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Studies on Phenelzine and its Acetyl Derivatives

by

Ashraf Mozayani 

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

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STUDIES ON PHENELZINE AND ITS ACETYL DERIVATIVES

SUBMITTED BY ASHRAF MOZAYANI

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

IN

PHARMACEUTICAL SCIENCES (TOXICOLOGY).

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To my family,

Masoud, Farrokh and Nikou,

who have given me unending love and support

throughout my studies.

ABSTRACT

Phenelzine [(2-phenylethyl)hydrazine, Pz], a monoamine oxidase inhibitor (MAOI) has been employed clinically as an antidepressant drug for over twenty years. However, knowledge of the *in vivo* metabolism of this substance is limited.

Indirect evidence suggests that Pz may undergo acetylation *in vivo* and that differences between fast and slow acetylator phenotype patients may influence the course of the clinical response. However, N-acetylated metabolites of Pz have neither been detected in biological samples nor fully characterized chemically. In fact, there is no direct evidence to support the occurrence of Pz acetylation *in vivo*.

Experiments were conducted to determine the *in vivo* formation and pharmacological properties of the two possible monoacetylated metabolites of Pz (N^1-acetylphenelzine, N^1-AcPz; N^2-acetylphenelzine, N^2-AcPz). Procedures were developed for the separate synthesis of each amide which were then characterised by gas chromatography, mass spectrometry, infrared and nuclear magnetic resonance spectroscopy. Analytical procedures for Pz, N^1-AcPz and N^2-AcPz were developed and applied to extracts of rat tissue. In addition the effects of Pz, N^1-AcPz and N^2-AcPz on monoamine oxidase enzymes (MAO) and biogenic amines were assessed and compared.

The data from clinical studies indicate that N^1-AcPz was the sole product of Pz acetylation under aqueous conditions whereas N^2-acetylation occurred under anhydrous conditions. Spectroscopic evidence suggests that N-acetylated Pz exists as a mixture of rotational isomers.

In the rat, only N^2-AcPz occurred as a metabolite of Pz and this amide possessed MAO inhibiting properties independent of its hydrolysis to Pz. N^1-AcPz did not inhibit MAO and was not detected as a metabolite of Pz.

Blood levels of N^2-AcPz, after administration of a low dose which did inhibit MAO enzymes but did not increase brain levels of biogenic amines, were several-fold higher than those measured after administration of a 3.8-fold higher dose of Pz. Taken in conjunction with the reduced potency of N^2-AcPz as a MAO inhibitor, these data suggest that the metabolic acetylation of Pz is probably not pharmacologically significant in the rat.

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I wish to acknowledge the help and guidance given by my supervisors, Dr. R. T. Coutts and Dr. T. J. Danielson throughout this program. I also would like to convey my most sincere thanks to members of my committee, Dr. G. B. Baker and Dr. F. M. Pasutto, for their assistance during this work.

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GLOSSARY OF ABBREVIATIONS

AcPH	Acetylphenylhydrazine
anal.	Analysis
AM	Azomethane
AOM	Azoxymethane
br	Broad
BzH	Benzylhydrazine
calcd.	Calculated
$CDCl_3$	Deuterochloroform
COMT	Catechol-O-methyltransferase
DA	Dopamine
DAM	Diazomethane
1,1-DMH	1,1-Dimethylhydrazine
1,2-DMH	1,2-Dimethylhydrazine
D_2O	Deuterium oxide
0^o	Degrees Celcius
ECD	Electron capture detection
eV	Electron volt
GC	Gas chromatography
GC/MS	Gas chromatography/mass spectrometry
h	Hour
HPLC	High performance liquid chromatography
HRMS	High resolution mass spectrometry

Hz	Cycles per second (Hertz)
5-HT	5-Hydroxytryptamine
IS	Internal standard
ip	Intraperitoneal
IR	Infrared spectrometry
iv	Intravenous
J	Coupling constant
KBr	Potassium Bromide
kg	Kilogram(s)
m	Medium intensity
µL	Microliter(s)
MAM	Methylazoxymethane
MAO	Monoamine oxidase
MAOI(s)	Monoamine oxidase inhibitor(s)
Me_2SO-d_6	Hexadeuterodimethyl sulfoxide
mg	Milligram(s)
mg/L	Milligram per liter
MH	Methylhydrazine
MHPG	3-Methoxy-4-hydroxy-phenylglycol
min	Minute(s)
mL	Milliliter
mL/min	Milliliter per minute
mm	Millimeter
mmol	Millimole

MS	Mass spectrometry
n	Number of observations
N^1-AcPz	N^1-Acetylphenelzine
N^1-AcN^2-ECPz	N^1-Acetyl-N^2-ethoxycarbonylphenelzine
N^2-AcPz	N^2-Acetylphenelzine
N^1N^2-AcPz	N^1N^2-Diacetylphenelzine
NAT	N-Acetyltransferase
NE	Norepinephrine
N^1-ECPz	N^1-Ethoxycarbonylphenelzine
N^1-ECN^2-AcPz	N^1-Ethoxycarbonyl-N^2-acetylphenelzine
N^2-ECPz	N^2-Ethoxycarbonylphenelzine
N^1N^2-ECPz	N^1N^2-Diethoxycarbonylphenelzine
ng	Nanogram
ng/mL	Nanogram/milliliter
NMR	Nuclear magnetic resonance
PA	Phenylacetaldehyde
PAA	Phenylacetic acid
pg	Picogram
PE	2-Phenylethylamine
PFBA	Pentafluorobenzaldehyde
PFBC	Pentafluorobenzoyl chloride
PFPA	Pentafluoropropionic anhydride
PLP	Pyridoxal-5-phosphate
PPz	Pheniprazine

Pz	Phenelzine
q	Quartet
r^2	Correlation Coefficient
rt	Retention time
s	Singlet (NMR) or strong intensity (IR)
SEM	Standard error of the mean
sh	Shoulder
t	Triplet
TCA	Tricyclic antidepressant

1. INTRODUCTION

1.1. The Drug

1.1.1. History

Depression has been part of the human condition since antiquity. However, pharmacological treatment for depression became available only during the late 1950s. Prior to that time, the main treatments were psychotherapy for mild depression and electroconvulsive therapy for more severe depression. With the clinical introduction of monoamine oxidase inhibitors (MAOIs) and later the dibenzazepine tricyclic antidepressants, some optimism towards pharmacological management of depressive syndromes wasn generated. This optimism is supported by many controlled clinical studies which indicate that both groups of agents are effective antidepressants.[1-5]

MAOIs were originally viewed as less effective and more toxic than tricyclic antidepressants. The reason for this belief may be that, in early studies, subtherapeutic doses were often employed[6-9] and early reports of hypertensive crises during MAOI therapy did not anticipate the relative ease of dietary control. Use of MAOIs in the United States has become widespread over the past decade,[9-14] as the clinical responses to these agents have become better understood.

Phenelzine (Pz) was introduced in 1959 for treatment of atypical, neurotic or reactive depression.[5,15-17] It has subsequently been

shown to be possibly of superior clinical value to the tricyclic antidepressants (TCAs) in accurately diagnosed patients.[18] Some evidence suggests that Pz may have superior action to TCAs in bulimic patients.[19,20] There are also reports of beneficial response to Pz in patients suffering from migraine headaches,[21] aphthous ulcers of the mouth[22] or cocaine abuse,[23] although relationships to MAO inhibition have not been established.

Pz is also a particularly useful alternative in those patients who are either refractory to TCAs, intolerant of their side effects[24] or viewed as potentially suicidal.[25] This last point should not be underestimated since records maintained by the Chief Medical Examiner's Office indicate that during the past five years in the Province of Alberta approximately 150 individuals have died as a direct result of tricyclic overdose or tricyclic levels were sufficient to be judged as a contributing factor. Because of the rapid onset of cardiac symptoms after tricyclic overdosing and the high degree of tissue binding, intervention in the course of tricyclic toxicity is usually ineffective and intentional overdosing is almost always fatal.[26] Although some mortalities after Pz have been reported with very high doses, there is frequently a relatively long lag period prior to expression of toxicity (approximately 12 hours) during which hemodialysis can be instituted.[27]

1.1.2. Physicochemical Properties

The chemical structures of Pz [(2-phenylethyl)hydrazine], and its two possible monoacetyl conjugates are depicted in Fig. 1-1.

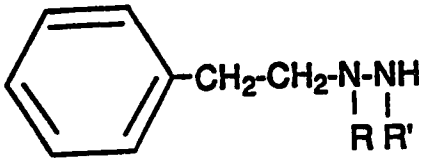
Pz base is an oil but the drug is employed as the acid sulfate,

$C_8H_{12}N_2.H_2SO_4$ with a molecular weight of 234.3.[28] This is a white or yellowish-white powder or pearly plates, and is soluble in water. It is however practically insoluble in ethanol, chloroform or ether. Pz is marketed in Canada under the trade name of Nardil.

1.1.3. Mechanism of Action

Pz is thought to produce its beneficial effects through inhibition of monoamine oxidase (MAO), an enzyme which plays an important role in the intraneuronal metabolism of norepinephrine (NE), dopamine (DA), serotonin (5-hydroxytryptamine, 5-HT) and the trace amines.[29] This enzyme is widely distributed and has been found in most mammals.[30] In humans, MAO is present in most tissues, with greatest activity in liver, blood and brain. Within the brain the greatest activity of MAO is found in the hypothalamus, nucleus accumbens and locus coeruleus.[31] Individuals with low brain and platelet MAO activity are thought constitutionally prone to suicide, alcoholism and unipolar or bipolar depression.[32]

MAO consists of two main isozymes referred to as MAO-A and MAO-B, which are distributed differently throughout the body.[33] For example, the intestinal tract of man and other animals contains almost exclusively MAO-A, whereas blood platelets contain almost exclusively MAO-B. In the brain one or the other type predominates according to species and brain region. MAO-A and MAO-B are also distinguished by their relative preferences for biogenic amine substrates[33] (Table 1-1). Thus, for example, MAO-A exibits a



Pz	R = H	R' = H
1 N-AcPz	R = C-CH3 (O)	R' = H
2 N-AcPz	R = H	R' = C-CH3 (O)

Fig. 1-1. Chemical structure of phenelzine and its monoacetylated derivatives.

strong substrate preference for NE and 5-HT, while MAO-B exhibits preference towards benzylamine and 2-phenylethylamine (PE).[34] Some substrates such as DA are degraded with equal efficiency by both isozymes.[35] Although strict substrate preferences may be lost at either high substrate or enzyme concentrations,[35] this classification is generally accepted and the MAOIs are hence also classified according to their relative abilities to distinguish between and selectively inhibit one or the other MAO-subtypes. For example, (-)-deprenyl is a selective type B inhibitor which blocks the metabolism of benzylamine and PE at concentrations far below those required to block deamination of 5-HT.[36] In contrast clorgyline is a type-A inhibitor, which blocks the degradation of 5-HT at very low concentrations, while affecting the metabolism of benzylamine only at very high concentrations.[37] In comparison Pz and tranylcypromine do not exhibit isozyme preference and are considered to be non-selective MAOIs.[10]

The most notable action of MAOIs including Pz, is their ability to interact with and inhibit MAO enzymes in vivo. This inhibition results in decreased degradation of NE, 5-HT and DA and a significant accumulation of these substrates in brain.[38-41] In addition, effects on biogenic amine re-uptake or release mechanisms may also contribute to net effects on biogenic amine metabolism.[42] It has also been demonstrated that a decreased density of β-adrenergic, α-adrenergic and 5-HT binding sites occurs during chronic MAOI treatment.[42] Although the significance of this decreased binding is not fully understood, it has also been reported with other forms

Table 1-1. Substrates and Inhibitors of Monoamine Oxidase*.

	MAO-A	Mixed	MAO-B
Substrates	epinephrine	m,p-tyramine	2-phenylethylamine
	norepinephrine	dopamine	phenylethanolamine
	metanephrine	octopamine	o-tyramine
	serotonin (5-HT)	synephrine	benzylamine
		tryptamine	
		N-methyltryptamine	
		N,N-dimethyltryptamine	
Irreversible inhibitors	clorgyline	phenelzine	(-)deprenyl
	Lilly 51641	tranylcypromine	Lilly 54781
		isocarboxazid	MDL 72145
		pargyline	AGN 1133
		(more B)	AFN 1135
Reversible inhibitors	harmine		
	amiflamine		
	cimoxatone		
	brofarmine (CGP 11305A)		
	Ro 11-1163		
	MD 780515		
	FLA 336(+)		

* adapted from ref. 37.

of antidepressant therapy including TCAs and electroconvulsive therapy,[43] and the latency in its development appears to correspond to the delayed development of an antidepressant response.

Pz, by nature of its chemical similarity to PE, may also act in an entirely unique manner. Biochemical data indicate that Pz is degraded *in vivo* to PE and the stimulant properties of this metabolite may also provide some antidepressant effect.[41,44-45]

The dominant effect of MAOIs, however, is their ability to inhibit MAO-A and MAO-B, and several authors have employed inhibition of platelet MAO as an index of brain MAO inhibition.[6,15,46] Thus, Robinson *et al.*[6,15] studied the relationship between platelet MAO inhibition and clinical improvement during Pz therapy. These investigators observed a direct relationship between the degree of platelet inhibition and clinical outcome. Thus, whereas only 44% of patients with less than 80% inhibition showed any improvement, 68% of those with greater than 80% inhibition improved. Of those with 90% inhibition, 79% improved. In addition, they found, with 60 mg Pz per day, maximum MAO inhibition did not occur until after two weeks; a point which may explain the frequent inability to demonstrate clinical benefit from Pz in earlier studies of less than three weeks duration and when lowerr doses of Pz were employed.[6-8] In further support of a relationship between MAO inhibition after Pz administration and clinical response, Gupta *et al.*[47] have reported decreased blood levels of 3-methoxy-4-hydroxy-phenylglycol (MHPG), a MAO-dependent metabolite of NE (Fig. 1-2), corresponding to decreased platelet MAO activity.

1.1.4. Side Effects and Toxicity

Early unfavorable publicity on two drugs contributed to the devaluation of the MAO inhibitors as clinical agents. Iproniazid, currently not available on the U.S. market, inhibits oxidative metabolism via the cytochrome P-450 system in addition to effects on MAO enzymes.[48] This drug was also shown to covalently bind to hepatic macromolecules and to induce hepatic necrosis in approximately 1% of treated patients.[46] Similarly, the occurrence of hepatic necrosis after treatment with Catron [pheniprazine, (1-methyl-2-phenylethyl)hydrazine] contributed to the disfavour of MAOIs.[48] Observations of hypertensive crisis in MAOI-treated patients who subsequently ingested sympathomimetic amines also contributed to their withdrawal from common use.[49-51] However, Blackwell et al.[50] have since shown that patients ingesting food rich in sympathomimetic amines rarely experience adverse hypertensive crisis, suggesting that this risk may have been exaggerated.

More specific to Pz, autonomic effects including orthostatic hypotension,[52] sexual dysfunction[48-50] and edema[10,11,15] are not uncommon. These effects are not always dose related and may disappear during the course of continued therapy.

Weight gain during Pz therapy[53] can be related to a craving for carbohydrates and appears to be indicative of therapeutic response.[54] However, 45 cases of weight gain (in excess of 6.8 kg in 32 of the cases),[53] resulting in discontinuation of therapy, have been reported since 1963. It is important to ensure that the duration of therapy is sufficient to allow distinction between weight

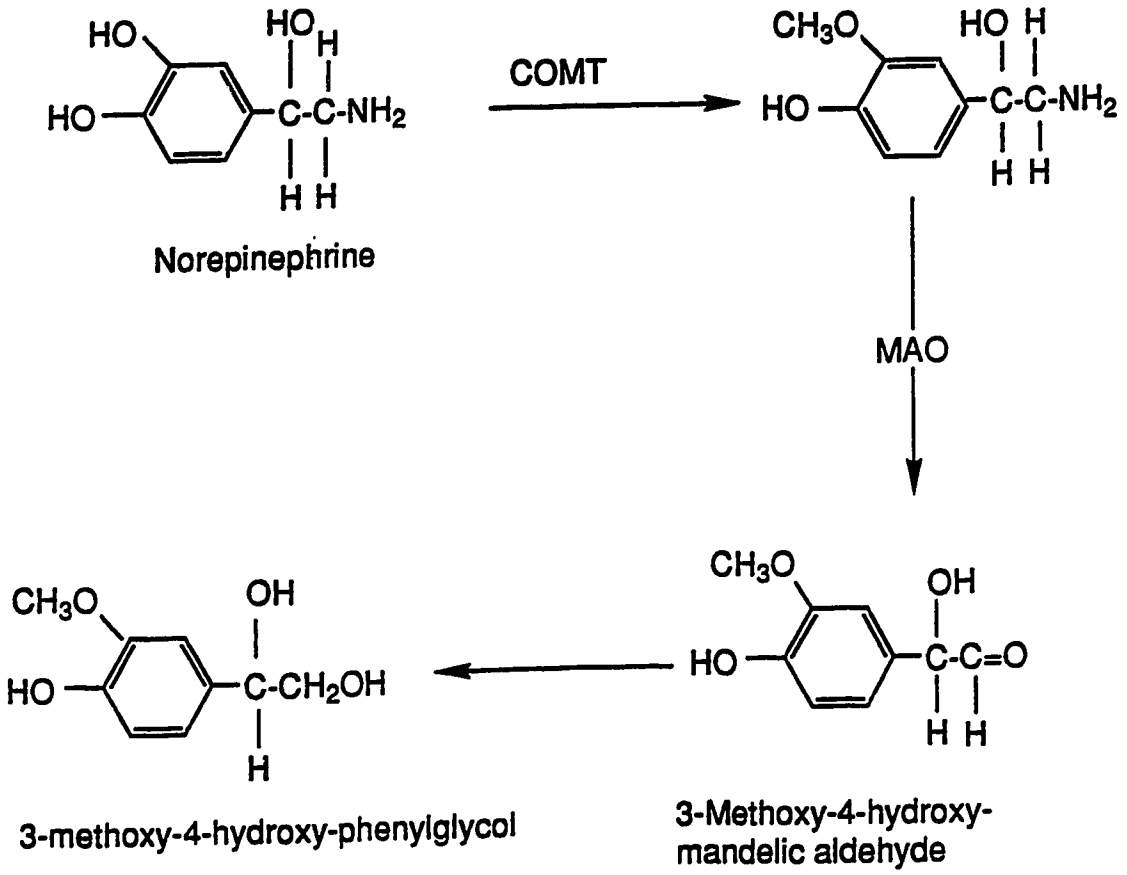


Fig. 1-2. Conversion of norepinephrine (NE) to its metabolite 3-methoxy-4-hydroxy-phenylglycol (MHPG).

gain due to changes in the affective state rather than effects directly on appetite.

Edema after Pz often subsides without special intervention within a week of starting treatment. However, electrolytes should be monitored during persistent edema because of the possibility of effects by Pz on secretion of antidiuretic hormone.[55] Pz has also been associated with hypersensitivity, pheochromocytoma, congestive heart failure, urinary retention and blurred vision and there is some small risk of hepatocellular-type liver damage.[56,57]

A National Institute of Mental Health collaborative study using 110 patients on maintenance Pz found no adverse hypertensive reactions (with proper dietary discretion), 1 case of elevated liver function test and a small number of anticholinergic side effects.[35] This study again suggests that the prevalence of MAOI side effects may initially have been overestimated. Other studies have yielded similar data and also suggest that anticholinergic side effects appear gradually during the first therapeutic week but then decline.[49] Similarly, in a comparative study of Pz, nortriptyline (a TCA) and placebo, Georgot et al.[58] reported that approximately equal antidepressant responses were observed in each drug-treatment group. Anticholinergic symptoms were greater in the TCA group whereas orthostatic effects were similar in each group.

In addition to inhibition of MAO, several MAOIs have been demonstrated to inhibit hepatic enzymes which catalyse N-dealkylation, O-demethylation and hydroxylation of both endogenous and exogenous substrates.[59] This suggests that the pharmacokinetics

and clinical action of other agents may change during MAOI therapy even though they are not directly metabolised by MAO enzymes. The classic drug interaction with MAOIs is that experienced during simultaneous treatment with TCAs. Although some evidence suggests that such combined therapy may be beneficial to refractory patients,[60] extreme care must be exercised to avoid potentially fatal cardiovascular responses.[61] Metabolic interactions also have been demonstrated between MAOIs and other drugs. For example, conjoint MAOI treatment may modify phenothiazine metabolism and potentiate hypertensive and extrapyramidal reactions.[49] Similar potentiation of the actions of some sympathomimetics,[62-63] barbiturates,[64] ethanol and narcotics[56] have also been reported.

Finally, Pz, like other hydrazine-derived drugs, can interfere with vitamin B_6 metabolism through reaction with the aldehyde function of pyridoxal-5-phosphate (PLP),[65] (Fig. 1-3), resulting in depletion of this coenzyme and inactivation of the drug. This effect by Pz has been observed in rats as an increased urinary excretion of xanthurenic acid after a tryptophan load[65] (Fig. 1-4) and occasionally in humans as a peripheral neuropathy during long-term Pz therapy.[66] Some authors[67] have even suggested that because of the reactive nature of PLP with hydrazine the possibility of subclinical vitamin B_6 deficiency should be considered even in the absence of overt clinical signs.

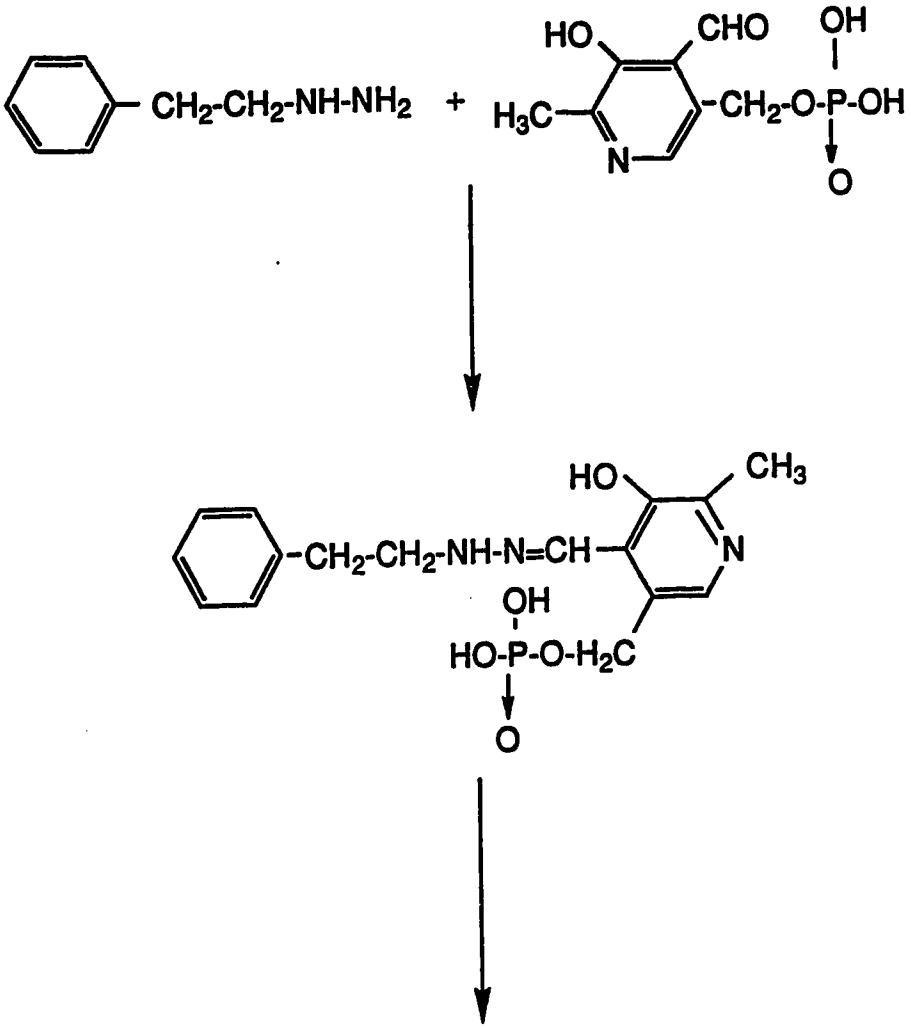


Fig. 1-3. Mechanism of phenelzine and vitamin B_6 (pyridoxal) interaction.

