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UNIVERSITY OF ALBERTA

EFFECTS OF IPRINDOLE ON FENFLURAMINE AND NORFENFLURAMINE IN RAT BRAIN AND LIVER

BY

KATHLEEN M. HEGADOREN

A THESIS

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

IN

NEUROCHEMISTRY

DEPARTMENT OF PSYCHIATRY

EDMONTON, ALBERTA

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FACULTY OF GRADUATE STUDIES AND RESEARCH

THE UNDERSIGNED CERTIFY THEY HAVE READ, AND RECOMMEND TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH FOR ACCEPTANCE, A THESIS ENTITLED **EFFECTS OF IPRINDOLE ON FENFLURAMINE AND NORFENFLURAMINE IN RAT BRAIN AND LIVER** SUBMITTED BY KATHLEEN M. HEGADOREN IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE (PSYCHIATRY) IN NEUROCHEMISTRY.

RT Coutts

Date: December 4th, 1990

DEDICATION

I would like to dedicate this thesis to Dr. Glen Baker for believing in me and to Dale for his understanding.

ABSTRACT

A novel assay procedure was developed for simultaneous analysis of fenfluramine and norfenfluramine. The assay involved homogenization of brain or liver tissue in 5 volumes of ice-cold 0.1 N perchloric acid, centrifugation to remove the protein precipitate and retention of the resultant supernatant for analysis. An aliquot of the supernatant was basified and the drugs extracted with ethyl acetate. The organic extract was taken to dryness under a stream of nitrogen and the residue reacted with pentafluoropropionic anhydride. The reaction mixture was partitioned between toluene and sodium borate buffer and an aliquot of the toluene layer was injected on a gas chromatograph equipped with a fused silica capillary column and an electron-capture detector.

The assay procedure was applied to a time study on brain and liver tissue taken from rats pretreated with iprindole or saline vehicle and subsequently injected with (±)-fenfluramine. Brain and liver levels of fenfluramine were much higher and norfenfluramine much lower in the iprindole/fenfluramine-treated rats than in the vehicle/fenfluramine-treated rats, suggesting that iprindole may block N-dealkylation in addition to its known effects on blockade of ring hydroxylation. It is proposed that iprindole pretreatment may provide a useful means of studying the neurochemical and/or behavioural effects of fenfluramine in the absence of norfenfluramine.

ACKNOWLEDGEMENT

I would like to thank Dr. Ron Coutts and Dr. Alan Carroll for sharing their knowledge and expertise with me.

I would also like to acknowledge Jordyce van Muyden and Gail Rauw for their invaluable support in the lab, Sally Omura for her time and patience in typing this thesis and all my fellow graduate students for their tolerance.

Funds were provided by the University of Alberta Graduate Research Assistanceship Fund and by the Provincial Mental Health Advisory Committee.

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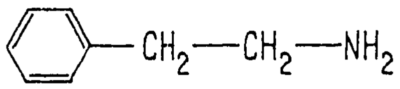
A. INTRODUCTION

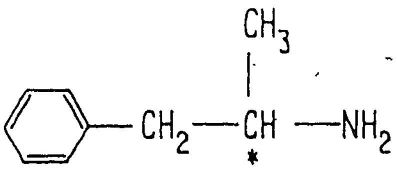
Fenfluramine [N-ethyl-3-trifluoromethylamphetamine] (FEN) was developed by Beregi et al. (1970) at the Laboratories Servier in an effort to retain the anorexiant properties but decrease the stimulation and abuse potential seen in the parent drug, amphetamine (AMPH). Since then, FEN has been widely used clinically as an adjunct in the treatment of obesity.

Fenfluramine has been extensively studied using a variety of animal models both to examine the mechanisms of FEN action and to further elucidate physiological mechanisms involved in hunger, satiety, obesity and diet selection. This drug has proved to have complex behavioural and neurochemical effects. Researchers have used FEN as a tool to examine a number of central nervous system (CNS) parameters. Clinical applications other than obesity treatment have been attempted; perhaps the best known is the use of FEN in the syndrome of infantile autism, a pervasive developmental disorder diagnosed in early childhood.

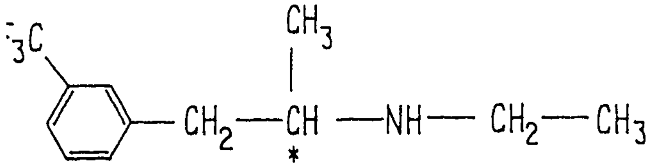
The rapid metabolism of FEN into norfenfluramine (NORFEN), which has biological activity that equals or surpasses that of FEN, has made it difficult to collect data about specific FEN effects. While there have been studies examining in vivo effects of NORFEN alone, attempts to isolate FEN effects have encountered methodological problems. Knowledge about the separate effects of FEN and NORFEN is important in view of ongoing concerns regarding possible neurotoxic effects of FEN and/or NORFEN.



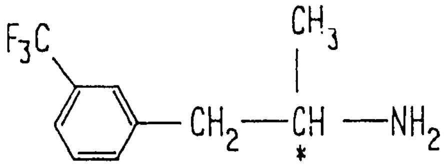
β- Phenylethylamine



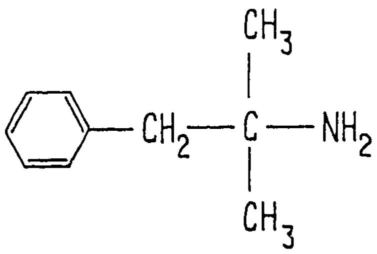
(±)Amphetamine



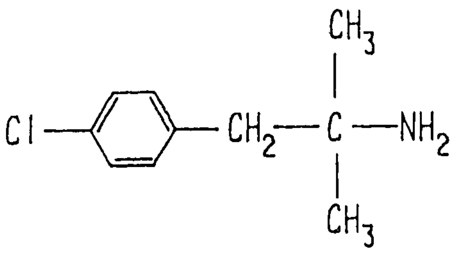
(±)Fenfluramine



(±)Norfenfluramine



Phentermine



p-Chlorophentermine

* Denotes chiral centre

Figure 1: ß-Phenylethylamine analogues.

A.1 OBJECTIVES

Although N-deethylation of FEN to NORFEN has been demonstrated in several species (further details are given later), much still remains unknown about the metabolism of FEN.

The objectives of this thesis were two-fold. One was to develop a relatively simple assay for FEN and NORFEN in rat whole brain and liver which would be compatible with gas chromatography (GC) analysis. The other was to apply the method to examine the effect of iprindole on the levels of FEN and NORFEN in brain and liver to discern a potential role of ring hydroxylation in the metabolic profile of FEN.

A.2 STRUCTURE OF FENFLURAMINE

Fenfluramine is a N-ethyl m-trifluoro-substituted 2-phenylethylamine analogue, classed together with other analogues sharing anorexiant properties (Figure 1), the best known of which is (+)-AMPH. Fenfluramine is unique to the anorexiant drugs in that it produces CNS sedation rather than CNS stimulation (Pinder et al., 1975).

A.3 STEREOCHEMISTRY

Fenfluramine exists as an enantiomeric compound. The (+)- and (-)-enantiomers and their respective metabolites, (+)- and (-)-NORFEN, have differing patterns of biological activity and kinetic properties. Most of the earlier studies reported in the literature used the racemic mixture, which has caused difficulties in separating the various effects and in interpreting conflicting data. In general, it is believed that the (+)-isomer of FEN acts primarily via the serotoninergic system, while the (-)-isomer appears similar to dopamine antagonists in its effects (Invernuzzi et al., 1986).

(+)-Fenfluramine decreases brain concentrations of 5-hydroxytryptamine, (5-HT, serotonin) and 5-hydroxyindole-3-acetic acid (5-HIAA), the major metabolite of 5-HT, through a mechanism which is thought to involve blockade of 5-HT uptake and enhancement of 5-HT release in nerve terminals. The normal pathways of synthesis and breakdown of 5-HT are outlined in Figure 2. de Simoni et al. (1988) used recordings from microelectrode implants in rats to show the biphasic nature of the effects of (+)-FEN and (+)-NORFEN on the level of extraneuronal 5-HIAA in the nucleus accumbens and hippocampus. An increase of 5-HIAA levels is observed initially after administration of FEN or NORFEN, probably as a result of the metabolism of the increased 5-HT released. The marked decrease of 5-HIAA concentrations seen over time indicates that the 5-HT reuptake blockade effect of the drugs predominates as the intraneuronal stores of 5-HT become depleted. In these experiments, (+)-NORFEN was 3 to 5 times more effective both in vivo and in vitro than (+)-FEN in stimulating 5-HT release. (+)-Norfenfluramine also retained its 5-HT-releasing properties in reserpine-treated animals while the releasing effects of (+)-FEN were lost.

Sebban et al. (1989) compared electroencephalogram (EEG) signals from prefrontal and sensorimotor transcortical electrodes after intraperitoneal injections of (-)-FEN or haloperidol, a dopamine antagonist, in adult male rats. There were

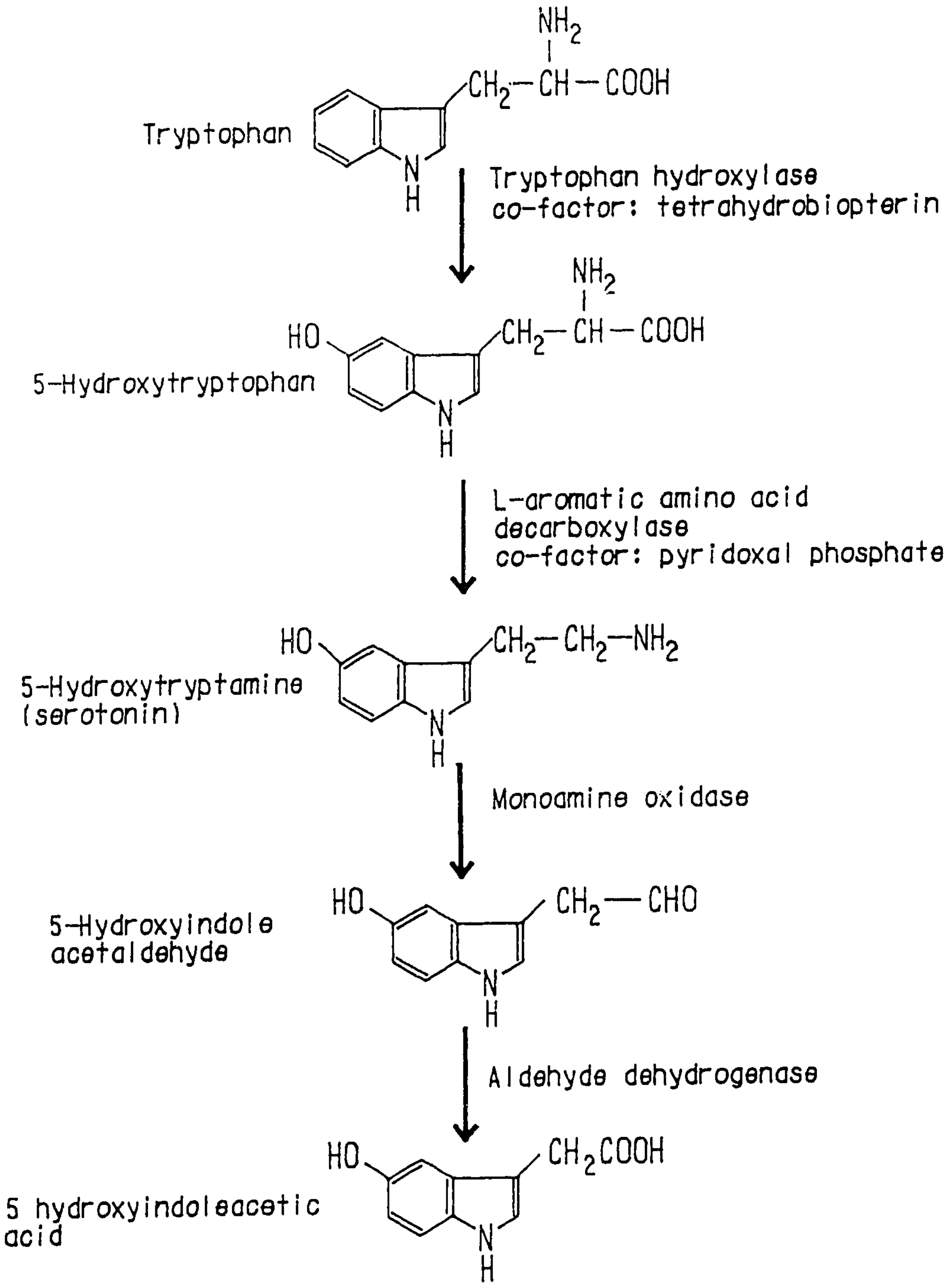


Figure 2: Principal pathways in the synthesis and breakdown of 5-hydroxytryptamine.

similar net effects but differing patterns of power changes over specific frequencies. Jori et al. (1974) also used adult rats to compare intraperitoneal administration of haloperidol and (-)-FEN and found that both drugs decreased striatal dopamine (DA) levels and increased those of the DA metabolites homovanillic acid (HVA) and 3,4-dihydroxyphenylacetic acid (DOPAC). Both drugs antagonized stereotypies induced by high doses of AMPH but only haloperidol antagonized the increased sniffing and gnawing produced by apomorphine. (-)-Fenfluramine is also less effective than is haloperidol in stimulating acetylcholine (ACh) release in striatum. Similarity of action is also suggested by the cross-tolerance to the DA-releasing effect develops between haloperidol and (-)-FEN (Invernuzzi et al., 1989). However, according to results from in vitro binding studies, neither (-)-FEN nor its metabolite have strong affinities for D_1 or D_2 dopamine receptors. Thus, while the net dopaminergic effects of (-)-FEN and haloperidol are similar, the site of action and the precise mechanisms of action do not appear the same. It is noteworthy that (-)-NORFEN was more potent than its parent compound in blocking DA-dependent behaviour (Bendotti et al., 1980).

Garattini et al. (1987) studied in vitro binding by the 4 stereoisomers to a number of different receptors. He found that the two (-)-isomers were somewhat more potent in displacing radioligand from 5-HT_1 binding sites whereas (+)-NORFEN was more active at 5-HT_2 sites. Both of the enantiomers of NORFEN had weak effects on binding to ß-noradrenergic sites. The (-)-isomers also showed some ability to bind to α_2-noradrenergic sites.

Unless an enantiomer is specified in the following review of the literature, the racemate of FEN and/or NORFEN was employed.

A.4. BEHAVIOURAL EFFECTS

4.1 In Animals

Taylor and Goudie (1975) compared FEN, NORFEN and 780 SE (another anorexiant drug) to AMPH in their ability to affect different rat behaviours. They found that NORFEN at both 3 and 9 mg/kg decreased rearing, walking and grooming while sniffing was increased. Fenfluramine at both 3 and 9 mg/kg had an inhibitory effect only on rearing. Administration of AMPH produced the opposite effects, with increased rearing and walking and decreased sniffing. Administration of high doses (10-30 mg/kg) of FEN produced a consistent dose-related abnormal pattern of backward walking, whereas similar doses of AMPH produced predominantly head swaying. Kutscher (1987) also showed that FEN and AMPH produced opposite effects: FEN decreased the acoustic startle response when given for 22 days intraperitoneally (IP) at a dose of 10.12 mg/kg, whereas AMPH increased the animals' responsiveness to the same test.

Wurtman and Wurtman (1979) reported that acute administration of FEN to rats reduced the selective intake of carbohydrate (CHO) relative to protein. These workers have postulated that this selective effect on nutrient choice is related to the neurochemical consequences of eating a CHO-rich, protein-poor meal; CHO stimulates insulin secretion which increases the ratio of serum tryptophan to the other large neutral amino acids, which in turn increases brain tryptophan levels, thus accelerating 5-HT synthesis. The resultant enhancement of 5-HT transmission decreases subsequent CHO intake, allowing central 5-HT levels to return to normal. Other researchers (Rowland and Carlton, 1986, 1988; Blundell, 1983; McArthur and Blundell, 1983) have not been able to consistently replicate Wurtman and Wurtman's selective sparing of protein experiments. Rowland and Carlton (1988) cited methodological problems involved in self-selection paradigms and proposed alternative interpretations for some of Wurtman and Wurtman's data. Rowland and Carlton (1988) calculated the DI_{50} (50% inhibition of food intake relative to vehicle-injected controls) of (+)-FEN in a number of different feeding paradigms. They found that overfeeding paradigms were more sensitive to (+)-FEN than were deprivation setups. Stress-induced eating was also sensitive to (+)-FEN. Data gained from animal models on self-selection of food must be extrapolated to humans with a great deal of caution as food selection in humans is a result of a complex dynamic interaction of physiological, social and psychological factors.

Robinson et al. (1988) summarized their series of studies in which they examined possible links between changes in gastric function and undereating associated with cholecystokinin (CCK) and FEN. Cholecystokinin is an endogenous neuropeptide with potent inhibitory actions on feeding which are thought to be both peripherally and centrally mediated. Cholecystokinin receptors are found in the distal part of the circular muscle of the pyloric sphincter of the stomach and extensively throughout the CNS. Monkeys were given FEN intragastrically. There was inhibited gastric emptying of both saline and glucose solutions, suggesting that peripheral effects contribute to the mechanism of FEN action. Delayed gastric emptying has been proposed as being partially responsible for the potent ability of FEN to act as an unconditional internal cue for the formation of a conditioned taste aversion. The enduring and unpleasant sensation of fullness was considered an internal cue (Lorden et al., 1979).

Tolerance to the anorectic effects of FEN has been shown by many researchers using total food intake (Rowland and Carlton, 1986; Taylor and Goudie, 1975; Reuter, 1975; Blundell et al., 1988; Garattini et al., 1975). Despite the apparent tolerance that develops within 2-5 days in that food intake levels in FEN-treated animals return to control levels, some researchers have shown that absolute weight gain in FEN-treated animals remained lower than in control animals for up to 30 days, especially if the animals were made obese by enriched diets prior to the experiments (Rowland and Carlton, 1986; Rozen et al., 1988).

