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The Use of Toxicokinetics for the Safety Assessment of Drugs Acting in the Brain

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Abstract

Pharmacological and toxicological studies undertaken on drugs that affect the brain are frequently performed in disparate species under various experimental conditions, at doses often greatly in excess of those expected to be administered to humans, and the findings are extrapolated implicitly or explicitly with scant regard to differences in the biodisposition of the drugs. Such considerations are necessary since:

1. Species;
2. Strain;
3. Gender;
4. Route;
5. Dose;
6. Frequency and time of administration;
7. Temperature;
8. Coadministration of drugs; and
9. Surgical manipulation

are but some of the factors that have been shown to influence the kinetics and metabolism of drugs. This article, using MDMA and other phenylethylamines as examples, provides evidence for the need to measure the exposure of the drugs and their active metabolites in blood and brain (toxicokinetics) in order that conclusions based only on dynamic, biochemical, or histological evidence are more pertinent. Further, the combined use of toxicokinetic-dynamic modeling can lead to a better appreciation of the mechanisms involved and a more useful approach to the calculation of safety margins.

Index Entries: Pharmacokinetics; pharmacodynamics; metabolism; allometry; phenylethylamines, MDMA; mathematical modeling.

Introduction

The choice of dosage, frequency, and experimental design for neurotoxicological investigations of drugs of abuse in animals appears to have evolved by experimental trial and error. Once a dose and regimen are found, then they are used by others to replicate the study design and hopefully the results. Little consideration would seem to be made regarding the relevance of the chosen dose or the route of administration, either for the interpretation of the study or for the possible extrapolation to humans. Often, in the last paragraph of the conclusion, authors attempt to give their results some clinical perspective by a simple comparison of the doses used in animals to achieve their effect and those used in humans. Unfortunately, animals have different kinetic and metabolic profiles from those found in humans, and the extent of exposure of the active compounds may not simply be related to dosage. For example, the amphetamines generally have a half-life of approx 2 h, but are generally administered sc every 12 h for 4 d, whereas in humans, these drugs of abuse have a half-life of approx 10 h and are probably taken by mouth once or twice a week (Cook et al., 1992). In the past, such sophistication was hampered by the lack of suitable analytical techniques, but not so today, where relatively simple methods can measure drugs like MDMA and its metabolites with blood samples as low as 200 μL (Michel et al., 1993).

This brief article attempts to explain why the extrapolation of results from animals to humans based on dosage alone without concomitant measurement of blood or tissue levels can lead to the misinterpretation of data, particularly when the metabolites are probably more active than the parent drug. Recently, the importance of this concept has led to the drafting, and in principle acceptance, by regulatory agencies and industrialists alike of toxicokinetic guidelines on the need for measurement of drug levels in all toxicological or safety studies in animals so that more meaningful extrapolation to humans can be made (ICH2, 1993).

What Is Toxicokinetics?

Toxicokinetics has been defined as "the generation of pharmacokinetic data either as an integral component in the conduct of nonclinical toxicity studies or in specially designed supportive studies, in order to assess systemic exposure" (ICH2, 1993). In practice, samples of body fluids or tissues are taken to measure the drug or active metabolite level, often at the same time as other biochemical measures, to assess the rate and extent of exposure during all pivotal toxicological studies. Repeat sampling may in some cases be hazardous to the conduct of the study, and satellite groups held under similar conditions can be used to obtain sufficient samples. Sometimes it is difficult to take multiple samples from single animals to measure certain kinetic parameters, such as the area under the plasma curve, and techniques, such as composite sampling, may be employed. In this case, instead of taking four to six samples from each animal (serial sampling) or killing an animal at each time-point (destructive sampling), two to three samples are taken from each animal, but at different times to cover the whole profile. This reduces the stress on the serial animals and the total number of destructive animals (Campbell and Jochemsen, 1994). Another method of overcoming the problem is to use a microdialysis probe to sample plasma or tissue effluents, but sample size is small and analytical methods need to be particularly sensitive.

Why Is Toxicokinetics Necessary?

The extrapolation of animal data to humans has relied on the use of the highest dose in animals that produces a no effect (NOEL) or the lowest dose (LOEL) that produces a measurable toxic effect, if this can be defined. For many neurotoxins, this is understandable since there may be no "normal" plasma levels in humans, and it would be difficult to undertake studies to examine this. The same is true, to some extent, for the drugs of abuse, but often

some data do exist that could provide a basis for extrapolation. There are two basic reasons why simple dosage comparisons can be incorrect. First, there are frequently large differences in the rates and routes of elimination between species, which, as will be explained, is often dependent on the relative body weight of the species. Second, the higher doses and the routes of administration that are used in toxicological studies can dramatically alter the kinetics, leading to nonlinear drug exposure. In addition, the experimental procedure, coadministration of another drug, anesthetic, housing conditions, age of the animal, gender, naivety of animals, and so on, can also influence the biodisposition of any test compound let alone the effect.

This brief article examines the kinetic processes of absorption, distribution, and elimination during toxicological studies with particular emphasis on species and dosage differences (Table 1) explaining how they may influence their interpretation.

Table 1
Possible Effects of High Dosage and Species Differences on Kinetics and Brain Concentrations

Kinetics	Species	High dose	Brain levels
Absorption	+[a]	Saturation (act/pass)[b]	↓
Distribution			
Protein binding	+++	Saturation	↑
Tissue uptake	++[c]	Saturation (act)	↑[d] ↓[e]
Elimination			
Renal	+[c]	Saturation	↑
Metabolism	+++	Saturation	↑ ↓ (Metab)[f]

[a]↑ increase, ↓ reduce, + small, ++ large, +++ very large species differences can be expected.
[b]act = active transport, pass = passive diffusion.
[c]Insufficient information available.
[d]Uptake into the brain is saturated.
[e]Uptake into other tissues is reduced, allowing greater uptake into the brain.
[f]Active or toxic metabolite levels will be reduced if metabolism of parent drug is saturated.

Absorption

There are relatively few differences in the relative rates and extent of absorption between species, and often animal models are used with some success to compare new formulations (Clarke and Smith, 1984). Similarly, over a small range of doses, unless the drug is actively absorbed, this process is linear. At very high toxicological doses, a reduction in total absorption can occur, particularly if the compound has a poor dissolution or has a local effect, sometimes limiting the total amount of drug that can be put into the systemic circulation by this route. In neurotoxicological studies, many drugs are administered ip or sc, and, although this may overcome problems of dose-related reductions in absorption, by-passing the initial hepatic metabolism can lead to large differences in the relative proportion of drug to metabolite seen in oral dosing. Thus, for fenfluramine, the parent drug levels are increased fivefold after sc dosing in the rat compared to oral dosing, but without changing the norfenfluramine concentrations owing to marked, but disproportionate first-pass hepatic metabolism (Zaczek et al., 1990; Gordon, 1991 unpublished). Little is known regarding route-specific absorption or metabolism for other phenylethylamines.