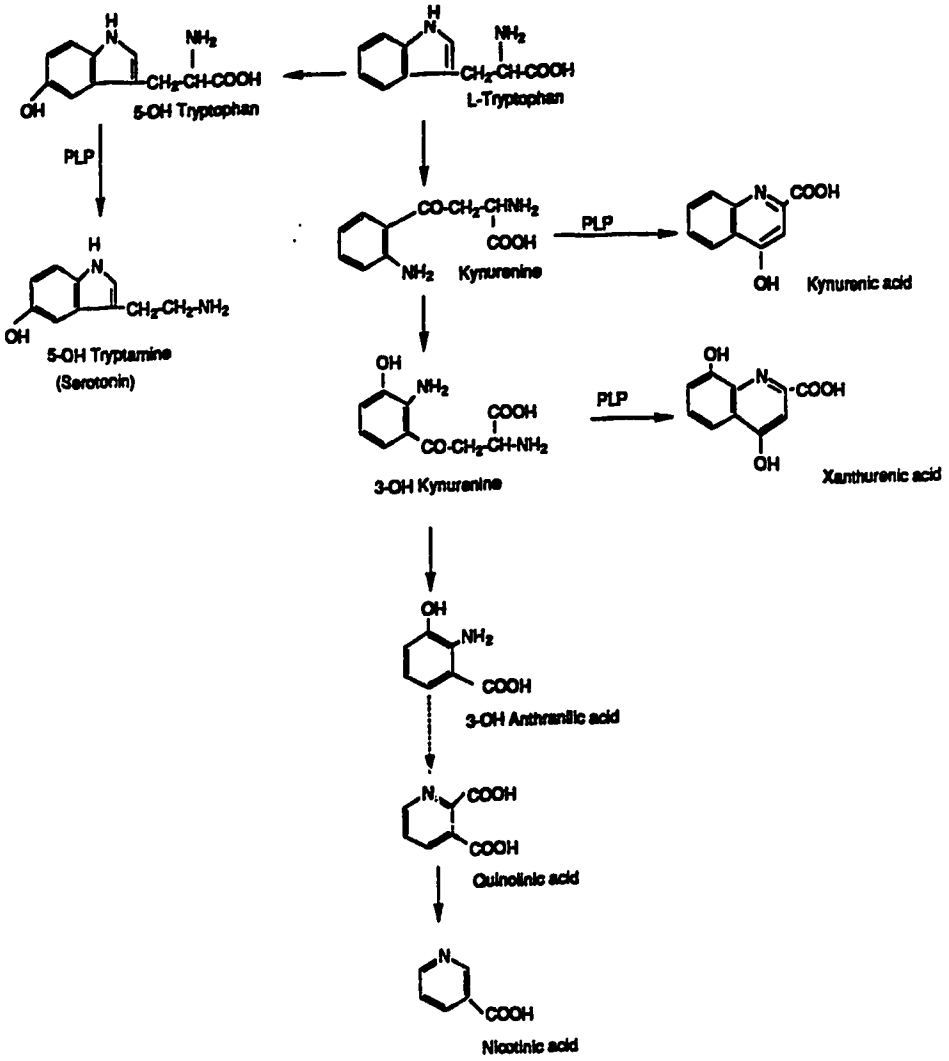


Fig. 1.4. Principal pathways of tryptophan metabolism which require pyridoxal-5'-phosphate(PLP) as coenzyme.

1.1.5 Metabolism and Pharmacokinetics of Phenelzine:

Data have been reported which indicate that simple hydrazine compounds are metabolised primarily by oxidation or conjugation. These pathways can also be applied to Pz metabolism and may support observations made during *in vivo* metabolic studies. These observations with simple hydrazines and their applications to Pz metabolism will therefore be discussed in an approximate chronological order.

1.1.5.1. Oxidative Metabolism of Hydrazines

In 1964, Miller[68] proposed that the carcinogenic properties of the glycoside cycasin (methylazoxymethanol-O-glucoside) (Fig. 1-5), were due to the spontaneous decomposition of its aglycone to diazomethane (DAM) and formaldehyde. He further proposed that azoxymethane (AOM) also could be metabolically transformed into DAM through similar pathways. These suggestions were confirmed by a series of reports from Reed *et al.*[69,70] These authors demonstrated that many hydrazines were metabolised by microsomal enzymes and that whereas mono-alkylhydrazines (e.g. 1-methylhydrazine, MH) were metabolically converted to hydrocarbons (e.g. methane) by the rat (Fig. 1-6), more highly substituted hydrazines [e.g. 1,1-dimethylhydrazine (1,1-DMH) or 1,2-dimethylhydrazine (1,2-DMH))] were converted to carbon dioxide via formaldehyde (Fig. 1-6). To explain these observations they proposed that an initial step in the metabolism of monoalkylhydrazines and 1,2-dialkylhydrazines was equivalent to dehydrogenation and yielded a mono- or a 1,2-di-

substituted diazine. These authors suggested that the marked instability of monosubstituted diazines led to their spontaneous free radical degradation to alkanes. In contrast the more stable disubstituted diazines did not decompose spontaneously and survived to undergo further metabolism leading to carbon dioxide. Reed et al. also suggested that because 1,1-disubstituted hydrazines were not structurally compatible with conversion to diazines, they were metabolised through separate oxidative pathways also leading to aldehydes (Fig. 1-6).

Similar studies by Fiala et al.[71,72] added to these observations and reconfirmed that 1,2-DMH was metabolically converted to azomethane (AM), AOM and methylazoxymethanol (MAM). Electron spin resonance studies have since demonstrated the generation of free radical intermediates during the metabolism of a wide variety of hydrazine substances.[73-76]

Similar conversions have been proposed and observed with Pz (Fig. 1-7). Thus, Clineschmidt and Horita[77,78] demonstrated that phenylacetic acid (PAA) or phenylacetaldehyde (PA) was a major metabolite of Pz and that MAO enzymes participated in this metabolism. However, Prough et al.[79] demonstrated that although monoalkylhydrazines could be metabolised by mitochondrial enzymes to generate aldehyde, metabolism by microsomal enzymes generated both aldehyde plus hydrocarbon. Thus ethylbenzene, a free radical-derived metabolite, has been identified as a major in vivo and in vitro metabolite of Pz in the rat[80-81] (Fig. 1-8) and C-centred radicals have been detected through electron spin resonance spin

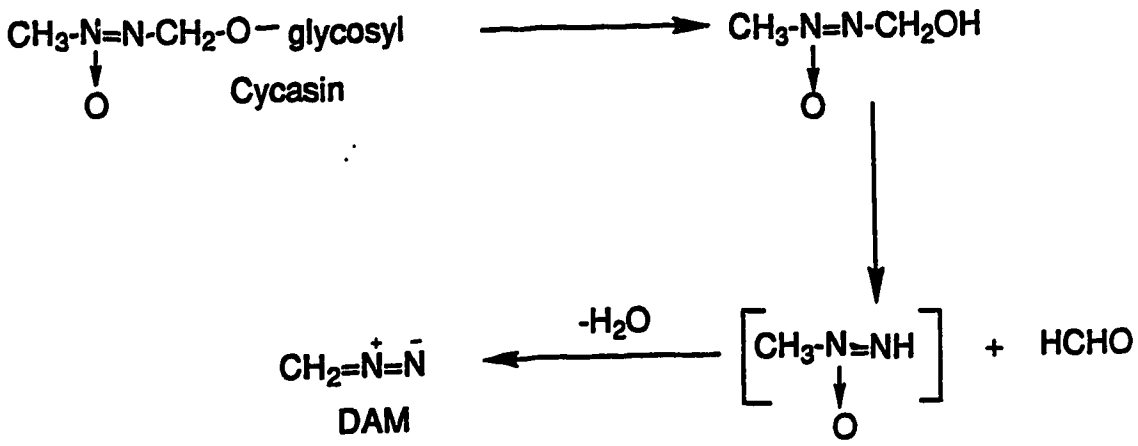


Fig. 1.5. Metabolic pathway of cycasin.

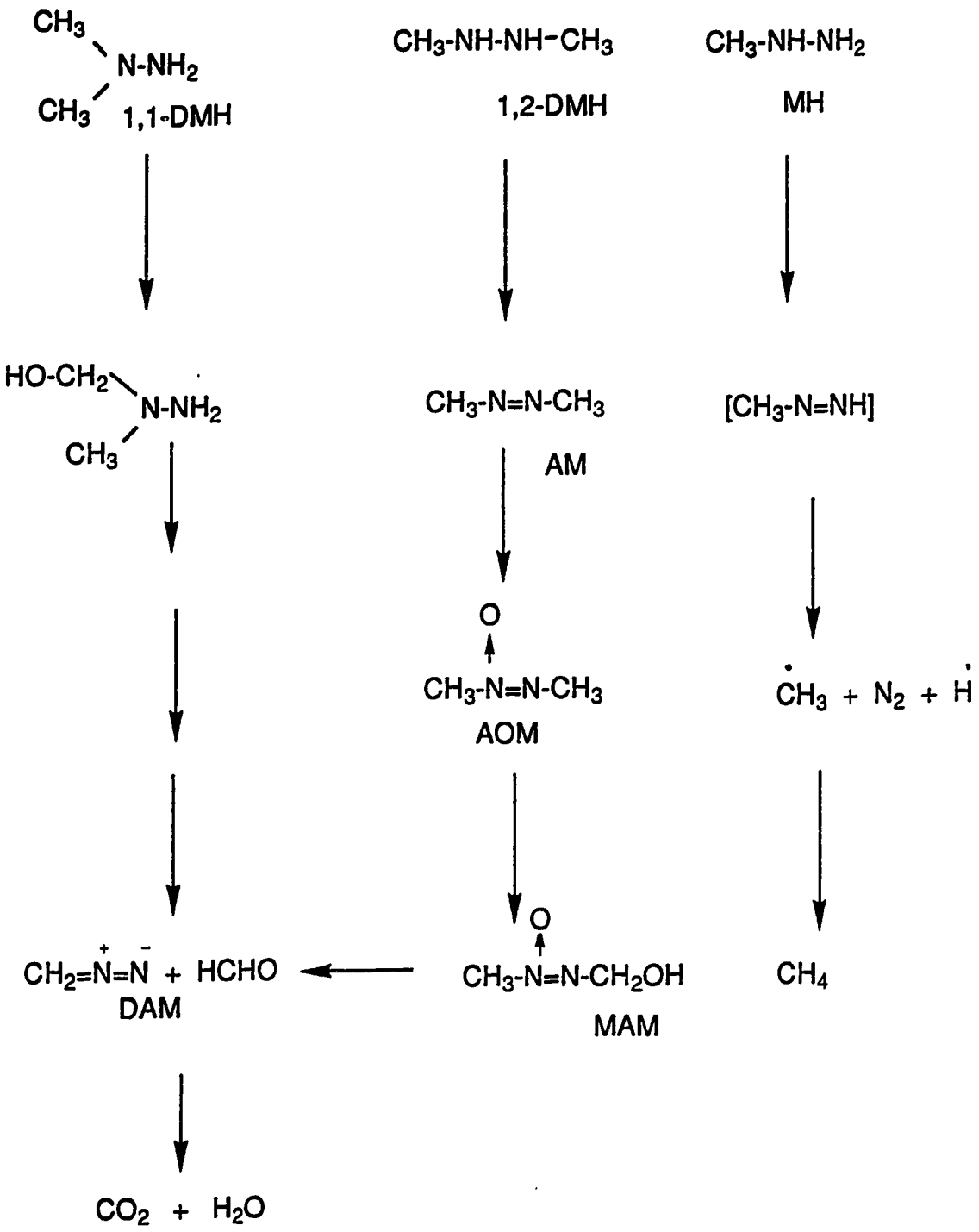


Fig. 1.6. Metabolic conversions of methylhydrazine (MH), 1,1-dimethylhydrazine (1,1-DMH) and 1,2-dimethylhydrazine (1,2-DMH).

trapping techniques.[75,76]

The exact sequence by which Pz is degraded has not been fully established. However, it has become customary to depict the initial oxidation step as occurring on the carbon alpha to nitrogen[82-83] (Fig. 1-7). This conclusion does not account for several observations. Thus, neither hydrazine nor acetylhydrazine has been identified as a metabolite of Pz.[84] In addition Fig. 1-7 also does not account for either the generation of ethylbenzene[80,81] or the apparent lack of kinetic isotope effects during the MAO catalysed degradation of deuterated Pz.[97,98] It is therefore possible that Fig. 1-7 is not entirely accurate and that the true sequence of Pz metabolism may be close to that depicted for the more simple aliphatic hydrazines (Fig. 1-8).

It has also been reported that Pz can undergo an <u>in vivo</u> deamination reaction leading to PE.[44,45] A similar reaction was earlier reported to occur with pheniprazine.[25,85] The mechanism of this deamination is not clear. However, the possibility of an initial N-oxidation and subsequent denitrosation of the N-nitrosamine cannot be excluded (Fig. 1-9).[86]

1.1.5.2. <u>Conjugation Reactions of Phenelzine.</u>

Similar to other hydrazines, such as isoniazid,[65] Pz can be expected to undergo condensation reactions with carbonyl compounds such as pyridoxal and α-ketoglutaric acid.[65,87] Such reactions have been demonstrated and an acquired pyridoxine deficiency has been reported in a patients treated with Pz.[66]

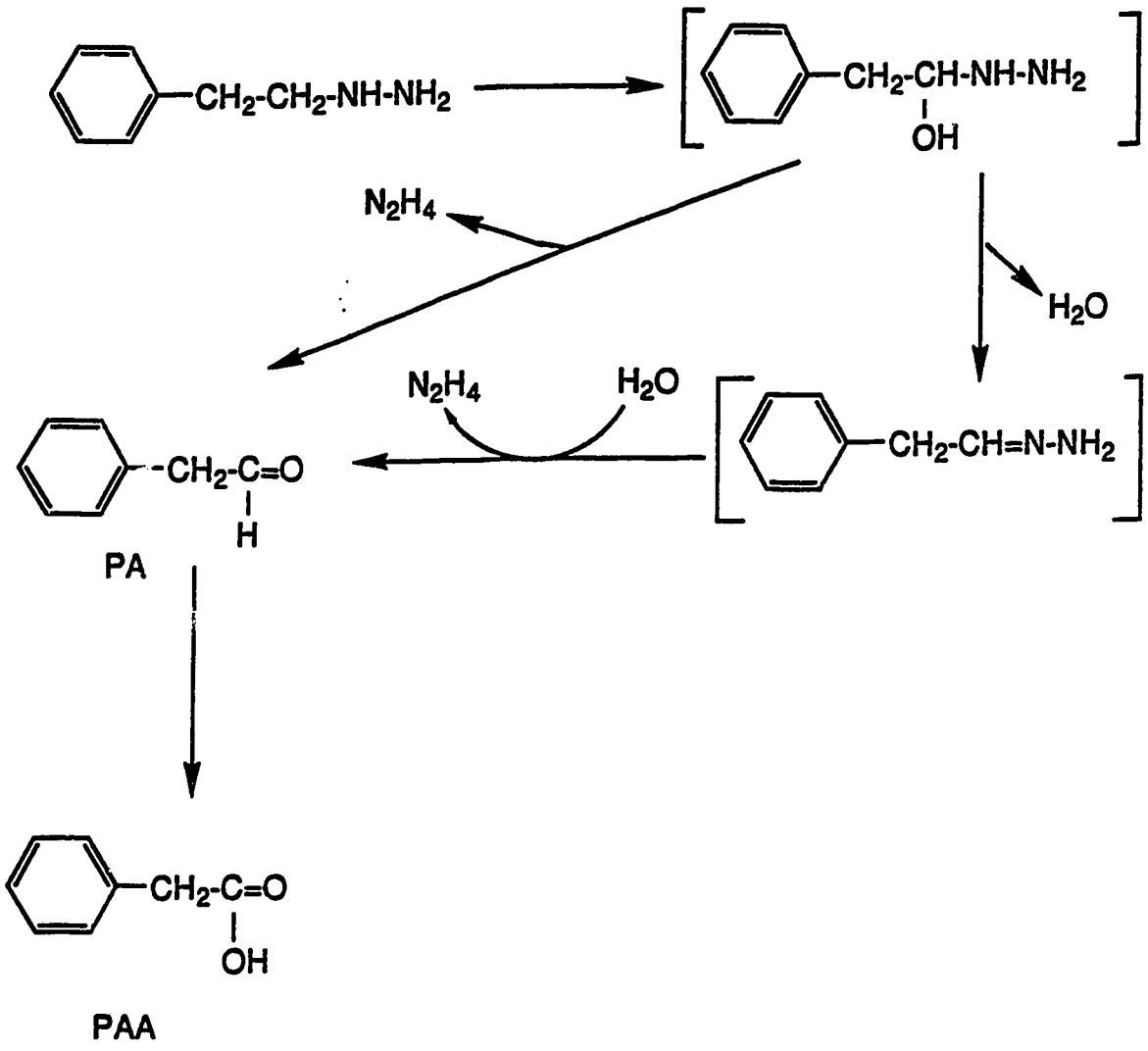


Fig. 1.7. α-C-Oxidation pathways of phenelzine. (adapted from Toth[83]; formulae enclosed in brackets represent proposed intermediates.)

A significant body of evidence also exists to suggest that Pz may be conjugated via metabolic acetylation. This possibility will be discussed separately.

1.1.5.3. Kinetics of Phenelzine.

Regardless of the route of metabolism, Pz is rapidly lost from human plasma after oral dosing.[88] A plasma half-life of 1.2 h after a single dose of 30 mg Pz sulfate has been reported.[89,90] Plasma levels from 1 to 10 ng/mL were found in 6 patients receiving a therapeutic dose (60 mg/day) of Pz. Steady state plasma concentrations of Pz appear to increase over the initial 6-8 weeks of chronic treatment[92,93] possibly due to inhibition of metabolising enzymes.

The time course of the response to Pz suggests a very long-lasting pharmacodynamic effect consistent with either irreversible enzyme inhibition or non-linear elimination kinetics. For example, urinary excretion of tryptamine was increased 8 to 13-fold by week 3 of continuous treatment and remained elevated for 2-3 weeks after the drug was stopped.[94,95] There are at least two possible explanations. First, it has been suggested that the drug or its active metabolites may inhibit Pz metabolism in liver and other tissues, causing drug accumulation.[92,93] Secondly, only a maximum 80% of an administered dose was accounted for even when given to chronically-treated patients.[84] This suggested that an irreversible binding of Pz to tissue components might account for a least

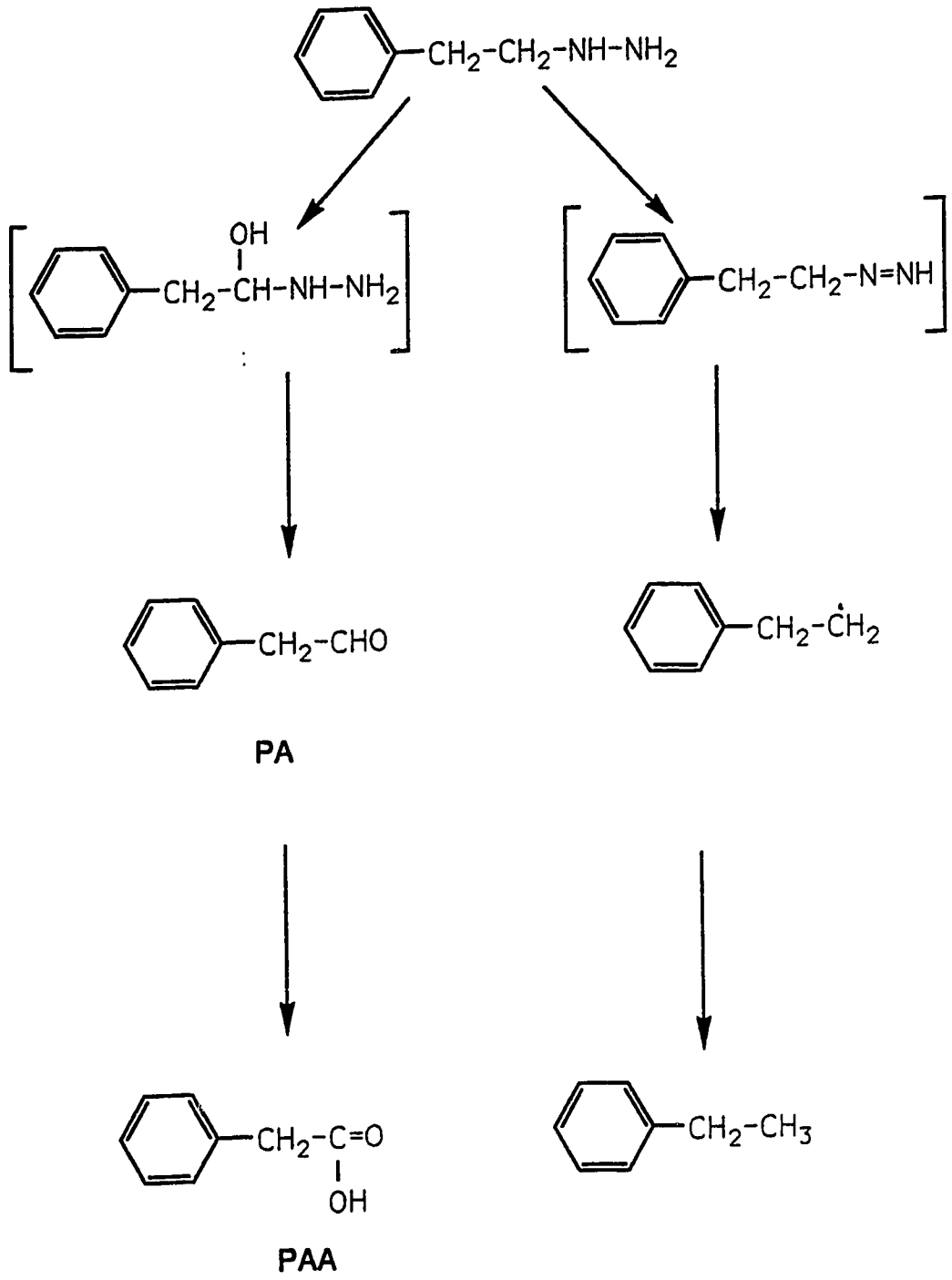


Fig. 1-8. Possible metabolic pathways of phenelzine.

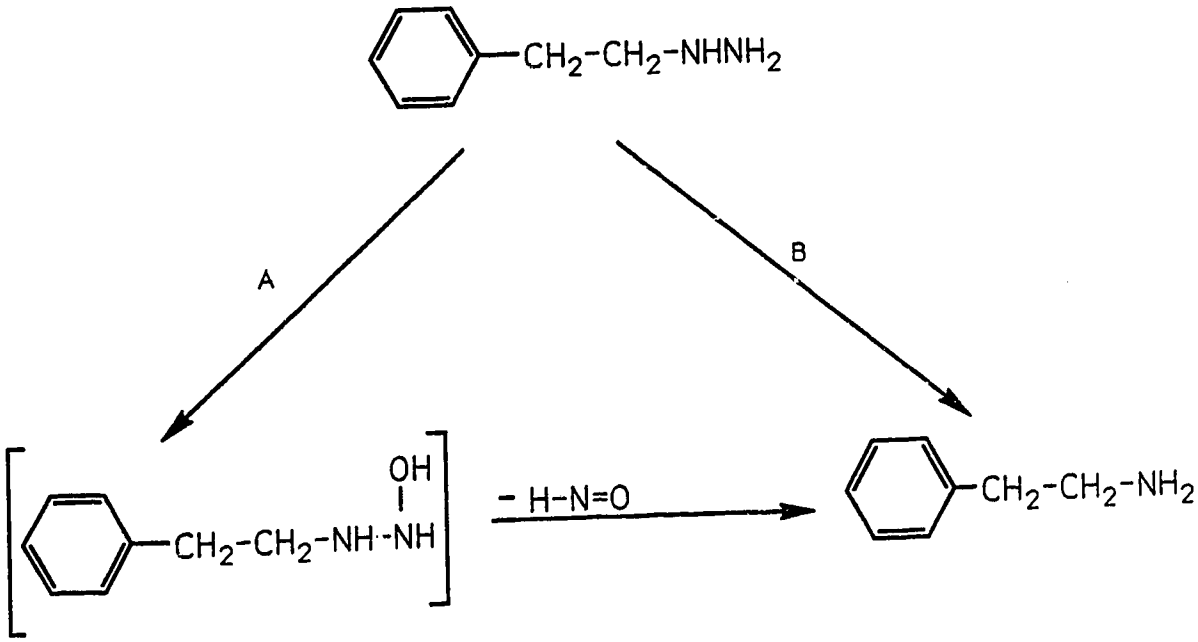


Fig. 1-9. Possible mechanism for the metabolic N-N bond cleavage of phenelzine. (adapted from ref. 86)

part of the remaining balance of the dose. These authors furthermore suggested that this protein/drug complex breaks down over days or weeks, allowing the drug to bind to the newly synthesized MAO.