4.2 In Man

Administration of FEN produces a dose-related body weight loss in obese patients when compared with placebo controls (Rowland and Carlton, 1986; Nathan and Rolland, 1987). The dose of FEN is usually increased over the first few days of treatment, building up to 60-120 mg/day or approximately 1-2 mg/kg/day. Weight losses of about 0.5 kg/week for up to 20 weeks can be expected if adequate plasma levels are obtained (Innes et al., 1977). The weight loss slows after several months, with a steady-state body weight about 10 kg below initial weight achieved by about 6 months.

The (+)-isomer has been studied more recently as the mediator of the weight reduction (Nathan and Rolland, 1987). The superiority of (+)-FEN over placebo was demonstrated. Finer et al. (1987) further demonstrated that (+)-FEN produced a mean weight loss that continued over the entire 6 month study with no evidence of tolerance. The (+)-FEN produced fewer reported side effects than the racemate, presumably due to the absence of the specific antidopaminergic activity of (-)-FEN.

Fenfluramine has been shown in pharmacological and clinical investigations to produce many peripheral effects that are related to overall body weight reduction (Macrae, 1975). There is a reduction of fat stores, altered fat metabolism mediated by the release of catecholamines, decreased serum levels of triglycerides and an increase in the uptake of glucose in skeletal muscle. Macrae (1975) summarized research showing that FEN significantly improved glucose tolerance, but not as a result of increased insulin production, suggesting a possible use in the treatment of maturity onset diabetes mellitus. Although less marked than with AMPH, FEN has a hypertensive effect via an indirect sympathomimetic action consistent with stimulation of α-adrenergic receptors

(Macrae, 1975). Delayed gastric emptying has also been demonstrated in man after ingestion of FEN (Robinson et al., 1988).

Day-time drowsiness is a frequently reported troublesome side effect of FEN (Pinder et al., 1975). This could be due in part to a poor sleep at night as FEN reduces rapid eye movement (REM) and increases transitions from deep to light slow wave sleep ["intrasleep restlessness"] (Lewis et al., 1971). Sudden withdrawal of the drug has led to sleep disturbances and mood changes (Rowland and Carlton, 1986).

Tolerance to the anorectic effects of FEN in humans has proved to be a difficult area to examine. Weight loss can continue slowly over 6 months while on FEN treatment. Weight gain that might occur while on extended period of FEN treatment would be difficult to interpret or separate between true tolerance and a number of social and psychological factors.

A.5 NEUROCHEMICAL EFFECTS

5.1 In Animals

Since FEN was introduced in 1970 as an anorexiant devoid of central stimulatory effects, researchers have been studying the neurochemical basis for the differences seen in FEN compared to other anorexiants such as (+)-AMPH.

In one of the original articles written on the central effects of FEN, Costa et al. (1971) studied ex vivo telencephalon and diencephalon, brain stem, and cardiac biogenic amine levels after treatment with varying doses of FEN. A selective depletion of 5-HT was demonstrated in the telencephalon and diencephalon lasting longer (still apparent at 48 h) than in keeping with the biological half-life of the drug. The 5-HT depletion did not extend to brainstem, stomach or heart stores of 5-HT. There was also an increased turnover rate of 5-HT seen only in the telencephalon and diencephalon. High doses (90 μmol/kg) produced catecholamine depletion in brain and heart but this effect was short-lived, with levels returning to normal within 24 h. These same authors showed that neither tryptophan hydroxylase nor aromatic amino acid decarboxylase was inhibited in vitro; rather, conversion of tyrosine to catecholamines and tryptophan to 5-HT was enhanced. In her review, Reuter (1975) added that NORFEN differs quantitatively from FEN, with the former producing a more pronounced and prolonged effect on catecholamine levels.

Controversy remains over what effects the administration of FEN has on tryptophan hydroxylase activity. Morgan et al. (1972) supported Costa's earlier work showing that the acute administration of FEN does not have an effect on tryptophan hydroxylase activity. However, Steranka and Sanders-Bush (1979) reported that long-term effects of FEN include a decrease in tryptophan hydroxylase activity. Sanders-Bush et al. (1972) demonstrated that p-chloroamphetamine (pCA) decreased central tryptophan hydroxylase activity. Comparisons between the mechanisms of action of pCA and FEN have been made (Harvey, 1978; Fuller and Hemrick-Luecke 1980). Harvey (1978) concluded that both pCA and FEN have similar mechanisms of action on serotoninergic neurons, with pCA being more potent than FEN at equimolar dosages.

Garattini et al. (1975) demonstrated that FEN had two distinguishable dose-dependent effects on 5-HT neurons: a Ca^{2+}-independent release of granular 5-HT and an inhibition of 5-HT uptake. The level of 5-HIAA was also decreased after FEN administration, although 5-HIAA depletion lags behind that of 5-HT depletion. Other compounds which are known to lower both 5-HT and 5-HIAA are p-chloroamphetamine and p-chlorophenylalanine, both of which inhibit 5-HT synthesis (Fuller et al., 1988). However, Garattini et al. (1975) showed that FEN increases 5-HT turnover. Thus, 5-HIAA decreases were felt to reflect FEN's inhibitory effect on 5-HT uptake, as the metabolism of 5-HT occurs mainly via intraneuronal monoamine oxidase A activity. Pretreatment with reserpine greatly attenuated the 5-HT release seen with FEN but not with NORFEN, suggesting that the latter releases 5-HT from reserpine-insensitive pools (Rowland and Carlton, 1986).

Garattini et al. (1975) also provided evidence that an intact 5-HT system was necessary for FEN's anorectic effects. Using electrodes situated in the dentate gyrus of anesthestized rats, Richter-Levin and Segal (1988) demonstrated that (+)-FEN caused a marked increase in population spike response to perforant path stimulation without affecting the slope of the excitatory postsynaptic potential. This increase was not present in rats depleted of 5-HT by p-chlorophenylalanine but could be restored using the 5-HT precursor 5-hydroxtryptophan, thus demonstrating that 5-HT must be present in terminals for (+)-FEN's action as a 5-HT releaser.

Fuxe et al. (1975) used spinal cord preparations to add supportive evidence that FEN is a releaser of 5-HT stores and a blocker of the membrane pump responsible for uptake of 5-HT. They postulated that the motivational control of food intake involves mainly the limbic system, whereas basal control involves primarily the hypothalamus. Fuller et al. (1988) used (+)-FEN to examine the different roles that release/depletion and uptake inhibition play in the overall effects of FEN. These authors concluded that (+)-FEN initially enhances serotoninergic function by carrier-dependent release of 5-HT and not by uptake inhibition. The early phase of 5-HT release was followed by a prolonged period of decreased 5-HT activity as indicated by decreased 5-HIAA. Fuller et al. (1988) used pCA, a known carrier-dependent 5-HT depleter used as a test for 5-HT uptake inhibitors, to examine (+)-FEN's uptake inhibition. At low doses, (+)-FEN failed to antagonize depletion of 5-HT produced by pCA and at higher doses mimicked the long term pCA-induced neurotoxic depletions of 5-HT. From these data, Fuller et al. (1988) proposed that (+)-FEN's affinity for the 5-HT uptake carrier is vital to its effects, not because it leads to inhibition of 5-HT uptake, but rather because it allows the accumulation of (+)-FEN necessary for 5-HT release.

Direct in vivo neurochemical confirmation of the 5-HT-releasing effects of FEN was provided by Schwartz et al. (1989). This group used microdialysis techniques to monitor 5-HT, 5-HIAA, HVA and DOPAC in the perifornical lateral hypothalamus of freely moving rats after systemically and locally applied (+)-FEN. Both systemic and locally applied (+)-FEN caused a rise in extracellular

5-HT, indicative of 5-HT release, and a decrease in 5-HIAA. Systemic (+)-FEN produced increased extracellular levels of DOPAC and HVA, suggestive of an increase in DA turnover. The results obtained regarding the DA metabolites differ from other reports. The authors suggest that the difference arises from their use of microdialysis to measure extracellular neurochemical levels whereas the other researchers used tissue homogenates which reflect primarily intracellular levels. They noted that a meal also increased 5-HT in the microdialysis perfusate.

Other indices of serotoninergic function are also affected by administration of FEN and NORFEN. Coutts et al. (1989) demonstrated that acute injections of FEN or NORFEN resulted in elevated tryptophan levels in rat whole brain samples. The increase was more profound after NORFEN, with tryptophan levels rising to 204% above control. Other research supports this finding (Tagliamonte et al., 1971; Morgan et al., 1972). The tryptophan increase was thought to be related to the drugs' inhibitory effects on tryptophan hydroxylase activity. Tryptophan hydroxylase is the rate-limiting enzyme in the synthesis of 5-HT. Thus, any decrease in enzymatic activity would have a significant impact on the neuron's ability to maintain 5-HT intracellular stores. Clineschmidt et al. (1978) administered an inhibitor of aromatic amino acid decarboxylase inhibitor to rats 8 days after acute administration of FEN. The level of 5-hydroxytryptophan (5-HTP) in brain was decreased, in contrast to the situation in rats pretreated with vehicle, adding supportive evidence that FEN affects the synthesis rate of 5-HT.

The 5-HT-releasing ability of FEN has led some researchers to use FEN as a test agent for the integrity and functionality of 5-HT neurons. For example, Richter-Levin and Segal (1989) used FEN to test the ability of embryonic raphé cells grafted into the hippocampus to release 5-HT as an indicator of function of these cells.

Concomitant with the release of 5-HT and decrease in 5-HIAA concentrations in brain with FEN administration, there is also a rise in striatal HVA without changes in striatal DA levels (Jori et al., 1973; Garattini et al., 1975). In demonstrating that FEN blocked apomorphine (DA agonist)-induced stereotypies, Garattini et al. (1975) provided evidence that FEN blocked DA receptors in a similar fashion to that of neuroleptic drugs. Although Garattini's group found the (-)-enantiomer of FEN mainly responsible for the striatal DA effects, Rowland and Carlton (1986) showed that (+)-FEN can produce an increase in HVA at least as great as that of an equal dose of (+)-AMPH.

Although there has been much evidence that anorexia produced by FEN is not mediated via central DA systems (Samanin et al., 1972; Garattini et al., 1975; Rowland and Carlton, 1986; Reuter, 1975), it must be remembered that food intake is mediated by a number of neurotransmitter (NT) systems and that the behaviour anorexia can be elicited by a large number of agents with different neurochemical and/or peripheral effects (Rusk et al., 1989; Robinson et al., 1988). Thus, a direct causal relationship between central 5-HT and FEN-induced anorexia would probably not accurately reflect the complexity of this behaviour in animal models and would be of limited application to humans.

5.2 In Man

There has been much less written about FEN-induced neurochemical changes using human subjects. Obese patients treated with FEN have not had routine analysis of blood and urine to indirectly examine changes in NTs and/or central NT metabolite levels. Decreases in whole blood 5-HT levels seen in autistic children administered 1.5 mg/kg/day of FEN are felt to reflect similar decreases in CNS 5-HT levels (Ritvo et al., 1986, 1984; Ho et al., 1986).

It has been shown in post mortem brains of completed suicide victims that the levels of 5-HT and 5-HIAA were decreased (Pare et al., 1969; Lloyd et al., 1974). Meyendorff et al. (1986) administered FEN (180 mg/day x 4 weeks) to a series of suicidal patients from a number of diagnostic categories, and examined 5-HIAA and HVA levels in cerebrospinal fluid (CSF) and platelet 5-HT uptake. A number of psychological scales used to assess suicidal ideation were also used to assess the patients. They found a significant decrease in 5-HIAA concentrations in CSF but no change in HVA levels. Platelet 5-HT uptake was reduced. Suicidal ideation was significantly attentuated by FEN, with reversal upon discontinuation of FEN. These data are difficult to interpret from known effects of FEN. Theoretically FEN should increase suicidal ideation if this behaviour correlates with low 5-HT and 5-HIAA levels. Targum and Marshall (1989) indicated that 2 of the 9 depressed patients in their study expressed acute suicidal ideation during acute FEN administration which abated as the drug effect wore off.

Levels of HVA and 5-HIAA in CSF have been examined after probenecid loading in non-obese patients with Huntington's chorea, tardive dyskinesia and spinocerebellar degeneration given FEN, 120 mg/day x 8 days (Chase and Shoulson, 1975). Fenfluramine led to a significant reduction in the level of 5-HIAA ranging from 53-90%. There was a tendency for the level of HVA to increase but the difference did not achieve statistical significance. Administration of FEN had no effect on involuntary movements in patients with Huntington's chorea.

Urinary HVA was demonstrated to be an indicator to distinguish between responders and nonresponders to FEN treatment in a population of autistic children (Bartholemy et al., 1989). While both responders and nonresponders showed a significant decrease of urinary 5-HT, responders had both lower pretreatment levels of HVA and a significant increase in HVA levels during treatment, whereas no significant changes occurred in nonresponders.

A.6 Neurohormonal Effects

Pituitary releasing factors and hormones have effector targets both in the periphery and on receptors in the CNS, producing complex effects ranging from direct activation to subtle modulation in concert with various NT systems. The homeostatic control of peripheral CHO and fat metabolism is in part regulated by a number of these pituitary agents.

The administration of FEN has been shown to affect a number of hormones. Plasma growth hormone levels decreased following FEN administration to human subjects (Sulaiman et al., 1975). Serri and Rasio (1987) did both in vivo and in vitro studies on anterior pituitary hormone release in rats. They found a dose-dependent (+)-FEN-induced rise in serum prolactin (PRL) and corticosterone concentrations. Growth hormone (GH) increased with low dosages but decreased at higher doses. Fluoxetine, a selective 5-HT uptake inhibitor, could partially block the PRL increase produced by (+)-FEN but showed the same ability as (+)-FEN to increase corticosterone. Using hypothalamic-pituitary preparations, these same authors showed evidence that (+)-FEN's effect on PRL secretion is exerted through the hypothalamus and is at least partially mediated by a 5-HT mechanism. Coccaro et al. (1988) showed a similar PRL response to FEN but failed to shown any FEN-induced elevation of thyrotropin-stimulating hormone (TSH). This result was interpreted to indicate that thyrotropin-releasing hormone (TRH) is not responsible for FEN-induced PRL release. However, Bowers et al. (1973) simultaneously examined the TSH and PRL responses to exogenous TRH and demonstrated that the responsiveness of these 2 hormones to TRH was highly correlated. More research examining the interrelationships between the various neurohormones is needed.

The ability of FEN to increase circulating PRL levels and the belief that PRL release is mediated, at least in part, by central 5-HT mechanisms have led some researchers to use FEN as an indirect agonist probe to examine serotoninergic functions. Siever et al. (1984) have shown blunting of the PRL response to FEN in depressed inpatients. López-Ibor et al. (1988) used an acute oral dose of FEN to investigate the role of 5-HT in a variety of depressed patients and found FEN-induced PRL increases did not correlate with any behaviours linked to central 5-HT activity such as suicidal behaviour and sleep disorders. However, these authors found a correlation between severity of illness and endogeneity and the ability of FEN to produce a rise in PRL level. They proposed that FEN could have possibilities as a marker for endogenous depression. The ability of imipramine to enhance 5-HT-mediated neuroendocrine responses was evaluated by Shapira et al. (1989) by testing its ability to attentuate the blunted PRL response to FEN. Targum and Marshall (1989) used the FEN-induced PRL response to examine a possible role of 5-HT in the provocation of panic attacks. Fenfluramine precipitated heightened panic and/or anxiety responses in patients with panic disorder but not with major depression or in control patients. Panic disorder patients had a significantly greater PRL response than did either the depressed or control groups. McBride et al. (1989) demonstrated that young adult male autistic patients also had a blunted PRL response to oral FEN administration. These authors also found a significant decrease in the mean number of platelet 5-HT_2 receptors in autistic patients.

A.7 Metabolism of Fenfluramine and Norfenfluramine

Fenfluramine differs in its metabolic profile from its analogue AMPH, both in the major metabolic pathway and in the interspecies variations seen with these two drugs.

Metabolism of AMPH has been studied extensively (Figure 3). The major metabolic pathway of AMPH varies with the species studied (Testa and Jenner,

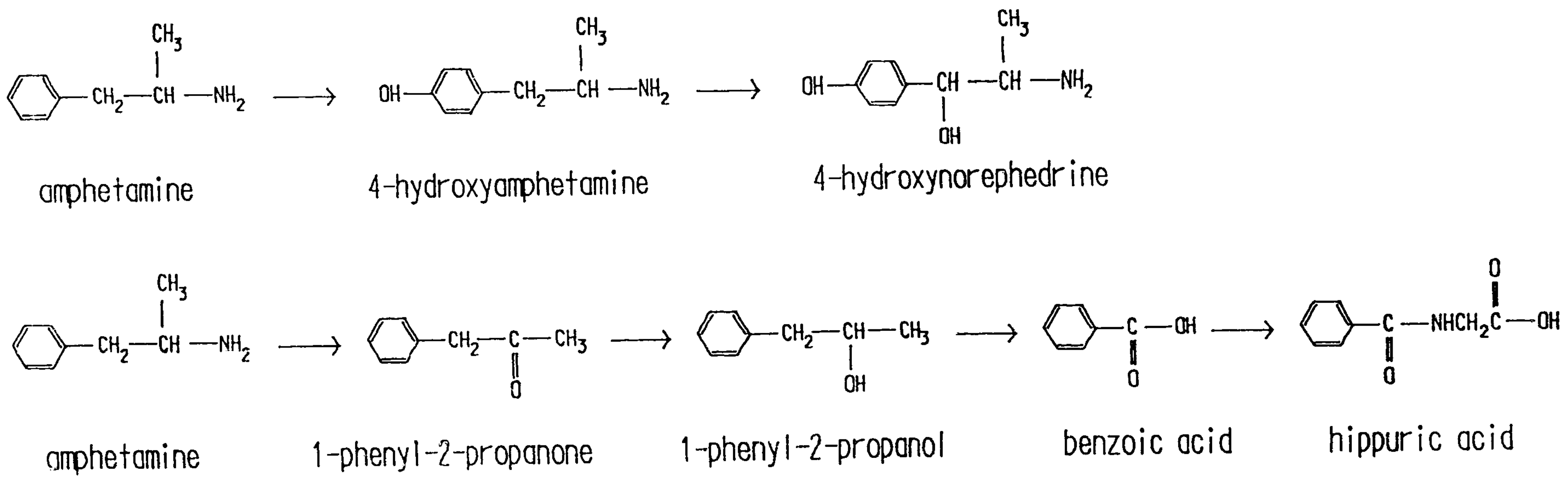


Figure 3: The major metabolic pathways of amphetamine based on information from Costa and Garattini (1970) and Gibson and Skett (1986). 4-Hydroxynorephedrine is excreted as a sulphate or glucuronate conjugate.