Distribution

Plasma Protein Binding

It is generally accepted that it is the free drug that is active, and Smith (1993) has shown that there can be more than a 20-fold difference in the unbound concentrations for certain drugs in animals compared to humans, and that these differences cannot easily be predicted. It would appear from those drugs studied that the free active levels of drug are generally lower in humans compared to mouse, rat, or dog. This can become very important if brain levels are not measured, particularly for those compounds with high plasma protein binding

where only small changes in binding, for example from 98 to 96%, can increase free levels by 200% and thus the potential for brain uptake.

In addition, the amount of free drug can increase with dosage owing to the saturation of binding sites, but this will depend on to which plasma protein the drug is bound. Protein binding is relatively low for the amphetamines (<50%) (Campbell, 1978), and because this is specific to albumin, which has high amounts present in plasma, more than 500 μg/mL of a drug with a mol wt of 200 would be needed before saturation occurs, assuming one binding site. For drugs that bind to the lower circulating levels of α_1-glycoprotein, the drug levels at which saturation may occur will be correspondingly lower (≈20 μg/mL). Similar considerations hold for drugs that bind to lipoprotein, but less information on these interactions is available. Unfortunately, these considerations are rarely taken into account when comparing exposure of drugs and can potentially lead to big discrepancies in the calculation of safety margins.

Tissue Uptake

For drugs that act as central neurotoxicants, it has been assumed that the uptake and distribution into the brain are similar between species. This concept has rarely been challenged, since it is not common practice to measure brain levels in animals, and because of the extreme difficulty in extrapolating the results to humans. Recent results with fenfluramine and norfenfluramine using postmortem tissue after overdosage (30–40 mg/kg) suggest that the uptake into the human brain (Fleisher and Campbell, 1969; Holmes and Gordon, 1989) is as much as seven times lower than that found in other species (Caccia et al., 1982; Zaczek et al., 1990; Caccia, personal communication), and that the brain plasma ratio seems to be related to the body weight of the animal, although some variation is seen in primates (Fig. 1). Interestingly, in all these species studied and particularly in monkeys, there is a tendency for norfenfluramine levels to be higher

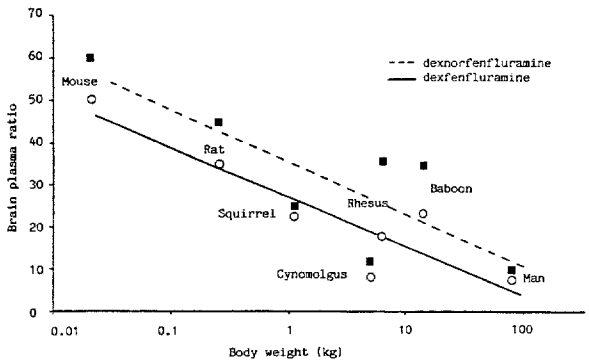


Fig. 1. Relationship between the brain plasma ratio of *d*-fenfluramine (○), *d*-norfenfluramine (■), and body weight for different species.

in brain than the parent drug, which is unexpected since the partition coefficient, and, therefore, lipid-solubility, is lower for the metabolite, but without a significant difference in the plasma protein binding (Campbell, 1978). This would suggest a specific transport or binding for the metabolite. Similar species differences in brain uptake have been reported for fluoxetine and norfluoxetine, using the more physiological technique of MRS in humans after therapeutic dosing (Karson et al., 1992; Renshaw et al., 1992), and brain excision for rats (Caccia et al., 1992) and for at least one other drug, alapride, a novel nootropic agent (Lapka, 1991). These species differences, therefore, may be more common than previously thought.

There are a number of possible reasons for these observations, including species differences in:

1. Brain fat and protein composition;
2. Specific binding sites;
3. Cerebral blood flow, which could be expected to be greater for small animals in relation to brain size;
4. Specific and active carrier-mediated uptake into the brain, which has been previously reported for amphetamine (Pardridge and Connor, 1973);
5. An active removal of the drugs from the brain;
6. Differences in plasma protein binding; and

7. Sample dilution effects, since the human brain is 3–10 times larger, relative to body weight, compared to other animals (Boxembaum and Fertig, 1984).

Indeed, Dedrick (1986) has suggested that "the large primates are poor surrogates for the human" and that these interspecies differences in brain levels may explain the lower toxicity seen in humans compared to other animals for anticancer agents, such as carmustine, when administered by intracarotid infusion.

Metabolism

Species Differences

There are a number of examples of the qualitative differences in the metabolism of drugs between species, including a virtual absence of *o*-demethylation or acetylation in the dog, or glucuronide formation in the cat, or aromatization in New World but not Old World monkeys, but perhaps it is less well known that there are quantitative differences that can be predicted from the weight of the animal. Most physiological functions appear to be related to the size of the organ or tissues concerned, which in turn is proportional to the body weight. Thus, for the small rodents, processes, such as breathing, heart and metabolic rate, blood flow, and renal and hepatic clearances, all occur at a relatively faster rate than that found in larger animals and humans. Since longevity is also proportional to weight, the smaller animal will live for a shorter time, but the total number of physiologicals event will be approximately the same for all mammals (Mordenti, 1986). This means that for most drugs, the total body clearance will be considerably faster in the rat compared to humans, producing a much shorter half-life and reduced exposure for the same dosage expressed in terms of mg/kg. This size proportionality is known as allometric scaling, and a linear relationship can be found for a large number of drugs when the log of the body weight is plotted against the log of the clearance, half-life, or volume of distribution (Boxembaum, 1982), according to the formula:

$$Y = aW^b$$
$$\log y = \log a + b \cdot \log W$$

where y is the kinetic parameter, W the body weight, b the slope of the line, and a a constant related to each drug. The slopes of these regressions have been found to be very similar for a large number of drugs, with b for clearance approx 0.7, the half-life 0.25, and the volume of distribution 1.0 (Campbell, 1994a). Thus, if the kinetics is known in one or more species, the value in humans, in principle, can be calculated. For example, the half-life in the rat for amphetamine and methylamphetamine is 2 h, and using the above formula, the value in humans can be expected to be 8 h, which is similar to that actually found (Campbell, 1978; Cook et al., 1992). Although this approach may be applicable to a number of drugs with high clearance or those renally eliminated, for others, the prediction to humans is unreliable (Boxembaum, 1982), particularly for those with a low metabolic clearance (Campbell, 1994c). The human lives longer than can be expected from allometric scaling considerations compared to all other mammals, and the use of a life-span correction factor has been shown to provide better estimates, particularly using data from rats and Old World Monkeys (Campbell, 1994c). From these allometric considerations, it follows that parent drug exposure will often be lower in animals (approx 12 times for rats and 3.5 times for cynomolgus monkeys) compared with humans when the same dosage is administered in terms of mg/kg and therefore, toxicity should be correspondingly lower. This is often not the case. The opposite can be true perhaps owing to the more extensive conversion to active and sometimes reactive and toxic metabolites in the liver or brain of experimental animals.