It has been suggested[15,78,90,96] that inhibition of MAO enzymes may reduce the rate of clearance of Pz. Thus metabolism by MAO may be of considerable significance *in vivo*. Furthermore it has been observed that substitution of deuterium for hydrogens on the α,α-position and in the α,α,β,β-positions of the alkyl side chain of Pz increased the *in vivo* potency of Pz without increasing its *in vitro* potency.[45,97,98] A tentative explanation of these observations is that deuterium substitution slows the metabolic degradation of Pz by non-MAO pathways, thus resulting in increased central concentrations of deuterated Pz.

1.1.6 Acetylation of Phenelzine:

1.1.6.1 The Acetylation Question

Acetylation is a common pathway in the metabolism of aromatic amines[99,100] and it has been observed that the clinical response of patients treated with these drugs can vary according to the rate at which acetylation proceeds.[99-102] With this in mind, various tests of "acetylator status" have been derived in which patients are dosed with an acetylatable drug, and plasma or urine ratios of parent drug versus acetylated metabolite are determined. Results of these determinations indicate that in humans, acetylator status is genetically determined and that interindividual variation is considerable.[103,104] Nevertheless, it is clear that acetylation proficien-

cy is bimodally distributed and the human population can be divided into "rapid" and "slow" acetylator groups. Although it has not been established, indirect evidence has been collected to suggest that Pz may also be metabolised by acetylation and that acetylator status can be important to the clinical outcome of individuals treated with Pz.

Evidence regarding the role of acetylation of Pz is confusing and often contradictory. Although neither of the two possible mono-acetylated metabolites of Pz had been identified *in vivo*, the similarity of structure of Pz and other substances such as isoniazid, hydralazine and hydrazine itself[99,102] provided the first indirect link between Pz and biological acetylation.[105] Tilstone *et al.*[105] and Hein and Weber[106] have demonstrated *in vitro* acetylation of Pz by human and rabbit N-acetyltransferase (NAT) enzymes, respectively. However, the inability to detect any acetylated metabolite *in vivo* has prompted several researchers[84,107] to comment negatively regarding the *in vivo* significance of Pz acetylation.

This question of Pz acetylation is not clear-cut and is complicated by several factors. Firstly Pz contains two basic nitrogen atoms and metabolic production of either N^1-acetylphenelzine (N^1-AcPz) or N^2-acetylphenelzine (N^2-AcPz) are at least theoretical possibilities. Thus although acetylation at the primary nitrogen might be anticipated through analogy to isoniazid, secondary nitrogen may also be a site of acetylation.[108] In other words the preferred site of Pz acetylation is unclear. A lack of anticipation of the formation of the second isomer may have

prevented its identification because of differences in chromatographic behavior from that of the anticipated N^2-AcPz. In fact, examination of the literature indicates that only N^2-AcPz is known[109] and that N^1-AcPz has not been synthesized or characterised. Although each of the studies so far reported[8,107,109,110] have purported to search for only N^2-AcPz, it is unclear which compound was actually prepared as a standard reference substance.[107]

A second complicating point is the complete lack of information regarding the kinetics of either possible isomer in biological systems. This point is highly relevant since chemical detection is based upon the premise that detectable levels will have accumulated. Therefore, if the rate of metabolic clearance is similar to that of formation, chemical detection may be difficult, even though a high flux of acetylphenelzine exists.

1.1.6.2. N-Acetyltransferase Enzymes

The N-acetylation reaction utilizes acetyl-CoA as a cofactor and is catalyzed by cytosolic NAT (E.C.2.3.1.5) enzymes. The molecular weight of the enzyme is approximately 35,000.

Two isozymes of NAT have been isolated. One isozyme of NAT is located predominantly in hepatic parenchymal and gut mucosal cells[111,112] and its genetic variation leads to the phenomenon of polymorphic acetylation.[104] The established substrates for the polymorphic NAT enzyme are procainamide, dapsone, sulphapyridine, sulphadimidine (sulphamethazine), nitrazepam amine and most carcino-

genic aromatic amines.

The second isozyme is predominantly extrahepatic,[113] with highest activities in the duodenum and lung. This enzyme is also found in the thymus, ovary, spleen, uterus, adrenal gland, leukocytes, kidney, bone morrow, salivary gland, pancreas, pineal gland, erythrocytes, bladder mucosa and in brain.[114,115] NAT activity is not present in plasma, colon, esophagus, stomach, skeletal muscle and fat.[114] This extrahepatic isozyme is not polymorphically distributed and acetylates substrates such as p-aminosalicylic acid, p-aminobenzoic acid and sulphanilamide.

These substrate preferences and isozyme locations are based upon studies with human, rabbit and mouse models.[116-121] However, there is evidence to suggest that preferred substrates for polymorphic acetylation may be somewhat species dependent and that different species may have additional NAT enzymes. Thus, for example, hamsters appear to acetylate p-aminobenzoic acid and p-aminosalicylic acid polymorphically, whereas isoniazid and procainamide are monomorphic substrates.[122,123]

Furthermore, it has been shown[124-125] that although rat liver contains NAT activity toward the amine substrates, PE, tyramine, 5-HT, tryptamine, tyramine, octopamine, normetanephrine and isoniazid, at least isoniazid and 5-HT are acetylated by different NAT enzymes.[125] In the human, however, a single NAT catalyses acetylation of both 5-HT and isoniazid.[126] In addition Yang and Neff[127,128] have reported that the NAT enzyme of rat brain is similar to that found in rat liver, in contrast to the clear

substrate differences in humans, rabbits and mice.[116-121] Furthermore this enzyme also appears to acetylate many of the indole- and phenylethylamines.[124]

NAT activities can be inhibited by the MAOIs harmine and iproniazid and by reserpine.[125] Interestingly harmine potently inhibited liver NAT in the hamster and rat[129] but not in the mouse.[130] Both isozymes, hepatic and brain, were inhibited by harmine only in the rat.[129]

1.1.6.3. Clinical Evidence of Acetylation

Since 1965, a large number of studies have been reported with the intent of determining the relationship between acetylator status and clinical response to Pz. These studies differ widely in research design and patient populations and require very careful interpretation. Several of the studies were conducted prior to determination of ideal dose regimens or identification of those patients most likely to respond to Pz. It may be appropriate, therefore, to reexamine these studies in light of current knowledge of the clinical pharmacology of Pz.

In the first of these studies, Evans et al.[7] treated a mixed group of fifty endogenous or neurotic depressed patients with Pz three times daily (total dose, 45 mg/day) for four weeks. Psychiatrists, blind to acetylator status, then rated the antidepressant response and the frequency and severity of side-effects. The data did not indicate any relationship between acetylator status and clinical outcome but did indicate an increased frequency and severity

of side effects among the slow acetylator group. Although this may seen contradictory, later research provides an explanation for this apparent contradiction between clinical outcome and side-effects. Firstly, Pz has been shown to be of significantly greater benefit to atypical or neurotic depressives than to endogenous depressives.[9,15,108] Secondly the relatively low dose of Pz (45 mg/day) employed in this study may be subtherapeutic.[8,15,131] These two factors may have combined to increase the variation among small individual responses and thereby mask any slight differences between clinical outcome of fast and slow acetylators. Nevertheless, side effects, which would not be expected to differ between types of depression, were greater among slow acetylators.

In later studies[88,110,132] it was reported that a higher dose of Pz (90 mg/day) produced a greater response among slow acetylator atypical depressions although side effects did not vary according to acetylator phenotype.

Other studies have been less convincing. For example, Davidson et al.[133] did not observe any relationship between acetylator status and clinical outcome after treatment of primary unipolar depression or depression secondary to anxiety state with Pz (90 mg/day).

Similarly Marshall et al.[134] failed to observe any relationship between clinical outcome and acetylator status possibly because of subtherapeutic dose or mixed patient groups. Finally Tyrer et al.[8,131] reported only a slight and non-significant tendency for fast acetylators to respond to Pz better than slow acetylators.

1.1.7. Analysis of Phenelzine

Several reports on the quantitative analysis of Pz in biological matrices have been published. Development of these assays has been complicated by poor recoveries of Pz during extraction at alkaline pH and its propensity to form multiple derivatives under acylation conditions.

In 1976 and 1977, Caddy et al.[135,136] reported two assay procedures for the analysis of Pz in urine. These authors reported that Pz was unstable at basic pH and could not be extracted from an aqueous solution. Their solution was therefore to chemically modify Pz prior to extraction. In the first of these manuscripts[136] Pz was reacted quantitatively with acetone to yield a hydrazone which was then extracted and assayed by gas chromatography/mass spectrometry (GC/MS). This assay lacked sufficient sensitivity (2 µg/mL) for application to plasma and was applied only to urine. However, the concept of in situ stabilization through generation of a hydrazone has been adapted to several subsequent assay protocols.

In the second of these assays[136] Pz was measured after periodate oxidation to 2-phenylethanol (Fig. 1-10). This assay was also relatively insensitive and again was only applied to urine. In addition, the assay did not distinguish between endogenous 2-phenylethanol and that derived from Pz, or its metabolites. No further application of this "degradative" stabilization has been reported.

Using a somewhat different approach to stabilization prior to extraction, Cooper et al.[89] extracted Pz at a slightly acidic pH

(6.8) and derivatized with heptafluorobutyric anhydride in a non-aqueous medium (Fig. 1-11). Although recoveries and assay sensitivity were acceptable, multiple derivatives were obtained because of variable reactions at each nitrogen. In a similar assay, Narasimhachari et al.[137] also identified limitations due to variable derivatization.

Although each of these procedures was limited either by sensitivity or specificity, they did clearly point out an important fact for subsequent analysts. Thus, the need for stabilization, via derivatization prior to extraction, was established. This point has not been ignored by later analysts and four procedures incorporating the principle of prior derivatization have been reported.

In the first of these truly specific assays, Jindal et al.[91] reacted Pz in plasma with pentafluorobenzaldehyde and obtained a quantitative yield of the corresponding hydrazone (Fig. 1-12). Using hepta-deuterated Pz as internal standard, these hydrazones were extracted and assayed by GC/MS. This procedure provided the first realistic assessment of the plasma profile of Pz in treated patients. Direct application of this procedure in other laboratories has not been reported, perhaps because of the difficulties associated with synthesis of the deuterated internal standard or because of the requirement for highly sophisticated instrumentation. However, a similar mass spectrometric procedure was developed by Dyck et al.[41] whereby Pz was extracted from rat brain after its stabilization as the dansylated acetone hydrazone (Fig. 1-13). This assay was applied to the analysis of Pz in rat brain

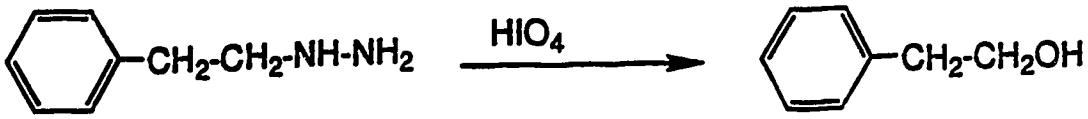


Fig. 1-10. Oxidation of phenelzine to 2-phenylethanol (ref. 136).

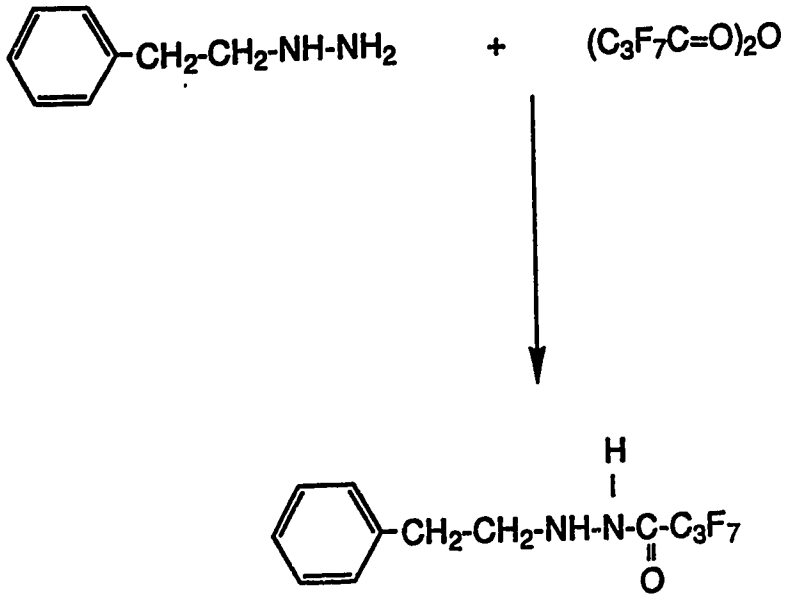


Fig. 1-11. Reaction of phenelzine with heptafluorobutyric anhydride (ref. 89).

but was not extended to the analysis of clinical samples.

Finally, two highly specific and sensitive gas chromatographic assays have been reported. Each of these procedures has been applied to the measurement of Pz in biological fluids, although somewhat different approaches to chemical stabilization have been taken. The first truly applicable gas chromatographic analysis with electron-capture detection (ECD) of Pz was reported by Rao et al.[138] in 1987. In this procedure Pz and an internal standard, 2-(4-chloro-phenyl)ethylamine, were exhaustively acylated with pentafluorobenzoylchloride prior to extraction (Fig. 1-14). Highly linear standard curves were generated over a broad concentration range and Pz was assayed in rodent and human samples. A very interesting aspect of this method is the fact that Pz was acylated under alkaline conditions in an aqueous solvent without undergoing extensive auto-oxidative loss. This may suggest that reports of extreme sensitivity to autooxidative degradation under these conditions[25] may have been misleading and that poor recoveries may be only a characteristic of Pz solubility. In spite of extreme efforts varying degrees of mono- and di-acetylation of Pz occurred during this derivatization, again demonstrating great difficulty of controlling this variable.

The second of these chromatographic assays was reported by Lichtenwalner et al.[139] who incorporated a reaction with aqueous acetylacetone to generate a cyclized derivative (Fig. 1-15) which was then extracted and assayed by nitrogen/phosphorus sensitive gas chromatography. This assay was applied to the analysis of Pz in

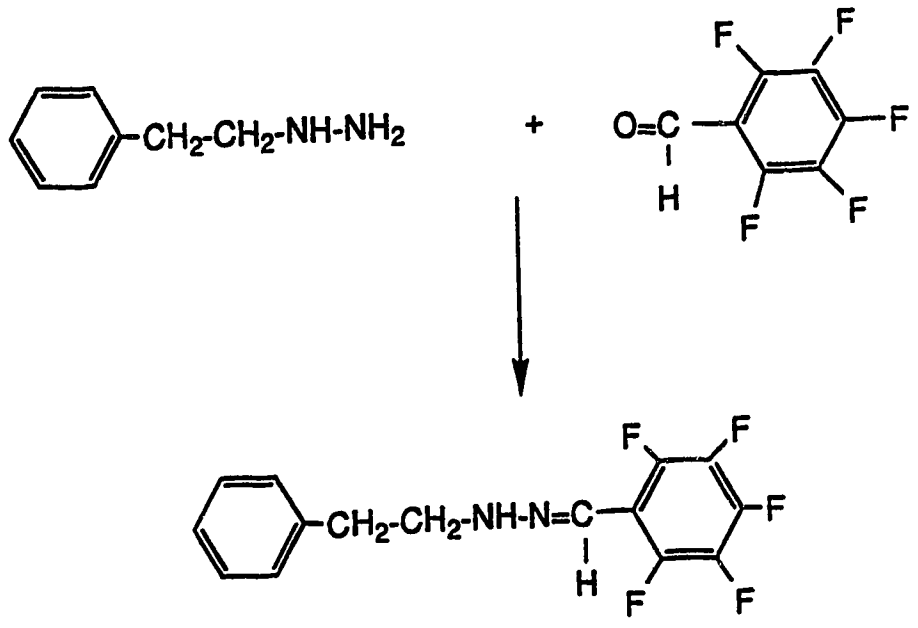


Fig. 1-12. Reaction of phenelzine with pentafluorobenzaldehyde (ref. 91).

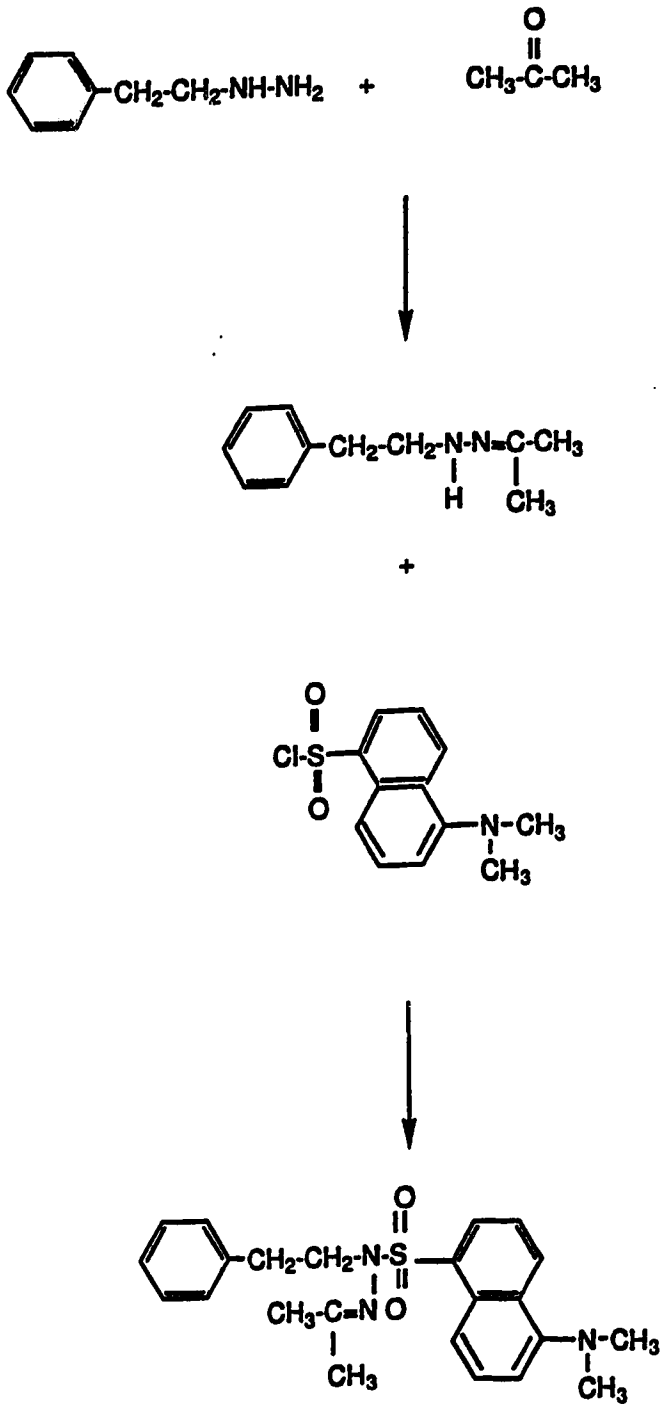


Fig. 1-13. Reaction of phenelzine with acetone and dansyl chloride (ref 41).

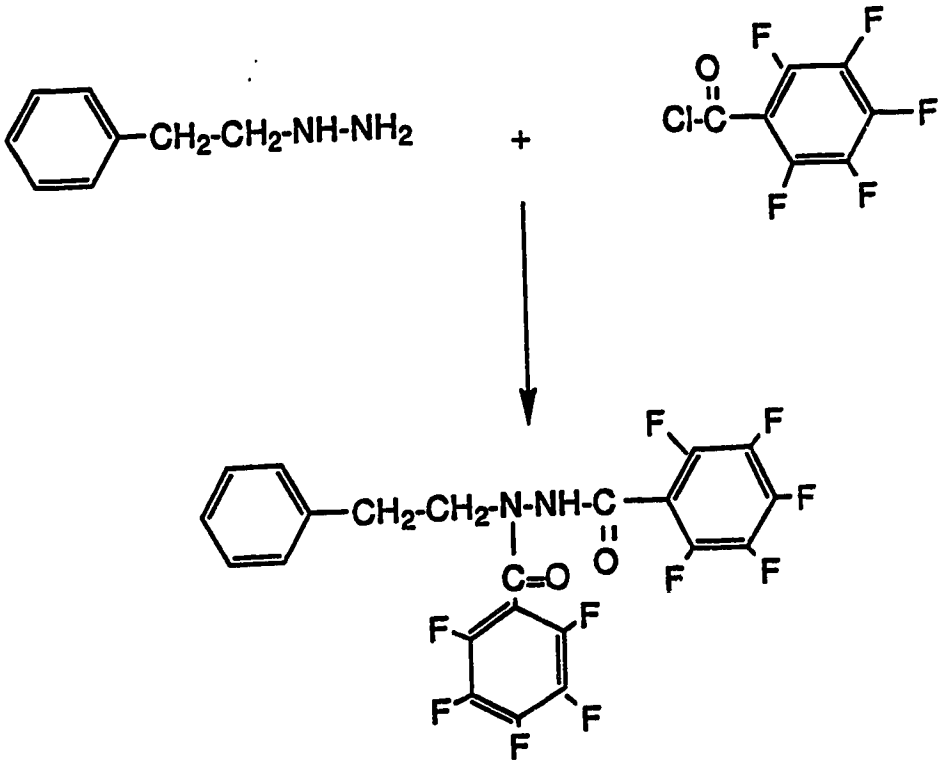


Fig. 1-14. Reaction of phenelzine with pentafluorobenzoyl chloride (ref. 138).

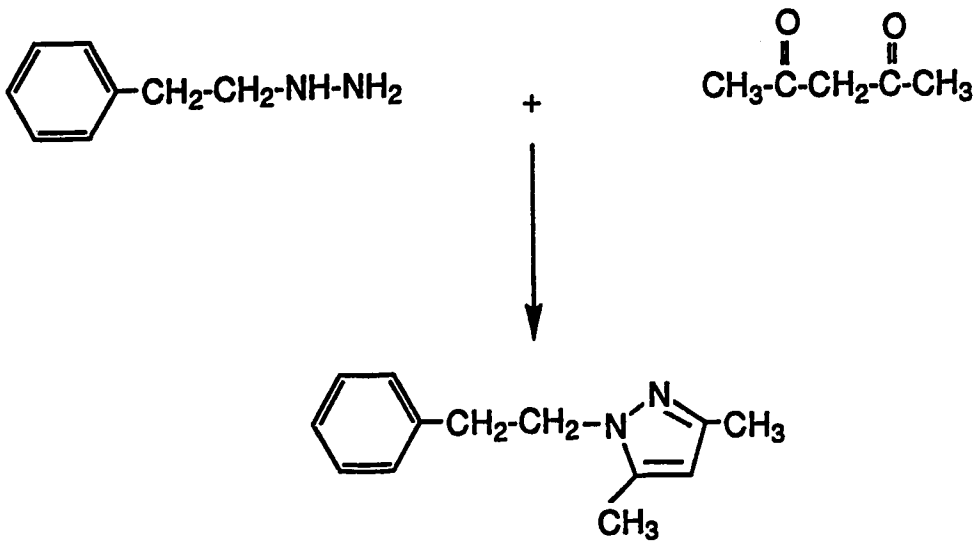


Fig. 1-15. Reaction of phenelzine with acetylacetone (ref. 139).

human fluids.