1976; Garrod and Beckett, 1978; Costa and Garattini, 1970). In the rat, ring hydroxylation is the primary metabolic route, with para-hydroxyamphetamine making up to 60% of the urinary metabolic products (Hucker, 1970). In the rabbit, the Rhesus monkey and humans, deamination and N-oxidation are the preferred pathways for AMPH metabolism (Gibson and Skett, 1986). There are numerous minor pathways which also vary among species. The speed of metabolism between the two AMPH enantiomers also differs among species.

The metabolism of a drug to allow conjugation is termed Phase I metabolism. Phase I metabolic reactions include oxidation, reduction, hydrolysis and hydration, as well as isomerization. Garrod and Beckett (1978) used metabolism of AMPH to describe Phase I metabolic reactions in man. Oxidative reactions such as C- and N-hydroxylation, dealkylation and deamination occur via a mixed function oxidase system of the hepatocytic endoplasmic reticulum, comprised of at least four enzymes: cytochrome P-450 and b_5 with their respective reductases. These reactions require molecular oxygen (O_2) and microsomal nicotinamide adenine dinucleotide phosphate hydrogen (NADPH), a reducing coenzyme. Fenfluramine utilizes this enzymatic system as does AMPH. However, the cytochrome P-450 enzyme system contains a number of isozymes which show selectivity to certain drugs. One of the most studied isozymes is the P-450 db1 isozyme system, originally named for its role in the metabolism of debrisoquine to its 4-hydroxylated metabolite (Kalow, 1986). It has since been shown that the P-450 db1 isozyme system is involved in the metabolism of many drugs, including propanolol, tricyclic antidepressants and dextromethorphan.

Brosen and Gram (1989) suggest that oxidative reactions involving aromatic and alicyclic metabolic ring hydroxylation are often under the influence of the P-450 db1 isozyme but side chain metabolic dealkylation and deamination are not. Thus, FEN and AMPH could be metabolized via different isozymes within the cytochrome P-450 system.

The major metabolic pathway for FEN is consistent among species, but the velocity of the metabolic reactions and the many minor pathways vary (Rowland and Carlton, 1986) (Figure 4). In man, the major metabolic pathway for elimination of FEN is N-dealkylation to yield NORFEN (Beckett and Salmon, 1972). Norfenfluramine is subsequently converted to 3-trifluoromethylhippuric acid which accounts for up to 90% of the urinary output of the drug. The specific amount varies with urinary pH (Bruce and Maynard, 1968). Bruce and Maynard (1968) found that in dogs 3-trifluoromethylbenzoic acid was formed as an intermediate step between NORFEN and 3-trifluoromethylhippuric acid. The two acid metabolites are considered inactive. Norfenfluramine, as indicated earlier, shares potent anorectic and other properties with its parent compound.

The electron-withdrawing property of the trifluoromethyl constituent on the aryl ring of FEN stabilizes the ring, thus reducing the likelihood of ring hydroxylation, but this has not been investigated thoroughly. However, Midha et al. (1983) used gas chromatography and mass spectrometry to identify two hydroxylated metabolites of FEN in human urine. Metabolic hydroxylation was reported to produce a propanol and a propanediol derivative, both inactive metabolites (Figure 4).

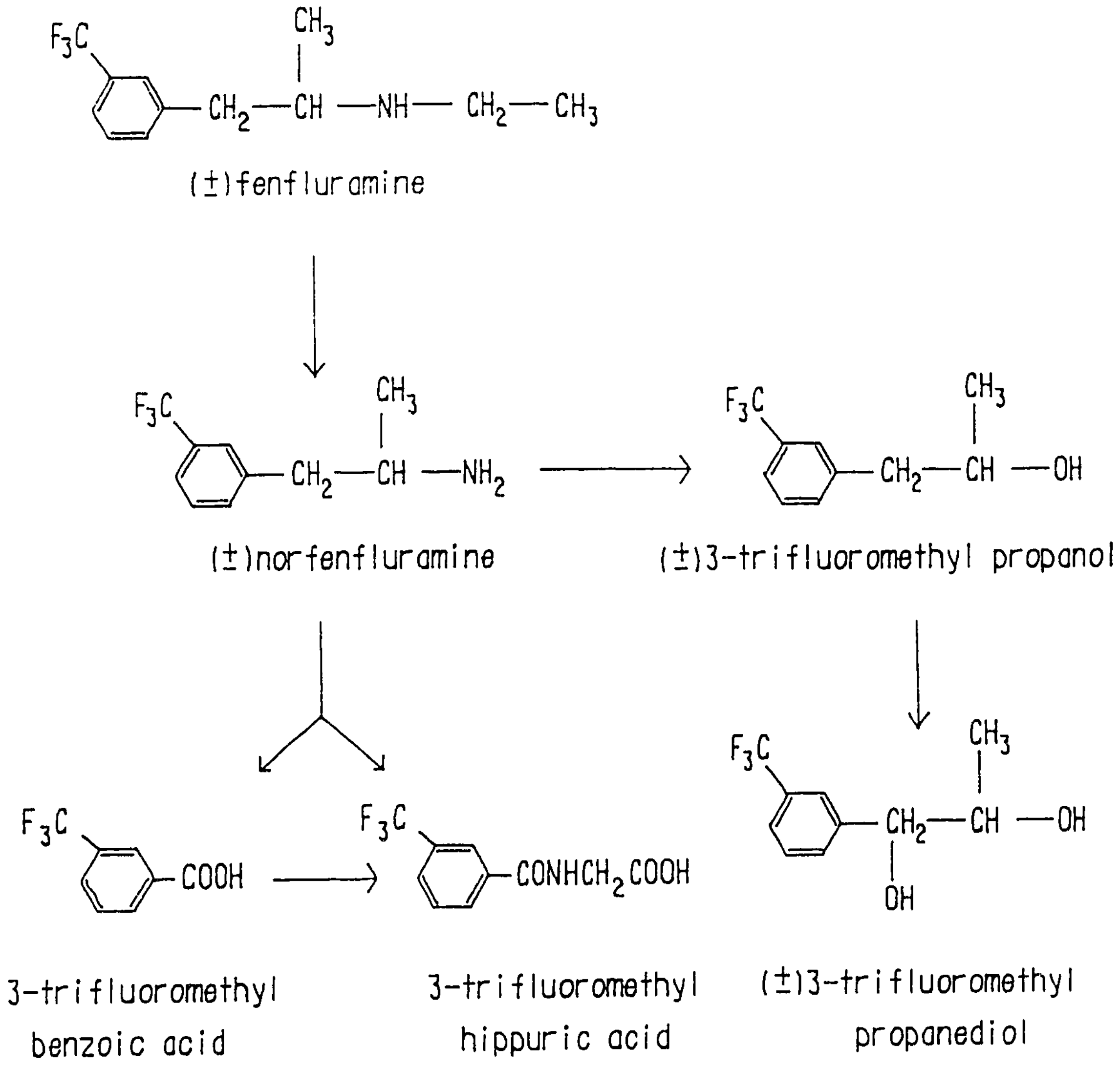


Figure 4: Identified metabolites of fenfluramine, based on information from Bruce and Maynard (1968) and Midha et al. (1983).

Beckett et al. (1973) proposed that N-ethyl-α-methyl-α-(m-trifluoromethyl-benzyl)nitrone was the major metabolite of FEN in guinea pigs in vitro using liver microsomal fractions. Schuster et al. (1986) used Beckett's proposed metabolic route in guinea pigs to conclude that it was FEN alone that was responsible for the long-term depletion of central 5-HT. However, Fuller et al. (1988) were able to show extensive NORFEN formation in guinea pig whole brain following administration of FEN (12.5 mg/kg i.p.).

In the rat, the metabolic rates of the enantiomers differ in that (-)-FEN is dealkylated to (-)-NORFEN faster than (+)-FEN is dealkylated to (+)-NORFEN (Morgan et al., 1972). In human plasma after a single dose of FEN, the concentrations of FEN always exceed those of NORFEN despite the slightly longer half-life of the latter (Rowland and Carlton, 1986). However, in rats, plasma half-life is only about 2 h for FEN, while NORFEN has a half-life of at least 12 h. By 2-6 h post-administration of FEN, NORFEN levels exceed those of FEN. In Rowland and Carlton's summary of research regarding plasma concentrations of FEN and NORFEN after a single dose of FEN, the authors state that no animal study has reproduced the plasma profile of FEN and NORFEN found in man, but mice came closest.

A.8 Receptor Binding Studies

Acute administration of FEN has yielded disparate results in binding studies on 5-HT receptors in different tissues and also in studies examining 5-

HT receptor subtypes. The lack of consistent results could arise in part from some researchers using racemic FEN while others used one or the other enantiomer, using a wide range of dosages of FEN, and a lack of selectivity in many of the radioligands used to examine the various subtypes of 5-HT receptors (Rowland and Carlton, 1986).

It has been shown by a number of earlier reports (Rowland and Carlton, 1986; Duhault et al., 1980; Saminin et al., 1980) that ^{14}C-FEN does bind to central 5-HT receptors. However, these studies do not differentiate pre-synaptic from post-synaptic receptors. Fenfluramine inhibits the in vitro specific binding of 5-HT to brain membranes. Mennini et al. (1985) reported the concentration which inhibits binding of 5-HT by 50% (IC_{50}) is highest for (+)-FEN (about 7 μM) and (+)-NORFEN (4 μM) with (-)-FEN/NORFEN being effective at about 2 μM. Fuller et al. (1980) calculated IC_{50} values of 7.1 μM for racemic FEN and 6 μM for racemic NORFEN.

A number of 5-HT agonists and antagonists have been employed to differentiate the receptors involved in FEN's action. Ketanserin, a selective 5-HT_2 receptor antagonist, produced a dose-dependent antagonism of the anorectic effect of FEN, leading the authors to conclude that FEN-induced anorexia is mediated by 5-HT_2 receptors (Hewson et al., 1988). However, Garattini et al. (1986) used (+)-FEN and found that the nonselective antagonist metergoline produced a maximal inhibiton whereas ritanserin, another 5-HT_2 selective antagonist, produced very little inhibition. Samanin et al. (1989)

provided supportive evidence that (+)-FEN-induced anorexia is not affected by ritanserin.

Some 5-HT_1 receptor-specific drugs such as m-chloropiperazine are potent anorexiants (Hewson et al., 1988). The 5-HT_{1A} autoreceptor agonist 8-hydroxy-2-(di-n-propylamino) tetralin (8-OH-DPAT), which has a hyperphagic effect on its own, reversed FEN-induced reduction of intake of sweetened wet mash (Dourish, 1988). Willoughby et al. (1988) used indwelling bilateral cannulae to demonstrate that activation of 5-HT_1 receptors in the medial basal hypothalamus stimulated PRL secretion in rats. These authors showed that 5-HT, FEN, and 8-OH-DPAT produced dose-related rises in plasma PRL. The PRL release could be blocked by metergoline but only partially by ketanserin.

Chronically decreased transmitter release can lead to up-regulation; the development of compensatory receptor changes as the CNS attempts to reestablish synaptic homeostasis. Two of these receptor changes are post-synaptic receptor supersensitivity (increased affinity as expressed by the K_D in binding studies) and an increase in receptor number (B_{max}) (Hrdina, 1986). However, Samanin et al. (1980) reported a decrease in binding of $[^3H]$5-HT to some brain regions of rats treated for 28 days using (+)-FEN, suggesting down-regulation. Rowland and Carlton (1986) could not reproduce these results using (±)-FEN.

Glennon (1990) reviewed work done on the various 5-HT receptor/binding sites and the increasingly more selective 5-HT agents available to explore the diverse 5-HT system. From this pharmacological review, Glennon provided some clinical implications for agents that are involved in a number of behaviours thought to be influenced by 5-HT. These behaviours include feeding, thermoregulation, sexual behaviour, cardiovascular/hypotensive effects, sleep, anxiety, agression, depression and hallucinogenic activity. In terms of feeding behaviours, Glennon provided evidence to support involvement of 5-HT$_{1A}$ receptors, 5-HT$_{1B}$ and 5-HT$_{1C}$ receptors working in tandem and 5-HT$_{2}$ receptors in some indirect manner.

A.9 Pharmacokinetic Properties

Beckett and Salmon (1972) proposed a three-compartment open pharmacokinetic model for the biological processing of FEN and NORFEN, with one peripheral compartment rapidly equilibrating with the central compartment and the second (tissue) compartment only slowly attaining equilibrium. Differences between the pharmacokinetic parameters obtained after oral and intravenous FEN were indicative of significant N-dealkylation in the intestine or on a first-pass through the liver in the former case.

There are wide subject-to-subject and species variations in rates of biotransformation and elimination of FEN, and the rate of urinary excretion depends on urinary flow rate and pH (Beckett and Salmon, 1972; Pinder et al., 1976; Caccia et al., 1982; Rowland and Carlton, 1986). Caccia et al. (1982) studied plasma and brain levels of (+)-FEN and (+)-NORFEN in a number of animal models and human subjects after administration of (+)-FEN. These authors found that the plasma half-life ($T_{1/2}$) of (+)-FEN was 2.6 h in rats, 4.3 h in mice and 17.8 h in man. The $T_{1/2}$ of (+)-NORFEN was longer than that of the parent drug in all species. Despite the longer $T_{1/2}$ of NORFEN, the plasma concentrations of FEN in humans always exceeded those of NORFEN after acute administration of FEN. However, in rats, plasma concentrations of NORFEN exceeded those of FEN by 3-4 h after administration of FEN. A similar pattern was shown in brain concentrations in rats (Rowland and Carlton, 1986). In rat brain, (+)-FEN had a longer $T_{1/2}$ and gave a larger area under the curve (AUC) than the (-)-isomer, indicative of stereoselective N-deethylation of the (-)-isomer (Garattini et al., 1979).

Fenfluramine is rapidly absorbed by the intestine and is highly lipophilic, which accounts for the higher brain than plasma concentrations and also for the sequestration of FEN in fat stores about the body. Brain concentrations are often 10-40 times the corresponding plasma levels (Rowland and Carlton, 1986).

A.10 Clinical Applications

As previously noted, FEN is most widely used as an adjunct in the treatment of obesity. In 1982, Ritvo et al. (1984) began using FEN with autistic children, as it had been shown that up to 1/3 of autistic children have elevated plasma 5-HT levels. Since then, there has been a ongoing multicenter study involving 9 North American clinical centers and 81 autistic children in which FEN effects on a number of behavioural and neurochemical parameters have been investigated. In a review of FEN in the treatment of autism, Aman and Kern (1989) reported a consistent 50% reduction of whole blood 5-HT compared to baseline levels regardless of initial levels. These authors also reported that the early reports of FEN enhancement of IQ were not substantiated but there was evidence of enhanced social relatedness, reduced stereotypic behaviour and hyperactivity and improved attention span in some autistic children given FEN.

Given that obsessive-compulsive disorder patients respond to antidepressants such as clomipramine and fluoxetine with potent 5-HT reuptake blocking ability, Hollander *et al.* (1988) administered FEN to a group of obsessive-compulsive patients. There was some improvement in the severity of their symptoms but their improvement was not significant when compared to placebo.

Donnelly *et al.* (1989) compared FEN and (+)-AMPH in their ability to reduce motor activity in male children with attention deficit disorder with hyperactivity and found that FEN lacked the therapeutic effects of (+)-AMPH despite some common overall effects on catecholamine metabolism and similar effects on weight.

Workers in the area of schizophrenia research have become interested in a possible role of 5-HT in the negative symptoms associated with schizophrenia. Alphs *et al.* (1989) conducted a clinical trial of FEN at 60, 120 and 180 mg/day in a group of long-term chronic schizophrenics in a locked unit of a veterans' psychiatric facility. A trial of 240 mg of FEN had to be abandoned due to severe hypotensive effects, and up to 20% of the 120 and 180 mg/day doses were missed for similar hypotensive side effects. The authors suggest that the hypotensive effects of FEN might be partially due to drug interactions in this patient population who have been chronically treated with a number of neuroleptic agents. Although there was no significant change in negative symptoms, there was anecdotal note made of 2 patients who showed drastic improvement in communication skills while on the FEN protocol.

A.11 Neurotoxicity

The controversy as to whether FEN is neurotoxic to serotoninergic neurons via a similar mechanism to that of pCA has yet to be resolved. After a single injection of pCA the brain 5-HT level in rats is decreased and remains decreased for months. There are concurrent reductions in 5-HIAA concentrations, tryptophan hydroxylase activity and high affinity 5-HT uptake, all of which imply depletion with possible neuronal destruction (Sanders-Bush et al., 1972). Harvey (1978) showed histological evidence to support neuronal destruction, detailing cell body changes specifically in the ventral midbrain tegmentum, an area designated B-9. The cell bodies had a shrunken, pyknotic appearance with a dense staining of Nissl substance throughout the cytoplasm and nucleus and a prominent perineuronal space, increased staining of glial cells and evidence of glial reactions and neuronophagia.

Fuller and Hemrick-Leucke (1980) cited two characteristics of pCA which they felt were necessary for the neurotoxic effects: persistence of pCA due to its slow rate of metabolic removal and ongoing active accumulation of the drug into neurons because of its high affinity for the uptake system on the neuronal membrane. In comparison, FEN is rapidly metabolized but NORFEN is not. Both FEN and NORFEN are taken up into 5-HT neurons by the 5-HT uptake system (Fuxe et al., 1975). Fuller et al. (1987) concluded that the 5-HT depletion following FEN administration to rats was likely due mainly to NORFEN as brain levels of NORFEN are higher than those of FEN. These authors could find no data showing 5-HT depletion without the presence of NORFEN in brain after FEN administration to any species. Wong (1990) studied whole brain levels of 5-HT and its precursor amino acid tryptophan 0.5, 1 and 2 h after administration of equimolar doses (0.037 mmol/kg ip) of FEN and NORFEN and found that NORFEN produced a much larger decrease in 5-HT levels and a markedly greater elevation of tryptophan levels, suggesting that NORFEN is a potent inhibitor of tryptophan hydroxylase and/or a stimulator of amino acid transport into the brain. Further studies by Wong (1990) at the same dose indicated that NORFEN also increased brain levels of several large neutral amino acids (valine, leucine, isoleucine) that are thought to share the same transport system as tryptophan.

