows for measurement of hydrazine and PEH in samples, corresponding to SIM at m/z of 112.9 and 174.9, respectively. The limits of detection and quantitation for PEH and hydrazine were 10 and 40 pmol, respectively. Intra-assay variability was 2.8% for PEH and 1.1% for hydrazine. The standard curves for both compounds were linear ($r^2 > 0.99$) and are represented in **Figure 6-8**.

6.3.4 Comparison of hydrazine content in PEH samples between assays

Samples containing 4 nmol PEH (n = 6) were analyzed using both derivatization methods, with hydrazine content compared between the two assays. Percent hydrazine content was calculated by dividing the quantified amount of hydrazine (based on hydrazine standard curves for each respective assay) in each sample by the amount of PEH in the sample (4 nmol). The results are demonstrated in **Figure 6-9**, with PEH samples derivatized using acetone and PFBA containing 2.52 ± 0.05 % and 74.30 ± 2.86 % hydrazine content, respectively. Differences were evaluated statistically using an unpaired two-tailed t-test. The difference in hydrazine content between the derivatization procedures was statistically significant ($P < 0.001$).

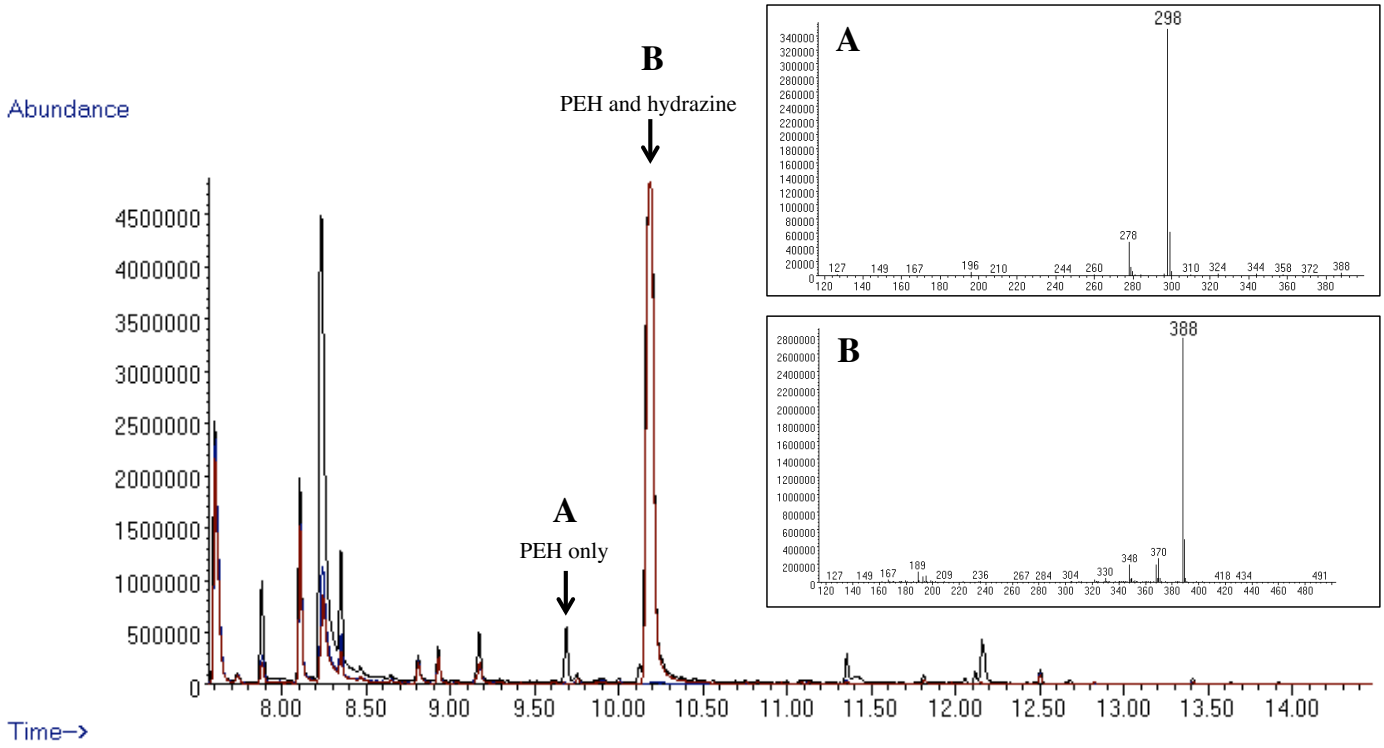


Figure 6-6. Methanol spiked with PEH (6 nmol) or hydrazine (4 nmol), derivatized with PFBA and analyzed by GC-MS in NCI mode. The chromatogram represents an overlay of a methanol blank with PEH- and hydrazine-spiked methanol.