Metabolic Pathways

Many of the drugs of abuse are relatively lipid-soluble, little is excreted unchanged in

the urine, and they will undergo extensive metabolism in the liver. However, because they are weak bases with pK_a values of approx 9, the absolute amount of renal excretion and therefore metabolism will depend on the urinary pH with acidic urine increasing the renal involvement up to fivefold. This may have implications on the rate and, to a lesser extent, routes of metabolism when different animals with different diets are studied under varying external conditions. Little has been done to study these effects, but they may partially explain some of the interspecies differences reported on amphetamine metabolism (Dring et al., 1970; Caldwell et al., 1972). Although for many drugs, metabolic degradation is a deactivation process to more polar compounds that can be more easily excreted, it is becoming clearer that for others, particularly those with long-term toxicities, such as carcinogenicity, metabolism can produce reactive intermediates, which may be present in the body for relatively short periods, making measurement and identification difficult (Mansuy, 1989). An example of this is the conversion of the neurotoxin 1-methyl-4-phenyl-1, 2, 3, 6 tetrahydropyridine (MPTP), to the free radical 1-methyl-4-phenylpyridine (MPP^+), which is cleared more slowly in primate brains compared to the rodents (Johannessen et al., 1985) and could explain the greater sensitivity in monkeys (Boyce et al., 1984). Similarly, it has been suggested (Molliver et al., 1986; Paris and Cunningham, 1991) that long-lasting depletion of 5-HT when 3-4 methylenedioxy methylamphetamine (MDMA), and 3-4 methylenedioxy amphetamine (MDA) are administered systemically, but not when injected intraventricularly, is caused by the formation of toxic metabolites. Formation of chemically reactive metabolites had been previously hypothesized for parachloroamphetamine (PCA) (Ames et al., 1977; Sherman et al., 1975) and reactive intermediates found in brain microsomal preparation (Miller et al., 1986), but the search for such compounds is generally hampered by their relatively low levels compared with other metabolites and their rapid clearance owing in part to their reaction with tissues. For example, MDMA in the rat is metabolized by at least seven different reactions:

1. Ring hydroxylation;
2. Demethylenation of the methylenedioxy bridge;
3. With subsequent *o*-methylation of the hydroxy group; or
4. Oxidation to quinoline;
5. Demethylation of the alkyl side chain;
6. Cyclization of the trihydroxy derivatives to the corresponding indoles;
7. Oxidative deamination of the nitrogen to polar inactive metabolites; and finally
8. Glucuronidation and sulfation of hydroxyl groups, the mechanism and intermediates and, indeed, the importance of which remain unknown (Fig. 2) (Lim and Foltz, 1988, 1991b; Hiramatsu et al., 1991; Patel et al., 1991; Lin et al., 1992)

Table 2 attempts to summarize the possible involvement of each of the putative metabolites so far identified. In brief it would appear that all compounds with an intact methylenedioxy bridge have little direct toxic effect, as measured by prolonged 5-HT depletion, even though they readily enter the brain after peripheral injection (Lim and Foltz, 1988). Conversion from MDMA to the more active MDA was not thought to be important, since pretreatment with the cytochrome P450 inhibitor, piperonyl butoxide, had no effect on the acute depletion of 5-HT (Schmidt and Taylor, 1987), but without actual measurements on the formation of MDA in the brain and organ specificity of this inhibitor (Murray and Reidy, 1990), these results must be interpreted with caution. The more polar catechols and *o*-methylated derivatives formed by hepatic demethylenation by the cytochrome 2D isozymes (Tucker et al., 1994) and ring hydroxylation by cytochrome 2B4 and 2D1 (Kumagai et al., 1992b,c) may not be able to cross the blood–brain barrier, but are found in cerebral tissue, since metabolism of the parent drug also occurs in the brain (Lim and Foltz, 1988; Lin et al., 1992). However they do not appear to have any 5-HT-depleting effects when injected intraventricularly. Similarly, β-

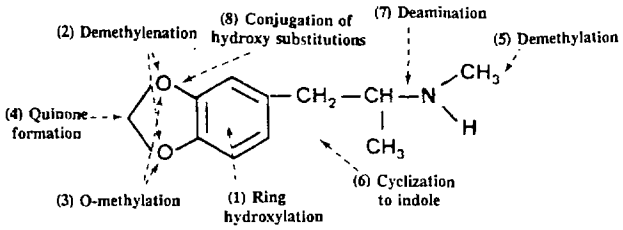


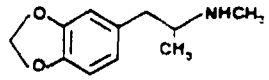
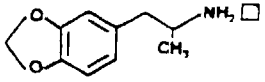
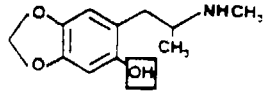
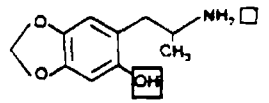
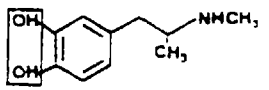
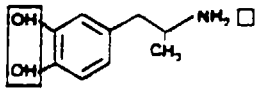
Fig. 2. Proposed metabolic routes of MDMA.