Administration of FEN produces decreases in 5-HT-related neurochemical parameters which persist longer than is expected from the $T_{1/2}$ of FEN. Different researchers have studied effects of chronic administration of FEN and long-term effects after acute high doses of FEN to evaluate possible neurotoxicity. Studies have yielded conflicting results and differing interpretations.

Harvey (1978) compared (±)-pCA and FEN in the rat using the same dosing regimen (.0125 mmol/kg or 0.1 mmol/kg x 10 days) and found at the higher dose FEN produced similar histological changes in cell bodies in the B-9 region as did pCA. Clineschmidt et al. (1978) presented evidence that the major site of FEN's action is serotoninergic nerve terminals. This group used FEN, 0.6 mmol/kg, given as a single dose to rats and found that 5-HT and 5-HIAA concentrations reached lowest levels at 5 h after drug administration, remained at this low level for 16 days and then began to recover. Recovery was not complete even 64 days later. In this study, NORFEN caused greater decreases in 5-HT concentrations than did FEN. Kleven et al. (1988) performed regional analysis of 5-HT and 5-HIAA decreases induced by FEN and found that there was partial recovery in the striatum and hypothalamus after 8 weeks but that hippocampal 5-HT and 5-HIAA remained depleted. Appel et al. (1989) administered FEN, 24 mg/kg, subcutaneously twice daily x 4 days and demonstrated a profound decrease in several brain regions in density of fine-caliber 5-HT-like immunoreactive fibers and terminals, with relative sparing of the thicker caliber axons and interneuronal axons. The effect was especially marked in cerebral cortex, hippocampus, medial hypothalamus and striatum. However, cell bodies in the midbrain raphé nuclei and spinal cord fibers and terminals appeared unaffected. Two weeks later the thickening and gross varocosity-type deformities in the fibers and terminals were no longer evident, but the decrease in density remained.

Schaechter and Wurtman (1989) administered (+)-FEN to rats, using doses of 1.25, 2.5, 5 or 10 mg/kg/day x 10 days and studied hypothalamic 5-HT levels and the ability of (+)-FEN-treated hypothalamic slices to spontaneously release endogenous 5-HT. They found no change in 5-HT levels in rats receiving 1.25 to 5 mg/kg and a 22% decrease at the highest drug dose. In addition, there was no difference between (+)-FEN-treated and control animals in the ability of hypothalamic nerve terminals to either spontaneously release 5-HT or to respond to electrical field stimulation. These authors concluded that doses of FEN required to produce anorexia in rats do not cause long-lasting decreases in hypothalamic 5-HT levels nor is there any loss of terminal functionality.

A.12 Drug Combinations

The administration of one or more pharmacological agents in conjunction with the drug under study provides another avenue to explore the studied drug's pharmacological and physiological effects. This technique can be employed in a variety of ways. Agents whose chemical structures are similar to that of a given drug can be administered. Bendotti et al. (1980) conducted a series of experiments with rats aimed at evaluating the relative potency of FEN enantiomers and their metabolites in their ability to counteract the locomotor stimulant effects and stereotypy induced by AMPH or apomorphine. The (+)-FEN and (+)-NORFEN were more potent than the (-)-enantiomers in reducing the stereotyped licking and/or biting induced by apomorphine. However, (-)-FEN was as potent as (+)-FEN and more potent than (-)-NORFEN in counteracting the AMPH-induced stereotypy. As part of their series of experiments, Bendotti et al. (1980) administered (+)-FEN or (+)-NORFEN to rats as pretreatment 15 min prior to (+)-AMPH and showed that both (+)-FEN and (+)-NORFEN increased brain levels of (+)-AMPH. This elevation of brain (+)-AMPH was lost when larger doses of (+)-AMPH were used.

Agents whose behavioural and/or neurochemical effects are known can be administered to validate predicted effects. Broekkamp et al. (1988) studied the effect of FEN on catalepsy induced by morphine or haloperidol. 5-Hydroxytryptamine was thought to play a key role in morphine-induced catalepsy. Haloperidol is a neuroleptic whose primary action is one of dopamine antagonism. However, these authors wished to examine a possible modulatory role for 5-HT in haloperidol-induced catalepsy. The administration of FEN enhanced the morphine-induced catalepsy but had no effect on the haloperidol-induced catalepsy. Jori et al. (1974) administered (-)-FEN to rats pretreated with the DA agonist apomorphine to explore the mechanism of (-)-FEN effect on striatal DA. Pretreatment with apomorphine antagonized the increase in striatal HVA induced by (-)-FEN. There was also a dose-dependent reduction of apomorphine stereotypy after the administration of (-)-FEN. Aulakh et al. (1988) administered acute IP injections of FEN to rats receiving concomitant short- or long-term continuous administration of the MAO inhibitor clorgyline. They then studied clorgyline's effects on FEN-induced suppression of food intake and locomotor activity. Although the food intake after the FEN injection was not less in clorgyline-treated animals relative to saline-treated animals, body weight gain was significantly less in clorgyline-treated animals. Clorgyline did not affect FEN-induced suppression of locomotor activity. Other examples of the use of drug combinations include known antagonists or reuptake blockers of biogenic amines which can be administered either as a pretreatment or concomitantly with FEN (Costa et al., 1971; Garattini et al., 1975; Rowland and Carlton, 1986). Fuxe et al. (1975) studied the influence of FEN on the 5-HT depletion caused by the tryptophan hydroxylase inhibitor α-propyldopacetamide and found that FEN enhanced the 5-HT depletion.

Knowledge about metabolism and pharmacokinetic properties of a drug can also be gained by employing drug combination techniques. Thus, Fuller et al. (1987) used SKF 525-A, a microsomal inhibitor to block N-dealkylation in an attempt to ascertain whether pCA formation is necessary for the serotoninergic depletion effects after administration of N-alkylated pCAs. However, the authors were not able to block N-dealkylation to a sufficient extent to provide data.

The ability of one drug to influence the enzymatic metabolism of another drug is well established. This influence can take the form of competitive inhibition in which both drugs utilize the same enzyme system. An example of non-competitive inhibition is one drug binding to an enzyme, thus rendering the enzyme inactive and preventing the metabolism of the second drug. Certain drugs such as phenobarbital cause enzymatic induction, enhancing metabolic processes (Hansten, 1985). Arvela (1973) administered FEN, 25 mg/kg or 50 mg/kg, via a stomach tube for 8 weeks and examined effects on drug-metabolizing enzymes in rat liver. Both dosage levels of FEN caused an increase in the relative liver weights but no morphogical changes were seen on histological examination. There were increases in levels of N-methylaniline demethylase (MAD) and cytochrome P-450. Maximal enzymatic induction was seen at 3 weeks.

There are many well studied examples of drugs involved in enzymatic inhibition. Cimetidine is known to inhibit metabolism of many drugs, including anticoagulants, tricyclic antidepressants, benzodiazepines, phenothiazines, phenytoin and quinidine (Hansten, 1985). Quinidine is also a cytochrome P-450 enzyme inhibitor. Leeman et al. (1986) looked at polymorphic oxidation of metoprolol and showed that quinidine blocked metoprolol metabolism by binding to the same isozyme system as that involved in debrisoquine metabolism. Neuroleptics are known to inhibit the metabolism of pethidine (Clark and Thompson, 1972), tricyclic antidepressants (Hansten, 1985) and tranylcypromine (Baker et al., 1986). The antidepressant tranylcypromine binds to mephenytoin hydroxylase, another P-450 isozyme involved in the metabolism of mephenytoin and perhaps other anticonvulsants. Belanger et al. (1982) showed that both tranylcypromine and phenelzine inhibited competitively the N- and O-demethylations of aminopyrine and p-nitroanisole respectively. Phenelzine was, however, a noncompetitive inhibitor of N-demethylation of N,N-dimethylaniline. Thus, phenelzine's inhibitory effect on aminopyrine demethylase and aniline hydroxylase was that of a mixed type of inhibition, involving more than 1 isozyme system.

There has been less written about the enzymatic inhibitory properties of the antidepressant iprindole. Iprindole has the distinction of being the first of the so-called 'second generation' antidepressants, the classical tricyclic antidepressants being termed the first generation. Two of the rings of the standard tricyclic configuration are replaced by an indole moeity which renders the drug nearly devoid of activity on MAO or on blockade of reuptake of biogenic amines (Zis and Goodwin, 1979) (Figure 5). Controlled clinical trails of iprindole in unipolar depression showed it superior to placebo and equivalent to imipramine in therapeutic efficacy, while producing milder anticholinergic effects than imipramine (Cassidy and Henry, 1987). de Montigny (1985) demonstrated that non-responders to iprindole improved with the addition of lithium. Interest in the clinical use of iprindole waned due to a lack of documented superiority to the tricyclic antidepressants and because of clinicians' familiarity with the classic tricyclics. However, the ability of iprindole to block aromatic ring hydroxylation has lead researchers to use this drug to study in more detail the metabolism of such drugs as AMPH.

Freeman and Sulser (1972) demonstrated a marked increase in brain levels of AMPH in iprindole-pretreated rats up to 24 h post treatment with AMPH. Levels of the ring-hydroxylated metabolites p-hydroxyamphetamine and p-hydroxynorephedrine were decreased, providing indirect evidence that the mechanism of the action of iprindole on AMPH was a blockade of p-hydroxylation. The same authors showed that desmethylimipramine (DMI) also blocked p-hydroxylation of AMPH.

Thus, the metabolic clearance of AMPH is slowed following pretreatment with iprindole, allowing significant amounts of AMPH to accumulate in dopaminergic neurons. As a result, chronic dopaminergic changes indicative of neurotoxic effects became evident. Fuller and Hemrick-Luecke (1980) showed that the decrease in striatal DA concentration after a single injection of AMPH in iprindole-pretreated rats persisted up to 1 week. Steranka (1982) had similar findings,

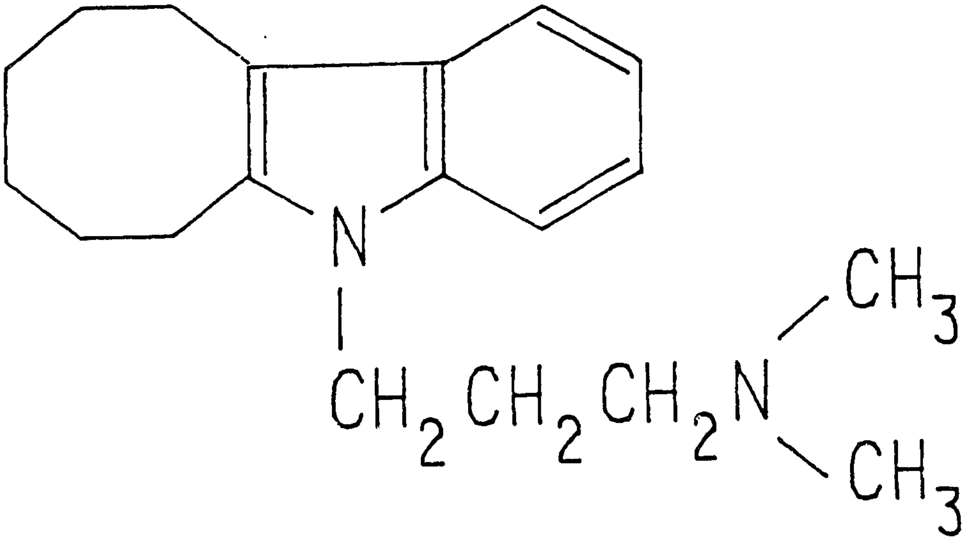


Figure 5: Structure of iprindole (5-[3-dimethylaminopropyl]-6,7,8,9,10,11-hexahydro-5*H*-cyclooct[*b*]indole).

with decreases in DA levels lasting at least 4 weeks. Uptake blockers such as amfonelic acid given concurrently with AMPH prevented the striatal DA, DOPAC and HVA decreases when brains were examined 1 week later (Steranka, 1982). Giving the amfonelic acid 4 or 8 h later than the AMPH continued to provide protection from the dopaminergic changes. However by 12 h, amfonelic acid no longer showed any preventative effect on the AMPH-induced decreases.

In rat liver preparations, ring hydroxylation of ß-phenylethylamine (PEA) to produce p-tyramine is carried out by a microsomal membrane-bound (100,000 g pellet), NADPH-requiring cytochrome P-450 enzyme system. Iprindole has been shown to block the reaction in a competitive manner (Mosnaim et al., 1989). This liver enzyme system is also involved in the hydroxylation of (+)-AMPH, such that (+)-AMPH competitively inhibits the formation of p-tyramine from PEA.

Iprindole has also been shown to inhibit phosphatidylinositol synthetase (Li et al., 1988). Green et al. (1989) demonstrated that iprindole provided partial protection to mouse brain MAO from the irreversible MAO inhibitor, phenelzine. The authors suggest that iprindole reversibly inhibited MAO-A and MAO-B in vivo.

A.13 Gas Chromatography

In the studies described in this thesis, gas chromatography (GC) was used to simultaneously separate and quantitate FEN and NORFEN. This technique has the advantages of simplicity, economy, speed and relatively high sensitivity.

13.1 Principles in Gas Chromatography

Gas chromatography is used extensively in analytical laboratories as it allows for efficient separation, quantitation and identification of minute amounts of individual components of a sample mixture. Separation is accomplished when a carrier gas (mobile phase) is allowed to pass through a column containing a non-volatile liquid (the stationary phase) coated onto an inert solid support material into which the sample mixture in a suitable solvent is injected. The components in the mixture are separated according to their partition coefficients between the mobile phase and the stationary phase. The rate at which each component moves through the column will depend on the extent of its distribution between the two phases. The column is housed in an oven. The column temperature can be held constant throughout the analysis, allowing an isothermic separation of components, or can be programmed to rise at a preset rate. Temperature programming provides greater resolution and shorter analysis time when analyzing samples containing components with a wide range of boiling points (Coutts and Baker, 1982).

When each component elutes from the column, it makes contact with the detector, setting up an electronic signal which is displayed as a peak on the chart recorder. The retention time of a peak is taken from the point of injection to the apex of the peak. Retention times are characteristic of, but not unique to, the component which produced a given peak under specific GC conditions.

13.2 Columns

Columns are composed of tubing, a solid support and the stationary liquid phase. The tubing is usually made of glass, but stainless steel, copper, nickel, aluminium and glass-lined metal have also been used (Coutts and Baker, 1982). Glass is a popular choice because of its inert nature and ease with which visual inspection can be made.

The solid support of a packed column is inert and provides a large surface area for the liquid stationary phase. Two types of support materials are available. Chromosorb® W (White) is a low density support, retaining as much as 30% stationary phase (Coutts and Baker, 1982). Chromosorb® P (Pink) is stronger but loses some of its retentive ability. Polymer beads (Porapak®) were first introduced as packing material by Hollis in 1966. The beads serve as both the solid support and the stationary phase.

Polarity can be used to classify types of stationary liquid phase in use, from the polar polymers of polyethylene glycol to the semipolar phenylmethylsilicone polymers to the nonpolar polymers of dimethylsilicone. There is a huge assortment of liquid phases. The choice is usually based on an empirical process, keeping in mind separations are best achieved by matching the polarities of the solute and liquid phase.

The size of the column can also vary. Packed columns are usually 1-2 m in length and 2-4 mm in internal diameter. Capillary columns are longer, 10-75 m in length, and narrower, 0.25-0.50 mm, in diameter. Capillary columns are commonly used in analysis of trace amounts, as the resolution of analytes obtained is superior to that of packed columns.

Capillary columns are generally made of borosilicate glass or fused silica; the latter material has become much more popular because of increased flexibility and durability. Two types of capillary columns are in common use - the wall-coated open tubular (WCOT) type in which the liquid phase has been deposited directly onto the inner glass surface of the column and the support-coated open tubular (SCOT) type in which the inner surface of the column is covered with a thin layer of a solid support that is coated with the liquid phase.

13.3 Detectors

There are several detectors in use, of which the nitrogen-phosphorus detector and the electron-capture detector are but two. The nitrogen-phosphorus detector is a specialized type of flame ionization detector developed to be highly sensitive to nitrogen- and/or phosphorus-containing compounds. With the addition of rubidium salt, a low-temperature plasma is formed rather than a discrete flame (Coutts and Baker, 1982). An electrical field is established between a negatively charged hydrogen burner jet and a positively charged electrode. Column effluent is ionized during combustion and the negative ions are collected at the anode. The amount of ions generated is proportional to the amount of the combusted compound. Although this detector can detect low picogram quantities of certain compounds, it was found to be of limited value in quantitating low nanogram concentrations of FEN in the present study.

The electron-capture detector (ECD) is one of the most selective and sensitive detection systems, especially for the analysis of trace amounts of compounds containing halogen, nitro, hydroxyl and carboxyl groups (Baker et al., 1981). The ECD functions by sensing a decrease in the number of free electrons in the detector chamber due to their capture by electrophoric sample molecules. A radioactive isotope with a continuous ß decay (usually Ni^{63}) provides the electron supply by bombarding the carrier gas. The decrease in the "standing current" is amplified and recorded as a peak. When the electrophore leaves the detector, the current returns to the original value of standing current.

The mass spectrometer can also be used as a selective detector and operates on the principle that functional groups direct the fragmentation of a molecule under electron bombardment in such a way that, knowing the structure of a molecule, it is possible to predict the fragmentation pattern and conversely, knowing the fragmentation pattern, a possible structure of the original molecule can be proposed (Clarke, 1978). For proper functioning of the mass spectrometer, carrier gas from the GC is removed prior to the column effluent passing into the ionization chamber of the mass spectrometer, where ionization of the compound occurs as a result of bombardment by high energy electrons (electron impact mode) or collision with an ionized reactant gas (chemical ionization mode) such as methane, isobutane or ammonia. The positively charged fragments are collected and passed into an electron multiplier that generates an electric current which is then amplified and recorded. The mass spectrometer can be programmed to amplify a specific current produced by an ion of a particular mass-to-charge ratio, called single ion monitoring, or can be set in the scanning mode (Reid, 1976).