In summary these reports indicate that aqueous derivatization prior to extraction is an important step in the analysis of Pz. Two approaches have been taken. The most frequently employed procedure has been to react Pz with a ketone prior to extraction to give a single hydrazone derivative; however, aqueous acylation without prior hydrazone formation has also been employed providing that steps to control, and compensate for, variable derivatization are taken.

1.1.8. Rationale

Despite over 30 years of clinical application, the metabolic fate of Pz is only poorly understood. Analogies drawn from chemically related substances suggest that alpha-C- as well as N-oxidation are important processes, and the corresponding Pz metabolites have in fact been isolated.

Acetylation, although also suggested by analogies to other hydrazines,[102,104] has not been demonstrated *in vivo*. Since N^2-AcPz does possess MAOI properties both *in vitro* and *in vivo*,[109,140] and retains activity as a counteractant to reserpine,[141] the contribution of this substance to the overall Pz response may be significant. Positive proof of its existence could help answer this question.

Furthermore, the alternate possibility of acetylation of Pz at N^1 is intriguing and worthy of investigation.

1.2. HYPOTHESIS

1) Pz and its acetylated metabolites can be assayed in biological fluid.
2) Monoacetylphenelzine is a metabolite of Pz.
3) An acetylated metabolite of Pz may contribute to the pharmacological response to Pz.

1.3. OBJECTIVES

1. Develop sensitive and specific qualitative and quantitative assays for Pz and its monoacetylated derivatives in plasma and tissue.
2. Investigate the chemical acetylation of Pz and prepare authentic samples of each possible monoacetylated derivative.
3. Determine the pharmacological and gas chromatographic properties of each monoacetylated Pz.
4. Identify either, or both, monoacetylated derivatives of Pz in rats treated with Pz and compare their kinetics to those of Pz.
5. Draw conclusions as to the possible pharmacological significance of any acetylated Pz metabolites.

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2. SYNTHESIS AND CHARACTERIZATION OF ACYL DERIVATIVES OF PHENELZINE*

2.1. INTRODUCTION

The phenomenon of selective acylation of methylhydrazine (MH) has been observed by several researchers.[1-3] In one of these studies,[3] acetylation of MH with acetic anhydride and with ethyl acetate involved two different mechanisms. This report examines the selective acylation of the monoamine oxidase (MAO) inhibitor phenelzine (Pz), a hydrazine derivative, using acetic anhydride or ethyl chloroformate as the acylating agents in aqueous and non-aqueous media. The selectivity was found to be medium-dependent.

The primary aim of this study was the preparation of Pz derivatives for evaluation as monoamine oxidase inhibitors (MAOIs) and as potential prodrugs of Pz. Other objectives were a study of the selectivity of the acylation reaction and an investigation of any conformational isomerism displayed by the mono- and di-acylated products. For these purposes, eight mono- and di-acylated derivatives of Pz were synthesised (Fig. 2-1) and characterized by their gas chromatographic (GC) retention times (rt) and their mass spectral (MS) fragmentation , infrared spectra (IR) and nuclear magnetic resonance (NMR) spectra.

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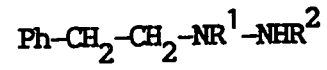

	$\underline{R}^1$	$\underline{R}^2$
Pz	H	H
N^2-AcPz	H	$COCH_3$
N^1-AcPz	$COCH_3$	H
N^1N^2-AcPz	$COCH_3$	$COCH_3$
N^2-ECPz	H	$COOC_2H_5$
N^1-ECPz	$COOC_2H_5$	H
N^1N^2-ECPz	$COOC_2H_5$	$COOC_2H_5$
N^1-AcN^2-ECPz	$COCH_3$	$COOC_2H_5$
N^1-ECN^2-AcPz	$COOC_2H_5$	$COCH_3$

Fig. 2-1. Structures of phenelzine and acylated derivatives.

2.2. EXPERIMENTAL

GC retention times and MS fragmentations were determined on a Hewlett Packard Model 5710A gas chromatograph interfaced with a Hewlett-Packard Model 5980A mass spectrometer and Model 5934A data system. The injection port and transfer line temperatures were 300°C. The operating conditions for the mass spectrometer were: electron beam energy, 70 eV, emission current, 35 ma and source temperature, 180°C. A 10 m DB-1 fused silica GC column (J & W Scientific, Palo Alto, CA. U.S.A.), 0.32 mm i.d., was employed. The carrier gas (helium) flow rate was 2 mL/min, and the oven temperature was programmed from 100°C to 280°C at 8°C/min.

Exact mass measurements were made on an AEI MS-50 mass spectrometer operated by personnel in the Department of Chemistry, University of Alberta, who also performed the elemental microanalyses. NMR spectra were determined for solutions in $CDCl_3$ or DMSO-d_6 with TMS as internal standard on a Bruker AM-300 or Varian EM-390 spectrometer.

Melting points were obtained with a Thomas Hoover apparatus and are uncorrected. IR spectra were recorded on a Nicolet 5DX spectrophotometer. Pz sulfate was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and acetic anhydride and ethyl chloroformate were obtained from Aldrich (Milwaukee, Wisconsin, U.S.A.). Carbon-14-labeled 5-hydroxytryptamine and 2-phenylethylamine were purchased from Dupont Canada Inc (Mississauga, Ontario, Canada).

2.2.1. 2-Acetyl-1-(2-phenylethyl)hydrazine (N^2-AcPz)

This compound was prepared by two different procedures:

a) a solution of phenylacetaldehyde (120 mg; 1 mmol) and acetylhydrazine (74 mg; 1 mmol) in isopropyl alcohol (10 mL) was heated under reflux for 4 h. The solvent was removed under reduced pressure and the intermediate hydrazone, 2-acetyl-1-(2-phenylethylidene)hydrazine, thus obtained was dissolved in methanol (5 mL) and reduced with sodium cyanoborohydride (200 mg) according to the method of Danielson et al.[4] to yield N^2-AcPz (56%) (Fig. 2-2) as colorless crystals, m.p. 70-71°C [Reported[4] m.p. 69-70°C].

IR (KBr): 3287 (s, N-H); 3221 (m, N-H); 1646 cm^{-1} (s, C=O). ^1H NMR ($CDCl_3$) major (minor) rotamer: 1.96 (2.12) (s, 3H, $COCH_3$); 2.84 (t, J=7 Hz, 2H, $PhCH_2$); 3.16 (3.10) (t, J=7 Hz, 2H, NCH_2); 4.26 (3.70) (br s, 1H, NHNHCO, exchanges with D_2O); 7.12 (6.90) (br s, 1H, NHCO, exchanges with D_2O); 7.32 (m, 5H, Ph); major:minor rotamer ratio = 6:1.

b) acetic anhydride (100 µL) was added dropwise with stirring to a suspension of Pz sulfate (1 mg) in ethyl acetate (500 µL) at 70°C for 15 min. as described by Narasimhachari et al.[5] A 2 µL aliquot of the reaction mixture was examined by GC/MS. The product had a GC retention time and a mass spectrum (Table 2-1.) identical to those of the product obtained by method a).

2.2.2. 1-Acetyl-1-(2-phenylethyl)hydrazine (N^1-AcPz)

A solution of acetic anhydride (102 mg; 1 mmol) in chloroform (15 mL) was added dropwise with stirring in 5 portions at 15 min

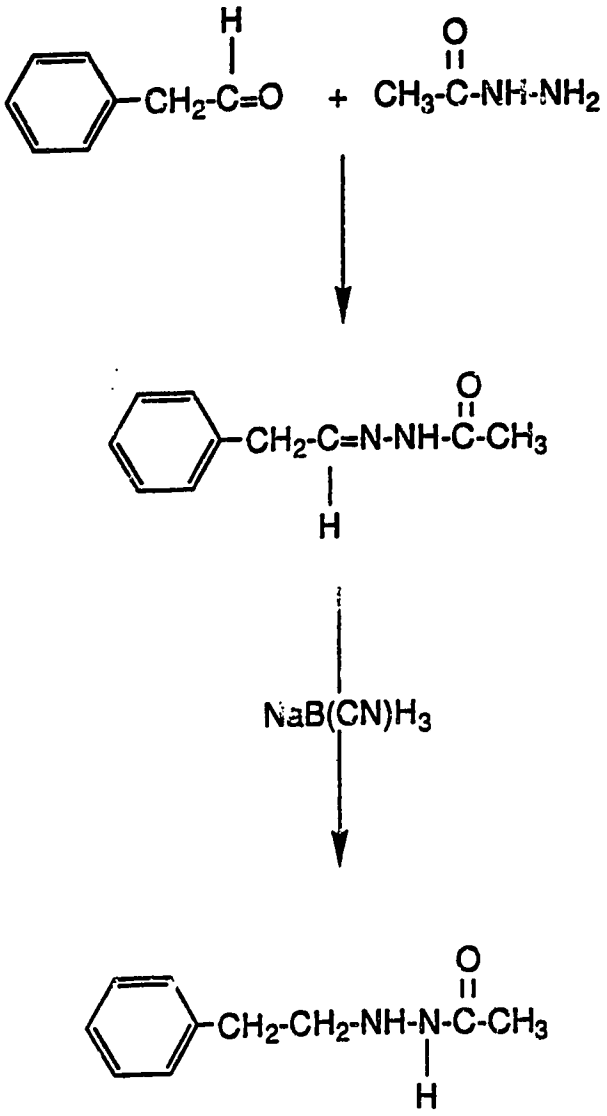

Fig. 2-2. Preparation of 2-acetyl-1-(2-phenylethyl)hydrazine (N^2-AcPz) from phenylacetaldehyde and acetylhydrazine.

intervals to a s[illegible]on of Pz sulfate (234 mg; 1 mmol) in water (15 mL) containing sodium bicarbonate (84.0 mg). After the final addition, the mixture was stirred at room temperature for a further 30 min. The organic layer was separated and dried (anhydrous sodium sulfate), and the solvent was removed under reduced pressure to yield N^1-AcPz (123 mg) which was recrystallized, 69%, from hexane as colorless crystals, m.p. 67-69°C.

IR (KBr): 3312 (m, N-H); 3213 (m, N-H); 1622 cm^{-1} (s, br, C=O). ^1H NMR ($CDCl_3$) major (minor) rotamers: 1.82 (2.20) (s, 3H, $COCH_3$); 3.00 (2.95) (t, J=7 Hz, 2H, Ph-CH_2); 3.70 (3.84) (t, J=7 Hz, 2H, CH_2NCO); 4.30 (3.54) (s, br, 2H, NH_2, exchanges with D_2O); 7.36 (m, 5H, Ph); major:minor rotamer ratio = 2:1.

Anal. calcd. for $C_{10}H_{14}N_2O$: C, 67.39; H, 7.92; N, 15.72; found: C, 67.00; H, 7.91; N, 15.61. Exact mass, calcd. for $C_{10}H_{14}N_2O$: 178.1106; found [(high resolution mass spectrometry (HRMS)]: 178.1107.

2.2.3. 1,2-Diacetyl-1-(2-phenylethyl)hydrazine (N^1N^2-AcPz)

Acetic anhydride (204 mg; 2 mmol) was added to N^1-AcPz (178 mg; 1 mmol) in chloroform (10 mL) and the solution was stirred at room temperature for 4 h. The solvent was evaporated and the solid residue was recrystallized from hexane as colorless crystals (130 mg; 59%), m.p. 61-63°C.

IR (KBr): 3394 (br, m, N-H); 1653 cm^{-1} (br, s, C=O). ^1H NMR ($CDCl_3$) major (minor) rotamers: 1.94 (1.90) (s, 3H, $NHCOCH_3$);

Table 2-1.: Gas Chromatographic Retention Times (rt) and Mass Spectrometric Fragmentation Data of Mono- and Di-acylated Derivatives of Phenelzine

Compound	rt (min)	MS Data (% Relative Abundance)
N^2-AcPz	6.1	178(2), 105(10), 91(20), 87(100), 45(43), 43(12).
N^1-AcPz	5.4	178(12), 136(8), 105(11), 104(11), 91(38), 87(31), 74(49), 45(100), 43(35).
N^1N^2-AcPz	9.0	220(1), 178(6), 116(22), 105(9), 91(19), 87(100), 74(10), 45(17), 43(20).
N^2-ECPz	6.5	208(2), 163(3), 117(100), 105(19), 104(12), 103(13), 91(45), 89(74), 79(13), 78(13), 77(17), 71(79), 65(16), 44(14).
N^1-ECPz	5.4	208(38), 163(4), 117(87), 105(14), 104(13), 103(11), 91(100), 89(8), 79(12), 78(10), 77(22), 65(31), 45(98), 44(33).
N^1N^2-ECPz	10.0	280(0), 235(1), 189(6), 176(78), 163(5), 145(8), 117(89), 105(29), 104(30), 103(12), 91(65), 89(100), 79(16), 78(11), 77(19), 71(55), 65(10), 45(6), 44(9).
N^1-AcN^2-ECPz	9.2	250(2), 208(17), 146(29), 117(100), 105(11), 104(20), 91(20), 89(32), 71(17), 65(4), 43(45).
N^1-ECN^2-AcPz	9.2	250(0), 208(3), 192(8), 117(22), 105(23), 104(44), 91(58), 87(100), 79(15), 78(13), 77(24), 65(16), 45(44), 43(45).

2.00 (2.08) (s, 3H, -NCOC$\underline{H}_3$-NHCOCH_3); 2.90 (t, J=7 Hz, 2H, PhC$\underline{H}_2$); 3.84 (br, t, J=7 Hz, 2H, C$\underline{H}_2$N); 6.90 (7.54) (s, 1H, N-$\underline{H}$, exchanges with D_2O); 7.26 (m, 5H, Ph); major:minor rotamer ratio = 4:1. Exact mass, calcd. for $C_{12}H_{16}N_2O_2$: 220.1211; found (HRMS): 220.1208.

2.2.4. 2-Ethoxycarbonyl-1-(2-phenylethyl)hydrazine (N^2-ECPz)

This compound was prepared from phenylacetaldehyde and ethyl carbazate by the method described by Anderson et al.[6] (Fig. 2-3), and recrystallized from hexane as a colorless compound, m.p. 58-59°C, identical to the reported m.p..[6]

IR (KBr): 3280 (s, N-H); 3263 (s, N-H); 1703 cm^{-1} (s, br, C=O). ^1H NMR ($CDCl_3$) : 1.24 (t, J=7 Hz, 3H, OCH_2C$\underline{H}_3$); 2.78 (t, J=7 Hz, 2H, PhC$\underline{H}_2$); 3.15 (t, J=7 Hz, 2H, NC$\underline{H}_2$); 4.00 (br, 1H, N$\underline{H}$NHCO, exchanges with D_2O); 4.15 (q, J=7 Hz, 2H, OC$\underline{H}_2$CH_3); 6.24 (br, 1H, N$\underline{H}$CO, exchanges with D_2O); 7.26 (m, 5H, Ph).

2.2.5. 1-Ethoxycarbonyl-1-(2-phenylethyl)hydrazine (N^1-ECPz)

This compound was synthesized from Pz sulfate (1 mmol) in a similar way to compound N^1-AcPz except that ethyl chloroformate (1 mmol) was used instead of acetic anhydride. The product (131 mg; 63%) was converted to its hydrochloride salt, m.p. 135-136°C when recrystallized from chloroform.

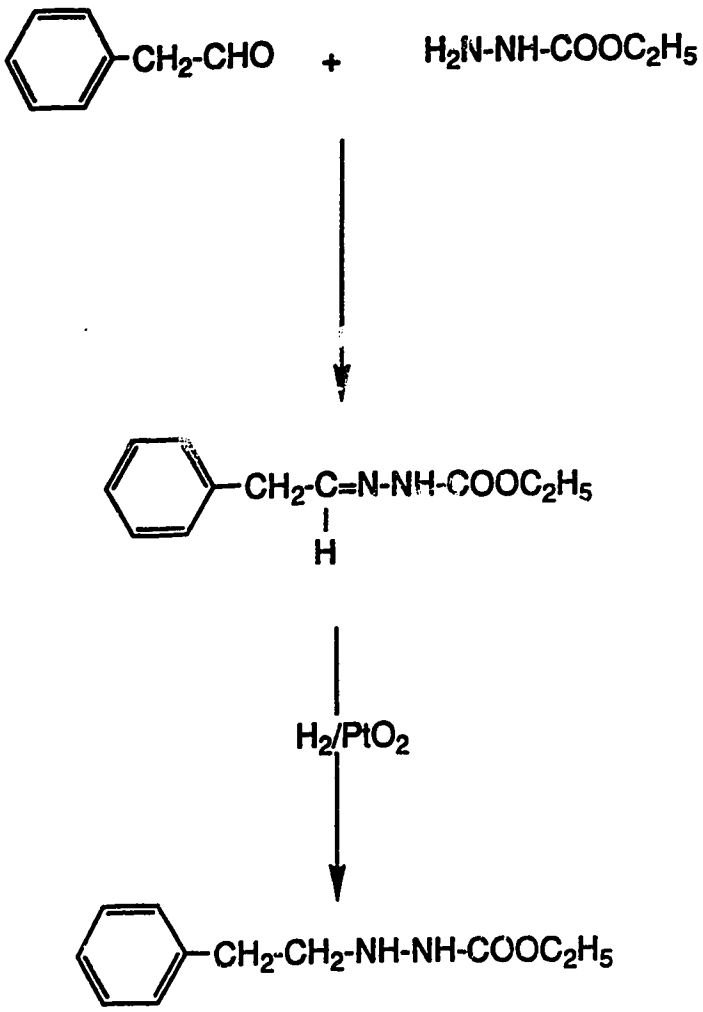

Fig. 2-3. Preparation of 2-ethoxycarbonyl-1-(2-phenylethyl)-hydrazine (N^2-ECPz) from phenylacetaldehyde and ethyl carbazate.

IR (KBr) of HCl salt: 2840 (s, br, NH_3^+); 1729 cm^{-1} (s, br, C=O). ^1H NMR (DMSO-d_6) of HCl salt: 1.13 (t, J=7 Hz, 3H, $CH_2\underline{CH_3}$); 2.90 (t, J=7 Hz, 2H, $PhC\underline{H_2}$); 3.76 (t, J=7 Hz, 2H, $\underline{CH_2}N$); 4.03 (q, J=7 Hz, 2H, $OC\underline{H_2}$), 3.20-4.20 (br, 3H, NH_3^+, exchanges with D_2O); 7.26 (m, 5H, Ph). Exact mass, calcd. for $C_{11}H_{16}N_2O_2$: 208.1212; found (HRMS): 208.1213.

2.2.6. 1,2-Diethoxycarbonyl-1-(2-phenylethyl)hydrazine (N^1N^2-ECPz)

a) A solution of N^1-ECPz (208 mg; 1 mmol) and ethyl chloroformate (217 mg; 2 mmol) in chloroform (5 ml) was stirred at room temperature for 4 h. The solvent was evaporated to yield an oil (190 mg; 68%) which did not solidify on standing.

IR (neat): 3296 (m, br, NH); 1740 (s, sh, C=O); 1720 cm^{-1} (s, br, C=O). ^1H NMR ($CDCl_3$): 1.22 (t, J=7 Hz, 3H, $NHCOOCH_2\text{-}\underline{CH_3}$); 1.28 (t, J=7 Hz, 3H, $NCOOCH_2\underline{CH_3}$); 2.94 (t, J=7 Hz, 2H, $PhC\underline{H_2}$); 3.78 (br, t, J=7 Hz, 2H, $\underline{CH_2}N$); 4.16 (q, J=7 Hz, 2H, $NHCOOC\underline{H_2}CH_3$); 4.20 (q, J=7 Hz, 2H, $-N\text{-}COOC\underline{H_2}CH_3$); 6.43 (br, s, 1H, $N\underline{H}$ exchanges with D_2O); 7.22 (m, 3H, o- and p-Ph); 7.30 (m, 2H, m-Ph). Exact mass, calcd. for $C_{14}H_{20}N_2O_4$: 280.1418; found (HRMS): 280.1410.

b) The reaction described immediately above was repeated except that N^1-ECPz was replaced with N^2-ECPz (208 mg; 1 mmol). The IR, NMR, GC and mass spectral properties of the product were identical to

those of the product obtained in reaction a).

2.2.7. 1-Acetyl-2-ethoxycarbonyl-1-(2-phenylethyl)hydrazine (N^1-AcN^2-ECPz)

a) A solution of N^1-AcPz (178 mg; 1 mmol) and ethyl chloroformate (217 mg; 2 mmol) in chloroform (10 mL) was stirred for 3 h at room temperature. The solvent was removed and the residue recrystallized from hexane as colorless crystals (187 mg; 75%), m.p. 72°C.

IR (KBr): 3229 (m, NH); 1738 (s, carbamate C=O); 1640 cm^{-1} (s, amide C=O). ^1H NMR ($CDCl_3$): major (minor) rotamers: 1.26 (t, J=7 Hz, 3H, $OCH_2\underline{CH_3}$); 2.06 (1.90) (s, 3H, $COCH_3$); 2.90 (br, 2H, $PhCH_2$); 3.80 (br, t, J=7 Hz, 2H, CH_2N); 4.25 (br, 2H, OCH_2CH_3); 6.28 (6.65) (br,s, 1H, NH, exchanges with D_2O); 7.30 (m, 5H, Ph); major:minor rotamer ratio = 4:1. Exact mass, calcd. for $C_{13}H_{18}N_2O_3$: 250.1318; found (HRMS): 250.1318.

b) A solution of N^2-ECPz (208 mg; 1 mmol) and acetic anhydride (204 mg; 2 mmol) in chloroform (10 mL) was stirred at room temperature for 3 h. Removal of the solvent gave the title compound which had IR, NMR, GC and mass spectral properties identical to those of the product prepared by method a) immediately above.

2.2.8. 1-Ethoxycarbonyl-2-acetyl-1-(2-phenylethyl)-hydrazine (N^1-ECN^2-AcPz)

a) A solution of N^1-ECPz (208 mg; 1 mmol) and acetic anhydride (204 mg; 2 mmol) in chloroform (10 mL) was stirred at room

temperature for 3 h. The solvent was evaporated to give the title compound as an oil that did not solidify on standing.

IR (neat): 3279 (m, br, NH), 1720 (s, br, carbamate C=O); 1679 cm^{-1} (s, br, amide C=O). ^1H NMR ($CDCl_3$): 1.16 (t, J=7 Hz, 3H, $OCH_2\underline{CH}_3$); 1.94 (s, 3H, $CO\underline{CH}_3$); 2.88 (t, J=7 Hz, 2H, $Ph\underline{CH}_2$); 3.76 (t, J=7 Hz, 2H, $N\text{-}\underline{CH}_2$); 4.10 (q, J=7 Hz, 2H, $O\underline{CH}_2$); 7.26 (m, 5H, Ph); 8.30 (br, s, 1H, $N\underline{H}$, exchanges with D_2O). Exact mass, calcd. for $C_{13}H_{18}N_2O_3$: 250.1318; found (HRMS): 250.1314.

b) A solution of N^2-AcPz (178 mg; 1 mmol) and ethyl chloroformate (217 mg; 2 mmol) in chloroform (10 mL) was stirred for 3 h at room temperature. The solvent was removed to yield an oil which had IR, NMR, GC and mass spectral properties identical to those of the product prepared by method a), immediately above.

2.3. RESULTS

When Pz sulfate was reacted with an equimolar amount of acetic anhydride in a biphasic medium (aqueous sodium bicarbonate and chloroform), a single monoacetylated product was obtained. In contrast, when the same acetylation reaction was repeated in ethyl acetate or chloroform, and in the absence of water, another monoacetylated product was obtained. The latter monoacetate was identical to the product obtained by the reduction of 2-acetyl-1-(2-phenylethylidene)hydrazine; it is unequivocally identified, therefore, as N^2-acetylphenelzine (N^2-AcPz). The product of the

reaction in the biphasic medium must therefore be the isomeric N^1-acetylphenelzine (N^1-AcPz). These isomeric monoacetylphenelzines are readily distinguished by their IR, NMR, GC and mass spectral properties. They were also distinguished by their conversion to N-ethoxycarbonyl derivatives (N^1-AcN^2-ECPz and N^1-ECN^2-AcPz) which are discussed below. N^1,N^2-Diacetylphenelzine (N^1N^2-AcPz) was readily obtained by acetylation of N^1-AcPz with excess acetic anhydride in a nonaqueous medium.