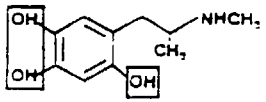
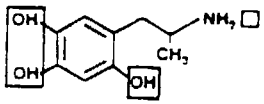
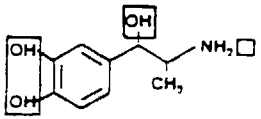
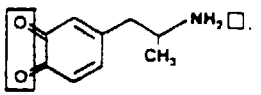
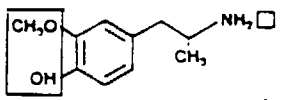
hydroxylation of the side chain-producing α-methyl norepinephrine does not change 5-HT levels, but production of this metabolite, if it occurs, could contribute to the hemodynamic changes seen in acute toxicity, such as hypotension and bradycardia (Yeh and Hsu, 1991), or even the mood-elevating effects. In contrast, the ring trihydroxy compounds have a greater effect on dopamine after icv injection (Elayan et al., 1992; Johnson et al., 1992), and these metabolites could be responsible for the long-term depletion in this catecholamine observed after MDMA administration. However, although they can be formed in the liver, because of their polarity, it is unlikely that they would cross the blood–brain barrier, and there is no evidence to suggest so far that they are actually metabolized in the brain. Further work is necessary to see if they are present in the brain after large doses, although this is inherently difficult because of the low levels, difficulties of analysis, and poor stability. Similarly, the quinone formed by reduction of the dihydroxy compounds forms glutathione adducts, which can be blocked by oxygen-free radical inactivators, such as superoxide dismutase, highly suggestive of a reactive intermediate. However, although this has been shown to occur in liver microsomes from DMA, information of the same metabolic activation in the brain is lacking. Certainly, the brain does have the ability to metabolize drugs using the CYP2D6 isozyme, and this may have evolved as a protective mechanism against plant alkaloids ingested by early humans (Britto and Wedlund, 1992). Although cerebral metabolic activity may be <1% compared to the liver, when high doses of these compounds are administered, metabolism may be sufficient to produce toxic levels of metabolites in small, but sensitive areas. However, until definitive measurement is made in brain tissue of those compounds known to be toxic, together with some form of kinetic-dynamic analysis, the relevance of the active metabolite hypothesis will remain unresolved.

In addition to the suggestion that these compounds in themselves may be metabolized to reactive metabolites, there is also evidence suggesting that they may release such large quantities of catecholamines, particularly from the peripheral circulation, that the catabolism in the brain is saturated, leading to toxic pathways forming known neurotoxins, such as 6-OH DA, from dopamine after methylamphetamine (MA) (Seiden and Vosmer, 1984; Stone et al., 1988) or 5,7-DHT from serotonin after PCA (Berger et al., 1992), but again definitive studies are awaited.

These detailed studies on one drug highlight the difficulties in the interpretation of toxicological studies without the benefit of drug and/or metabolite levels to assess exposure. Even this approach may be invalidated if highly reactive toxic metabolites and free radicals are formed, and are only present in blood or tissues for a few seconds, with little possibility of them being measured. Once formed, they can damage the lipid membranes of nerve terminals or inactivate key synthetic enzymes, such as tryptophan hydroxylase (Stone et al., 1989). Indeed, the smaller animals with faster rates of metabolism may actually be more susceptible to toxicity because of these reactions. Much of the studies described above have used the rat, and although some metabolic studies have been undertaken in mice and humans (Lim and Foltz, 1989, 1991a), little is known about the metabolism in other species. Some authors have suggested that the reduced sensitivity of MDMA seen in the mice compared with other species could be a consequence of different rates or routes of metabolism (Stone et al., 1987; Lim and Foltz, 1991a), and that primates are the most suitable species to compare with humans since it is suggested that they

Table 2
The Activity of MDMA and Various Putative Metabolites on 5-HT Depletion

Compound	Metabolic conversion	Location	5-HT depletion	DA depletion	Reference
 **MDMA**	--	All	-icv	-icv	(E) Molliver et al. (1986) (E) Paris and Cunningham (1991)
			+++per	+++per	(E) Schmidt (1987) (E) Cummins et al. (1987
 **MDA**	Demethylation	All			(M) Lim and Foltz (1988)
			-icv +++per	-icv +++per	(E) Molliver et al. (1986)
 **6-OH-MDMA**	Ring hydroxylation	Liver brain plasma			(M) Lim and Foltz (1991a) (M) Kumagai et al. (1992a)
			-icv -per	-icv	(E) Elayan et al. (1992) (E) Zhao et al. (1992)
 **6-OH-MDA**	Ring hydroxylation	Liver brain (sc) plasma			(M) Lim and Foltz (1991a)
			-icv	-icv	(E) Elayan et al. (1992)
 **3,4-di-OH-MA (N-Me-α-Me-DA)**	Demethylenation	Brain liver			(M) Kumagai et al. (1992b) (M) Chu et al. (1992) (M) Lim and Foltz (1991b) (M) Lin et al. (1992) (M) Hiramatsu et al. (1989b) (M) Tucker et al. (1994)
			-icv		(E) Johnson et al. (1992) (E) Steele et al. (1987)
 **3,4-di-OH-A (α-Me-DA)**	Demethylenation, demethylation	Brain			(M) Lim and Foltz (1988) (M) Lin et al. (1992) (M) Chu et al. (1992)

			–icv –per +(cell)		(E) McCann and Ricaurte, 1991 (E) Yeh and Hsu (1991) (E) Cho et al. (1993)
 **3,4,6-tri-OH-MA** **(2,4,5-tri-OH-MA)**	Demethylenation, ring hydroxylation	Rabbit microsome			(M) Lim and Foltz (1991a)
			++icv	+++icv	(E) Johnson et al. (1992) (E) Elayan et al. (1992)
 **3,4,6-tri-OH-A** **(2,4,5-tri-OH-A)** **(6-OH-Me-DA)**	Demethylenation, demethylation, ring hydroxylation				(M) Lim and Foltz (1991b)
			++icv	+++icv	(E) Johnson et al. (1992) (E) Elayan et al. (1992)
 **3,4-di-OH-α-OH-A** **(α-Me-NE)**	Demethylenation, demethylation, side chain hydroxylation		–per		(E) Yeh and Hsu (1991)
 **"MDMA Quinone"**	Demethylation, ring hydtrolation, and reduction	Rat microsome			(M) Hiramatsu et al. (1990)
			+[2]		(E) Cho et al. (1993)
 (I) **3-methoxy-4-OH-A**		Liver brain plasma			(M) Lim and Foltz (1988)
			+per		(E) Yeh and Hsu (1991)

(Compound) = Alternative nomenclature, □ = positions of metabolism, MD = methylene dioxy, MA = methylamphetamine, A = amphetamine, DA = dopamine, OH = hydroxy, Me = methyl, NE = norepinephrine, E = epinephrine, (M) = metabolism, (E) = effect, – = no effect, + = small effect, ++ = intermediate effect, +++ = large effect, [1] = 3-methoxy-4-OH I-MA and 4-methoxy-3-OH derivatives, [2] = indirect evidence of toxicity since it forms a GSH adduct, icv = intraventicular, per = peripheral administration.

may have a more similar metabolism to humans (Ricaurte, 1989). Unfortunately, the only substituted amphetamine that has been systematically studied, fenfluramine, has shown marked differences in the rates of metabolism between animals and humans (Caccia et al., 1982; Marchant et al., 1992), in particular, the conversion to the more active norfenfluramine. Thus, for most animals, with the exception of the mouse, the relative exposure of norfenfluramine to fenfluramine expressed as the areas under the plasma concentration time curve (Table 3) is 3- to more than 30-fold greater than in humans, with the primates being the least similar to humans. Work in our laboratories has also shown that only fenfluramine and norfenfluramine can be found in the brain of rodents, but an additional as yet unidentified metabolite observed in plasma may be present in the squirrel monkey's brain (Fig. 3).