B. MATERIALS

B.1 Animals

Male Sprague-Dawley rats (Bioscience Animal Services, Ellerslie, Alberta, Canada) weighing 216-365 g were used. Weights did not differ significantly between groups within individual experiments. All animals were housed two per cage under 12 h light/dark conditions at a room temperature of 21°C. Food and water were available ad libitum. Animals were fed Lab-Blox Feed (Wayne Food Division, Continental Grain Co., Chicago, IL, USA), composed of 4.0% crude fat (min), 4.5% crude fibre (max) and 24% crude protein (min). The experimental protocols involving animal experiments were approved by the the Health Sciences Animal Welfare Committee of the Faculty of Medicine, University of Alberta. Guidelines as set out by the Canadian Council on Animal Care were followed in all experiments.

B.2 Chemicals

The following list contains all chemicals used in this study as well as their supplier or manufacturer.

Chemical	Supplier/Manufacturer
(±)-fenfluramine	A.H. Robins Co. Inc. Research Laboratories 1211 Sherwood Avenue Richmond, Va. 23220

	U.S.A.
(±)-norfenfluramine	A.H. Robins Co. Inc.
p-chlorophentermine	synthesized by R.T. Coutts Faculty of Pharmacy & Pharm. Sci. University of Alberta
pentafluorobenzoyl chloride	Aldrich Chemical Co. Inc. P.O. Box 355 Milwaukee, WI 53201 U.S.A.
pentafluorobenzenesulfonyl chloride	Aldrich Chemical Co. Inc.
trichloroacetic anhydride	Pfaltz and Bauer Inc. Waterbury, CT 06708 U.S.A.
iprindole	Wyeth Research (U.K.) Ltd. Huntercombe Lane South Taplow Maidenhead Berkshire, SL6 0PH U.K.
perchloric acid (60%)	Fisher Scientific Company Fair Lawn, N.J. 07410 U.S.A.
potassium carbonate	Fisher Scientific Company
ethyl acetate	Fisher Scientific Company

potassium carbonate	Fisher Scientific Company
nitrogen gas	Union Carbide Canada Ltd. Edmonton, Alberta
pentafluoropropionic anhydride, 99%	Aldrich Chemical Co. Inc.
toluene (glass distilled)	BDH Chemicals 9527 - 49 Street Edmonton, Alberta
sodium borate	Fisher Scientific Company
helium	Union Carbide Canada Ltd.
5% methane in argon	Union Carbide Canada Ltd.
(S)-(-)-N-(trifluoroacetyl)-L-prolyl chloride	Aldrich Chemical Co. Inc.
trichlorobenzenesulfonyl chloride	Pfaltz & Bauer Inc.
sodium bicarbonate	Fisher Scientific Comparny
acetonitrile	BDH Chemicals
chloroform	BDH Chemicals
2-methylbutane (isopentane)	BDH Chemicals
n-pentane	BDH Chemicals
di-(2-ethylhexyl)phosphate (DEHPA)	Sigma Chemical Company P.O. Box 14508 St. Louis, MO 63178

B.3 Analytical Instrumentation and Equipment

3.1 Gas Chromatograph Used in Study

A Hewlett-Packard (HP) 5880A gas chromatograph equipped with a fused silica SP 2100 capillary column (15m, 0.25 mm ID, 0.25 μm phase thickness) supplied by Supelco (46-220 Wyecroft Road, Oakville, Ontario, Canada, L6K 3V1) and an ECD was employed. The instrument was interfaced with a HP 5880A Series GC Terminal (level 4) integrator to measure peak heights. All samples, 1 μl each, were injected manually. Helium (2 ml/min) was used as the carrier gas and 5% methane in argon (35 ml/min) was used as the make-up gas at the detector. The injector port and detector temperatures were 200°C and 300°C respectively. The initial oven temperature was set at 105°C and then programmed to rise at a rate of 10°C/min after an initial time delay of .5 min. At 8 min, the oven temperature increased to 230°C and was held at that temperature until the individual run stopped at 11 min.

3.2 Mass Spectrometry

A Hewlett-Packard (HP) model 5985A quadrupole mass spectrometer was used in the EI mode. Operating conditions were as follows: ion source temperature, 200°C; interface temperature, 275°C; column pressure, 10 p.s.i.; accelerating voltage, 2200 eV; ionization voltage, 70 eV; scan speed, 100 amu/sec; and dwell time, 200 msec. The same column as described above was used for GC-mass spectrometry.

3.3 Washing of Glassware

All glassware was soaked in a solution of Sparkleen® (Fisher Scientific Co.) and then sonicated three times for 20 min each in a solution of 2% Contrad 70® (Canlab, American Hospital Supply Canada Inc., Mississauga, Ontario) in water. The glassware was then cleaned in a cold water-hot water-distilled water wash-and-rinse cycle (Miele dishwasher; Miele Electronics, F.R.G.). Finally the glassware was air dried at 100°C-150°C for 1 h in a Precision® mechanical convection oven, model 18EM (Precision Scientific Group, 3737 West Courtland Street, Chicao, Ill., 60647).

3.4 Balances

Tissue samples, drugs and chemicals were weighed on a Mettler AE160 (Mettler Instrumente Ag, Ch-8606, Greifensee-Zurich, Switzerland) electronic weighing balance with 0.1 mg sensitivity. Animals were weighed on a 700 series triple beam balance with a 2610 g capacity (Ohaus, Florham Park, N.J., U.S.A.).

3.5 Tissue Homogenizer

Tissue samples (brain and liver) were homogenized with a Tri-R Stir-R® Model 563C homogenizer (Tri-R Instruments, Rockville, N.Y., U.S.A.) using a Teflon® pestle and a glass mortar (with a clearance of 0.1-0.15 mm). The rotor shaft has a maximum speed of 12,000 rpm with a ten-speed setting. The speed setting routinely began at 5 then was increased to 7. Brain tissue was homogenized using 12 up- and down-strokes while liver tissue required 15 strokes.

3.6 Centrifuges

All low speed centrifugations (up to 1500 x g) were performed on a Sorvall GLC-2B benchtop centrifuge (Dupont Instruments). Precipitation of protein from tissue homogenates was performed on a Damon IEC B-20A refrigerated centrifuge (Damon/IEC, Needham Hts., MA, U.S.A.).

3.7 Shaker-Mixers

A 36-sample IKA-IBRAX-VXR® (Janke & Kunkel GmBH, Sweden) tube shaker was employed for extraction purposes. Mixing and vortexing of individual tubes were performed on a benchtop Thermolyne Maxi Mix 1® (Sybron Corporation, Dubuque, IA, U.S.A.).

3.8 Pipettes

Pipetting was done using either variable sample size (20, 200, 1000 μl capacity; 1-5 ml capacity; Gilson, France), or repeated sample (1.25, 5.0, 12.5 ml capacity; Eppendorf, U.S.A.) pipettes. Transfer of layers was accomplished using single-use 5-3/4" and 9" Pasteur Capillary Pipettes (Fisher Scientific Co.).

3.9 Heating Block

A 20 sample Canlab Temp-Blok® module heater (model Cat. H2025-1; 115V, 50/60 Hz, 100W) (Lab-Line Instruments Inc., Melrose Park, Ill., U.S.A.) was used to heat the final derivitization mixture.

C. METHODOLOGY

C.1 Experimental Design

Six animals were assigned at random to each of 5 different treatment groups. To compensate for any possible handling effects, two single-injection treatment groups were included: single saline and single FEN injections. The other treatment groups were: saline injection followed 1 h later with FEN or iprindole followed 1 h later with saline or with FEN. The iprindole/saline treatment group was included to examine the possibility that a metabolite of iprindole had a similar retention time to that of derivatized FEN or NORFEN. The animals were sacrificed at 1, 2, 4, or 8 h after the last injection. Doses of FEN and iprindole used were 6.7 mg/kg (0.025 mmol/kg) and 11.2 mg/kg (0.039 mmol/kg) respectively.

C.2 Drug Administration

Acute drug administration was done via intraperitoneal injection using a tuberculin 1 cc syringe equipped with a 27G 1/2" needle (Becton Dickinson, Closter, N.J., U.S.A.). All injected drugs were dissolved in saline solution (0.85% w/v sodium chloride) (Fisher Scientific Co.) such that the rats received 2 ml/kg. Animals were injected in random order.

C.3 Tissue Collection

Animals were killed by cervical dislocation and decapitation. Whole brain and liver were removed, frozen immediately in isopentane over solid carbon dioxide and stored at -80°C until the time of analysis. Gas chromatographic analyses of the samples were conducted within 2 weeks of collection.

C.4 Tissue Preparation

Whole brain samples weighing approximately 1.8-2.0 g were divided in half, using either the left or right half at random. Samples of brain and liver were weighed, and 5 volumes of .1N $HCl0_4$ were added. The samples were homogenized and then centrifuged in the refrigerated centrifuge (10 min at 15,000 x g). A portion (200 μl) of the supernatant was used in the assay.

C.5 Assay Development

This assay was developed to quantitate simultaneously the amounts of FEN and NORFEN in brain and liver tissue, using a relatively rapid and easy protocol.

Derivization was attempted with a number of different reagents: pentafluorobenzoyl chloride (PFBC), pentafluorobenzenesulfonyl chloride (PFBS), trichloroacetic anhydride, (S)-(-)-N-trifluoroacetyl-L-prolyl chloride (a chiral reagent), trichlorobenzenesulfonyl chloride (TCBS) and pentafluoropropionic anhydride (PFPA). These reagents were dissolved in ethyl acetate:acetonitrile (9:1), toluene, n-pentane or ethyl acetate alone. Reagents used to basify the tissue supernatant included solid potassium bicarbonate, solid sodium bicarbonate, solid potassium carbonate and potassium carbonate in solution (a two-step process using a 25% solution then a 2.5% solution).

C.6 Assay

To each tube, containing 200 μl of either 0.1 N $HClO_4$ or tissue in 0.1 N $HClO_4$, 25% K_2CO_3 (100 μl) was added. To this was added 700 μl of 2.5% K_2CO_3 to make up the volume to 1 ml. The solution was vortexed briefly. The internal standard (I.S.) chosen was p-chlorophentermine (another halogenated AMPH analogue). Twenty μl of I.S. (100 ng per μl solution) was added to all samples except the blank. Ethyl acetate (2 ml) was added and the samples were shaken (5 min) and centrifuged (at 2,000 rpm for 3 min). The top layers were transferred to smaller test tubes and taken to dryness in a warm water bath under a gentle stream of nitrogen. Ethyl acetate (25 μl) and PFPA (75 μl) were added. The mixture was vortexed and the samples were placed in a heating block for 30 min at 60°C. The samples were allowed to cool at room temperature for 10 min, and toluene (300 μl) and saturated sodium borate (3 ml) were added to each. After vortexing briefly, the tubes were centrifuged and the phases separated. The toluene layer was retained in each case and an aliquot (1 μl) employed for GC analysis. Figure 6 demonstrates the assay procedure and Figure 7 outlines the derivatization reaction. All samples were refrigerated and analyzed the following day.

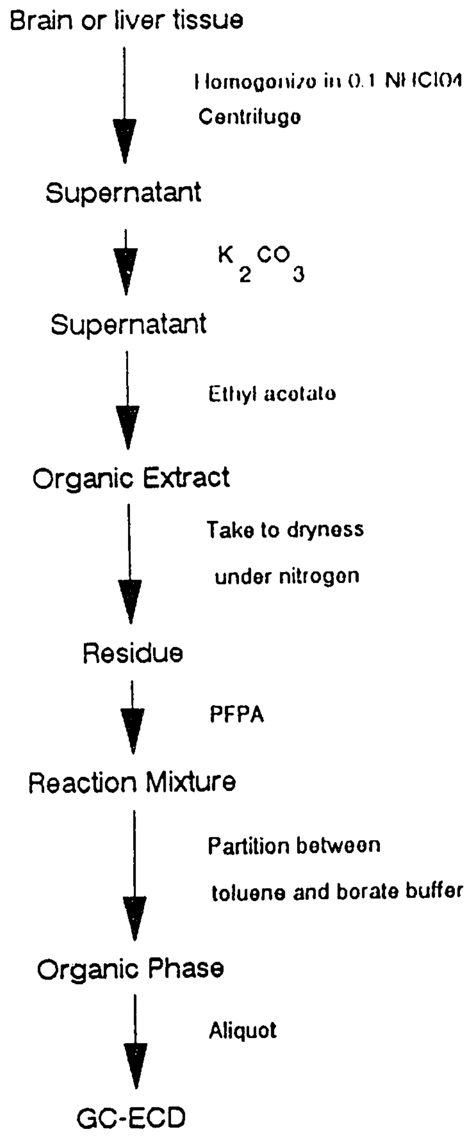


Figure 6: Flow sheet demonstrating the assay procedure developed for simultaneous analysis of fenfluramine and norfenfluramine in brain and liver tissue.

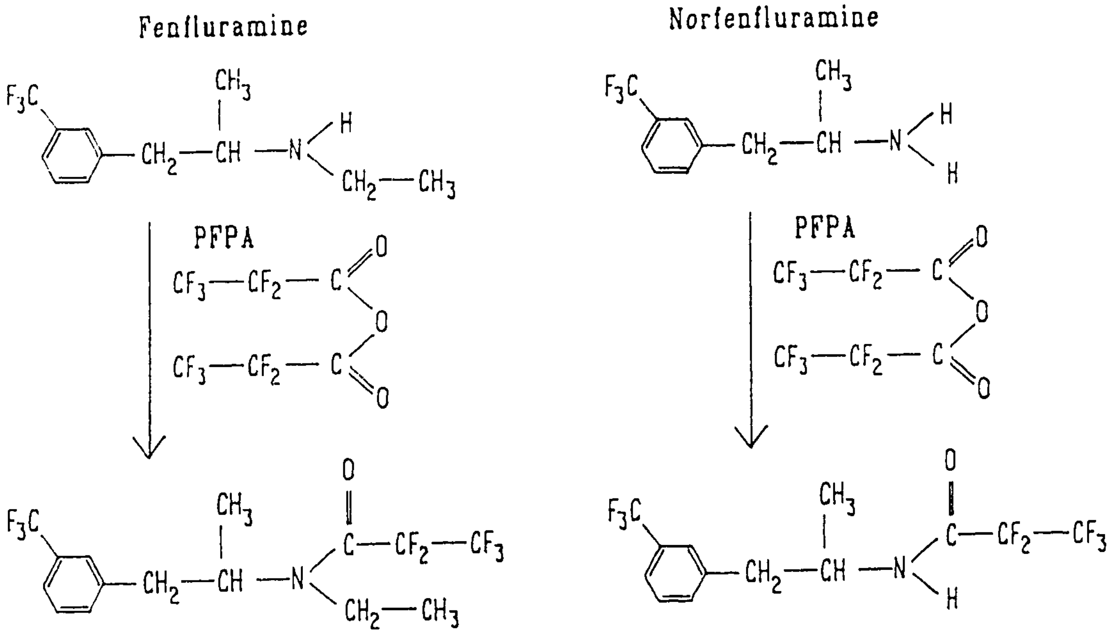


Figure 7: Derivatization of fenfluramine and norfenfluramine with pentafluoropropionic anhydride.

A set of authentic standards was run in parallel through the procedure to provide a calibration curve. This curve was prepared by adding varying amounts of FEN and NORFEN and a fixed amount of I.S. (the same amount as added to the tissue extract samples) to a set of tubes. At the end of the assay, the peak height ratios of derivatized FEN and NORFEN to that of derivatized I.S. were determined and plotted. Using the same peak height ratios in the tissue samples and extrapolating on the calibration curve, the amount of FEN and NORFEN in each sample was determined.

C.7 Statistical Analysis

The standard error of the mean (S.E.M.) is represented by error bars on all figures. Two-way analysis of variance was employed for pairwise comparisons between experimental groups. A pairwise post hoc comparison test, the Tukey test, was carried out to calculate the critical difference required to achieve statistical significance. For the calculations, the p value was 0.05 (Kiess, 1989).

D. RESULTS

Various reagents were investigated for their ability to produce detector-sensitive derivatives, and for the simplicity, specificity and reproducibility of the assay process. The derivatization of NORFEN with PFBC in toluene produced a derivative with good detector sensitivity using either a electron-capture or nitrogen-phosphorus detector. However, the sensitivity of the detectors to the derivative of FEN was poor. Using PFBC as the derivatization agent necessitated an additional step in the protocol -- a cleanup step using the liquid ion-pairing compound di-(2-ethyl-hexyl)-phosphate (DEHPA) (2.5% in chloroform), thus increasing the time required for the assay procedure.

Trials with other derivatization agents were conducted. Detector sensitivities were poor for derivatives of both FEN and NORFEN when PFBS was used. Trichloroacetic anhydride also yielded poor detector sensitivities to the derivatives of FEN and NORFEN. The chiral reagent, (S)-(-)-N-trifluoroacetyl-L-prolyl chloride, yielded the two pairs of enantiomeric derivatives. However, at concentrations required for analysis in tissues, the two chromatographic peaks corresponding to the derivatized NORFEN enantiomers were hidden by interference peaks in the chromatograph originating from the reagent.

The use of PFPA in ethyl acetate gave products that proved to be superior in sensitivity, ease of assay and chromatographic quality of the resultant derivatives when compared with the above indicated derivatization agents.

The pentafluoropropionic (PFP) derivatives provided good sensitivity (< 20 pg "on-column") and stability and possessed good chromatographic properties (see Figures 8 and 9 for typical GC traces). The assay procedure was highly reproducible, with correlation coefficients > 0.99 obtained routinely and interassay coefficients of variation of < 10% were obtained in a study on 50 ng samples run in 10 separate experiments. Typical calibration curves for FEN and NORFEN are shown in Figures 10 and 11. Recoveries (based on comparing samples reacted directly with PFPA versus those carried through the entire extraction procedure) were quantitative for both FEN and NORFEN. Structures of the derivatives were confirmed by mass spectrometry. Figures 12, 13 and 14 show the proposed fragmentation of the derivatives of FEN, NORFEN and p-chlorophentermine, respectively. Although molecular ions were absent, the presence of other fragments allowed confirmation of structure in each case.

Results from the single FEN injection and saline/FEN injection test groups show no statistical difference in FEN and NORFEN levels between the two test groups. An examination of GC traces of the iprindole/saline test group showed no peaks at the retention times of derivatized FEN and NORFEN.