Similarly, the N^1-ethoxycarbonyl derivative of Pz (i.e., N^1-ECPz) was the product of the reaction of equimolar amounts of ethyl chloroformate and Pz sulfate in a water/chloroform biphasic medium, whereas the isomeric N^2-ethoxycarbonylphenelzine (N^2-ECPz) was unequivocally synthesized by the reduction of 2-ethoxycarbonyl-1-(2-phenylethylidene)hydrazine. The isomers (N^2-ECPz and N^1-ECPz) were also readily distinguished by their IR, NMR, GC and mass spectral properties, and by the identification of products of their acetylation. N^1-Acetyl-N^2-ethoxycarbonylphenelzine (N^1-AcN^2-ECPz) was clearly the product obtained when N^2-ECPz was acetylated with acetic anhydride, since the same product resulted from the interaction of ethyl chloroformate with N^1-AcPz. The structure of N^1-ECPz was confirmed in a similar way; N^2-acetyl-N^1-ethoxycarbonylphenelzine (N^1-ECN^2-AcPz) was the product of the acetylation of N^1-ECPz with acetic anhydride, and of the interaction of N^2-AcPz with ethyl chloroformate.

N^1N^2-Di(ethoxycarbonyl)phenelzine was obtained by reaction of either N^1-ECPz or N^2-ECPz with excess ethyl chloroformate in

chloroform.

Mass spectrometry was used to verify structures, and accurate masses of all fragment ions were obtained to confirm their molecular compositions. Structures of diagnostic ions are provided in Fig. 2-4. The molecular ions of all products were generally of low abundance or were absent, while ions of m/z 91 and 105 were present in all spectra, and an ion of m/z 104 of appreciable intensity was present in all spectra except the N^2-acetates, N^2-AcPz and N^1-N^2-AcPz. Two other fragment ions were also diagnostic. The ion of m/z 89, identified in Fig. 2-4, was dominant only in N^2-ethoxycarbonyl compounds (N^2-ECPz, N^1N^2-EC[illegible] N^1-AcN^2-ECPz); the corresponding m/z 87 fragment was the base peak in all three N^2-acetates (N^2-AcPz, N^1N^2-AcPz, N^1-ECN^2-AcPz). A particularly diagnostic fragmentation pathway was observed in the spectra of the three N^1-acetates (N^1-AcPz, N^1N^2-AcPz, N^1-AcN^2-ECPz); each molecular ion underwent a McLafferty rearrangement as indicated in Fig. 2-4.

2.4. DISCUSSION

Our results indicate that acetic anhydride and ethyl chloroformate reacted selectively with Pz to give either N^1- or N^2-monoacylated products, depending on whether the medium was aqueous or anhydrous. The isomeric monoacyl derivatives of Pz (N^2-AcPz and N^1-AcPz; N^2-ECPz and N^1-ECPz) are readily distinguished.

Their GC retention times differ, as do mass spectral fragmentation pathways (Table 2-1; Fig. 2-4).

Interpretation of their NMR spectra showed that some of the acylated phenelzine were obtained as mixtures of conformational isomers. Hindered rotation in amides is due to the partial double-bond character of the C-N bond which is dependent upon the nature of the substituents on the amide's nitrogen atom.[7] With hydrazides also, the barrier to rotation around the C-N bond may be sufficiently large to produce conformational isomers. NMR studies indicate that four acylated Pz, (N^2-AcPz, N'-AcPz, N^1N^2-AcPz, N^1-AcN^2-ECPz), all acetylated Pz (Fig. 1-1), exibit conformational isomerism. The ratio of major to minor conformer ratios differed in each compound. The effect of temperature on the two monoacetyl-phenelzines (N^2-AcPz and N^1-AcPz) was studied. Spectra were recorded at 235°K, 313°K, 323°K and 355°K. The NMR signals began to coalesce at 313°K in the spectrum of N^2-AcPz, and at 323°K in the spectrum of N^1-AcPz. With both compounds, signal coalescence was complete at 355°K.

2.4.1. Pharmacological Evaluation

The three monoacylated Pz derivatives (N^2-AcPz, N^1-AcPz, N^2-ECPz) have been evaluated[4-6,8,9] for their abilities to inhibit MAO isozymes MAO-A and MAO-B. Results obtained show that like Pz, N^2-AcPz is an MAO-A and MAO-B inhibitor both *in vitro* and *in vivo*, whereas N^2-ECPz is a weak *in vitro* inhibitor of MAO-A and

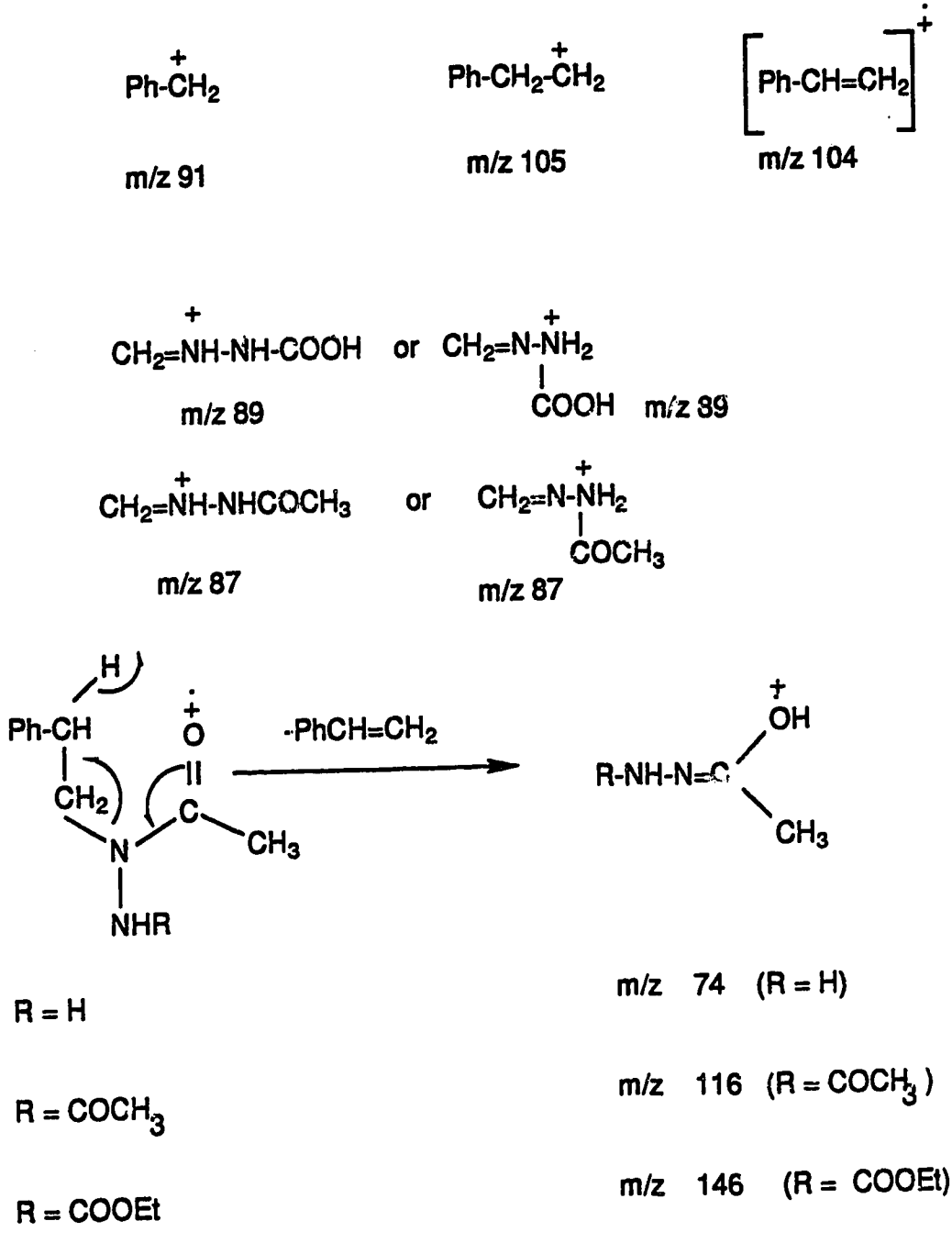

Fig. 2-4. Diagnostic fragmentation ions in the electron-impact mass spectra of acylated phenelzines.

MAO-B, but possesses the properties of a prodrug of Pz in rodents. In the present study, N^1-AcPz was evaluated in vivo for its MAO-inhibiting abilities in the rat model, using a reported method.[10] In this method [^{14}C]-5-hydroxytryptamine and [^{14}C]phenylethylamine, which were diluted with unlabelled compound, were used as specific substrate for MAO-A and MAO-B isoenzymes respectively. N^1-AcPz was virtually inactive. It appears that the presence of a hydrogen atom on the N^1-position of Pz and its derivatives is necessary to preserve MAO-inhibiting activities. The other four compounds await evaluation, but as they are all N^1-substituted, they are unlikely to be pharmacologically active compounds.

Further evaluation of N^2-AcPz is warranted. The antiarrhythmic drug, procainamide, is known to precipitate lupus erythematosus whereas N-acetylprocainamide is free of this toxic effect.[11] Pz has many undesirable side effects,[11] including a lupus-like reaction.[12] It is conceivable that while N^2-AcPz is comparable in MAO-inhibiting activity to Pz, the former may lack some of the toxic properties of Pz.

2.5. References

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3. GAS CHROMATOGRAPHIC ANALUSIS OF PHENELZINE*

3.1. INTRODUCTION

The analysis of phenelzine (Pz), a monoalkylated hydrazine, in biological samples is known to be complicated by poor extraction efficiencies possibly due to auto-oxidation and decomposition under alkaline conditions.[1,2,3] In addition, reactions of hydrazines with acylating reagents frequently give rise to multiple derivatives[4,5,6] and steps must be incorporated into assay procedures to compensate for these undesired reactions. Jindal et al.[1] have demonstrated that Pz reacts quantitatively in aqueous solution with pentafluorobenzaldehyde (PFBA) to form the corresponding hydrazone derivative, which can be assayed by a mass spectrometric procedure. Furthermore, Dyck[4] has demonstrated that the acetone hydrazone of Pz readily reacts with dansyl chloride to form a monodansylated hydrazone. These two observations suggested that monoalkylhydrazines might be conveniently analyzed in biological systems by converting them to PFBA hydrazones prior to their reaction with a perfluoroacylating reagent to produce single derivatives sensitive to gas chromatographic (GC) analysis with electron-capture detection (ECD).

Described below is an assay procedure based upon dual derivatization with PFBA and pentafluoropropionic anhydride (PFPA), by which Pz as well as simple monoalkylated hydrazines such as methylhydrazine (MH), pheniprazine (PPz) and benzylhydrazine (BzH)

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* A version of this chapter has been published: A. Mozayani, R.T. Coutts and T.J. Danielson. J. Chromatogr. 423,131-137 (1987).

can be determined in aqueous biological samples.

3.2. EXPERIMENTAL

3.2.1. Chemicals and Reagents

Pz sulfate and BzH dihydrochloride were obtained from Sigma Chemical Co. (St Louis, MO, U.S.A.). MH, PFBA and PFPA were obtained from Aldrich (Milwaukee, WI, U.S.A.). (±)-PPz hydrochloride was a gift from Merrell Pharmaceuticals (Concord, Ontario, Canada).

All solvents were ACS grade (Fisher Scientific, Fair Lawn, NJ, U.S.A.) and glass-distilled prior to use. Water was distilled and deionized using a Milli-Q reagent water system (Millipore, Bedford, MA, U.S.A.).

3.2.2. Instrumentation

Analyses were performed using a Perkin Elmer model 3B gas chromatograph equipped with an electron-capture detector (^{63}Ni) and a 20 m DB-5 fused silica capillary column (J&W Scientific, Palo Alto, CA, U.S.A.). Oven temperature was programmed from 110 to 250°C at a rate of 4°C/min. Carrier gas was helium, and argon-methane (95:5) was added as make-up gas.

Mass spectra were obtained on a Hewlett Packard model 5985 mass spectrometer in series with a Hewlett Packard model 5840A gas chromatograph which contained a 30 m DB-5 fused silica capillary column (Hewlett Packard, Palo Alto, CA, U.S.A.). Chromatographic condi-

tions were: initial temperature 80°C, increasing at 20°C/min to 300°C and held for 3 min.

3.2.3. Analysis of Methylhydrazine, Phenelzine, Benzylhydrazine and Pheniprazine in blood

To whole blood (0.6 mL) were added MH, BzH, Pz and PPz (MH 20 ng; others 100 ng). The solution was mixed for 30 seconds on an IKA- Vibrax-VXR multi-tube vortex mixer (Terochem, Edmonton, Canada), and 0.4 M HCO_4 (1 mL) was added to precipitate protein. After centrifugation for 10 min (Fisher microcentrifuge Model 235B at 10,000g), the supernatant was retained and the pH was adjusted to between 6.8 and 7.0 with solid sodium bicarbonate. After addition of 40 µL of PFBA (4%,v/v in dimethylformamide), the samples were shaken at room temperature for 30 min after which time excess solid sodium bicarbonate was added and the solution was extracted with ethyl acetate (5 mL). After shaking for 30 min, the organic layer was retained and 5 µL of n-dodecane was added to prevent complete evaporation of the solvent under a stream of nitrogen (cf. ref. 1). The volume was reduced under a gentle stream of nitrogen to 50 µL, and the organic phase remaining was transferred to a 1.5 mL polypropylene microcentrifuge tube and evaporated to dryness. PFPA (65 µL) and dry ethyl acetate (25 µL) were then added and acylation was allowed to proceed for 30 min at 70°C. The reaction mixture was evaporated to dryness and the residue was dissolved in 0.6 mL of toluene. After washing with 0.5 mL of 0.1 M ammonium hydroxide, a 1 µL portion of the toluene solution was injected into the GC.

3.2.4. Standard Curves

To prepare standard curves, BzH (internal standard, 100 µL of 1 µg/mL free base) and Pz (5, 50, 100, 250, 500, 750, 1000, 2000 µL of 1 µg/mL free base) were added to eight whole blood samples (1.0 mL) which were extracted as described above. A calibration curve was constructed by plotting Pz/BzH peak-area ratio against Pz concentration, using the regression equation (Fig. 3-1).

3.2.5. Analysis of Phenelzine in Blood

Rats (Sprague/Dawley, male, 200-300 g) were fast for 6 h, then anesthetized with ether. An incision (1.5-2.0 cm) was made above the right clavicle. When the jugular vein was exposed and ligated, the cannula was inserted towards the right atrium of the heart. The catheter was looped subcutaneously and exteriorized through an incision behind the right ear. The muscle layer and skin were closed with silk. The jugular cannulated rats were allowed to recover for one day before being administered Pz (15 mg/kg free base). Blood samples were withdrawn 30, 60, 90 and 120 min after drug administration. BzH (100 ng) was added to each sample and derivatization and extraction were carried out as described above.

3.3. RESULTS AND DISCUSSION

Earlier reports on the analysis of Pz have described steps necessary to minimize or compensate for poor extraction efficiencies and formation of multiple derivatives.[1,4-6] The procedure now

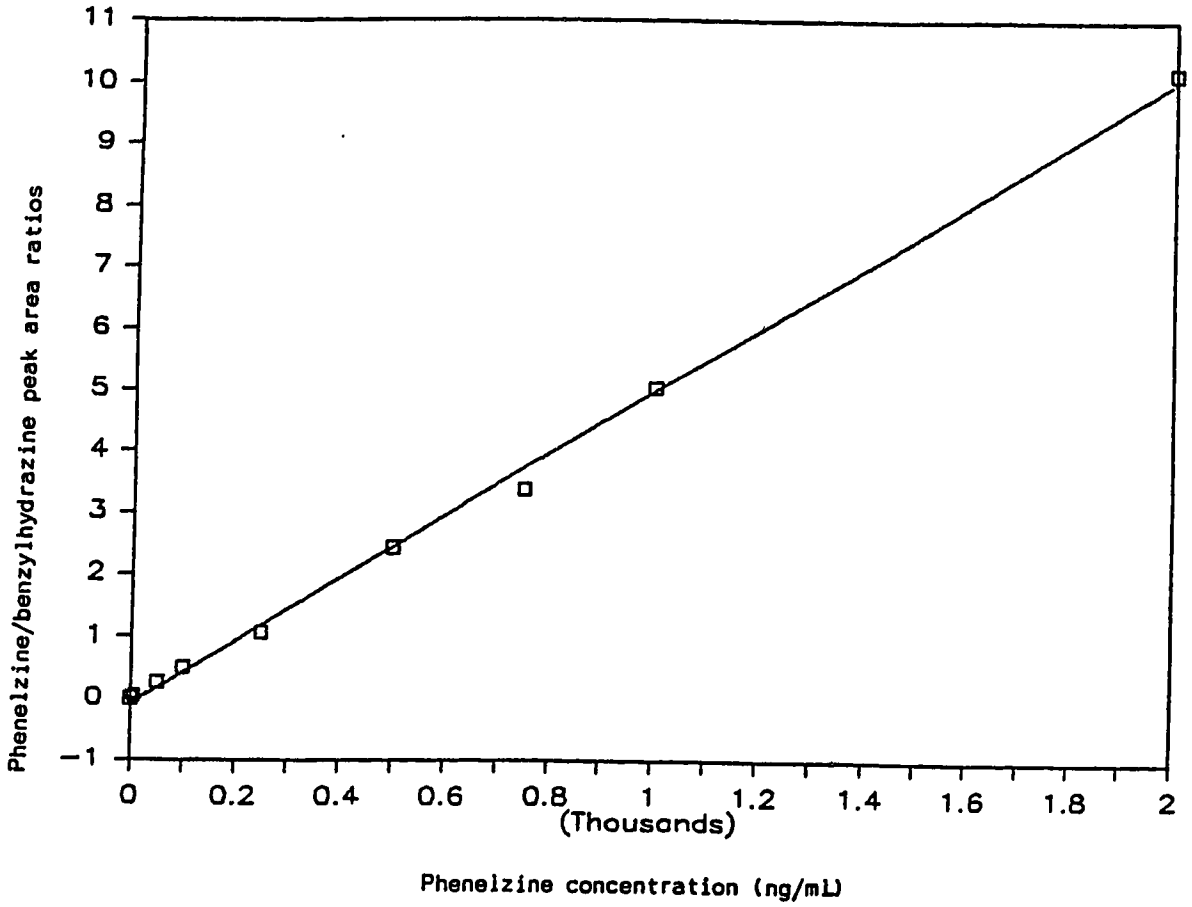

Fig. 3-1. Phenelzine calibration curve in blood.

described is superior to previously reported procedures in that a single, stable derivative of Pz, sensitive to GC-ECD, is obtained. Similar derivatives of three other monoalkyl hydrazines were also obtained; it is possible that this procedure may have broad application.

The reaction sequence involved in this procedure is depicted in Fig. 3-2. By reacting the hydrazine first with PFBA as described by Jindal *et al.*,[1] each was converted to an easily extracted, stable hydrazone. It was determined that these hydrazones were not sufficiently ECD-sensitive, and to increase this characteristic further, derivatization with PFPA was necessary. Whereas others[5,6] have observed the formation of multiple perfluoroacylated derivatives of monoalkylated hydrazines, single derivatives of MH, Pz, BzH and PPz were obtained using these reaction conditions. The initial formation of the hydrazone and the subsequent perfluoroacylating reaction are apparently quantitative. The identity of each derivatized hydrazine was confirmed by mass spectrometry. The electron-impact mass spectra of the four derivatized hydrazines are provided in Fig. 3-3. All spectra contained molecular ions of low abundance. The derivatives of Pz and PPz gave spectra that resulted from similar fragmentation pathways which are depicted in Fig. 3-4. The spectra of derivatized MH and BzH also contained diagnostic ions which are identified in Fig. 3-5.

After derivatization, all four model hydrazines employed in these experiments were chromatographically well separated, both from each other and from interfering substances (Fig. 3-6). GC of mixtures

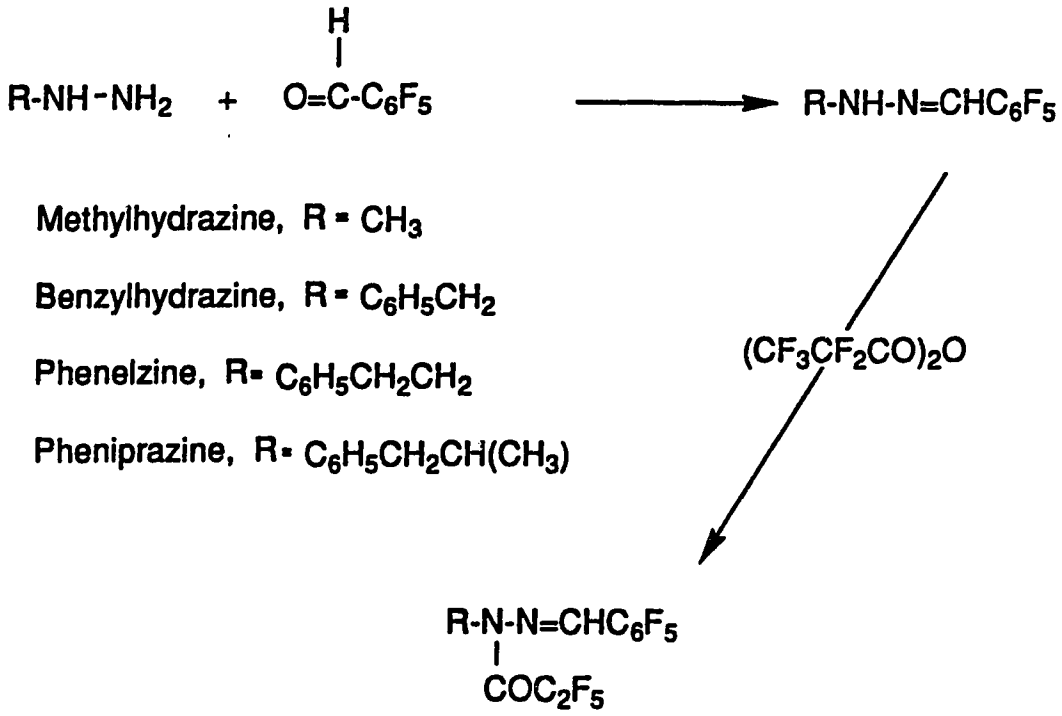

Fig. 3-2. Reaction sequence for generation of perfluoroacylated hydrazone derivatives of monoalkylhydrazines.

of MH, BzH, Pz and PPz, after derivatization, indicated greatest detector sensitivity towards MH and lowest sensitivity toward PPz. The reasons for these differences in detector sensitivity are not known. The high sensitivity towards MH may be of value in the analysis of the low levels of this hydrazine that are found in mushrooms[7] or in biological samples from patients who have ingested MH-containing mushrooms.

Standard curves for the analysis of Pz in whole blood were linear over the range 5-2000 ng/mL when BzH was used as internal standard. Averages of triplicate measurements were plotted; the coefficient of variation was less than 4% at all concentrations except in 5-ng levels for which the coefficient of variation was 10.8%. The line through the data points is described by $y = 0.00506x - 0.0778$ ($r^2 = 0.998$). The limit of detection was 2 ng/mL and sufficiently sensitive to study the kinetics of this antidepressant in human patients.[1] When applied to the analysis of Pz in rat blood, levels detected 30, 60, 90 and 120 min after intravenous injection of Pz (15 mg/kg) were 10.47 ± 1.54, 4.35 ± 0.87, 0.80 ± 0.17 and 0.42 ± 0.12 µg/g (n=4), respectively. These levels are appreciably higher than those reported by Rao et al.[6] in rat brain after a similar dose was administered intraperitoneally. These differences may be due to changes in route of administration, in sampling times or in tissues examined. Our results agree more closely with those of Dyck[4] who reported a Pz concentration of 500 ng/g in rat striatum 2 h after intraperitoneal administration of a higher drug dose.

In summary, it has been demonstrated that the analysis of simple

hydrazine substances in biological samples can be accomplished after conversion of each hydrazine to a hydrazone by treatment with PFBA, followed by perfluoroacylation with PFPA to give a diderivatized product. This procedure generates derivatives which are sensitive to GC-ECD and easily quantified. Using this method, Pz can be readily analyzed quantitatively in rat blood.