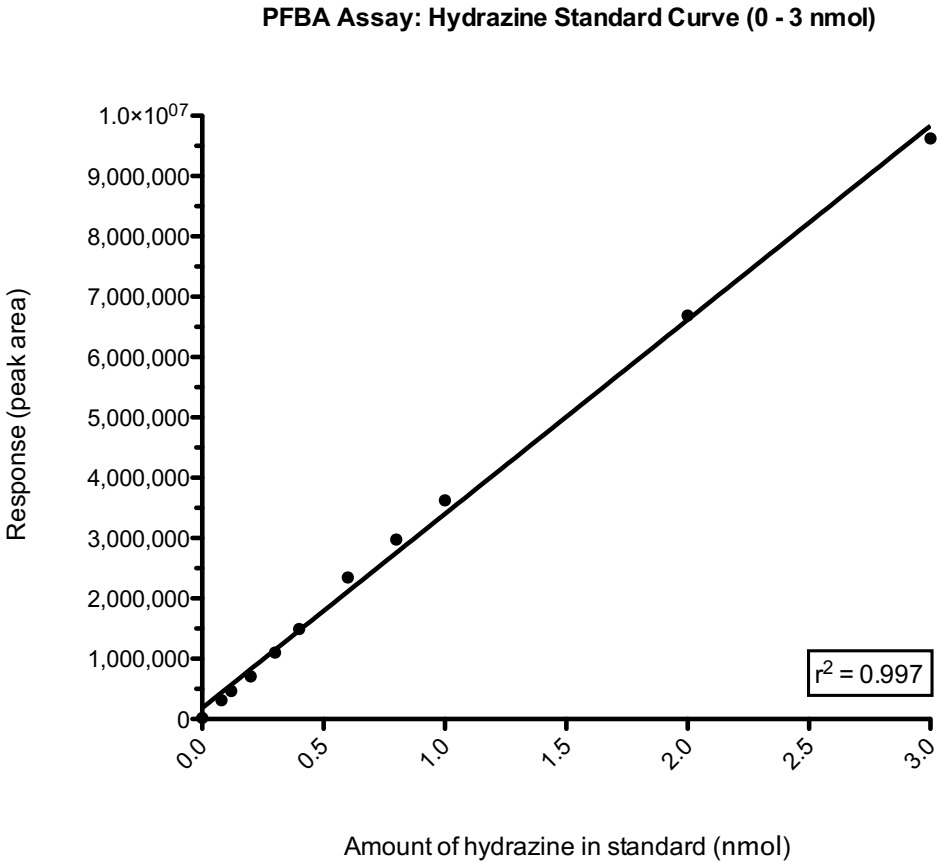
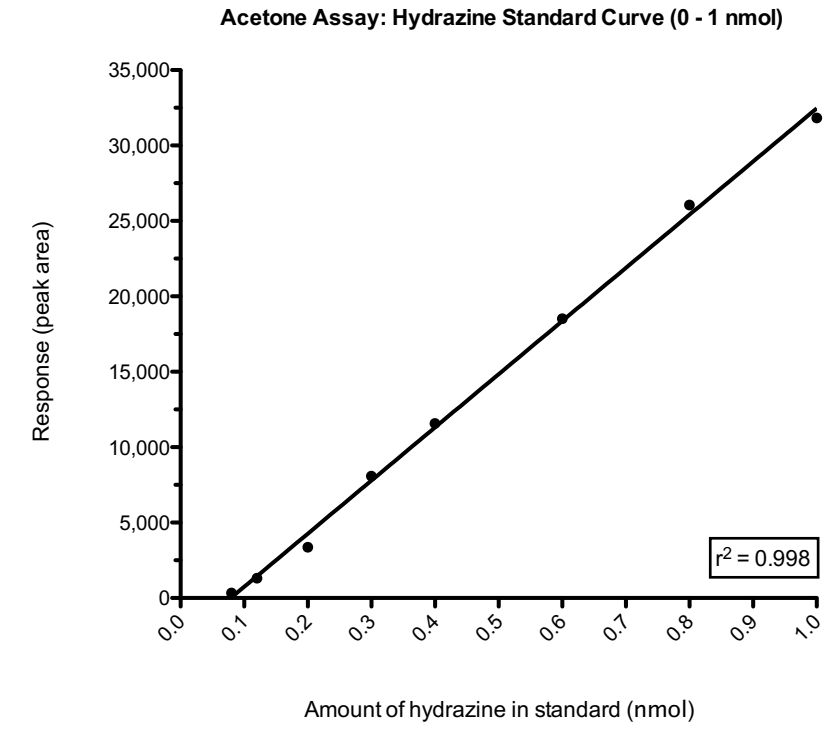


Figure 6-7. Standard curve of hydrazine following derivatization with PFBA. Hydrazine content was quantified using SIM at m/z of 388 by GC-MS in NCI mode.



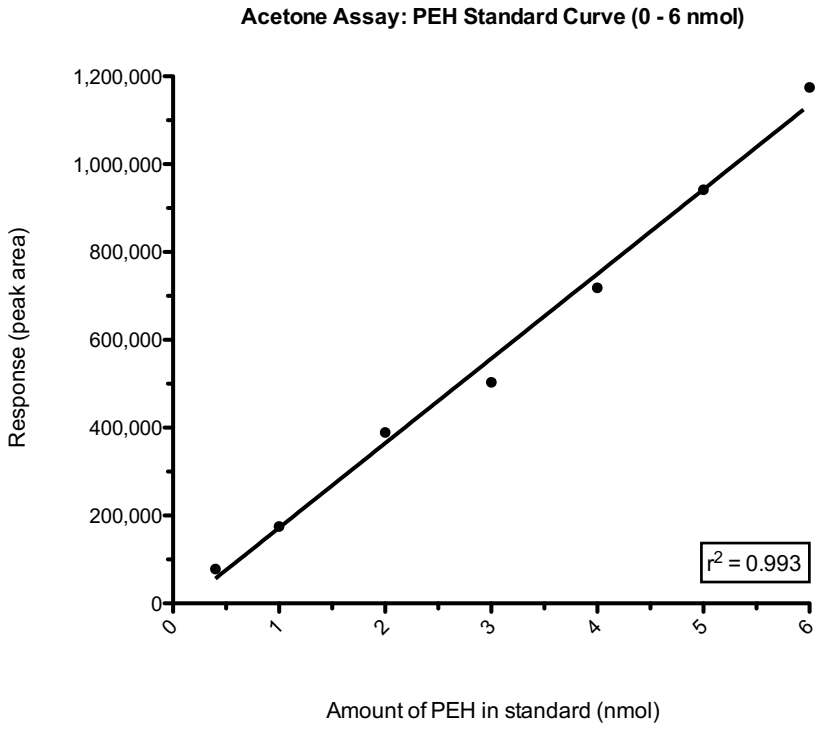


Figure 6-8. PEH and hydrazine standard curves following derivatization with acetone. Analysis was performed by SIM at m/z of 112.9 (hydrazine) and 174.9 (PEH) by LC-MS.