An additional complexity to take into account is that all the inferences described above have relied on the depletion of 5-HT as the index of toxicity, and recent work in mice has shown that toxicity, as measured by increased levels of GFAP, argyrophilia, and irreversible reductions in tryptophan hydroxylase, occurs at high doses of MDMA and MDA without necessarily large depletions of 5-HT, whereas fenfluramine at similar doses producing a reduction in 5-HT shows no toxicity, as measured by these indices (Miller and O'Callaghan, 1993; O'Callaghan and Miller, 1993). These differences may be the result of the ability of MDMA, but not fenfluramine, to deplete dopamine acutely, and if these findings are confirmed, it would appear that much of the previous screening that has been undertaken to examine the toxicity of the putative metabolites, using only 5-HT as a biochemical marker, may need to be repeated.

Table 3
Species Differences in the Metabolic Ratio—The Relative Amount of Norfenfluramine (NFen) to Fenfluramine (Fen) in the Body Following Oral Administration

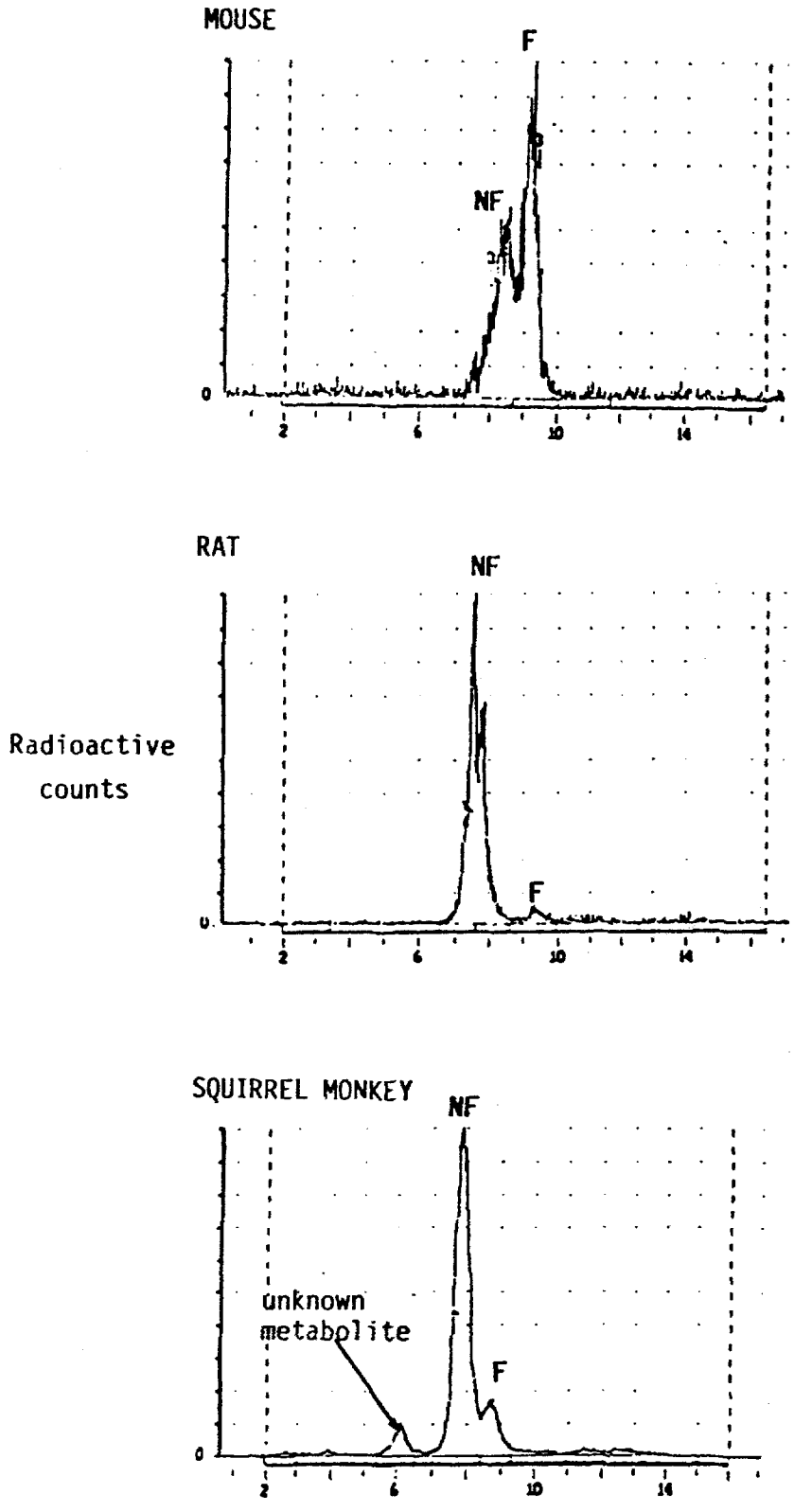
Species	Plasma AUC ratio NFen/Fen
Mouse	0.3
Humans	0.7
Rat	2.8
Pig	3.0
Dog	5.2
Guinea pig	9.5*
Squirrel	11.0
Cynomolgus	16.0
Rhesus	27.0
Baboon	37.0

Caccia et al. (1982) and unpublished.
*(Fuller et al. [1988] ip administration).