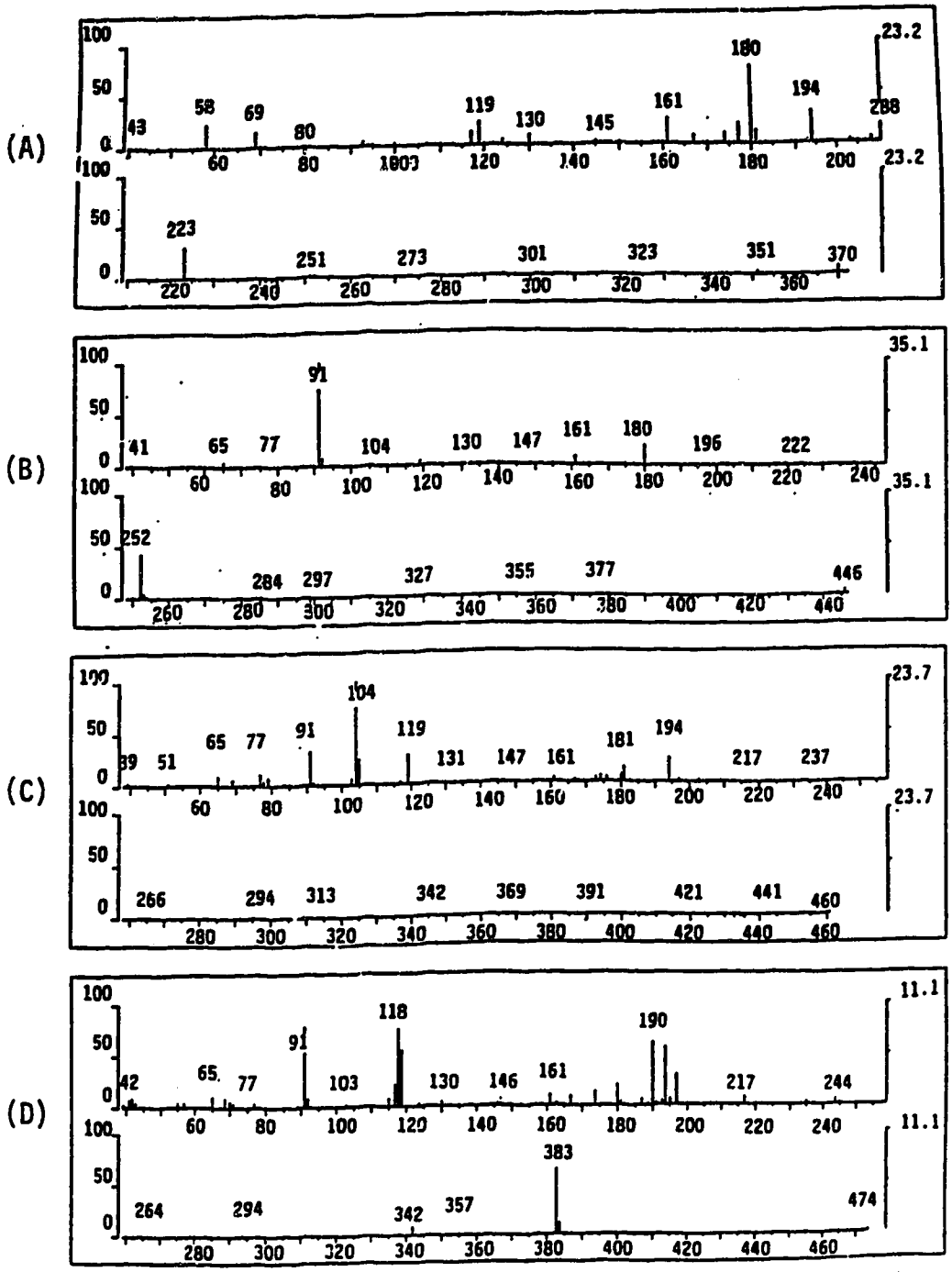

Fig. 3-3. Electron-impact mass spectra of (A) methylhydrazine, (B) benzylhydrazine, (C) phenelzine and (D) pheniprazine after derivatization with PFBA and PFPA. Molecular ions of the derivatives are of m/z 370, 446, 460 and 474, respectively.

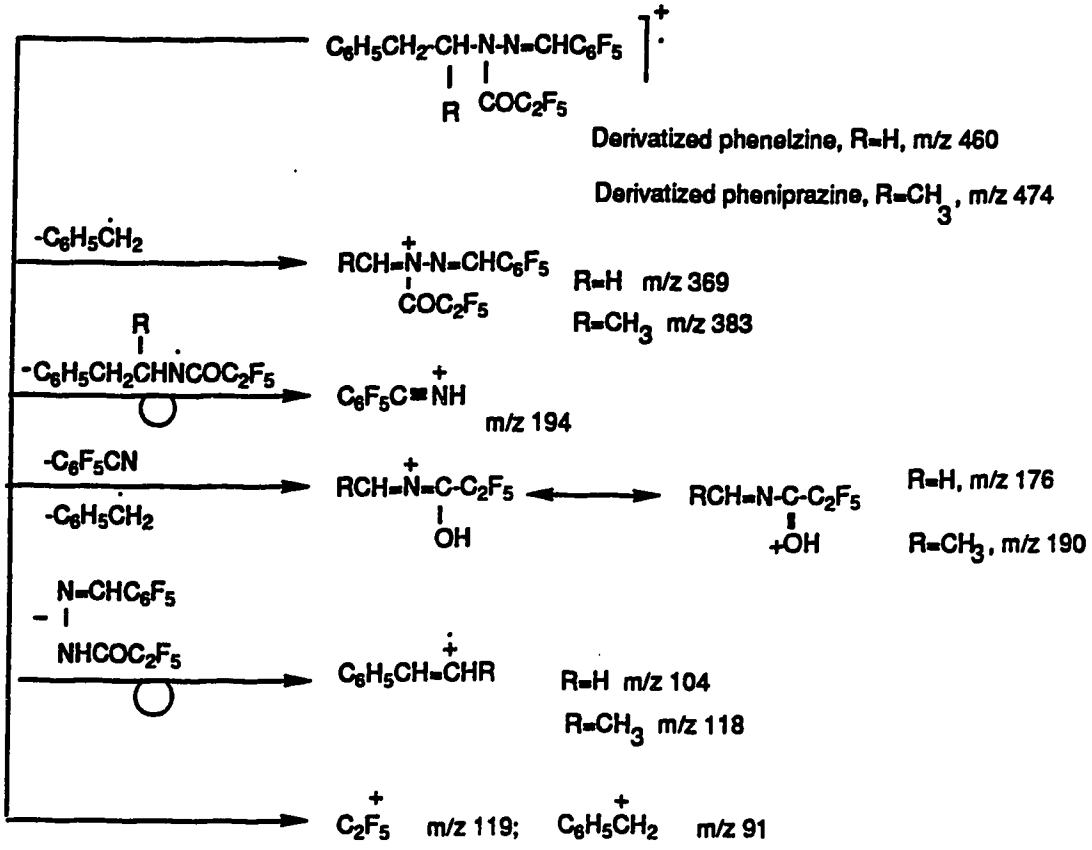

Fig. 3-4. Proposed fragmentation pathways in the electron-impact mass spectra of derivatized phenelzine and pheniprazine.

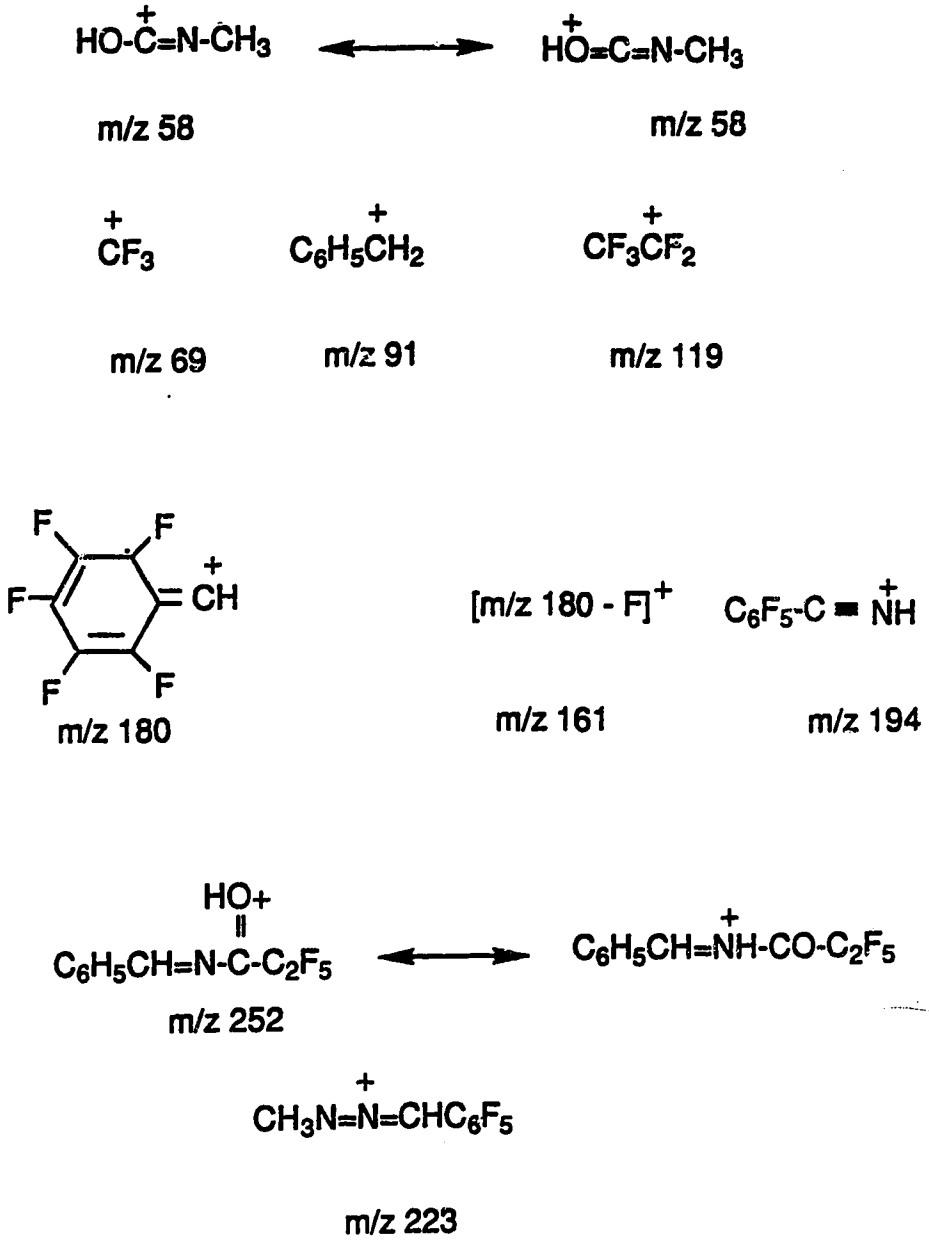

Fig. 3-5. Diagnostic fragment ions in the electron-impact mass spectra of derivatized methylhydrazine and benzylhydrazine.

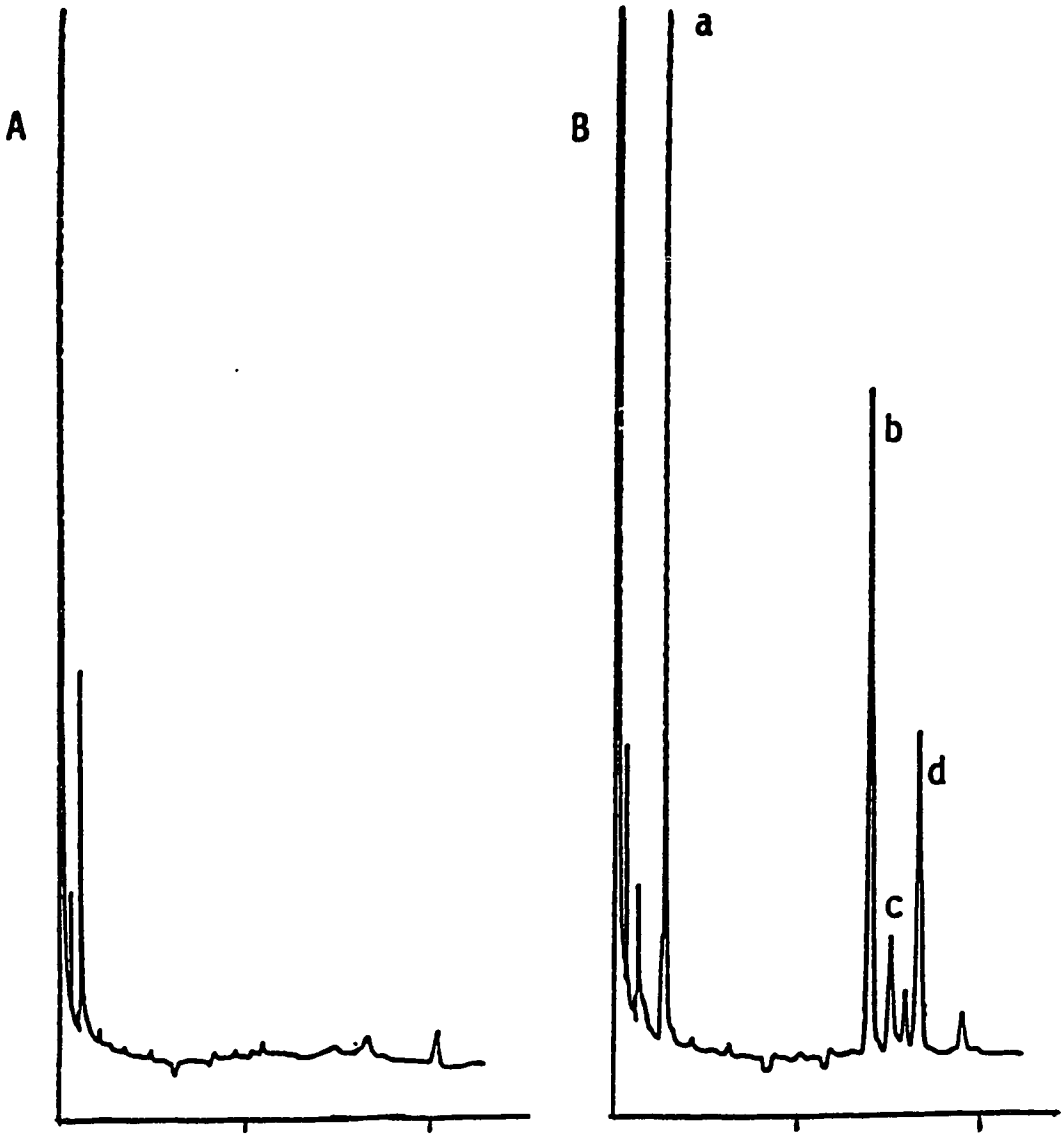

Fig. 3-6. Gas chromatographic traces of derivatized extracts of (A) blank blood and (B) blood containing (a) 20 ng/mL methylhydrazine, (b) 100 ng/mL benzylhydrazine, (c) 100 ng/mL pheniprazine and (d) 100 ng/mL phenelzine.

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4. Metabolic acetylation of phenelzine in microorganisms and rats*

4.1. INTRODUCTION

The antidepressant drug phenelzine [(2-phenylethyl)hydrazine, Pz], a potent inhibitor of monoamine oxidase (MAO) enzymes,[1] has been in clinical use for over 30 years, yet its metabolism in man and animal models is still poorly understood. It is often claimed to undergo metabolic acetylation in humans, based upon three observations: a) other drug substances that are derivatives of hydrazine, including isoniazid and hydralazine, are extensively acetylated *in vivo*;[2] b) Pz is a substrate for human[3] and rabbit[4] N-acetyltransferases *in vitro*; and c) patients who are of the slow acetylator phenotype tend to respond better to Pz but exhibit more frequent side effects than do patients in the fast acetylator group.[5-8]

Despite this circumstantial evidence in support of Pz's metabolism in mammals to an acetylated product, most investigators have concluded that N-acetylphenelzine (N-acetyl-Pz) is not a urinary or plasma metabolite of Pz in humans. The lack of sound evidence in favor of this acetylation pathway has been emphasized.[9] An earlier report[10] is difficult to appraise. While these authors concluded from their studies that N-acetyl-Pz was lacking in the plasma and urine of patients who received 60 mg of Pz daily, they also reported that when combined gas chromatography/mass spectrometry

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*. Versions of this chapter have been published:

1. A. Mozayani, R.T.Coutts, T.J. Danielson and G.B. Baker. Res. Commun. Chem. Pathol. Pharmacol. 62,397-406 (1988).
2. B.C. Foster, R.T. Coutts, F.M. Pasutto, A. Mozayani. Life Sci. 42,285-292 (1988).

(GC/MS, in the selected ion mode) was used as the analytical procedure, N-acetyl-Pz was detectable in the plasma of patients at levels between 0 and 10 ng/mL, and in 24 h human urine samples at levels between 0 and 100 μg/mL. They also claimed that traces of N^1N^2-AcPz were present in urine. Clearly, further information on the metabolic acetylation of Pz in mammals is required.

Metabolic N-acetylation involves the transfer of an acetyl moiety from acetyl coenzyme A. Substrates for this enzymatic reaction are generally believed to be confined to those containing a primary amine or amide function,[1] but there is at least one exception to this generalization; a major fecal metabolite of azaperone in the rat is an N^1-derivatized piperazine which apparently undergoes further metabolism at the secondary N^4-amino group to an N-acetyl derivative.[11] If indeed secondary amines can be substrates for N-acetyl transferase, it raises an interesting question regarding the metabolic acetylation of hydrazines such as Pz. Pz contains both a primary and a secondary amino group. It has always been assumed by investigators that if Pz undergoes metabolic acetylation, it will be converted to the N^2-acetate [$PhCH_2CH_2$-$NHNHCOCH_3$; N^2-AcPz]. However, the isomeric monoacetate [$PhCH_2CH_2N$-($COCH_3$)-NH_2; N^1-AcPz] is, at least theoretically, also a possibility.

A literature search for both monoacetates revealed that only the isomer acetylated on the primary amino group [N^2-AcPz] was known.[12] Therefore, it was decided to synthesize by unequivocal procedures, the two monoacetylated plus the diacetylated derivatives of Pz [N^1-AcPz, N^2-AcPz and N^1N^2-AcPz], in order to deter-

mine initially their gas chromatographic and mass spectrometric properties. When these properties were established, Pz was used as a substrate for metabolic studies with Mycobacterium smegmatis and in rats treated intraperitoneally with a high dose of Pz sulfate. Evidence that Pz is monoacetylated in this microorganism and in rats is presented.

4.2. EXPERIMENTAL

4.2.1. Chemicals

Pz sulfate was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). N^2-AcPz, m.p. 70-71°C was synthesized from phenylacetaldehyde and acetylhydrazine by the method reported in chapter 2.[13] N^1-AcPz, m.p. 67-69°C and N^1N^2-AcPz, m.p. 61-63°C were prepared from Pz and Pz monoacetate respectively, by reaction in an aqueous alkaline ($NaHCO_3$; pH 8) solution of acetic anhydride, as described in chapter 2. Acetylphenylhydrazine (AcPH) was purchased from Aldrich Chemicals (Milwaukee, WI, USA.). All solvents and reagents were analytical grade or higher.

4.2.2. Instrumentation

Combined GC/MS (electron-impact) analyses were performed on a Hewlett Packard Model 5980A mass spectrometer and Model 5934A data system. The injection port and transfer line temperatures were

300°C. The operating conditions for the mass spectrometer were: electron energy, 70 eV; emission current, 35 ma; source temperature, 180°C. A 15 m DB-5, 0.32 mm i.d., fused silica GC column (J & W Scientific, Palo Alto, CA, U.S.A.) was employed. The carrier gas (helium) flow rate was 2 mL/min, and the oven temperature was programmed from 130° to 280°C at 8°C/min. In this condition the retention times (rt) of N^1-AcPz, N^2-AcPz and N^1N^2-AcPz were 14.8, 15.2 and 17.8 min respectively.

4.2.3. Incubation Protocol

Mycobacterium smegmatis ATCC 14468 was maintained in nutrient broth (Difco Laboratories, Detroit, Michigan, U.S.A) at 4°C. Cultures were started by transferring 0.5 mL of stock into 125 mL Erlenmeyer flasks containing 25 mL of nutrient broth (8 g/L) and dextrose (16 g/L). Dextrose was added aseptically after autoclaving. Fresh medium was incubated with 1 mL of overnight culture and filter-sterilized solutions of the substrate (0.2 mg/flask) were added aseptically after a further incubation of 6 h. Cultures were incubated at 37°C in a New Brunswick Scientific Model G-25 gyratory shaker equipped with 45° angle brackets operated at 250 rpm. Culture controls were prepared by incubating the microorganism in the absence of Pz. Samples of each culture were plated on nutrient and Sabouraud dextrose agar (Difco). No contamination was observed. Substrate controls were prepared by incubating substrates in sterile medium.

4.2.4. Animals and Treatment

Two male rats (Sprague-Dawley), weighing 581 and 670 g, were housed in air conditioned animal quarters on a 12 h light-dark cycle, and fed food and water ad lib. Pz sulfate (50 mg kg^{-1}) in normal saline was injected intraperitoneally into these rats, and 1.0 h later they were sacrificed by cerebral dislocation.

4.2.5. Assay Procedures

The whole blood (about 8 mL) of each rat was collected separately in beakers containing solid citric acid (100 mg), and weighed. Whole brains were also collected, weighed, and kept in dry ice until analysis. Blood protein was precipitated by the addition of an equivalent volume of perchloric acid (0.4 N). Brains were separately homogenized in perchloric acid (10 mL). The precipitated protein was removed by centrifuging the samples (blood or brain) at 10,000 rpm for 15 min at 4°C in a Damon/IEC B-20A refrigerated centrifuge, and the blood and brain supernatants were collected in separate tubes, basified with excess solid sodium bicarbonate and extracted with ethyl acetate (5 mL) by shaking on a vortex mixer for 30 min at room temperature. After centrifugation, each organic layer was separated and evaporated under nitrogen. Ethyl acetate (10 µL) was added to each residue, and an aliquot (1.5 µL) was injected into the GC/MS instrument. A total ion trace, and selected ion traces (m/z 45, 74 and 87) were simultaneously recorded. As control, two male rats of similar weight received injections of normal saline and were then treated as described above for the rats

that received Pz sulphate.

4.2.6. Sampling protocol

Incubation broths were analyzed by withrawing 2 mL aliquots at various times up to 168 h. A 1 mL sample was made alkaline (solid potassium carbonate) and extracted three times with five volumes of a diethyl ether/methylene chloride mixture (55:45 v/v). The solvents were freshly distilled prior to use. The extracts were combined and concentrated at 50°C to approximately 50 µL. A portion of extract obtained by this method was reacted with an equal volume of acetic anhydride by a previously reported method.[14] The final extracts were then analyzed by GC/MS.

4.3. RESULTS

Extracts of incubation broths of the microorganism and blood and brain of rats treated with Pz were found to contain two metabolites and trace amounts of substrate when examined by GC and GC/MS. The mass spectrum of the metabolite contained diagnostic fragment ions (m/z 105, 91, 87,45 and 43) consistent with those expected for a mono-N-acetyl derivative of Pz. This was confirmed when a portion of the original extract of the M.smegmatis incubation broths was reacted with acetic anhydride and re-examined by GC. The peaks ascribed to Pz and its N-acetyl derivative were now absent from the GC trace, having been replaced by a single new peak which was found to have GC/MS properties identical to those of authentic N^1N^2-

AcPz. However, the position of the N-acetyl group in the mono-N-acetyl metabolite was not readily discernible from the MS fragmentation data. Two structures were possible, and further characterization of the metabolite was required. Both isomers were synthesized and found to possess different GC and MS properties (Table 2-1). The metabolite produced by M.smegmatis displayed properties identical to those of N^2-AcPz.

All incubation broths were made alkaline prior to extraction in order to recover neutral and basic metabolites and unmetabolized substrate. These extracts were analyzed by GC/MS to confirm that GC peaks of metabolites and substrates were free from interfering peaks. The levels of recovered metabolites were not sufficient to account for the total amount of added substrates. Instability of these substrates in alkaline solution, their reaction with media and cellular constituents, the possibility of additional metabolic pathways and autooxidation[15] may account for the portion not detected by the extraction/derivatization procedures employed.