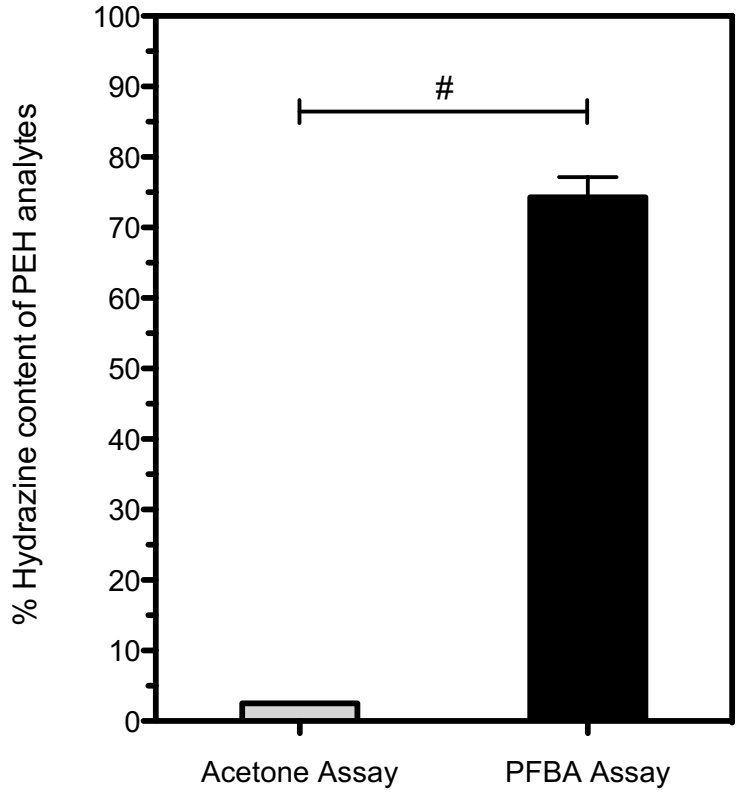


Figure 6-9. Comparison of hydrazine content in 4 nmol PEH samples (n = 6) between acetone and PFBA derivatization assays. GC-MS in NCI mode and LC-MS were employed for the PFBA and acetone assays, respectively. Data are normalized to amount of PEH present in samples, with values expressed as means ± SEM. Symbol shows that means are significantly different, as assessed by an unpaired two-tailed t-test, # $P < 0.001$.

6.4 DISCUSSION

The most prominent peak in PEH-spiked methanol, containing a base peak ion m/z of 388, is also present in hydrazine standards and likely corresponds to PFBA-derivatized hydrazine. Moreover, this is the largest peak in rat brain homogenate spiked with PEH.

According to the acetone assay, there appear to be trace amounts of hydrazine in PEH samples (approximately 2.5 %). Since the analyzed PEH samples were prepared in methanol and do not interact with aqueous media until contact with the mobile phase (15% water) in the analytical column, the hydrazine content of the samples is unlikely to be accounted for by PEH hydrolysis to hydrazine. As phenylacetaldehyde and hydrazine are the reagents used for synthesis of PEH, it is not surprising that small quantities of hydrazine may still be present in the compound stock solution. It is interesting that the percent of hydrazine content in PEH samples increases to approximately 74% when analyzed using the PFBA assay. As this significant amount of hydrazine formation cannot be attributed to hydrazine initially present in the PEH stock solution or hydrolysis of PEH (no interaction with aqueous media in the procedure), a significant portion of the PEH must be converted to hydrazine during the derivatization with PFBA.

The conversion of PEH to hydrazine during derivatization with PFBA makes interpretation of *ex vivo* results complicated. In Chapter 2, we have found that PEH hydrolyzes to hydrazine and phenylacetaldehyde in aqueous media over time. As such, it would be difficult to determine whether the hydrazine signal detected in samples is originating from PEH conversion to hydrazine during the derivatization process or from hydrolysis of PEH in the tissue.

6.5 CONCLUSION

In summary, the use of PFBA as a derivatizing reagent is not well suited for *ex vivo* determination of PEH levels in rat brain tissue due to conversion of PEH to hydrazine during the derivatization process. Derivatization with acetone works well for analysis of PEH levels in small volumes of incubates *in vitro*, but is not readily applicable to analysis in brain tissue due to interfering substances in the tissue. Thus, a search for a novel assay for PEH in brain tissue is continuing.

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CHAPTER 7.

General discussion and future directions

7.1 SUMMARY OF NOVEL FINDINGS

The work presented in this thesis has contributed to the elucidation of the mechanisms of phenelzine oxidation to β-phenylethylidenehydrazine (PEH) by human monoamine oxidase B (MAO-B). Using the acetone assay to directly measure concentrations of phenelzine and PEH, I was able to definitively show that PEH is a major metabolite of phenelzine *in vitro* (Chapter 2). There are older studies suggesting that PEH is formed from phenelzine (Tipton and Spires 1972; Patek and Hellerman 1974), but these experiments were not performed using human enzyme and employed methods that were either indirect or not quantitative and lacking sensitivity. The *in vivo* production of PEH in the rat brain following phenelzine administration was previously investigated in our laboratories (MacKenzie 2009); however, this assay was determined to be an indirect assay measuring PEH conversion to hydrazine as described in Chapter 6.