The brain concentrations of FEN and NORFEN after i.p. administration of FEN, 0.025 mmol/kg, are illustrated in Figure 15. By 1 h, there was significant FEN accumulation in the brain tissue. Also by this time, the level of NORFEN indicates that FEN was rapidly metabolized (Figure 15). The amount of NORFEN leveled off by 2 h and remained essentially at that level for the remaining periods studied. The corresponding hepatic levels of FEN and NORFEN are given on

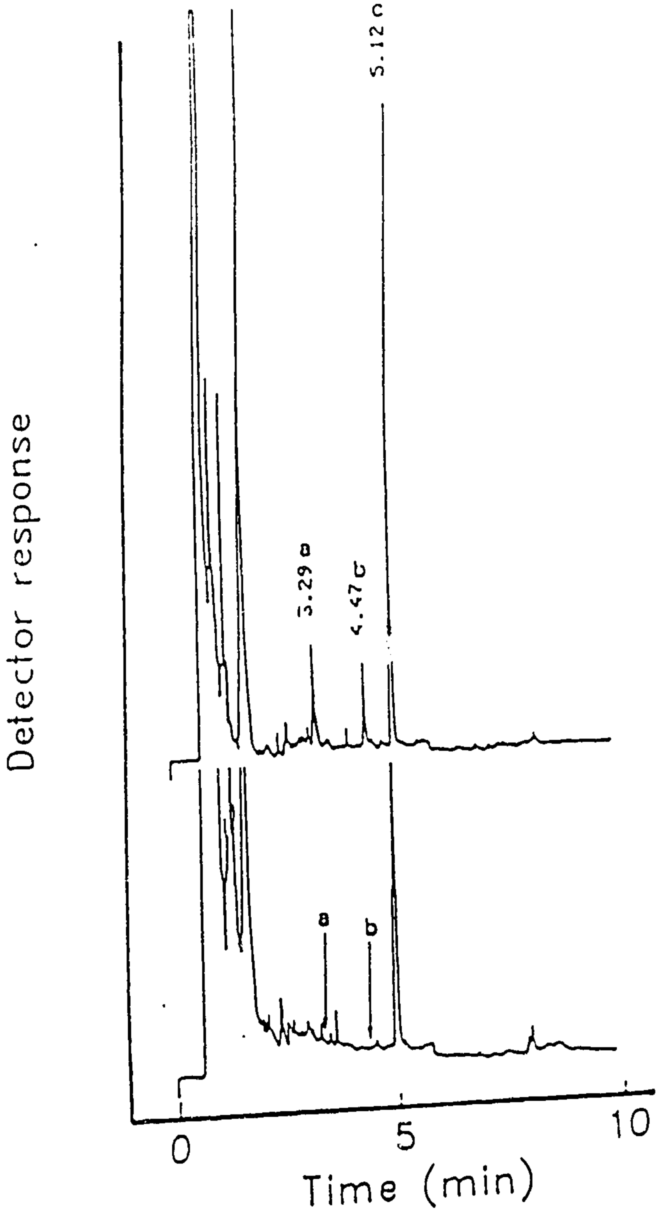


Figure 8: Typical GC traces of extracted brain tissue. Upper trace = extract from a rat treated with vehicle/fenfluramine; lower trace=extract from a rat treated with vehicle/vehicle. a, b and c are peaks corresponding to derivatized norfenfluramine, fenfluramine and p-chlorophentermine, respectively. No peaks corresponding to derivatized p-chlorophentermine were present in unspiked brain extracts.

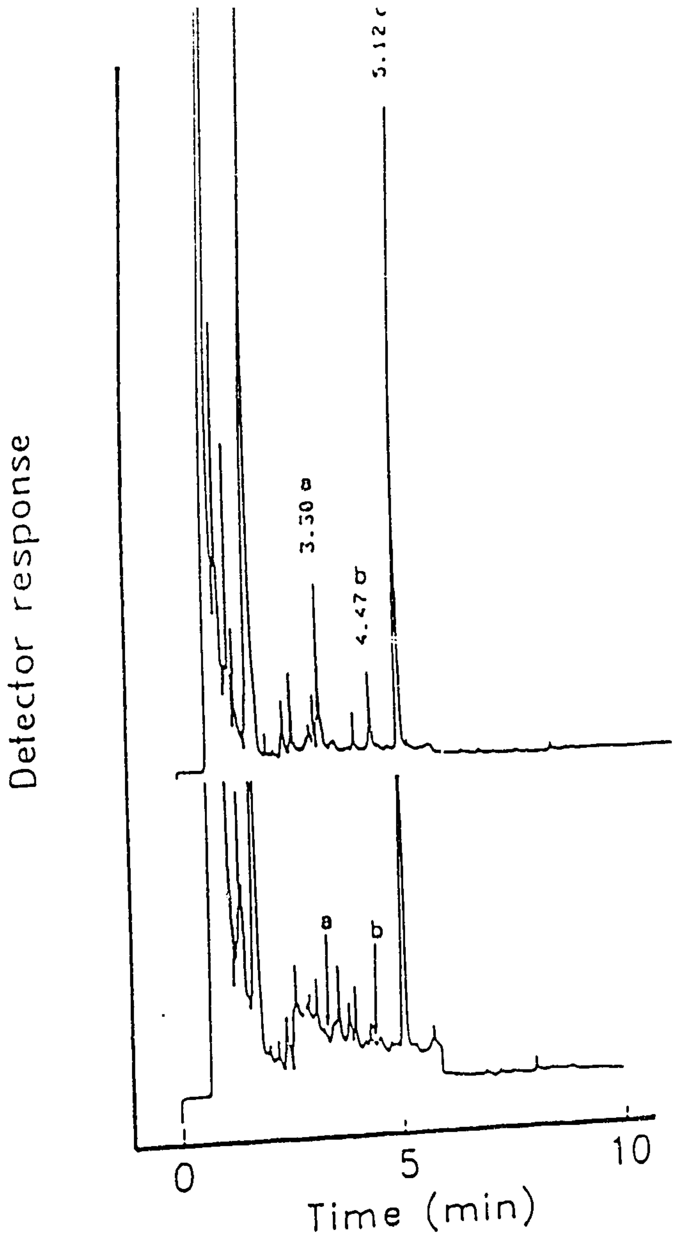


Figure 9: Typical GC traces of extracted liver tissue. Upper trace = extract from a rat treated with vehicle/fenfluramine; lower trace = extract from a rat treated with vehicle/vehicle. a, b and c are peaks corresponding to derivatized norfenfluramine, fenfluramine and p-chlorophentermine, respectively. No peaks corresponding to derivatized p-chlorophentermine were present in unspiked liver extracts.

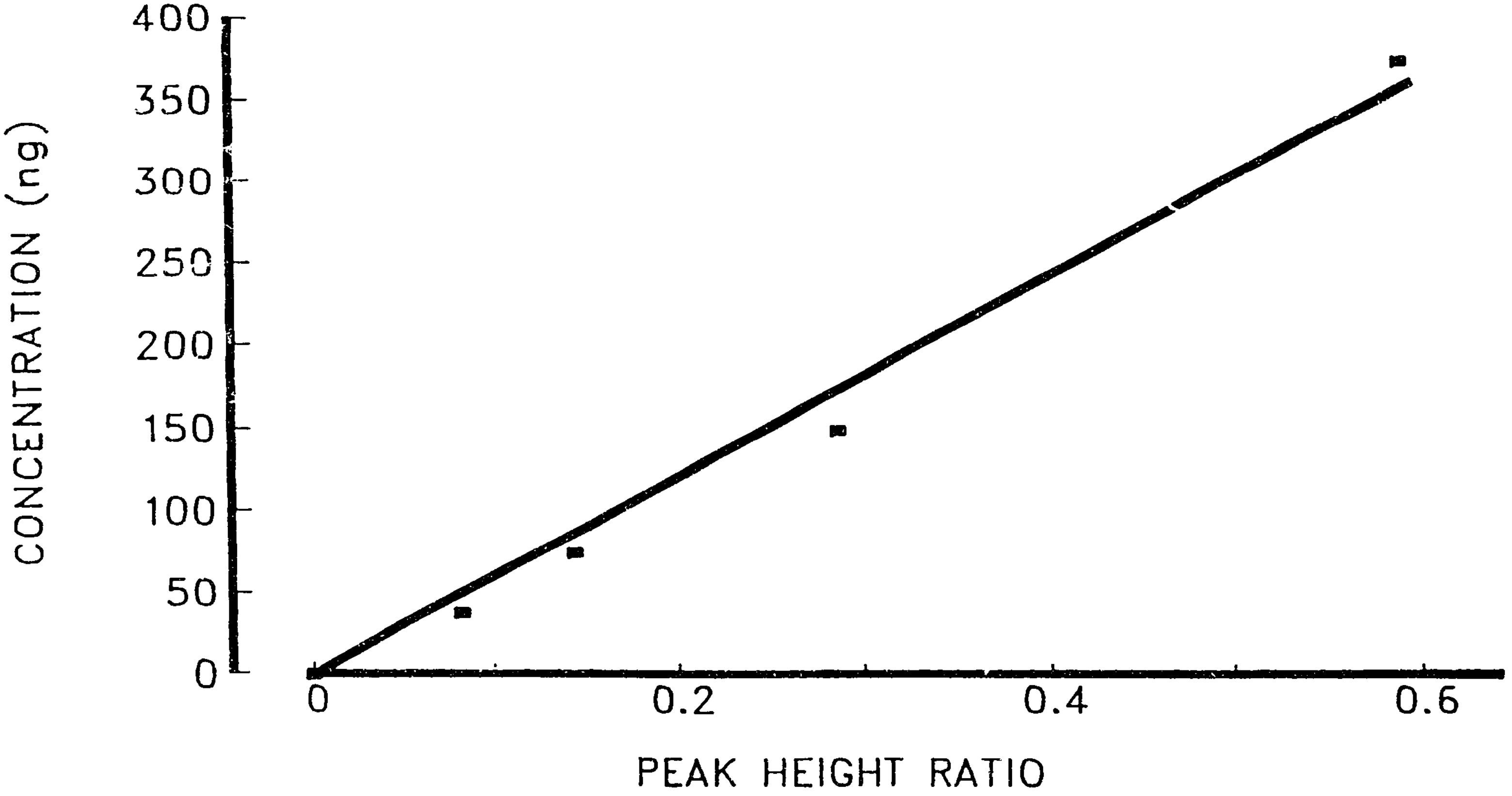


Figure 10: A typical standard curve in the usual working range for the fenfluramine assay.

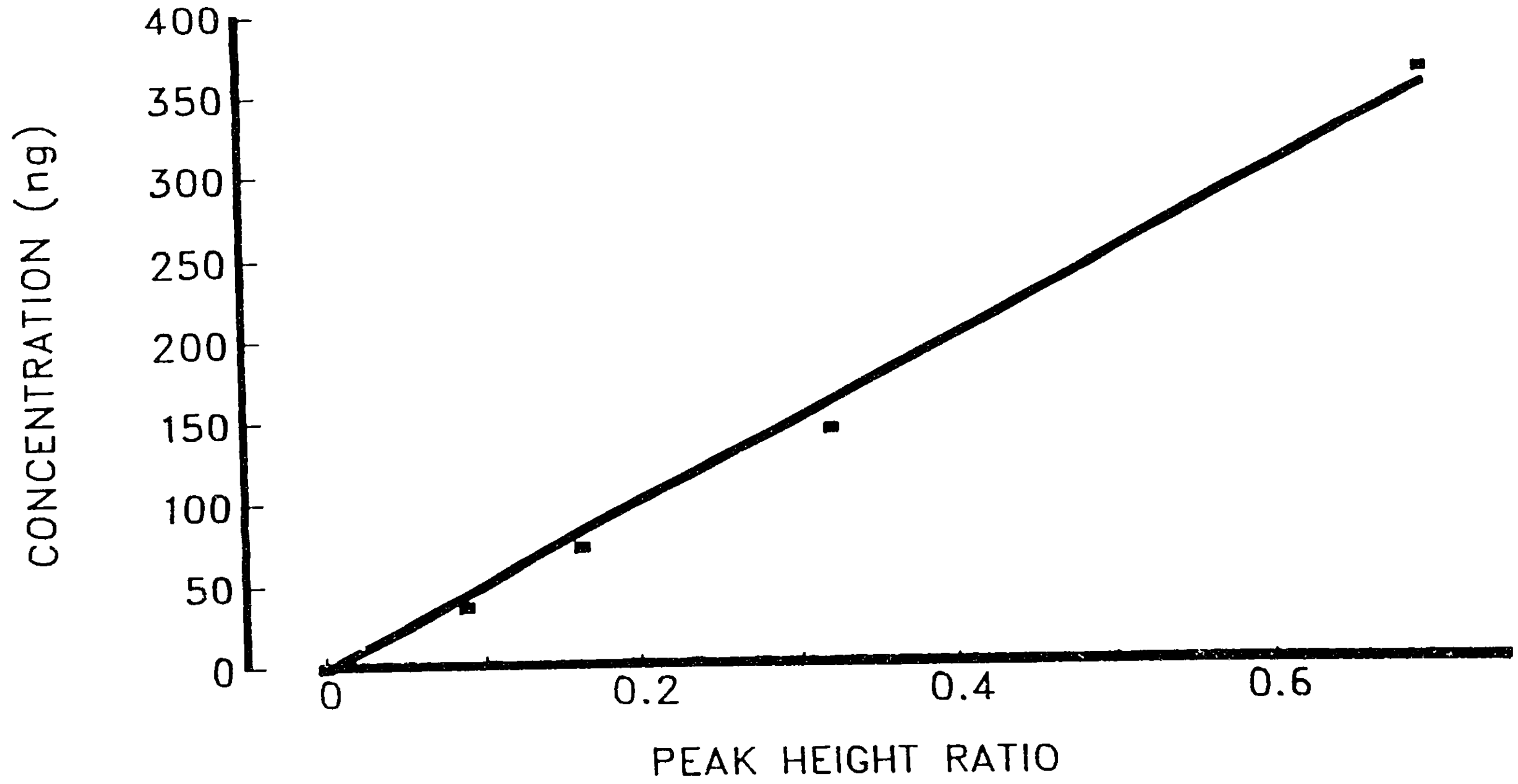


Figure 11: A typical standard curve in the usual working range for the norfenfluramine assay.

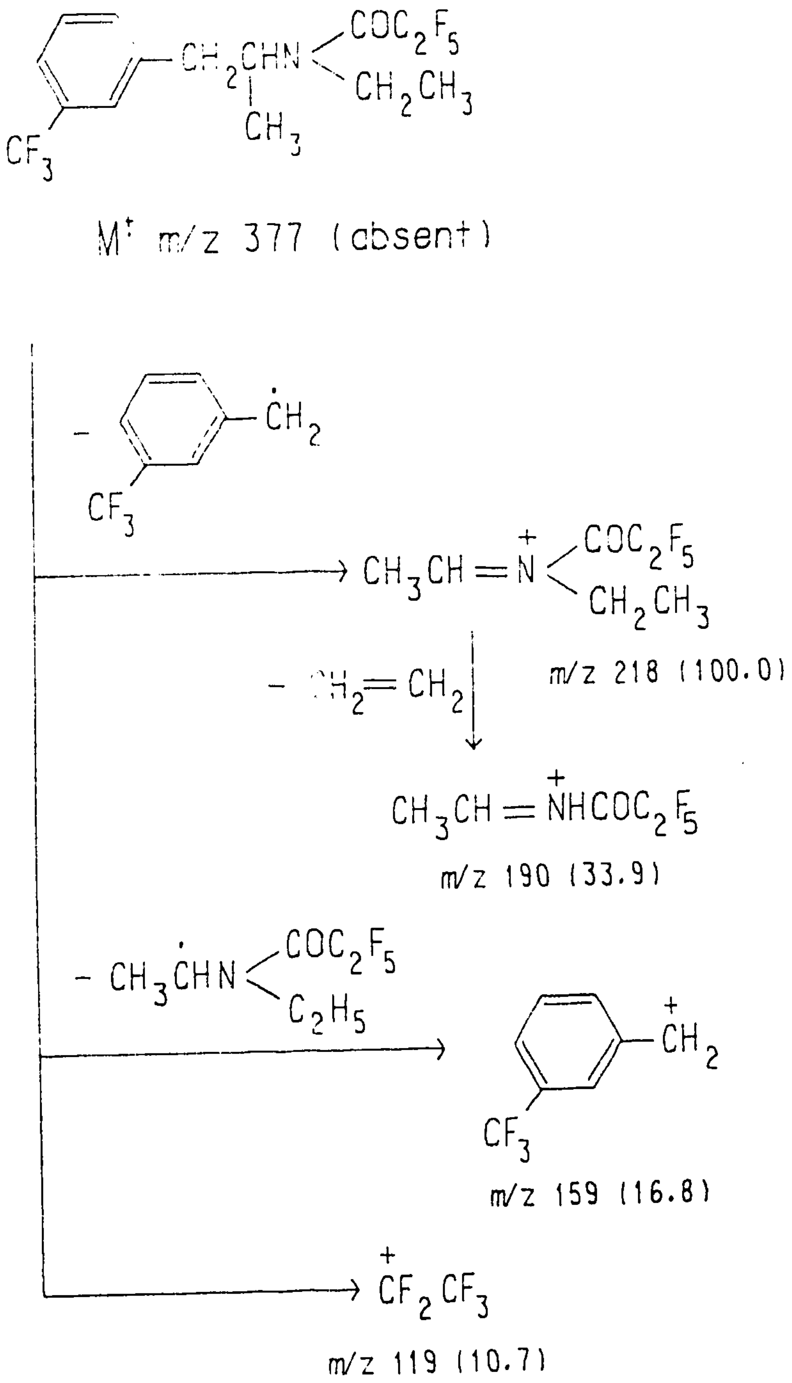


Figure 12: Proposed mass spectrometric fragmentation of the PFP derivative of fenfluramine.