A calibration graph was constructed by spiking control rat blood samples with various amounts of N^2-AcPz (0.10 - 50 µg/mL) and AcPH (20 µg; internal reference compound), and subjecting these samples to the same procedure used for the blood and brain samples obtained from drug-treated rats. Reference to this calibration graph showed that the blood and brain samples from one rat contained 41.0 and 84.5 ng/g, respectively, while those samples from the other rat contained 33.6 and 132.0 ng/g, respectively, of N^2-AcPz.

4.4. DISCUSSION

GC retention times and mass spectra of authentic samples of N^1-AcPz, N^2-AcPz and N^1N^2-AcPz were determined. The data indicate that these compounds differ with respect to retention times and fragmentation patterns. Thus, for example, the fragment ion, m/z 74, is a prominent ion in the spectrum of N^1-AcPz (49%, rt 14.8 min) and N^1N^2-AcPz (10%, rt 17.8 min), but is absent from the spectrum of N^2-AcPz (rt 15.2 min). In contrast the fragment ion, appearing at m/z 87 is the base peak in the spectra of N^2-AcPz and N^1N^2-AcPz, but is much lower abundance in the spectrum of N^1-AcPz. These differences were employed to monitor extracts, of either rat tissue or a microbiological incubate, for acetylated metabolites formed after exposure to Pz. Structures for these ions are suggested in Fig. 4-1.

The GC/MS selected ion and total ion current chromatograms of the final extract of the rat are shown in Fig. 4-2. Similar traces were obtained when brain or incubate broth were assayed. The mass spectrum of the componnt with a rt of 15.2 contained an ion at m/z 87, but not an ion at m/z 74, and that no peaks containing these ions were detected at retention times 14.8 or 17.8 min. These observations are consistent with the conclusion that N^2-AcPz was formed as the sole acetylated metabolite of Pz in each of these systems. The electron impact mass spectra of this compound was identical to that of authentic N^2-AcPz, confirming the identity of this metabolite. Chromatograms of tissue extracts from untreated rats did not contain any evidence of these compounds.

By unequivocal identification of N^2-AcPz as a metabolite of Pz in the rat, and in a microbiological incubate, the current data suggest that biological acetylation may be a common, albiet minor, metabolic route in a rqange of systems, possibly including man. In a similar vein, 2-phenylethylamine has also been identified as a metabolite of Pz in each of these systems, suggesting that deamination of hydrazines may also be a metabolic pathway shared by variety of species.[16,17] These observations may be of significance in view of the very different pharmacologies of these metabolites, compared to Pz itself.[13,16]

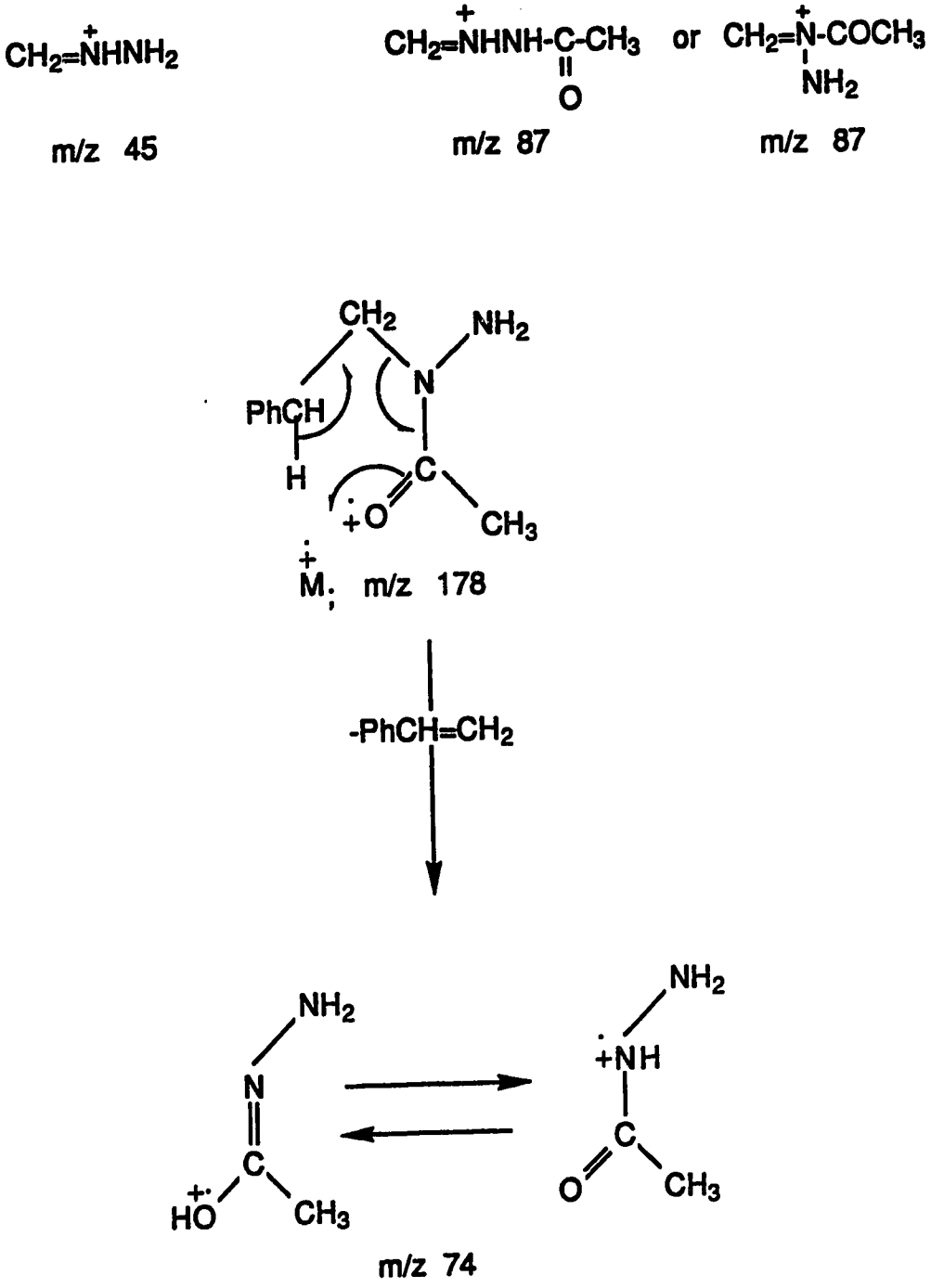

Fig. 4-1. Structures of abundant or diagnostic ions in the mass spectra of acetylated derivatives of phenelzine.

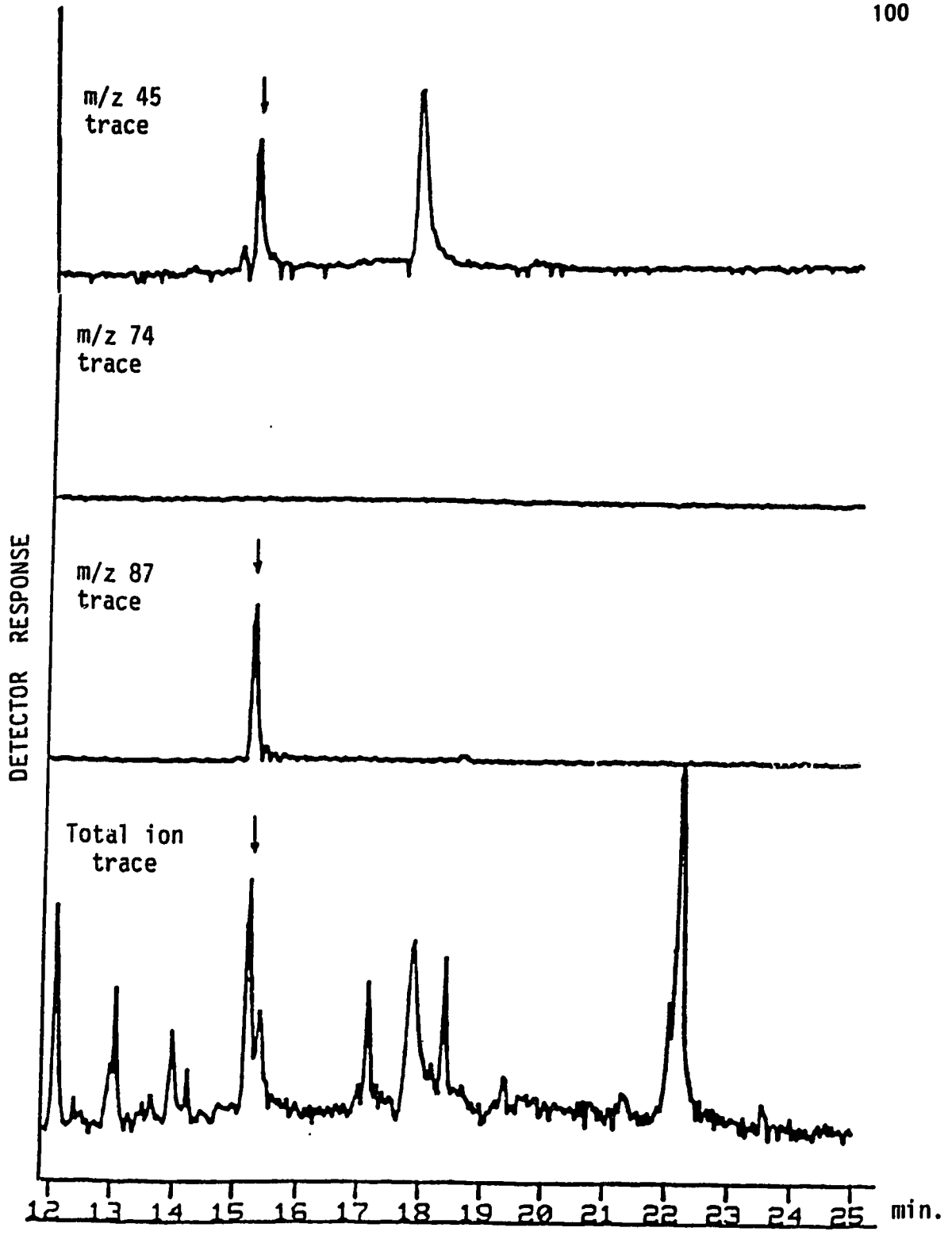

Fig. 4-2. Total and selected ion GC/MS traces of an extract of blood from a rat that received phenelzine sulfate. The arrows indicate the GC peaks of the metabolite, N^2-AcPz.

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5. TISSUE LEVELS AND SOME PHARMACOLOGICAL PROPERTIES OF AN ACETYLATED METABOLITE OF PHENELZINE IN THE RAT.

5.1. INTRODUCTION

The role of acetylation in the metabolism and clinical pharmacology of phenelzine [(2-phenylethyl)hydrazine, Pz] has been controversial since the observation was made that clinical response to Pz appeared to vary among acetylator phenotypes.[1-3] However, although Pz does act as a substrate for human[4] and rabbit[5] N-acetyltransferase in in vitro experiments, the ability of the human to acetylate phenelzine in vivo has been questioned.[6] It has been recently demonstrated that acetylation of Pz to N^2-AcPz does occur in vivo in the rat.[7]

Since this acetylated metabolite (N^2-AcPz) also possesses an ability to inhibit monoamine oxidase (MAO) enzymes[8,9] in rodents, it was considered necessary to try to estimate the pharmacological consequences of Pz acetylation in the rat, as a potential model of man.

5.2. MATERIALS AND METHODS

5.2.1. Drugs and Standards:

Phenelzine sulfate, (-)-norepinephrine (NE) hydrochloride, 5-hydroxytryptamine (5-HT), creatinine sulfate, 3,4-dihydroxyphenylethylamine (dopamine, DA) hydrochloride and pentafluorobenzoyl chloride (PFBC) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). N^2-Acetylphenelzine (N^2-AcPz) was synthesized

according to a previously published procedure.[8]

Acetylphenylhydrazine (AcPH), pentafluoropropionic anhydride (PFPA) and pentafluorobenzaldehyde (PFBA) were purchased from Aldrich Chemicals (Milwaukee, WI, U.S.A.).

N^1-Acetylphenelzine (N^1-AcPz) was prepared from Pz by its reaction with acetic anhydride in a two-phase (water/chloroform) system containing sodium bicarbonate.[10] Radiolabeled substrates (5-[2-^{14}C]-hydroxytryptamine binoxalate, 55.0 mCi $mmol^{-1}$ and β-[ethyl-1-^{14}C]-phenylethylamine hydrochloride, 50.2 mCi $mmol^{-1}$) were obtained from Dupont Canada Inc (Mississauga, Ontario, Canada). All reagents used were of the highest quality available. All solvents were "distilled-in-glass" grade.

5.2.2. In vivo Acetylation of Pz:

Male rats (Sprague-Dawley, weight 230 - 300 g) were administered a saline solution (2 $ml.kg^{-1}$) of Pz (0.38 $mmol.kg^{-1}$, free base) as a single intraperitoneal (ip) injection. Groups of five rats were sacrificed by decapitation at 0, 15, 30, 45, 60, 120 and 180 min after injection. Trunk blood (3-5) mL was collected on solid citric acid (100 mg) and was weighed. Liver tissue was removed and frozen by immersion in liquid nitrogen. Samples were stored in polyethylene vials at -80^{o}C for a maximum period of 14 days prior to analysis. Just prior to analysis each partially thawed sample was homogenized in cold perchloric acid ($HClO_4$, 0.1 N, 5 vol.) and centrifuged (12,000 rpm, 15 min, 4^{0}C). Each supernatant was divided into two portions, 1/3 and 2/3 of the total samples. The

smaller portion was assayed for Pz and the larger portion for N^2-AcPz as described below.

5.2.3. Inhibition of MAO, Effect on Biogenic Amines and Deacetylation of N^1-AcPz and N^2-AcPz in vivo:

Male rats (Sprague-Dawley, 230-300 g) were administered Pz, N^1-AcPz or N^2-AcPz (0.1 or 0.2 $mmol.kg^{-1}$) by ip injection of solutions (2 mL/kg) in dilute phosphate buffer (0.01 M, pH 7.4). After 90 min, animals were decapitated and trunk blood was collected into polyethylene vials and weighed. Brains were immediately removed and frozen in a dry ice/isopentane bath. All samples were stored at -80°C until analysis (within 14 days). Just prior to analysis, brain and blood samples were allowed to partially thaw. Entire blood samples were homogenized in cold perchloric acid ($HClO_4$, 0.2 N, 1 vol.). After centrifugation (12,000 rpm, 15 min, 4°C), the supernatants were assayed for Pz and N^2AcPz as described below. Brains were divided in half longitudinally. One half was weighed and then homogenized in cold isotonic KCl for determination of MAO activity in an assay system where [^{14}C]-hydroxytryptamine and [^{14}C]-phenylethylamine were employed as substrates, for MAO-A and MAO-B respectively.[9,11] The second half of each brain was also weighed and then was homogenized in $HClO_4$ (0.1 N, 5 volumes) containing disodium EDTA (0.1 g/L) and ascorbic acid (0.05 mM). After centrifugation (12,000 rpm, 15 min, 4°C), portions of the supernatants were assayed for biogenic amines by high pressure liquid chromatography (HPLC) with electrochemical detection,[12] or for Pz and N^2-AcPz as described below.

5.2.4. Analysis of Phenelzine :

Pz was determined according to the method described in chapter 3.[3]

5.2.5. Analysis of N^2-Acetylphenelzine:

Suspensions of whole homogenates of blood, brain or liver, prepared as described above, were used. Internal reference compound AcPH (100 µL of a 20 µg/mL solution in methanol) and excess solid $NaHCO_3$ were added to each supernatant prior to extraction with ethyl acetate (5 mL). The organic layers were transferred to clean glass tubes, PFBC (5 µL) was added, and acylation was allowed to proceed at room temperature for 20 min. Ethyl acetate was then evaporated and the residue remaining was dissolved in toluene (1.0 mL). Excess reagents were removed by extraction with a saturated aqueous solution of sodium borate (2 mL). A portion (1 µL) of the toluene layer was then injected into the GC.

The amount of N^2-AcPz was quantified against sets of standard samples prepared by spiking blank blood and homogenized liver or brain with N^2-AcPz. Final concentrations of N^2-AcPz were 10, 50, 100, 500, 1000 and 2000 ng/g. The solutions were subjected to analysis according to the method described for unknown samples.

Quantitation was achieved by comparison of sample peak area ratios (unknown/internal standard) to a plot of the peak area ratios obtained by analysis of standard samples, versus concentration. Calibration curves (Fig. 5-1) were prepared for each analytical series .

5.2.6. Instrumentation:

GC analyses were conducted using a Perkin Elmer Sigma 3 B gas chromatograph equipped with an electron-capture detector (^{63}Ni) and a fused silica capillary column (30 m, 0.32 mm I.D., DB-5). The helium carrier gas flow was adjusted to 2 $mL.min^{-1}$. Argon-methane (95:5, 35 mL min^{-1}) was added as make-up gas. The oven temperature was programmed to increase from 80 to 230°C at a rate of $10°C.min^{-1}$ for analysis of N^2-AcPz. For Pz analysis the temperature program was 110 to 250°C at 4°C min^{-1}.

Combined gas chromatography/mass spectrometry (GC/MS) was performed on a Hewlett Packard model 5985 mass spectrometer equipped with a Hewlett Packard model 5840-A gas chromatograph containing a DB-5 fused silica column (10 m, 0.32 mm I.D.). Chromatographic conditions were: initial temperature 80° (1 min), increasing at $20°C.min^{-1}$ to 300°, and maintained for 3 min.

5.3. RESULTS

The reaction involved in the assay of N^2-AcPz is depicted in Fig. 5-2. Mass spectrometric examination indicated molecular ions and fragmentation pathways consistent with assigned structures (Fig. 5-3 and Fig 5-4). Linear correlation between peak area ratios (N^2-AcPz/IS) and N^2-AcPz concentrations was found (r^2>0.995) over the N^2-AcPz concentration range of 10 to 2000 ng/g for blood, brain and liver tissues. The best fit lines passing through the data points were described using the peak area ratio (y), and the

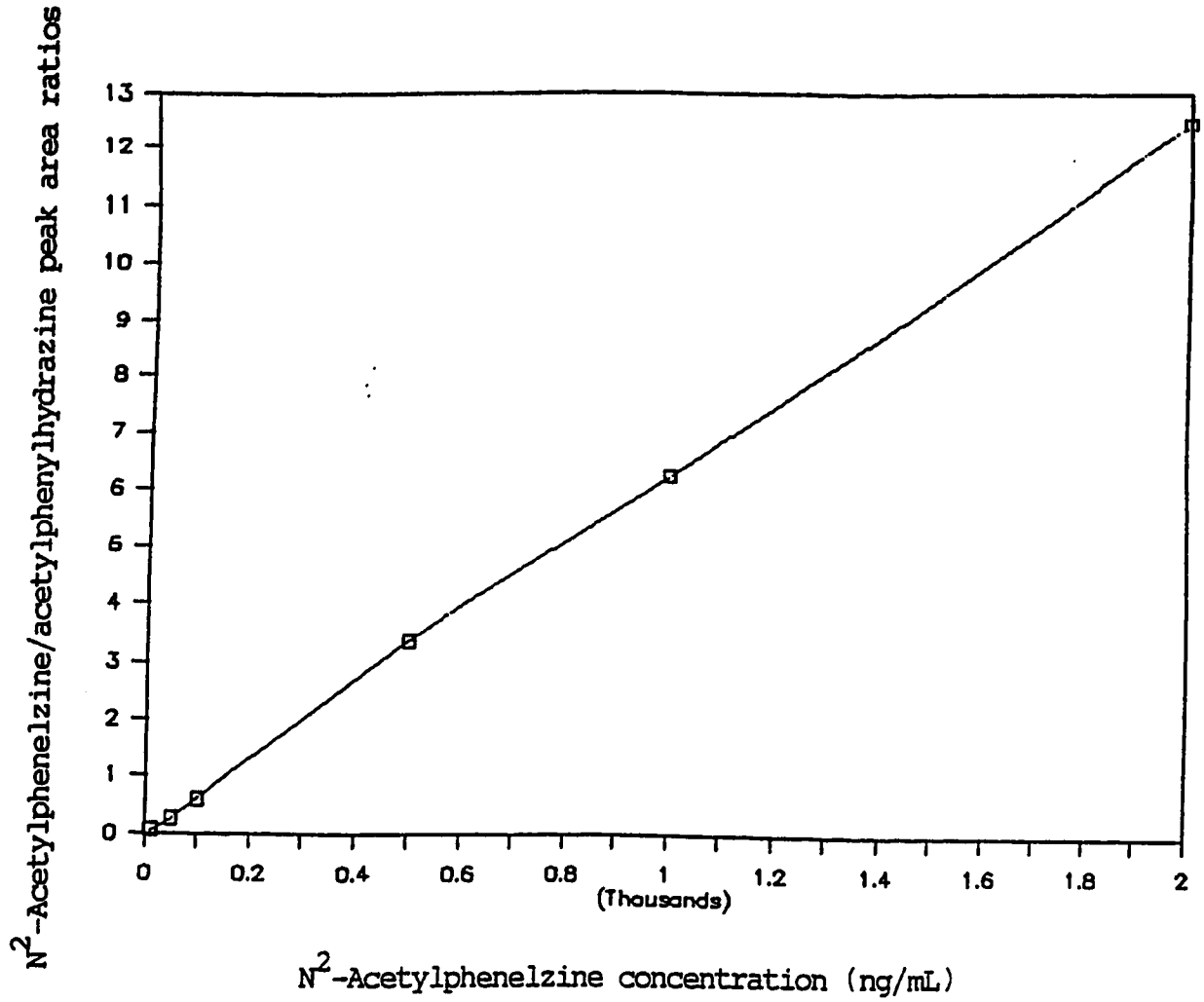


Fig. 5-1. N^2-Acetylphenelzine calibration curve in blood.

concentration in ng/g (x), where $y = .022x + .006$ and $y = .067x + .0026$ for N^2-AcPz concentration in blood and liver, respectively. After derivatization N^2-AcPz and AcPH (IS) were chromatographically well separated (Fig. 5-5) and on-column sensitivity was less than 5 pg for each compound.

Data in Table 5-1. indicate that Pz and N^2-AcPz were each present in blood and liver obtained from rodents treated with Pz. Peak levels of N^2-AcPz were only approximately 5-10% of the maximum concentration of Pz. Data in Table 5-1 also may suggest that the elimination of Pz from blood or liver is more efficient than that of N^2-AcPz. No chromatographic peak assignable to N^1-AcPz was observed in any tissue subsequent to treatment with Pz.

Data in Table 5-2. indicate the relative effects of Pz, N^1AcPz and N^2-AcPz on MAO enzymes. These data show that Pz and N^2-AcPz each produced 80% or greater inhibition at each dose level, 90 min after drug treatment. N^1-AcPz, in comparison, produced 16% or less inhibition, even at the highest dose tested (0.2 $mmol.kg^{-1}$).

Data in Table 5-3. are consistent with observations made in the MAO-inhibition studies described above. Thus, treatments with either Pz or N^2-AcPz at the 0.2 $mmol.kg^{-1}$ dose level each resulted in increased brain levels of the neurotransmitter amines (NE, DA and 5-HT). At the same dose level, N^1-AcPz failed to modify levels of these amines. The reduced effect of N^2-AcPz on biogenic amines at the 0.1 $mmol.kg^{-1}$ dose level is consistent with previous observation that N^2-AcPz was slightly less potent <u>in vivo</u> than Pz as an inhibitor of MAO (cf. Table 5-2.).