The work presented in Chapter 2 also addressed the stability of PEH as an imine product of phenelzine oxidation by MAO-B. As imines are generally regarded as unstable compounds that hydrolyze rapidly in water, it would be of importance to determine the rate of PEH hydrolysis to hydrazine and phenylacetaldehyde. With reports of phenylacetaldehyde and phenylacetic acid as the major metabolites of phenelzine administration *in vitro* (Yu and Tipton 1989) and *in vivo* (Clineschmidt and Horita 1969; Robinson et al. 1985), respectively, there was some doubt as to whether PEH is stable enough in solution to exert its neurochemical effects. The rate of hydrolysis was found to be pH-dependent, with only 20-25% of PEH hydrolyzed after 8 hours at a pH of 7.4. This provides support to the notion that PEH is slowly hydrolyzed at a neutral pH and is a relatively long-lived metabolite under the experimental conditions used.

Although it has been established that PEH is a relatively poor and transient inhibitor of rat brain MAO-A and MAO-B *ex vivo* (Paslawski et al. 2001; MacKenzie et al. 2008a), I have determined that this is also the case for human MAO-B. Furthermore, I have demonstrated that this inhibition is of a reversible and competitive nature. Although PEH has approximately a 20-fold lower affinity for the MAO-B active site in comparison to phenelzine, administration of sufficiently high doses of PEH may still cause MAO-B inhibition. These findings should be replicated for human MAO-A in the future, as this enzyme isoform is responsible for the increases in brain levels of catecholamines and serotonin, as well as the dietary interactions with tyramine. If PEH is also a competitive inhibitor of MAO-A, as is likely the case, administration

of this drug should not be associated with "the cheese effect" since high levels of tyramine should be able to outcompete PEH for access to the active site. The lack of potent MAO-A and MAO-B inhibition by PEH is further supported by the findings that PEH geometric isomers did not significantly increase rat whole brain levels of noradrenaline, dopamine and serotonin (Chapter 3).

The results presented in Chapter 2 also suggest that phenylethyldiazene, the metabolite of phenelzine considered to be responsible for irreversible MAO inhibition, leaves the active site prior to enzyme inactivation. This is in contrast to the established mechanism of phenelzine inactivation of MAO-B proposed by Binda et al. (2008), who have hypothesized that phenylethyldiazene stays in the active site and immediately alkylates the flavin adenine dinucleotide (FAD) cofactor of the enzyme. The release of phenylethyldiazene, a very reactive species prone to radical formation, into solution may possibly account for some toxic effects of phenelzine at high doses, such as the rare incidences of hepatotoxicity (Robinson and Kurtz 1987). The formation of phenylethyldiazene should not be associated with PEH administration, as PEH is either formed by a separate catalytic pathway or from phenylethyldiazene itself by intramolecular rearrangement.

The effects of phenelzine and PEH on rat brain levels of the amino acids alanine, γ-aminobutyric acid (GABA), glutamine, glycine, arginine and L-serine have been previously reported (Popov and Matthies 1969; Wong et al. 1990; Baker et al. 1991; McManus et al. 1992; Paslawski et al. 1995; Todd and Baker 1995; Tanay et al. 2001; Todd and Baker 2008; MacKenzie 2009; Kumpula 2013). In addition, previous investigations in our laboratory have demonstrated that PEH does not significantly alter rat brain levels of noradrenaline, dopamine and serotonin (MacKenzie 2009). To contribute to the existing literature, I examined the differences in neurochemical effects of the geometric isomers of PEH, namely (*E*)- and (*Z*)-PEH, with respect to their neurochemical effects on the aforementioned amino acids and biogenic amine neurotransmitters. This is relevant for potential therapeutic implications of PEH as the pharmacodynamic and pharmacokinetic properties of isomers can be markedly different, as was the case for stereoisomers of the selective serotonin reuptake inhibitor (SSRI) citalopram (Burke and Kratochvil 2002) and geometric isomers of the tricyclic antidepressant doxepin (Pinder et al. 1977). In Chapter 3, I have demonstrated that (*E*)- and (*Z*)-PEH are equivalent in their effects on

rat whole brain changes in levels of amino acids and lack of an increase in biogenic amine neurotransmitters. These results also reaffirm the previous observations that phenelzine and PEH produce substantial elevations in brain levels of alanine and GABA, while reducing levels of glutamine, glycine and L-serine. Whole brain levels of arginine were increased only by PEH isomers in the current study, whereas phenelzine, but not PEH, significantly elevated levels of this amino acid in previous experiments (MacKenzie 2009). The finding that both PEH isomers did not have significant effects on brain levels of biogenic amine neurotransmitters is in line with previous observations for racemic PEH (MacKenzie 2009).

Former investigations in our laboratory revealed that phenelzine and PEH can inhibit human primary amine oxidase (PrAO) with relatively equivalent potency *in vitro* (MacKenzie 2009). In addition, PEH and phenelzine were found to increase rat whole brain levels of methylamine, a physiological substrate of PrAO, to approximately 200% of controls at 3 hours following drug administration (MacKenzie 2009). In Chapter 3 of this thesis, I have confirmed this observation for phenelzine and both geometric isomers of PEH. Furthermore, a differential effect of phenelzine and PEH isomers on methylamine was noted by including later time points following drug administration. While phenelzine and PEH isomers produced comparable elevations in methylamine at 3 hours, methylamine levels continued to rise for both (*E*)- and (*Z*)-PEH at 6 and 12 hours whereas phenelzine-induced increases in methylamine were reduced at these time points. Since methylamine can be formed from MAO-catalyzed metabolism of adrenaline (Schayer et al. 1952; Yu et al. 1997), the attenuation of methylamine accumulation by phenelzine at the longer time points may be due to reduced production of methylamine from this metabolic pathway. Alternatively, the differential effects of phenelzine and PEH on rat brain methylamine levels may be due to pharmacokinetic differences between the drugs.