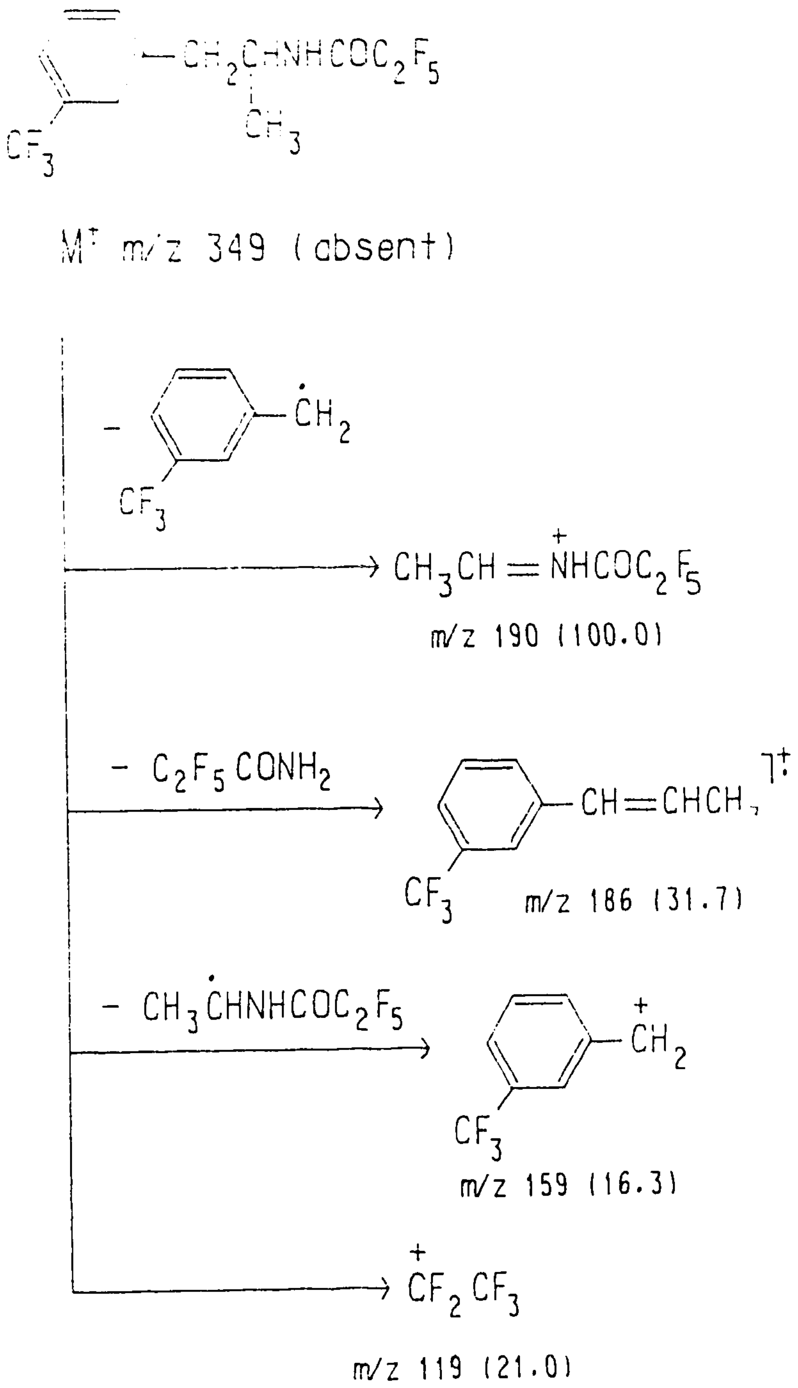


Figure 13: Proposed mass spectrometric fragmentation of the PFP derivative of norfenfluramine.

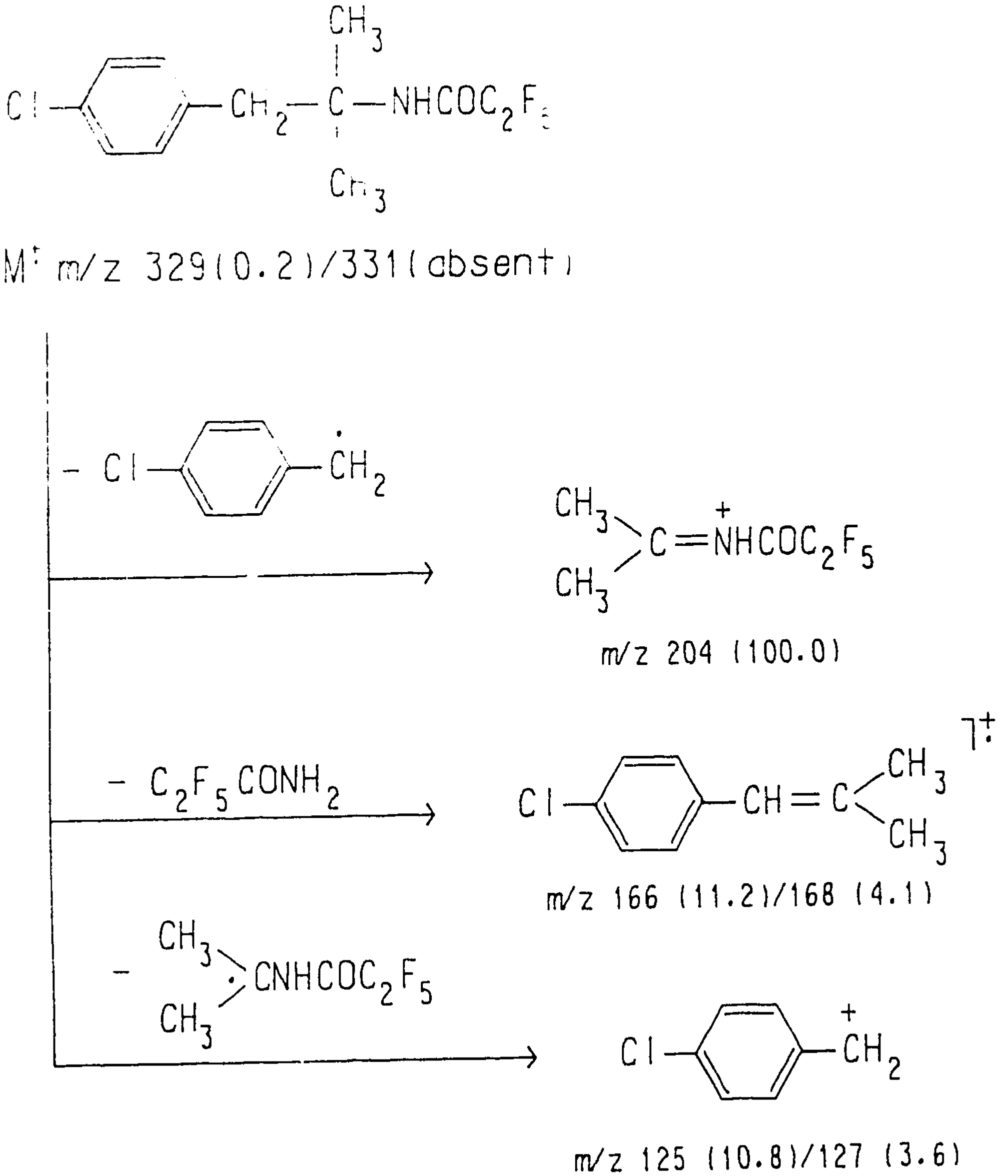


Figure 14: Proposed mass spectrometric fragmentation of the PFP derivative of p-chlorophentermine.

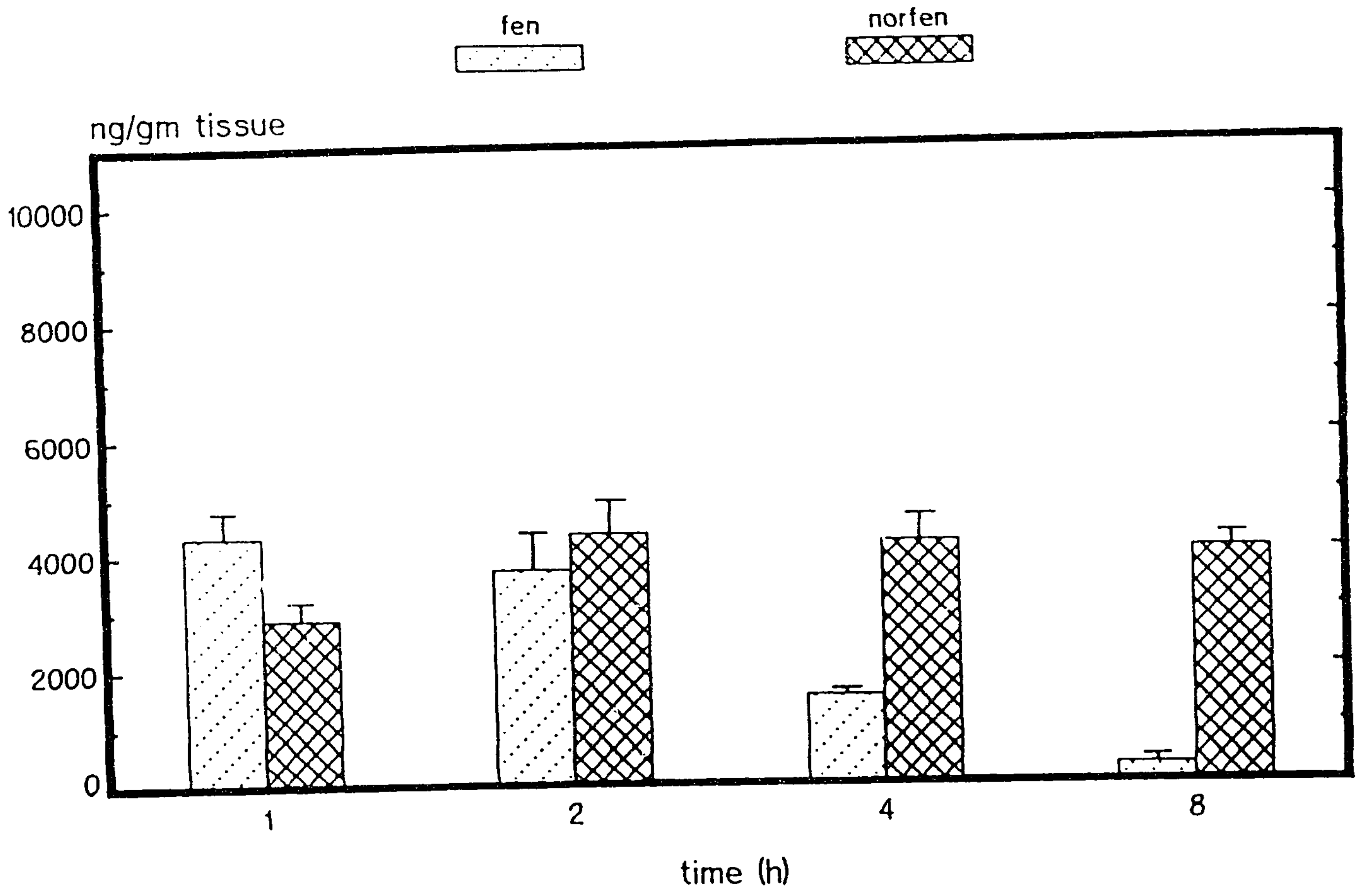


Figure 15: Whole brain levels of fenfluramine and norfenfluramine in saline pre-treated rats. The rats were treated with saline vehicle,then with (±)-fenfluramine (6.7 mg/kg) 1 h later; the rats were then killed at the times shown above after the (±)-fenfluramine administration. Values represent ng/g of tissue (mean ± SEM, n=6).

Figure 16. The pattern of declining levels of FEN over the 8 h period is similar to that in the brain. The initial level of NORFEN is higher, likely reflecting, in part, a first-pass metabolism effect. Again NORFEN is present at a significantly higher level than FEN by 2 h and remains so throughout the course of the experiment.

The brain concentrations of FEN and NORFEN after pretreatment with iprindole are shown in Figure 17. Statistically significant large increases (relative to the vehicle/FEN-treated rats) were seen in the levels of FEN, but, unexpectedly, the NORFEN levels were profoundly decreased. There was some recovery of NORFEN levels by 8 h, however the level of NORFEN still remained significantly below that of controls (i.e. vehicle/FEN-treated) at this time interval. Figure 18 shows the hepatic levels of FEN and NORFEN in the rats that were pretreated with iprindole and then given FEN. The same pattern is seen as with the brain in that maximum levels of FEN are seen at 2 h then gradually decreases at 4 h and 8 h. The 1 h level of FEN is higher in iprindole/FEN-pretreated rats than in the vehicle/FEN-treated rats but failed to reach statistical significance. NORFEN levels are again significantly decreased, with some recovery apparent at 8 h.

In comparing the combined FEN and NORFEN levels of the saline/FEN and the iprindole/FEN treatment groups (Figures 15 and 17), there is a significant difference over the study period. At 1 h, the combined FEN and NORFEN levels seen in both test groups are similar. By 2 h, the combined FEN and NORFEN level in the iprindole/FEN group has exceeded the level in the saline/FEN group. By 8 h, there is almost double the amount of combined FEN and NORFEN in

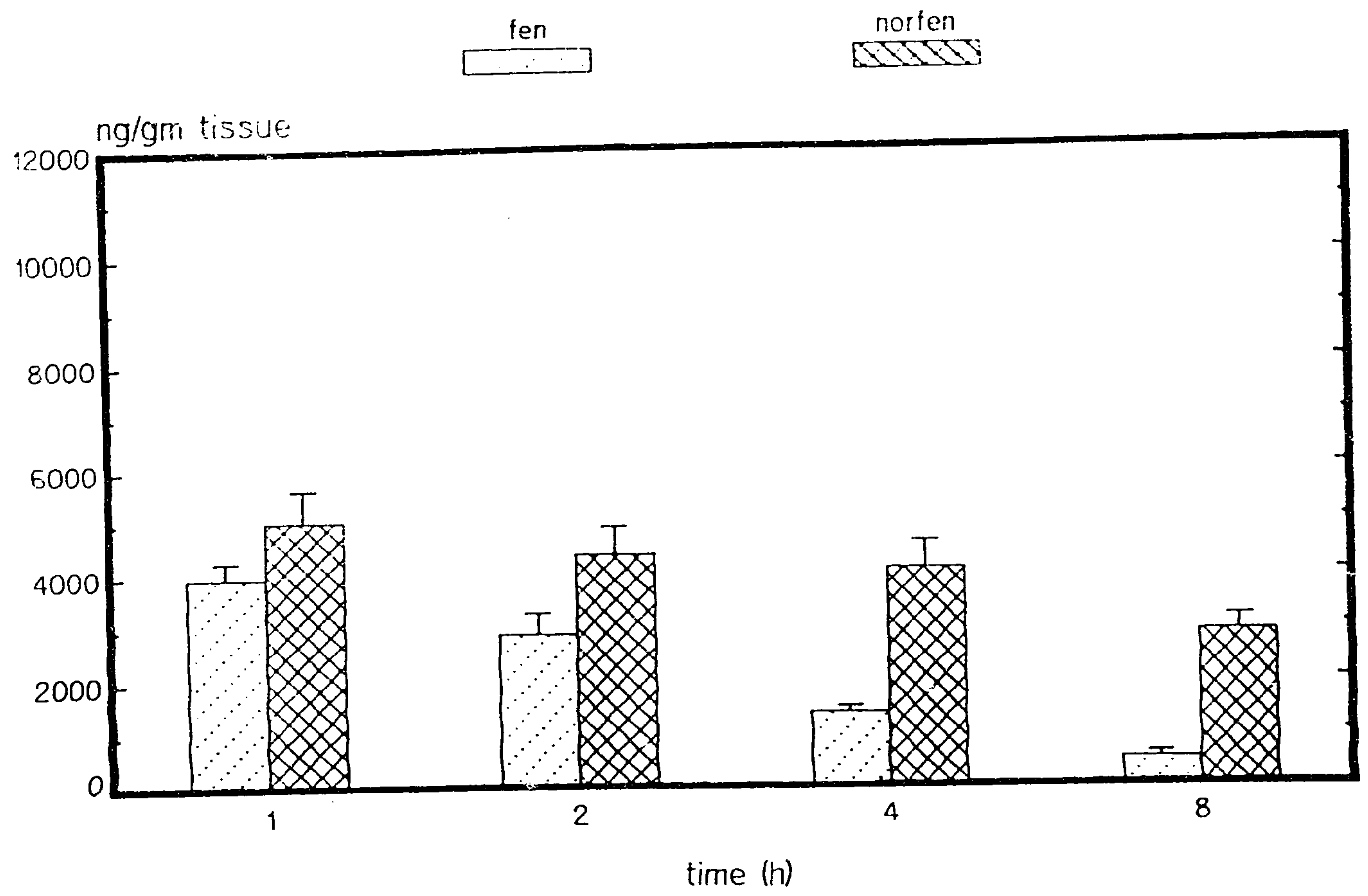


Figure 16: Hepatic levels of fenfluramine and norfenfluramine in saline pre-treated rats. Dosing schedule was as described in Figure 15. Values represent ng/g of tissue (mean ± SEM, n=6).