Nonlinearity

During toxicological investigations, doses are normally increased until a maximum tolerated dose is reached or a desired effect shown. It is often assumed that the kinetics is linear across the range of doses used. In practice, this is often the exception rather than the rule owing to a saturation of many kinetic processes, particularly those that are actively or enzymatically linked (Table 1). Thus, saturation of hepatic metabolism or renal elimination can lead to relatively large increases in blood levels, whereas on the contrary, saturation of absorption would lead to reduced exposure. Many processes of nonlinearity may be occurring simultaneously at high doses and at different times of dosing, confounding the interpretation of the data. The metabolism of fenfluramine becomes rapidly saturated in the rat at 4 mg/kg and this continues until 24 mg/kg, but thereafter remains constant (Zaczek et al., 1990). Exposure in these animals becomes five to six times higher than would be expected from simple dosage considerations.

Fig. 3. *(opposite)* The metabolic chromatographic patterns 2 h after the administration of (±) ^{14}C-fenfluramine (1 mg/kg) in the brains of mouse, rat, and squirrel monkey (F = fenfluramine, NF = norfenfluramine).


MOUSE
F
NF
0
2
6
10
14
RAT
NF
Radioactive
counts
F
0
2
6
10
14
SQUIRREL MONKEY
NF
unknown
metabolite
F
0
2
6
10
14

This saturation in metabolism is reflected by a fourfold increase in half-life, from 2.3 to 9.6 h found in the rat when the dose is increased from 5 to 40 mg/kg (Caccia et al., 1981). However, there appears to be less of a saturation of the metabolism of norfenfluramine, such that the AUC only increases by a factor of 1.5–1.9 over the same range of doses. There are also species differences in the saturation of metabolism, and longer half-lives are found in the dog at higher doses, but not in the mouse (Caccia et al., 1982). Although little data are available for other substituted phenylethylamines, it can be expected that similar saturation does occur, suggesting that investigators should be cautious when extrapolating doses within and across species.

Associated Treatments

It is common practice for various compounds to be coadministered with a test drug, for example, to block receptors, enzymes, or uptake, and if a positive result is found, to use this effect to suggest a mechanism of activity. Unfortunately, there may be kinetic or metabolic consequences of such an interaction that may well provide an alternative and more valid interpretation. For example, it has been assumed that animals that have been pretreated with the 5-HT uptake blockers, such as fluoxetine or paroxetine, show a reduction in the 5-HT depleting effects of MDMA and other substituted amphetamines owing to inhibition of 5-HT uptake into the neuron via the serotonin transporter (Schmidt, 1987). Although this may be true, another explanation exists. These compounds strongly inhibit metabolic pathways (Fuller et al., 1992), specifically the cytochrome 2D6 enzyme in humans (Brösen and Skjelbo, 1991; Bergstrom et al., 1992; Sindrup et al., 1992; Stevens and Wrighton, 1993), the enzyme now known to demethylenate MDMA to potentially toxic compounds (*see* Metabolic Pathways). Since both MDMA and MDA have high affinity for the CYP2D6 isozyme (K_m= 1–3 μM) (Tucker et al., 1994), it is likely that coadministration of these drugs could lead to significant kinetic interactions. A similar consideration would hold for the equivalent enzyme CYP2D1, which is responsible for the demethylenation of MDMA in rats (Kumagai et al., 1992b). Suggestions that this may be the case come from the work of Hashimoto and Goromaru (1990), who showed that coadministration of MDMA and paroxetine to rats leads to a significant reduction of paroxetine clearance with a subsequent elevation of blood and brain levels. In addition, it is known in humans that CYP2D6 exhibits genetic polymorphism, and it has been suggested that the severe reactions that occur in some individuals may be the result of a reduction in activity of this enzyme, which occurs in 8% of caucasians (Tucker et al., 1994). Another example of kinetic interaction is found with the coadministration of *d*-amphetamine and fenfluramine. Prior pretreatment of fenfluramine (Jori et al., 1978) or amphetamine (Hunsinger et al., 1985) will increase plasma and brain concentrations of the other compound by as much as 100%, perhaps because of the competition for the catabolic enzymes of deamination. Without the measurement of plasma and brain drug levels during such pharmacological interaction studies, it is not known which of several hypotheses may be correct.

The handling of the animals may also influence the kinetics, as can the time in the day when the drug is administered, since clearance is for certain drugs under circadian control leading to different toxicities (Lévi et al., 1994). Thus, amphetamine levels may be fourfold lower in stressed rats compared to rested ones (Pashko and Vogel, 1980), whereas anesthesia can significantly alter the uptake and distribution of drugs (Gordon et al., 1986; Büch et al., 1991). Recent findings have suggested that lowering the raised temperatures found after large doses of the substituted amphetamines reduces their toxicity with compounds that have a wide range of pharmacological activities. It is interesting to speculate what part kinetics may play in this effect, since it is well known that temperature can have a marked

effect on metabolic clearance and drug transport, with, for example, a doubling of methadone levels with a 10°C increase in temperature (Herr, 1989).

Similarly, surgical manipulation can markedly alter the kinetics of drugs. Adrenalectomy, for example, significantly augments the weight-reducing properties of fenfluramine (York and Maclean, 1992), and supplementary cortisone treatment reverses this effect. It has, therefore, been surmised that in some way the pituitary–hypothalamic axis plays an important modulatory role in the anorectic activity of fenfluramine. However, when plasma and brain levels of fenfluramine and norfenfluramine were measured, it was shown that both plasma, and more importantly brain concentrations, had increased approximately fivefold after adrenolectomy, and the additional activity could be completely explained by the increase in brain exposure. Indeed, when the combined brain levels of fenfluramine and norfenfluramine were plotted against the reduction in the weight loss in these animals, irrespective of the modulatory influence of adrenolectomy with or without cortisone, a direct curvilinear relationship was found (Fig. 4).

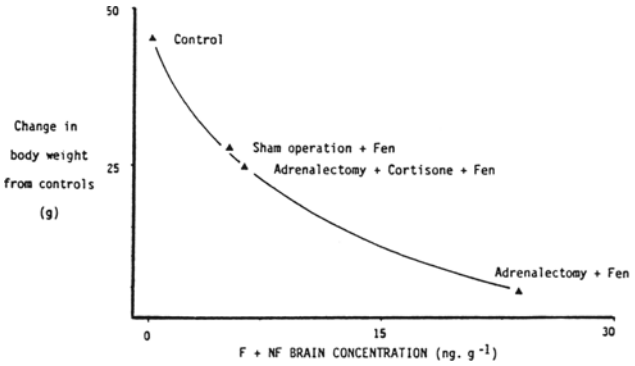


Fig. 4. Relationship between brain levels of fenfluramine (F) and norfenfluramine (NF) and corresponding changes in body weight following a dose of 10 mg/kg to rats, with or without adrenalectomy and cortisone supplementation.