In the literature, there has been a report of increased brain L-tyrosine (hereafter referred to as tyrosine) levels following administration of a high dose (100 mg/kg intraperitoneal [i.p.]) of phenelzine (Dyck and Dewar 1986). In Chapter 4, I have demonstrated that a 30 mg/kg i.p. dose of phenelzine, (*E*)- and (*Z*)-PEH produces robust elevations in rat whole brain tyrosine at 3 to 6 hours following injection. This effect is abolished for phenelzine, but not for PEH, by pretreatment with another MAO inhibitor (MAOI), suggesting that PEH formation is responsible for phenelzine's effects on rat brain tyrosine levels. This finding is in agreement with PEH being

the active metabolite responsible for several neurochemical properties of phenelzine, including elevation of brain levels of GABA, alanine and ornithine (Popov and Matthies 1969; Todd and Baker 1995; Paslawski et al. 2001; MacKenzie et al. 2008a; MacKenzie et al. 2008b; Todd and Baker 2008; MacKenzie 2009). The ability to increase brain levels of tyrosine may have therapeutic relevance in treatment of depression, anxiety and neurodegenerative disorders characterized by loss of catecholaminergic neurons. Thus, elevation of brain tyrosine levels may represent a novel mechanism contributing to the neuroprotective effects of both phenelzine and PEH.

With regard to toxic reactive aldehydes, phenelzine was previously demonstrated to protect rat primary cortical neurons and astrocytes against formaldehyde-induced toxicity (Song et al. 2010), exhibit a protective effect against 3-aminopropanal and acrolein toxicity in rat retinal ganglion cells (Wood et al. 2006) and attenuate 4-hydroxy-2-nonenal (HNE)-induced mitochondrial dysfunction in a rat model of traumatic brain injury (Singh et al. 2013). Furthermore, both phenelzine and PEH were demonstrated to sequester formaldehyde *in vitro* (MacKenzie 2009). In Chapter 5, I investigated the ability of phenelzine and PEH to sequester other relevant aldehydes *in vitro*, including acrolein, malondialdehyde and methylglyoxal. Both drugs were most effective at dose-dependently reducing free acrolein content, but also significantly sequestered malondialdehyde and methylglyoxal. Interestingly, it appears that phenelzine and PEH not only inhibit PrAO, but also sequester the reactive aldehyde products formaldehyde and methylglyoxal of methylamine and aminoacetone oxidation, respectively, by the enzyme. In mouse cortical neurons, phenelzine and, to a lesser extent, PEH were able to attenuate acrolein-induced toxicity when co-treated with the aldehyde. Although phenelzine has been previously demonstrated to exert a protective effect against aldehyde toxicity in several neuronal cell lines, this is the first report of PEH attenuating aldehyde-induced toxicity in neuronal cell culture. Furthermore, I investigated the effect of phenelzine and PEH administration on rat whole brain levels of extractable free acrolein and malondialdehyde. Neither drug had a significant effect on rat whole brain levels of extractable acrolein and only phenelzine was able to reduce brain levels of extractable malondialdehyde at 6 and 12 hours following injection. These results were surprising considering the efficacy of both drugs at sequestering acrolein and malondialdehyde *in vitro*; however, there were certain limitations associated with this experiment as described in Section 5.4. It may be possible that an acute 30

mg/kg dose of phenelzine or PEH is too low to produce a significant effect on reactive aldehyde content in the rat brain *ex vivo*; a chronic study with these drugs may be warranted. Overall, these results suggest that further investigation is needed on aldehyde sequestration by phenelzine and PEH, as both drugs have the potential to be protective against aldehyde toxicity and may be useful as adjunctive treatments in neurological disorders associated with increased aldehyde load.

An assay previously employed for measurement of PEH levels in brain and liver tissue was revisited in Chapter 6. It appears that this assay is actually detecting hydrazine, which is formed from PEH during the derivatization process. Since PEH is slowly hydrolyzed to hydrazine *in vitro* (Chapter 2), the use of this assay for pharmacokinetic studies of PEH may be limited. As such, there is a need for development of a novel approach for quantitation of PEH in brain tissue.

7.2 POTENTIAL THERAPEUTIC APPLICATIONS OF PHENELZINE AND PEH

The findings on phenelzine and PEH presented in this thesis and in the existing literature suggest that both drugs may be useful in treatment of a wide variety of neurological disorders. The therapeutic mechanisms of these drugs will be discussed in relation to depression, anxiety, epilepsy, cerebral ischemia, Alzheimer's and Parkinson's diseases.