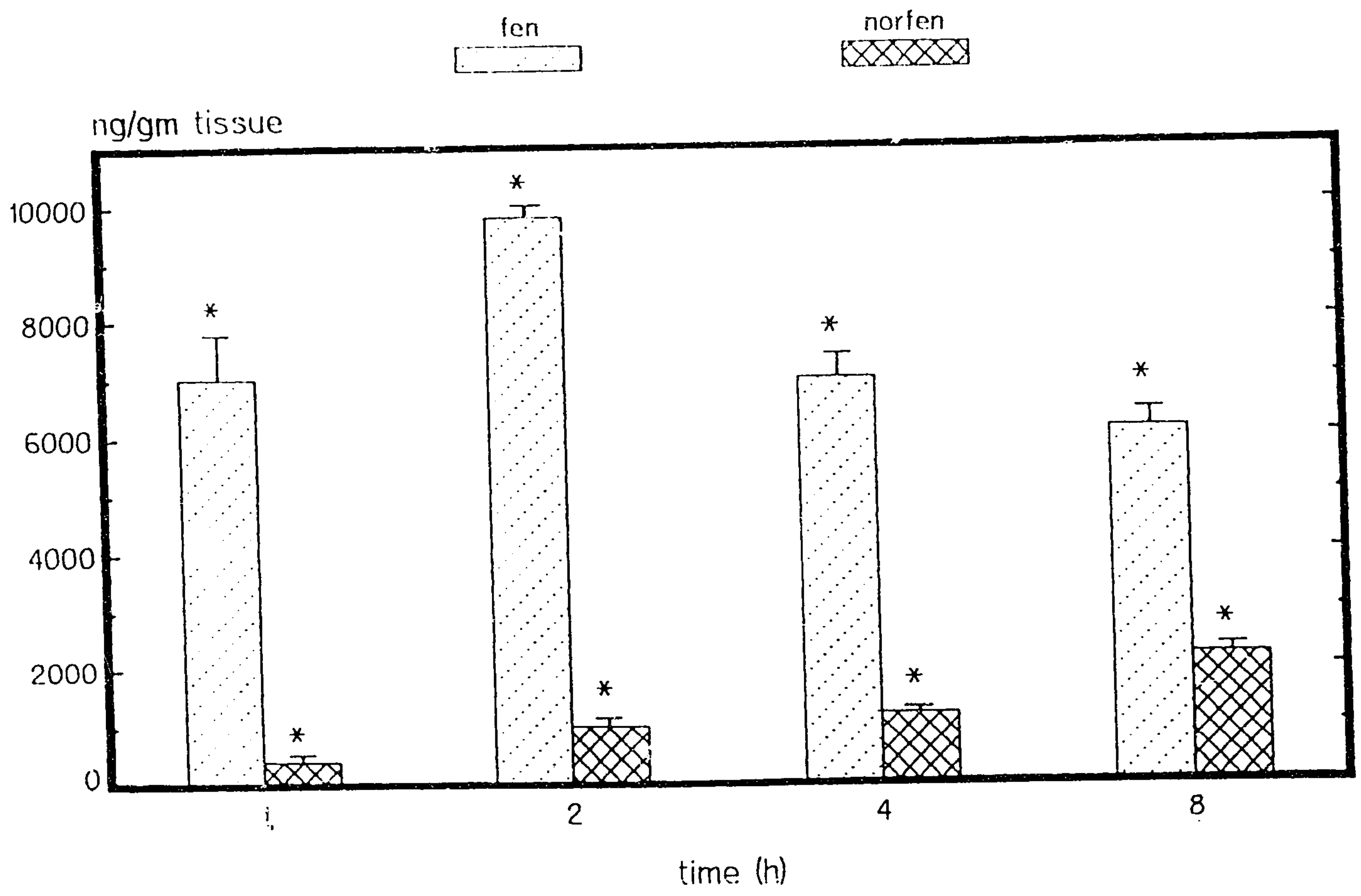


Figure 17: Whole brain levels of fenfluramine and norfenfluramine in iprindole pre-treated rats. Dosing schedule was as described in Figure 15 except that iprindole (11.2 mg/kg) was used instead of vehicle in the first treatment. Values represent ng/g of tissue (mean ± SEM, n=6).

*$p < 0.05$, Tukey test, pairwise <u>post</u> <u>hoc</u> comparison between indicated values and the corresponding values in saline/FEN-treated rats.

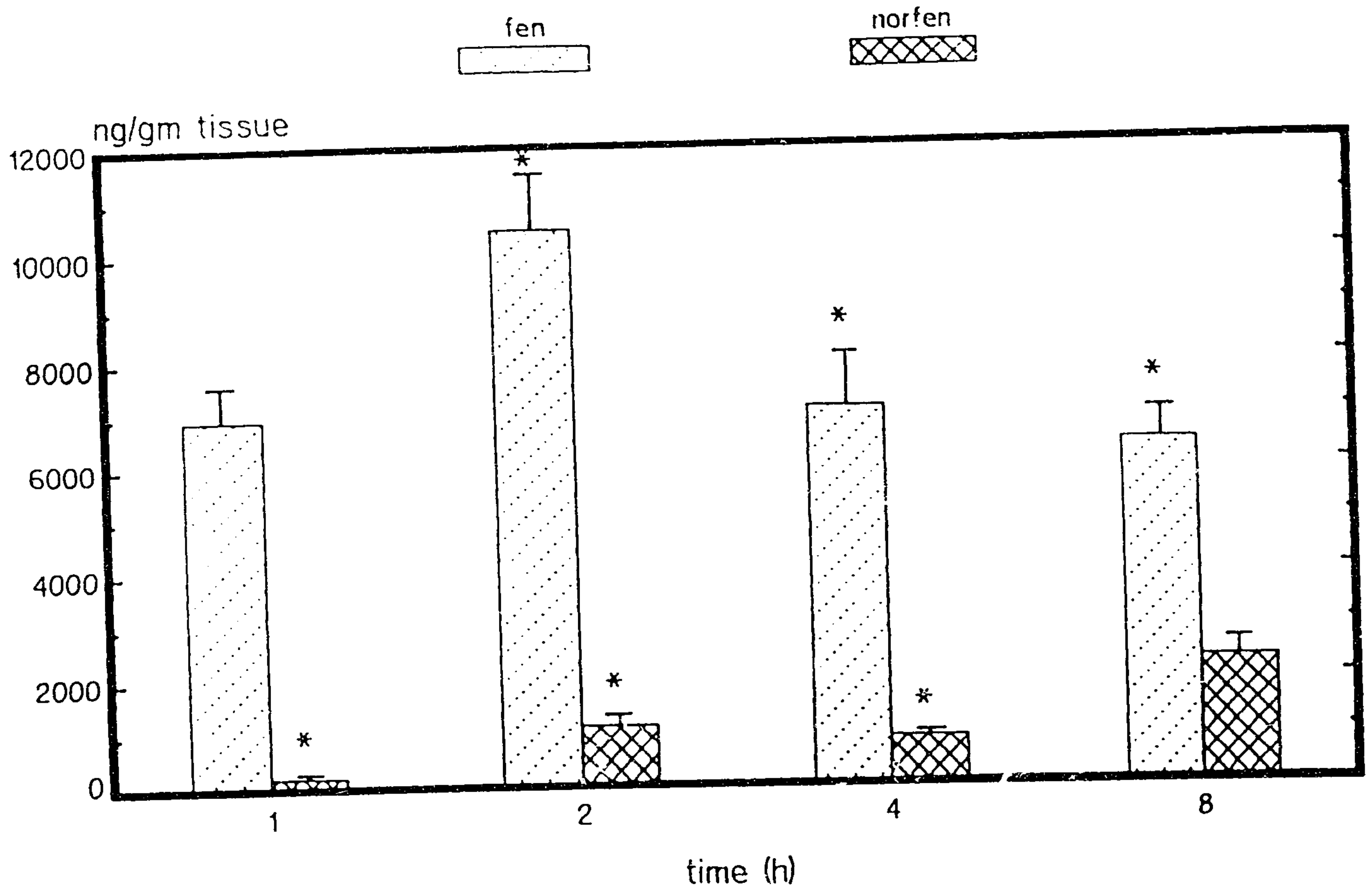


Figure 18: Hepatic levels of fenfluramine and norfenfluramine in iprindole pre-treated rats. Dosing schedule was as described in Figure 15. Values represent ng/g of tissue (mean ± SEM, n=6).
*p < 0.05, Tukey test, pairwise post hoc comparison between indicated values and the corresponding values in saline/FEN-treated rats.

the iprindole/FEN-treated animals compared to the saline/FEN group and NORFEN values have increased in the time period from 4 to 8 h in the former group.

E. DISCUSSION

The derivatizing reagents tested in this project have been utilized extensively in analysis of biogenic amines and amine-containing drugs related structurally to amphetamine (Coutts and Baker, 1982). Pentafluorobenzoyl chloride (PFBC) has been known for some time to import high sensitivity on ECD to compounds related structurally to ß-phenylethylamine (Cummings, 1971; Moffat et al., 1972; Matin and Rowland, 1972; Midha et al., 1979; Baker and Coutts, 1982), and PFBC and PFBS have been used recently for analysis of ß-phenylethylamine and tranylcypromine levels in tissues and body fluids (Baker et al., 1986b; Rao et al., 1986; Nazarali et al., 1987). A distinct advantage of these two reagents is the ability to perform the analysis under aqueous conditions (Baker et al., 1985). However, in the present study, while PFBC gave a derivative with NORFEN with good sensitivity on both the NPD and ECD, the corresponding derivative with FEN possessed poor sensitivity. The derivatives resulting from the reaction of PFBS with FEN and NORFEN displayed poor sensitivity. Reaction of trichloroacetic anhydride under basic aqueous conditions has been employed in the Neurochemical Research Unit for simultaneous analysis of amphetamine, N-(2-cyanoethyl)-amphetamine and 4-hydroxy-amphetamine in brain (Nazarali et al., 1984) and for simultaneous analysis of ß--phenylethylamine and tranylcypromine (Baker et al., 1985) but the corresponding reaction conditions with FEN and NORFEN resulted in derivatives with poor sensitivity. (S)-(-)-N-Trifluoroacetyl-L-prolyl chloride, a chiral agent, has the advantage of providing for the separation of enantiomers of drugs such as FEN (Singh, 1986) and tranylcypromine (Aspeslet, Baker and Coutts, unpublished), but unfortunately an interfering peak in the reagent prevents the attainment of adequate sensitivity with the resultant derivatives of the enantiomers of NORFEN.

The assay procedure developed in this project utilizing PFPA for derivatization is rapid and provides sufficient sensitivity to permit simultaneous analysis of FEN and NORFEN in relatively small amounts of brain and liver tissue.

The reagent PFPA, which has been employed extensively for analysis of a number of biogenic amines, including the catecholamines and 5-HT (Martin and Ansell, 1973; Baker and Coutts, 1982 for review), is unstable under aqueous conditions, and thus the reaction with FEN and NORFEN had to be conducted under anhydrous conditions after extraction of the drugs into an organic solvent. Following reaction of PFPA with the tissue extract, the reaction mixture was partitioned between toluene and saturated sodium borate. This partitioning procedure, in which unreacted reagent is neutralized in the aqueous phase, provides a rapid means of eliminating PFPA and retaining the final derivatives of the amines of interest in an organic phase suitable for injection on to the GC column (Martin and Baker, 1977; Baker et al., 1980).

The assay procedure developed here was readily applied to the study on levels of FEN and NORFEN in brain and liver samples of rats pretreated with vehicle or iprindole and then injected with FEN. To ensure that no interfering peaks corresponding to the retention times of those for FEN or NORFEN were produced by treatment with saline or iprindole, other sets of rats were treated with saline/saline or with saline/iprindole. In these latter two sets of rats, no peaks interfering with the FEN or NORFEN peaks were evident.

The pattern of decrease in the amount of FEN in the vehicle/FEN-treated rats over the 8 h study period is consistent with the reported $T_{1/2}$ of 4 h in brain tissue for this drug (Rowland and Carlton, 1986). By 4 h, the FEN level had dropped to less than 50% of the FEN level reported at 1 h. The rate of disappearance of NORFEN has been reported to be slower than that of FEN (Rowland and Carlton, 1986), and the results of the present study are in agreement with those reports.

It was expected that, if ring hydroxylation did contribute to metabolism of FEN and/or NORFEN, treatment with iprindole/FEN would result in an increase in concentrations of both FEN and NORFEN relative to those in rats treated with saline/FEN. There was, indeed, an increase in FEN levels observed in both brain and liver. However, surprisingly, there was a marked decrease in NORFEN levels, resulting in an elevated FEN/NORFEN ratio in the rats pretreated with iprindole, suggesting that iprindole blocks N-deethylation instead of, or in addition to, ring hydroxylation of FEN. It would be expected that if iprindole was blocking ring hydroxylation of FEN, levels of both FEN and NORFEN would increase since there would be more FEN available to be converted to NORFEN. The present results suggest that: (1) iprindole is blocking N-deethylation of FEN to NORFEN and/or (2) the increased FEN available after iprindole/FEN is in such a location in the brain that it is not readily available for N-deethylation. If (2) were the case, one would still expect to see similar amounts of NORFEN in the vehicle/FEN-treated animals and the iprindole/FEN-treated rats. However, NORFEN levels are much lower in the latter case than in the former, both in the brain and liver, particularly at 2 h (see Figures 13-16), suggesting that iprindole is causing considerable inhibition of N-deethylation of FEN. The FEN/NORFEN ratio was drastically increased during the time period studied, with partial recovery by the end of the 8 h period.

Inhibition of N-deethylation is not as commonly documented as other reactions reported in the drug-drug interaction literature. There are both research and clinical implications to these findings. The activities of drug metabolizing enzyme systems are under multiple dynamic factors including physiological, environmental and genetic factors, species-specific differences and the presence of another drug. Genetic studies have categorized humans into extensive metabolizers and poor metabolizers, using such drugs as debrisoquine, phenacetin and mephenytoin (Gibson and Skett, 1986). Species-specific metabolic pathways have also been examined. As stated earlier, the major and minor routes of metabolism of AMPH and FEN vary among species (Costa and Garattini, 1970; Gibson and Skett, 1986; Garrod and Beckett, 1978; Rowland and Carlton, 1986).

Neither the mechanism of inhibition nor the specific isozyme system involved can be demonstrated within the results of this study. However, as discussed earlier, iprindole inhibits the ring hydroxylation of AMPH, PEA and octopamine, suggestive of action via the P-450 dbl isozyme system. Perhaps the

N-deethylation of FEN is under a different isozyme system involved more in side chain and deamination reactions. Thus, the results of this study suggest that iprindole could have inhibitory effects on more than one isozyme of the cytochrome P-450 system.

If the interaction between iprindole and FEN in this study is indeed the result of inhibition of N-dealkylation, it is one of the few examples of such inhibition by a drug. Most of the other widely studied interactions in the literature show inhibition of aromatic hydroxylation reactions or of O-dealkylation.

Cimetidine has been shown to inhibit oxidation and hydroxylation reactions in a number of drugs and endogenous compounds by binding to the P-450 system. Oxidation of theophylline and 4-hydroxylation of aniline are inhibited by cimetidine (Gibson and Skett, 1986). Galbraith and Jellinck (1989) demonstrated that cimetidine inhibits the hydroxylation of estradiol in male rats. This effect was not seen in females, thus providing evidence that at least some components of the P-450 isozyme system are sex-linked. Cimetidine-induced inhibition of estradiol hydroxylation has also been demonstrated in human males (Galbraith and Michnovicz, 1989).

Both quinine and quinidine inhibit the aromatic hydroxylation of desipramine, with quinidine being the stronger inhibitor (Steiner et al., 1987). Via a similar isozyme system, quinine can inhibit the 4-hydroxylation of debrisoquine, the 0-methylation of encainide and the 4-hydroxylation of propanolol (Steiner et al., 1987; Gibson and Skett, 1986).

Dextromethorphan contains an N-methyl group but the major metabolic pathway in man is 0-dealkylation to produce dextrorphan (Woodworth, 1987). Sparteine undergoes N-oxidation as its major metabolic reaction (Eichelbaum et al., 1986). Both dextromethorphan and sparteine metabolism are under the influence of the P-450 db1 isozyme system and thus are likely to have their metabolism inhibited by drugs that inhibit debrisoquine metabolism.

Mephenytoin has a more complex metabolic profile and is metabolized via a different P-450 isozyme system (Guttendorf et al., 1990). The S-isomer of mephenytoin is rapidly 4-hydroxylated in extensive metabolizers and slowly N-demethylated in poor metabolizers. The R-isomer is slowly N-demethylated in both groups (Kupfer et al., 1982). The N-demethylation of diazepam and the 3-hydroxylation of demethyldiazepam have been shown to be related to the mephenytoin hydroxylation phenotype (Bertilsson et al., 1989). Thus, inhibitors of mephenytoin metabolism would similarly affect drugs that are degraded via the same isozyme system.

Although phenelzine, tranylcypromine and other MAO inhibitors do block N-dealkylation reactions, these drugs have been shown to inhibit many types of metabolic reactions involved in a variety of drugs (Belanger et al., 1982; Eade, 1970; Clark and Thompson, 1972). If iprindole can be shown to have more selective actions than tranylcypromine or phenelzine, there is potential use of this drug as a probe to explore the multifaceted P-450 system.

If the marked decrease in NORFEN levels produced by iprindole could be maintained over time, further work could be done examining specific neurochemical and behavioural effects of FEN separately from the usual presence of NORFEN. This would provide in vivo data important to the ongoing discussions surrounding tolerance effects, peripheral vs CNS effects and neurotoxicity. Racemic FEN was used in this study, but future experiments could examine various NT systems affected by either (+)-FEN or (-)-FEN. It is not known if iprindole affects the two enantiomers differently, or if recovery from metabolic inhibition occurs preferentially with one enantiomer.

The increases in the combined levels of FEN and NORFEN in the iprindole/FEN-treated animals compared with levels seen in the saline/FEN-treatment group and the rising levels of NORFEN in the 4 to 8 h period in the former group suggest that iprindole may also interfere with NORFEN metabolism. Further studies at longer time intervals appear to be warranted in order to examine the time course of the effects of iprindole on the FEN/NORFEN ratio.

Arvela (1973) reported that FEN appeared to have enzyme-inducing effects in the liver. Thus, the results obtained in the study reported in this thesis might reflect a complex interaction between effects of FEN on iprindole metabolism and effects of iprindole on FEN metabolism. As Coutts et al. (1990) have pointed out, very little is known about the metabolism of iprindole itself; these authors recently identified fourteen metabolites of iprindole in urine of iprindole-treated rats. The complexity inherent in any discussion of the interaction is heightened when using racemic FEN, because, in reality, there were four different drugs [the (+) and (-) enantiomers of FEN and NORFEN] interacting with iprindole. Further work in this area needs to be done, using the individual enantiomers and examining any effects FEN might have on iprindole metabolism.

There are clinical implications to the findings of this study. Obesity often coexists with depression in clinical populations, and the pharmacological treatment could include both FEN and iprindole or another antidepressant. As mentioned earlier in this thesis, tricyclic antidepressants such as desipramine have been reported to have marked effects on metabolism of other drugs. The resultant change in the FEN/NORFEN ratio could result in a dramatically altered therapeutic and/or side effect profile.

F. CONCLUSION

A novel GC procedure has been developed to permit simultaneous analysis of FEN and NORFEN. The resultant procedure was applied to rat whole brain and liver to study the effects of pretreatment with iprindole on the levels of FEN in these tissues.

Although the original intent of the project was to examine a possible role of ring hydroxylation in the metabolic pathway of FEN, a marked decrease in brain and liver levels of NORFEN was also observed after pretreatment with iprindole, suggesting that iprindole causes inhibition of N-deethylation of FEN.

These results show that more research needs to be focused on iprindole as a pharmacological agent to explore specific metabolic processes in the liver and brain. The results obtained here suggest the possibility of using iprindole as a vehicle to examine effects of FEN separately from those of NORFEN, using acute and chronic administration experiments.

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