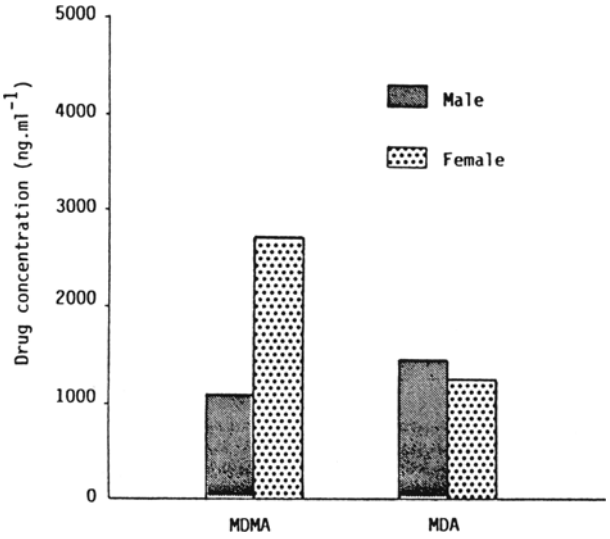


Fig. 5. The effect of gender on the levels of MDMA and its metabolite MDA, 4 h after 40 mg/kg sc MDMA (n = 5). (Redrawn from Fitzgerald et al. [1989.])

Gender Differences

It is becoming well documented that rodents and, in particular, rats often show gender-related differences in the metabolism of certain drugs, which is thought to be under endocrine control (Zaphiropoulos et al., 1989). This is, however, rarely seen in larger animals. The use of in vitro hepatic microsomal systems has shown that this mainly occurs for drugs that are metabolized by CYP3A, and that the female rat is deficient in this isozyme. Other metabolic routes may also show this gender difference. The enzymatic activity can be returned by the administration of testosterone or when the females pass menopause (Kato, 1974). Thus, plasma levels of both MDMA and MDA (Fitzgerald et al., 1989) (Fig. 5), various narcotics (Kato, 1974), and fenfluramine (Campbell, 1994b) are higher in females than males. Sometimes this difference is artificially lost, since dosage is nearly always based on mg/kg, and since males tend to be heavier, they actually get more drug. Since this is not necessarily taken up into the larger volume of distribution, the consequence is higher than expected plasma and brain levels in males compared with

females, thereby reducing any inherent gender differences. Again, because of the complexity of the situation, if gender differences in apparent sensitivity are observed, particularly in rats, then kinetic evaluation should be undertaken to negate this possibility.

Repeat Dosing Tolerance and Sensitization

Depending of the half-life of the drug and the frequency of administration, it can be expected that many drugs will accumulate in blood and tissues after repeated dosing according to the formula:

$$Rac = (1.44 \times half\text{-}life/dosing\ frequency)$$

where Rac is the accumulation index.

Thus, a drug with a half-life of only 2 h, but administered every 4 h will accumulate, but one with a half-life of 6 h given daily will not. The time plasma levels take to reach steady state normally occurs within 3–5 half-lives for all drugs with linear kinetics if dosed at every half-life, but for most of the substituted phenylethylamines administered to rats once or twice a day, steady-state levels of the unchanged drug are reached after the first dose. However, the half-lives of many primary amine metabolites are longer than the secondary or tertiary amine parent drug (Campbell et al., 1986; Caccia and Garattini, 1992). Presumably, dealkylation is more rapid than deamination, and for these where half-lives may be approx 10 h in rats, steady-state is reached in approx 2 d. In the case of fluoxetine, the half-lives of both parent drug and metabolite are only slightly longer than those observed with the phenylethylamines at lower doses, but at 15–20 mg/kg, doses that can reduce 5-HT levels, the half-lives increase to 13 h or more (Caccia et al., 1990), and up to 14 d of dosing are required to attain steady-state concentrations and maximal effect (Gardier et al., 1993). Some drugs on repeated administration can alter their own kinetics by auto-induction or auto-inhibition of hepatic enzymes, or the saturation of biodisposition processes. There is much evidence to show that repeated administration of the phenylethylamines leads to a reduction in activity measured by abnormal behavior or brain amines (Rebec and Segal, 1980), which could be the result of a reduction in plasma or brain levels. On the contrary, levels of amphetamine, for example, actually increase (Kuhn et al., 1978), as could be expected from normal accumulation. It would seem that this reduction in effect is more likely to be a biochemical adaptation rather than some change in kinetics. Similar considerations hold for cocaine, where levels accumulate after repeat dosing, but the effect diminishes (Cass and Zahniser, 1993). However, using an unusual dosing paradigm with rats treated with methylamphetamine (MA) every 6 h for alternate d for 7 d with escalating doses, when compared to the first day of treatment at the same final dose of 15 mg/kg, a 50% reduction in brain amphetamine and methylamphetamine levels was reported providing some rationale for the observed tolerance in CNS activity seen with prolonged administration (Schmidt et al., 1985). In contrast, for *d*-fenfluramine, although there were reductions in brain 5-HT levels, 15 and 28 d after semi-acute high dosing (10 mg/kg) for 4 d, escalating the dose to the same final amount over a 28-d period produced no change in brain amines, and the brain levels of fenfluramine and norfenfluramine were not significantly changed (Rose et al., 1993).

Stereochemistry

As many as 25% of all drugs used clinically are thought to have at least one chiral carbon atom and can exist as more than one enantiomer (Campbell, 1990a). Similar considerations are true for drugs of abuse, such as all the amphetamines, and those that are also used as pharmacological tools, such a paroxetine, fluoxetine, and fenfluramine. There can be important quantitative differences in their kinetics and metabolism that can become