For over 50 years, phenelzine has been used in the clinic for the treatment of depression and anxiety disorders. Originally developed as a MAOI, it now appears that several other properties of phenelzine may contribute to its antidepressant and anxiolytic effects. Patients with depression have been reported to exhibit a reduction of GABA levels in the cerebrospinal fluid (CSF), plasma and occipital cortex (Gold et al. 1980; Gerner and Hare 1981; Petty and Sherman 1984; Petty et al. 1990; Petty et al. 1992; Sanacora et al. 1999; Kugaya et al. 2003; Sanacora et al. 2004). As SSRI and electroconvulsive therapy treatment was demonstrated to normalize occipital cortex GABA levels in depression (Sanacora et al. 2002; Sanacora et al. 2003), the ability of phenelzine to produce robust elevations in brain GABA levels may contribute to its antidepressant efficacy. Moreover, it appears that phenelzine's anxiolytic properties are linked to increases in GABA since phenelzine administration to rats reduced signs of anxiety in the elevated plus-maze only at doses that increased brain GABA levels by at least two-fold (Paslawski et al. 1996). In the same study, administration of N^2-acetylphenelzine, a MAOI with

no GABAergic effects, did not produce an anxiolytic effect. This is not surprising as the anxiolytic mechanisms of barbiturates and benzodiazepines, drugs clinically used for treatment of anxiety disorders, involve the potentiation of GABA neurotransmission at the $GABA_A$ receptor. As discussed in Chapter 3, both PEH isomers, but not phenelzine, increased brain levels of arginine, the immediate precursor to nitric oxide (NO). However, previous work done in our laboratories has indicated that phenelzine is also capable of elevating rat brain levels of arginine (MacKenzie 2009). Interestingly, patients with depression were reported to have decreased plasma levels of NO and treatment with the SSRI paroxetine was shown to reverse this effect (Chrapko et al. 2006). Increases in brain levels of tyrosine may also contribute to the antidepressant and/or anxiolytic properties of phenelzine. Tyrosine supplementation was reported to improve cognitive performance and alleviate adverse moods in individuals exposed to a variety of environmental stressors (Banderet and Lieberman 1989; Deijen and Orlebeke 1994; Shurtleff et al. 1994; Deijen et al. 1999; Mahoney et al. 2007; O'Brien et al. 2007). In depressed patients, tyrosine treatment produced encouraging results in pilot studies (Gelenberg et al. 1980; Gelenberg et al. 1982) but failed to demonstrate an antidepressant effect in a larger trial (Gelenberg et al. 1990). Despite this result, the authors suggested that there might be a subset of depressed patients who would benefit from tyrosine supplementation. Taken together, the antidepressant and anxiolytic properties of phenelzine are not simply the result of MAO inhibition, but may also involve alterations in brain levels of the amino acids GABA, arginine and tyrosine.

It is not known whether PEH would demonstrate antidepressant effects since it is a poor inhibitor of MAO-A in the rat brain (Paslawski et al. 2001; MacKenzie et al. 2008a) and does not increase rat brain levels of noradrenaline, dopamine and serotonin (Chapter 3, MacKenzie 2009). However, PEH may be a promising candidate for the treatment of anxiety disorders as it produces robust elevations in both GABA and tyrosine. As such, it would be of interest to further investigate the ability of PEH to exhibit anxiolytic effects in an animal model of anxiety.

The potent GABAergic effect of phenelzine and PEH may also be of use in the treatment of epilepsy. Several established anti-epileptic drugs are believed to exert their function by augmenting $GABA_A$ receptor activation (benzodiazepines and phenobarbital), inhibiting GABA reuptake (tiagabine) or impairing GABA metabolism by inhibition of GABA transaminase

(vigabatrin) (Howard et al. 2011). Of interest, a 10 mg/kg dose of phenelzine was shown to increase rat brain GABA levels to a greater extent than a 1000 mg/kg dose of the anticonvulsant vigabatrin (Todd and Baker 2008). In addition, PEH was demonstrated to increase tissue GABA levels and dose- and time-dependently reduce epileptiform activity in rat hippocampal slices (Duffy et al. 2004). These findings support the rationale for investigation of both phenelzine and PEH as anticonvulsants.

Phenelzine and PEH have both been shown to reduce neuronal loss in a gerbil model of transient global ischemia (Todd et al. 1999). Following onset of ischemia, brain glutamate levels increase dramatically and the subsequent excitotoxicity is believed to play a central role in neuronal death (Choi and Rothman 1990; Kanthan et al. 1995; Lo et al. 2003). Following an ischemic insult, there also appears to be a long-term reduction in brain GABA levels that coincides with onset of neuronal damage (Shuaib et al. 1994; Mainprize et al. 1995; Shuaib et al. 1997). As the inhibitory GABAergic neurotransmission counterbalances excitatory glutamatergic neurotransmission, the combination of increased glutamate and reduced GABA following an ischemic event may intensify the neuronal damage due to excitotoxicity. Based on these observations, development of pharmacological approaches for treatment of cerebral ischemia included compounds that antagonized glutamatergic transmission (Muir and Lees 1995) or potentiated GABAergic transmission (Green et al. 2000). Increased brain GABA levels may counteract excitotoxicity by hyperpolarizing the presynaptic membrane (thus preventing glutamate release) and the postsynaptic membrane (thus preventing cell excitation by glutamate) (Schwartz-Bloom and Sah 2001). Of interest, the GABA transaminase inhibitor vigabatrin was shown to be protective against ischemic damage in a variety of gerbil animal models (Abel and McCandless 1992; Shuaib et al. 1992).