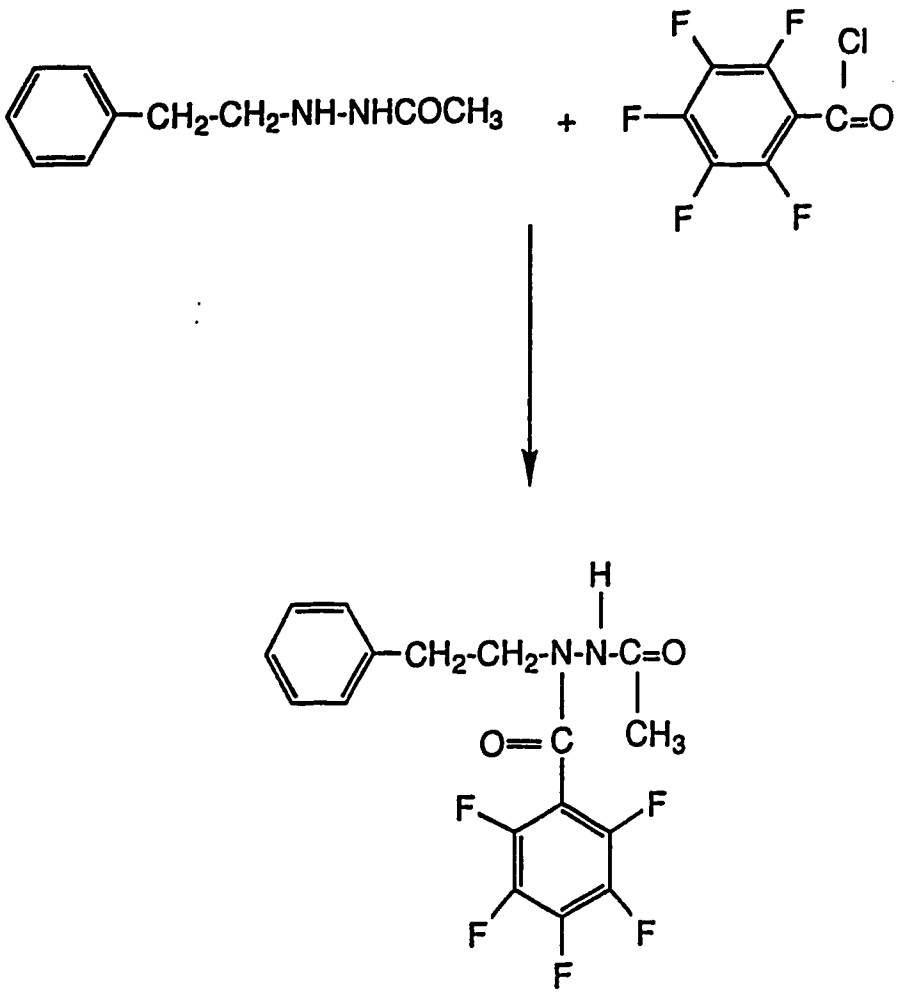
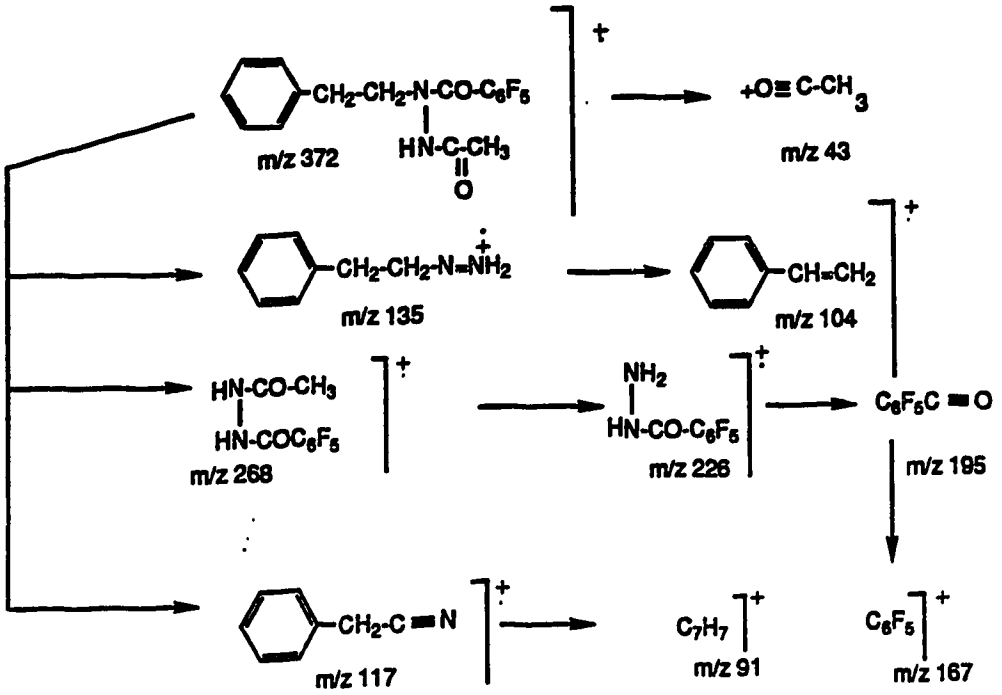


Fig. 5-2. Reaction of N^2-acetylphenelzine with pentafluorobenzoyl chloride.



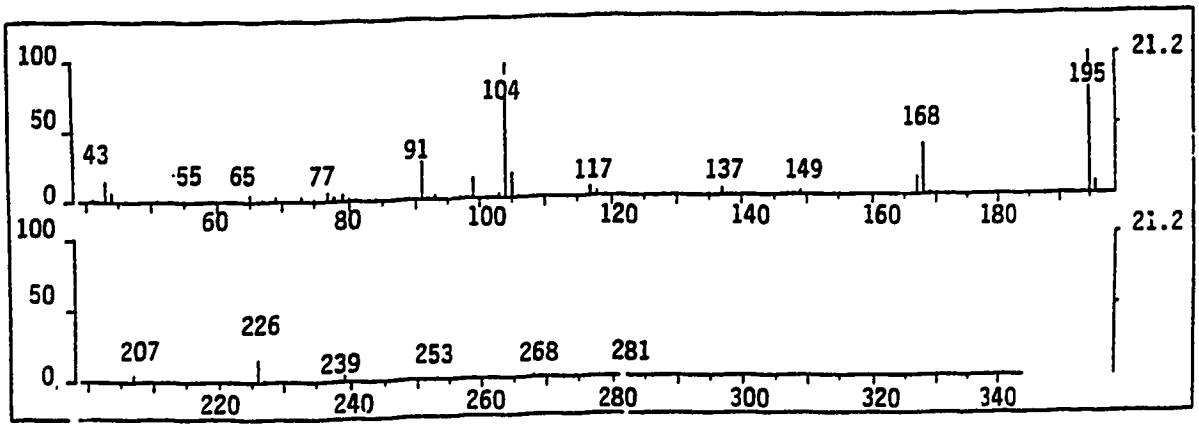
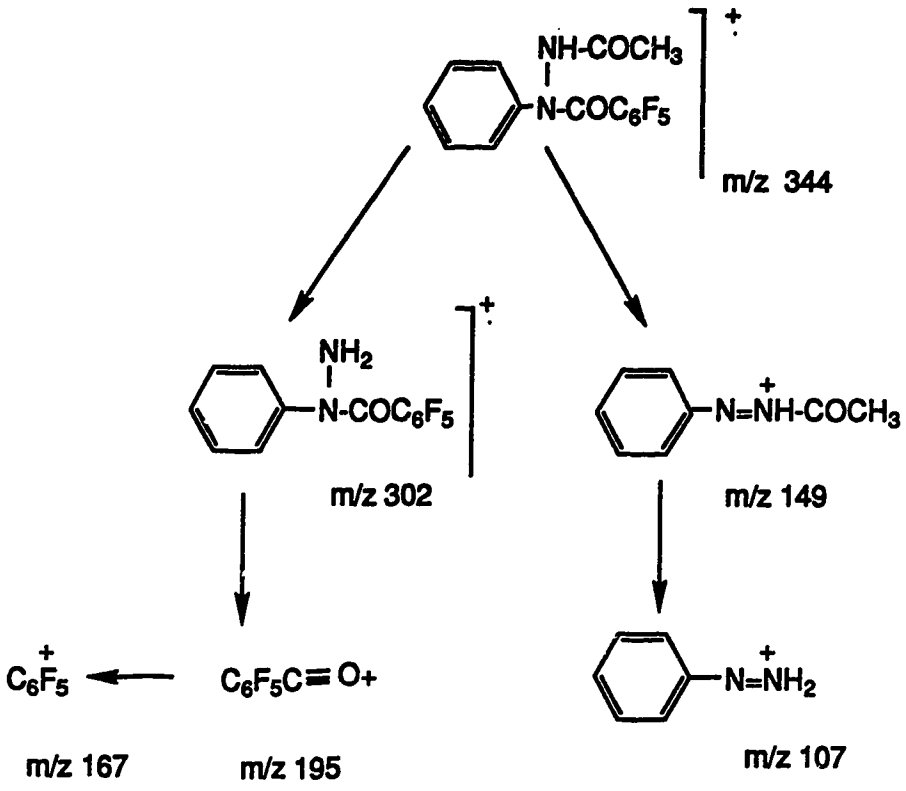


Fig. 5-3. Electron-impact mass spectra and proposed fragmentation pathways of N^2-acetylphenelzine after derivatization with pentafluorobenzoyl chloride.



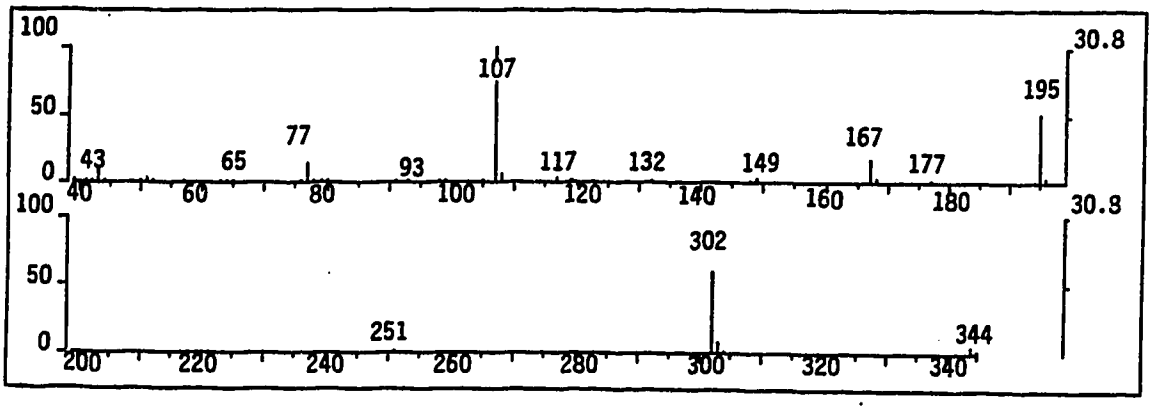


Fig. 5-4. Electron-impact mass spectra and proposed fragmentation pathways of acetylphenylhydrazine after derivatization with pentafluorobenzoyl chloride.

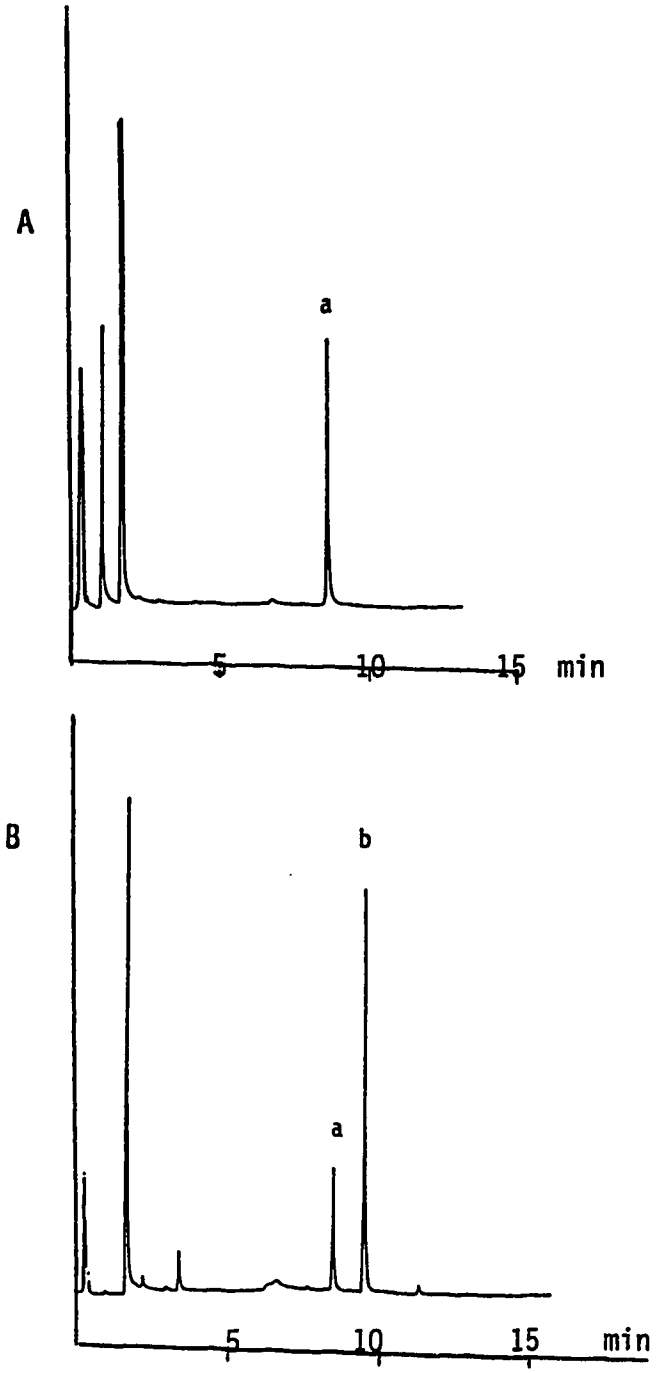


Fig. 5-5. Gas chromatographic traces of derivatized extracts of blood containing (A) 2 µg/mL acetylphenylhydrazine (peak a) and (B) 2 µg/mL acetylphenylhydrazine (peak a) plus 100 ng/mL N^2-acetylphenelzine (peak b).

Table 5-1. Levels of Phenelzine and N^2-AcPz in Rat Blood and Liver After ip Administration of Phenelzine (0.38 mmol.kg^{-1}; ng/g ± SEM; n=5).

Time	Levels Measured in Blood		Liver	
(min)	Pz	N^2-AcPz	Pz	N^2-AcPz
15	187±24	14±2	222±5	7±1
30	765±12	25±2	327±17	49±5
45	1144±3	59±3	958±91	103±8
60	1724±15	36±4	336±42	82±6
120	108±15	20±2	153±9	36±1
180	24±5	10±1	46±7	28±4

Table 5-2. Percentage Inhibition of Monoamine Oxidase Enzymes in Brain 90 Min After ip Administration of Phenelzine, N^1-AcPz or N^2-AcPz (mean ± SEM; n=5).

Drug	Dose ($mmol.kg^{-1}$)	MAO Type A	MAO Type B
Pz	0.1	97±2	83±2
	0.2	98±1	88±1
N^1-AcPz	0.1	4±2	5±2
	0.2	16±2	13±7
N^2-AcPz	0.1	90±2	80±8
	0.2	97±1	93±3

Data in Table 5-4. demonstrate that N^1-AcPz and N^2-AcPz were each hydrolyzed *in vivo* and generated blood levels of Pz approximately 10% of those measured after treatment with an equimolar dose (0.1 mmol.kg^{-1}) of Pz itself.

Regardless of whether it was derived from the administration of Pz or N^2-AcPz, ratios of the mean levels of Pz in blood *vs* brain were consistently between 1.1 and 2.2. In comparison, the ratio of the mean levels of N^2-AcPz in blood *vs* brain was greater than 7.0 after treatment with N^2-AcPz. This suggests that entry of N^2-AcPz into brain may be restricted and that Pz in brain after administation of N^2-AcPz may originate from hydrolysis in the periphery.

5.4. DISCUSSION

The data now reported were collected to meet two primary objectives. These were to confirm the previous observations[7] that N^2-AcPz was a metabolite of Pz in rat and to determine whether this metabolite might contribute to the overall response to Pz in that species. The data do confirm the metabolic generation of N^2-AcPz in the rat and also indicate that this metabolite possesses pharmacological properties similar to those of Pz but with reduced potency.

The contribution of metabolically-derived N^2-AcPz to the response to Pz by this species cannot be unequivocally established from the present data. Levels of N^2-AcPz measured in blood after administration of a high dose of Pz (0.38 mmol.kg^{-1}) approximated

to those measured after administration of a lower dose of N^2-AcPz (0.1 $mmol.kg^{-1}$) which inhibited MAO enzymes but only slightly increased brain levels of biogenic amines. On this basis it would appear unlikely that the even lower levels of N^2AcPz expected after treatment with a low, but still effective, dose of Pz could contribute in any pharmacologically significant way to the observed MAO inhibition.

As reported previously,[14-17] N^2-AcPz is less potent as an *in vivo* inhibitor of MAO enzymes than is Pz. This fact has previously been explained as a less than quantitative conversion to Pz. However, since N^1-AcPz and N^2-AcPZ did not appear to differ in any major way as sources of Pz *in vivo*, while only N^2-AcPz inhibited brain MAO enzymes, it would appear that this effect was due to N^2-AcPz itself, and did not require its hydrolysis to Pz. In addition, the data suggest that entry of N^2-AcPz into the brain is reduced relative to that of Pz. Therefore, reduced efficacy of N^2-AcPz as a MAO inhibitor may be a combination of reduced potency and restric- ted entry into the brain. Whether N^2-AcPz possesses other pharmacological activities remains to be determined.

Various authors[1-3,18-20] have reported conflicting results on the relationship between acetylator status and clinical outcome or side effects after treatment with Pz. In a previous report[9] it has been observed that N^2-AcPz induced a motor deficit in mice more readily than did Pz, possibly suggesting an increased tendency towards an unwanted effect with N^2-AcPz. In contrast, others have

suggested that acetylation of Pz and similar compounds might reduce the frequency of adverse reactions such as systemic lupus erythematosus[21] or pyridoxine antagonism.[10,22,23] The possibility of regional differences in the accumulation of N^2-AcPz within the brain has also not been addressed and could dramatically alter interpretation of the significance of metabolically- derived N^2-AcPz.

In conclusion, although metabolically-derived N^2-AcPz might not play a significant role in MAO inhibition after treatment with Pz, further experiments are required to fully define the pharmacological importance of this metabolite.

Table 5-3. Brain Levels of Biogenic Amines 90 min After ip Treatment of Rats with Phenelzine, N^1-AcPz or N^2-AcPz ($ng.g^{-1}$ ± SEM, n=6).

Drug	Dose ($mmol.kg^{-1}$)	NE	DA	5-HT
	0	389±24	685±53	411±26
Pz	0.1	516±28*	882±56*	1027±131*
	0.2	572±27*	940±56*	1088±48*
	0	371±19	634±25	428±18
N^1-AcPz	0.1	375±10	610±25	436±15
	0.2	370±13	627±24	476±39
	0	352±14	639±36	405±19
N^2-AcPz	0.1	397±19	667±25	636±68*
	0.2	462±26*	870±22*	902±28*

Asterisks indicate significant ($p<0.05$) increases over respective controls. Results were analyzed by ANOVA followed by the Newman-Keuls test.

Table 5-4. Levels of Pz in Rat Blood and Brain 90 min After Treatment with Pz, N^1-AcPz or N^2-AcPz ($ng.g^{-1}$ ± SEM;n=5).

Drug	Dose ($mmol.kg^{-1}$)	Blood	Brain
Pz	0.1	152±2	92±12
N^1-AcPz	0.1	15±4	<10
	0.2	33±2	15±4
N^2-AcPz[a]	0.1	18±2	11±1
	0.2	47±7	44±3

a) Blood and brain levels of N^2-AcPz 90 min after treatment with N^2-AcPz (0.1 $mmol.kg^{-1}$); 148±36 and 20.3±0.5 $ng.g^{-1}$, respectively (n=4).

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GENERAL DISCUSSION AND CONCLUSIONS:

Beliefs that Pz can undergo a metabolic acetylation and that formation of this metabolite is clinically significant are common and have contributed to a potential misunderstanding of the clinical pharmacology of this important psychiatric tool. This concept has never been proven by fact but has arisen through a series of circumstantial observations. Thus, for example, close analogies have been drawn between Pz and other amine or hydrazine drugs (e.g. isoniazid, hydralazine) already known to undergo biological acetylation. Furthermore, Pz has been shown to act as a substrate for human and rabbit N-acetyltransferase enzymes in vitro and patients of the slow acetylator phenotype have been reported to respond better to Pz but to exibit somewhat greater tendencies towards side effects. These latter two points have however been questioned and several research groups have failed to demonstrate any consistent relationship between acetylator status and clinical outcome.

The present study was therefore undertaken to determine firstly the occurrence of an acetylated metabolite of Pz in tissues collected from rodents treated with Pz and, secondly, whether this metabolite could contribute significantly to a Pz response. This study therefore constitutes a detailed examination of the chemistry and pharmacology of each of the two possible monoacetylated metabolites of Pz.

Metabolic acetylation of Pz is complicated by the presence of two basic nitrogen atoms in the molecule. Although acetylation at the primary nitrogen would be predicted through analogy to other

hydrazines, acetylation of secondary amines has been demonstrated and this possibility with Pz cannot be excluded. This prospect could be tested only after unambiguous synthesis of authentic samples of each respective amide for use as a standard in a subsequent assay protocol.

Literature surveys revealed that the preferred site of chemical acetylation of hydrazines varied with the reagent and that direct reaction with acylation reagents resulted in mixtures consisiting predominantly of the secondary amide. This present study further demonstrates that the reaction of these aliphatic hydrazines with one equivalent of an acylating reagent is also medium dependent and that careful selection of the medium allows for the selective synthesis of each amide. Taking advantage of this observation a variety of mono- and diacylated analogues of Pz were prepared, characterised and examined as potential inhibitors of MAO. Results of these experiments indicate that each of the acetylated Pz could be distinguished on the basis of chromatographic and spectroscopic differences.
Those observation were applied to study the metabolism of Pz in microorganisms. Results of this study indicated that N^2-AcPz, but not N^1-AcPz is a metabolite of Pz. Although N^1-acetylation largely eliminated MAOI activities, N^2-acetylation had an attenuating effect on MAOI activities.

Examination of the nuclear magnetic resonance spectra of these analogues indicated that each of the acylated hydrazines exhibited rotational isomerism similar to that previously observed with other amides. The role of rotational isomerism in MAO inhibition was not

assessed.

Several analytical procedures suitable for the analysis of Pz, or related hydrazines, have been reported in the literature. Although each of these assay systems was satisfactory for the researcher's needs, they were often hampered by the generation of multiple derivatives (N^1- plus N^2-acylation) or by comparatively low sensitivities. Gas chromatographic procedures were therefore developed for the analysis of Pz and each of its two possible monoacetylated amide analogues. It was observed that reaction with pentafluoropropionic anhydride after an initial condensation with pentafluorobenzaldehyde converted Pz into a single, mixed derivative which could be assayed at low levels in rat tissue. The applicability of this assay to the analysis of other similar hydrazines in blood was also demonstrated. Similarly, each of the two mono-acetylated amides was detected in spiked tissue after extraction and acylation with pentafluorobenzoyl chloride. Results of these experiments indicate that, although N^2-AcPz did occur as a metabolite of Pz in the rat, levels were probably not sufficient to contribute in any pharmacologically significant way to MAO-inhibition.

Data collected during these experiments were also relevant to the interpretation of previously reported observations. Thus for example, in an examination of the properties of N^2-AcPz and N^2-ECPz, it was concluded that while the latter did possess prodrug characteristics, the former possessed properties dissimilar from those of Pz. This was interpreted to indicate that N^2-AcPz acted in its own right and that some simple amides of Pz might possess

activities independent of hydrolysis to Pz. The present data support this conclusion since they confirm that deacetylation is only a very minor route of N^2-AcPz metabolism in the rat.

Several aspects of the potential significance of N^2-AcPz to Pz have not been addressed in this work but are none-the-less worthy of consideration. Thus, it is possible that the distribution of N^2-AcPz in brain may not parallel that of Pz or may exibit regional differences. Significantly enhanced accumulation of this amide in specific brain regions of fast acetylators may result in an increased pharmacological effect. Secondly, these experiments have been conducted in an animal model which may, or may not, reflect the degree of acetylation to be expected in man. A more complete understanding of the significance of biological acetylation in man might therefore be anticipated to result from application of these assay procedures to plasma samples collected from human patients exposed to Pz.