Although the ability of phenelzine and PEH to increase brain levels of GABA has likely contributed to their protective properties in an animal model of cerebral ischemia, inhibition of PrAO and sequestration of reactive aldehydes may also play a role. In the acute phase following onset of cerebral ischemia, levels of PrAO were found to be reduced in the cerebral vasculature but increased in patient plasma (Airas et al. 2008). The authors hypothesized that cerebral vessels high in PrAO content may be more susceptible to immediate vascular damage, resulting in the observed reduction of PrAO-positive vessels following the ischemic insult. Moreover, the PrAO

inhibitors aminoguanidine and LJP-1207 were found to have protective effects in rat models of cerebral ischemia (Cockroft et al. 1996; Xu et al. 2006). In addition to formaldehyde and methylglyoxal produced by PrAO, the reactive aldehydes 3-aminopropanal and acrolein can be generated as by-products of the oxidation of the polyamines spermine and spermidine to putrescine by polyamine oxidase and spermine oxidase. In rat models of cerebral ischemia, brain levels of polyamine oxidase, putrescine and 3-aminopropanal were significantly increased following ischemic insult (Ivanova et al. 1998; Liu et al. 2005). Furthermore, there are reports of elevated levels of spermine oxidase and acrolein in the plasma of stroke patients (Tomitori et al. 2005) and HNE in the striatum of rats following cerebral ischemia (Matsuda et al. 2009). Given that phenelzine was previously shown to be protective against 3-aminopropanal and acrolein toxicity in rat retinal ganglion cells (Wood et al. 2006) and both phenelzine and PEH were demonstrated to attenuate acrolein-induced toxicity in mouse cortical neurons in this thesis, both drugs may be able to exert a protective function in cerebral ischemia due to their aldehyde-sequestering properties.

There is potential for phenelzine and PEH to be useful adjunctive drugs in the treatment of Alzheimer's disease. Since there is considerable evidence that Alzheimer's disease pathogenesis involves an excitotoxic component (Ong et al. 2013; Revett et al. 2013), elevation of brain GABA levels may be a useful pharmacological approach to counteract pathological increases in glutamate. In the literature, there is also support for the role of reactive aldehydes in the disorder. As reviewed in Chapter 5, levels of the reactive aldehydes are increased in several relevant brain regions of individuals with established and preclinical Alzheimer's disease (Marcus et al. 1998; Lovell et al. 2001; Williams et al. 2006; Bradley et al. 2010; Nam et al. 2010). These aldehydes may exacerbate Alzheimer's disease pathogenesis by potentiating amyloid-β (Aβ) oligomerization and protofibril formation (Chen et al. 2006), producing alterations in amyloid precursor protein processing (Huang et al. 2013) and inducing *tau* hyperphosphorylation and aggregation into fibrils (Gomez-Ramos et al. 2003; Kuhla et al. 2007; Li et al. 2012). Furthermore, Alzheimer's disease patients were reported to have increased PrAO activity and/or overexpression in the plasma and cerebral vasculature, as well as co-localization of PrAO with cerebrovascular Aβ deposits (Ferrer et al. 2002; del Mar Hernandez et al. 2005; Unzeta et al. 2007; Jiang et al. 2008). Increased production of the reactive aldehydes formaldehyde and methylglyoxal by PrAO may contribute to the deposition of Aβ in the brain

vasculature, as was demonstrated *in vitro* for PrAO-mediated oxidation of methylamine to formaldehyde (Jiang et al. 2008). Thus, phenelzine and PEH may exert protective effects in this disorder by increasing brain GABA levels, directly sequestering a variety of reactive aldehydes and inhibiting PrAO. In addition, phenelzine may also provide a therapeutic effect by inhibiting MAO-B; the activity of this enzyme was demonstrated to be increased in Alzheimer's disease brains (Adolfsson et al. 1980; Oreland and Gottfries 1986; Reinikainen et al. 1988; Jossan et al. 1991; Sparks et al. 1991; Sherif et al. 1992; Saura et al. 1994) and may contribute to the production of hydrogen peroxide, ammonia and toxic aldehyde products.

There is also rationale for use of phenelzine and PEH in treatment of Parkinson's disease. Due to the substantial loss of nigrostriatal dopaminergic neurons in the disorder (Bernheimer et al. 1973), increasing brain tyrosine levels may represent a means of increasing dopamine production. Supplementation with tyrosine was shown to be effective at increasing striatal dopamine release in 6-hydroxydopamine rat models of the disorder (Melamed et al. 1980; During et al. 1989) and in potentiating dopamine turnover in Parkinson's disease patients (Growdon et al. 1982). In addition, the ability of phenelzine to increase brain dopamine levels due to irreversible inhibition of MAO-A and MAO-B may be of use in the disorder. There have also been numerous reports of increased reactive aldehyde levels in the plasma, CSF and substantia nigra of patients with established Parkinson's disease (Dexter et al. 1989; Ilic et al. 1999; Shamoto-Nagai et al. 2007; Chen et al. 2009; Serra et al. 2009; Baillet et al. 2010), as well as in several brain regions of patients with early Parkinson's disease (Dalfo et al. 2005). Moreover, reactive aldehydes were found to interact with α-synuclein and potentiate its oligomerization (Shamoto-Nagai et al. 2007; Dalfo and Ferrer 2008). Taken together, phenelzine and PEH may be useful adjunctive drugs in Parkinson's disease due to their ability to sequester reactive aldehydes and increase brain dopamine levels due to elevation of tyrosine and, for phenelzine, inhibition of MAO-A and MAO-B.

As reviewed here, phenelzine and PEH may be useful in treating a variety of neurological disorders. The current pharmacological options available for cerebral ischemia and neurodegenerative disorders often exhibit poor clinical efficacy and severe adverse effects, increasing the urgency for development of novel neuroprotective drugs. Since phenelzine has been used in the clinic for over 50 years, its therapeutic window and adverse effect profile has

been clearly established. While associated with possibility of weight gain, peripheral edema, orthostatic hypotension, sexual dysfunction and very rare cases of hepatotoxicity (Robinson and Kurtz 1987; Kennedy et al. 2009), the main reason why phenelzine is seldom used in the clinic is due to the fear of dietary interactions with tyramine. However, some have argued that the risks of "the cheese effect" are overstated as only 25 cases of hypertension and 1 death were documented with MAOIs after implementation of tyramine-restricted diets in 1967 (Gillman 2011). This author also notes that tyramine content of foods and beverages is now lower than in the past and that the risks of using phenelzine are very low with proper patient education. As PEH is a weak inhibitor of rat MAO-A and MAO-B (Paslawski et al. 2001; MacKenzie et al. 2008a) and human MAO-B, it is likely also a weak inhibitor of human MAO-A (although this should now be confirmed). Thus, PEH may be used without the fear of dietary tyramine interactions and could represent a safer alternative to phenelzine. Moreover, PEH should not be associated with production and release of phenylethyldiazene into the cytosol, an event that may contribute to some of phenelzine's toxicity at high doses. In the animals used for this thesis, we have not observed any overt signs of toxicity due to PEH or phenelzine administration. However, PEH, but not phenelzine, produced a significant reduction in cell viability of mouse cortical neurons at the highest dose used. As such, there is a need to conduct comprehensive toxicity studies with PEH.

7.3 FUTURE DIRECTIONS

Due to a lack of human MAO-A availability, the *in vitro* experiments in Chapter 2 dealt solely with human MAO-B. As such, there is a need to replicate these findings with human MAO-A. Although the two enzyme isoforms share 70% sequence homology, they differ in terms of substrate and inhibitor specificities, as well as some structural properties (Youdim et al. 2006). For example, phenelzine was reported to bind to and inactivate MAO-A with 6-fold greater efficacy than MAO-B (Binda et al. 2008). As such, it would be of importance to determine the mechanism of PEH inhibition of MAO-A (reversible or irreversible) and the affinity of PEH for the active site of MAO-A in comparison to phenelzine. Furthermore, the results suggesting that phenylethyldiazene leaves the active site prior to enzyme inactivation should be replicated with MAO-A. Since irreversible inhibition of MAO-A by phenelzine is responsible for the antidepressant properties of the drug and the possibility of dietary interactions with tyramine-

containing food and drinks, confirmation of PEH being a reversible inhibitor of human MAO-A is required for a thorough interpretation of its potential clinical effects.

The effects on rat whole brain tyrosine levels were determined following an acute administration of phenelzine or PEH in Chapter 4. As chronic administration of phenelzine, albeit at low doses, was reported to have no effect on tyrosine levels in the striatum, whole brain or plasma (Dyck et al. 1988; Paetsch and Greenshaw 1991), it may be important to investigate the effects of chronic phenelzine and PEH treatment at the 30 mg/kg dose on rat whole brain levels of tyrosine. It is possible that long-term administration of phenelzine may inhibit MAO to such an extent where only a negligible amount of PEH is formed, resulting in a drop of tyrosine towards control levels. In addition, it would be of interest to confirm that PEH elevates brain tyrosine levels due to inhibition of brain tyrosine aminotransferase, as suggested in this thesis.

Although phenelzine and PEH were effective at sequestering the reactive aldehydes acrolein, malondialdehyde and methylglyoxal *in vitro* and attenuating acrolein-induced toxicity in mouse cortical neurons, the ability of both drugs to reduce rat whole brain levels of extractable aldehydes *ex vivo* was not as promising. Neither drug reduced brain levels of extractable acrolein and only phenelzine was able to lower brain levels of extractable malondialdehyde. It may be argued that the single dose of either drug used in the experiment (30 mg/kg) was too low to exert an appreciable effect on rat brain aldehyde levels and that the effect of chronic treatment with phenelzine and PEH should be investigated. As this study was also conducted on healthy adult rats, it may also be of interest to determine the effects of both drugs on brain aldehyde levels in animal models mimicking oxidative stress conditions seen in neurodegenerative disorders. Furthermore, levels of protein-bound aldehydes and extractable aldehyde levels in different brain regions before and after administration of phenelzine and PEH should also be examined.

7.4 CONCLUDING REMARKS

The work performed in this thesis has contributed to the understanding of the mechanisms of phenelzine metabolism by human MAO-B and the neurochemical properties of phenelzine and its active metabolite PEH. Even though phenelzine has been studied for over 50 years, the mechanism of its metabolism by MAO is still relatively unclear. I have demonstrated that PEH is a major metabolite of phenelzine *in vitro* and clarified the interactions of both drugs

with human MAO-B. The results obtained in this thesis also suggest that phenylethyldiazene, the inhibitory metabolite of phenelzine responsible for MAO inhibition, may leave the active site prior to enzyme inactivation, a finding that is in contrast with the currently established mechanism of MAO inhibition by phenelzine. Moreover, I have demonstrated that geometric isomers of PEH are relatively equivalent in their effects on rat whole brain levels of amino acids, biogenic amine neurotransmitters and methylamine. Some of these neurochemical changes are different from those induced by phenelzine, such as the inability of PEH to increase rat brain levels of noradrenaline, dopamine and serotonin, as well as the more robust longer-term effects of PEH on methylamine concentrations. The finding that PEH is responsible for phenelzine-induced increases in rat brain tyrosine concentrations further reaffirms the notion that PEH is the active metabolite responsible for many of phenelzine's actions on amino acids. The increase in tyrosine levels may have potential therapeutic applications in depression, anxiety and Parkinson's disease. Finally, I have investigated the ability of both drugs to sequester toxic reactive aldehydes *in vitro*, as well as in a neuronal cell culture and in the rat brain *ex vivo*. The reduction of aldehyde toxicity by phenelzine and PEH may have implications for treatment of a variety of neurodegenerative disorders. Overall, both phenelzine and PEH possess numerous neurochemical properties that may contribute to neuroprotection. Further investigation of these drugs as adjunctive treatments in a number of neurological disorders is warranted.

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