important when the activities of the compounds and/or their active metabolites also exhibit stereoselectivity. Thus, although in vitro, the enantiomers of fluoxetine have equal potency as an uptake inhibitor, the metabolites have different activities (Wong et al., 1985, 1990; Fuller et al., 1992). In mice, S-fluoxetine is more slowly converted to the active metabolite S-norfluoxetine in contrast to the more extensive formation of the relatively inactive R-norfluoxetine from the R-enantiomer (Fuller and Snoddy, 1993). This could provide a rationale regarding why the R-enantiomer has been shown to have a reduced protected activity on depletion of 5-HT by PCA (Robertson et al., 1988) owing to the relative temporal differences in the kinetics of the active isomers of both drug and metabolites. Stereotaxic direct injection of (+) MDMA into the dorsal or medial raphe does not change 5-HT levels in the hippocampus or striatum (Paris and Cunningham, 1991). However acute sc administration with the (+) or (–) enantiomer of MDMA produces a reduction in 5-HT and 5HIAA in the striatum (Schmidt et al., 1987) and cortex (Schmidt, 1987) within 3 h, which remain reduced after 7 d for the (+) isomer, but not the antipode. Similarly, Johnson et al. (1988) found that at lower repeated doses (3–5 mg/kg), the d-isomers of either MDMA or MDA were more active on reducing 5-HT compared to the l-isomers, but this difference was lost at higher dosage (5–10 mg/kg). The plasma profiles and kinetics are similar for both enantiomers of MDMA (Cho et al., 1990; Hiramatsu et al., 1991; Lim et al., 1993), but the levels of the metabolite MDA were approximately three times higher after (+) MDMA, compared to (-) MDMA and these stereoselective differences in pharmacokinetics and metabolism (Hiramatsu et al., 1989a) may provide some explanation for these differences in toxicological findings. Differences in the activity of the optical isomers of PCA have been reported, which in part may be explained also by differences in kinetics (Stekerke et al., 1975). For fenfluramine, the situation is more complex, since although most animals tend to metabolize the S(+) active dextroenantiomer more rapidly, at high doses in the rat, the clearance of R(–)laevo fenfluramine is faster (Caccia et al., 1982). This may not be important, since neither the l-enantiomer of the drug nor the metabolite is active at low doses. However, at high dosage (≈40 mg/kg) there is a greater saturation in the kinetics of the l-isomer of both parent drug and metabolite and combined levels are higher than the d-antipodes (Caccia et al., 1981). At these very high levels, the l-enantiomers start to produce an activity in reducing 5-HT (Kleven et al., 1988). It is clear that if racemic mixtures are administered during pharmacological or toxicological investigation, particularly over a wide dosage range, and when active metabolites may be chirally selective themselves, then a knowledge of the kinetics of the individual isomers is important (Campbell, 1990a; Tucker and Lennard, 1990).

Extrapolation to Humans

There have been many attempts to relate the general toxicity observed in animals with that expected in humans (Kimmel, 1990; Dourson and Derosh, 1991), and more recently the problem of neurotoxicity extrapolation has been addressed (Slikker, 1991; Federal Register, 1993). There are many difficulties and unknowns, including species differences in sensitivity, biodisposition, environment, and so forth. There is a need to evaluate a dose–response relationship in animals to determine the no effect dosage (NOEL), to compare this with the exposure measured by simulations or directly expected in humans, and divide this figure by an uncertainty factor bringing together all these unknowns. Thus, a 10-fold uncertainty factor (UF) is used when relevant animal data are compared to known prolonged exposure in humans to account for individual sensitivity differences; another 10-fold UF is used if available data are inadequate for humans; a further 10-fold UF is used for inadequate animal data, e.g., short-term dosing. A final 10-fold factor is used when converting from a NOEL to a LOEL, bringing a

possible divisible factor to 100,000 (Barnes and Dourson, 1988). Clearly, there are problems with this approach, and various authors have attempted to improve these estimates by using either a benchmark dose, which is the lowest effective dose that would produce a measurable change of, for example, 10% of maximal observed response, which is used as a baseline starting point for factorization (Crump, 1984), or quantal responses where the individual dose–response data points are joined by some mathematical model to identify doses that can cause an effect outside those that have been measured (Kimmel, 1990). This can be linked to measurable biomarkers, such as 5-HT, dopamine, and their metabolites, after MDMA administration (Ali et al., 1991; Gaylor and Slikker, 1992). Although these approaches may be improvements, they still do not take into consideration one of the largest uncertainty factors, that of kinetic differences. The use of discrete kinetic dynamic mathematical models can overcome some of the limitations described above.

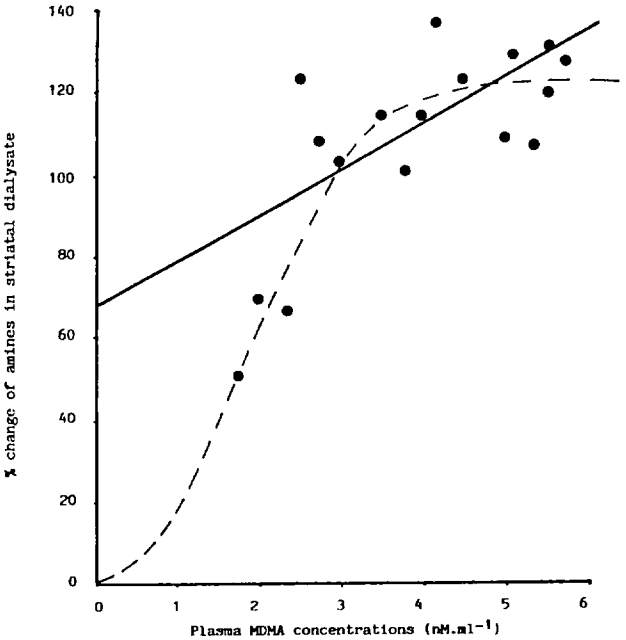


Fig. 6. Relationship between changes in amine efflux in striatal dialysate and plasma MDMA concentration. (— Line drawn in original paper, – – – line drawn assuming a receptor-based saturable interaction assuming reversible Michaelis-Menton kinetics). (Redrawn from Hiramatsu and Chu [1991.])

Kinetics Dynamic Relationships

Measurement of the plasma or brain levels is clearly a more useful assessment of exposure when comparing across dose, species route, and so on. However, it assumes that there is some simple direct relationship between the measured concentration and the activity. Often this relationship is presumed to be linear, and, although never stated, implicit in this belief is that a doubling of the dosage will lead to a doubling of the effect. For example, in Fig. 6 where MDMA plasma concentrations have been plotted against percentage change in striatal 5-HT dialysate levels, the authors (Hiramatsu et al., 1991) attempted to draw a straight line through the data points whereas an asymtopic curve reflecting a saturable receptor binding would be more appropriate and provide more useful information (Fig. 6), such as a maximal effect (E_{max}) at approx 5 n*M*/mL, and a potency effect at 50% of E_{max} (EC_{50}) of ≈2 n*M*/mL. Even at the simplest level of a reversible receptor or enzymatic interaction, a linear relationship can only partially describe the binding, and then only at low concentrations (Campbell, 1990b). For more complex interactions, a direct linear relationship cannot be expected, particularly if more than one receptor is involved or the reaction is irreversible. An example of such possible complex interactions can be seen with the effect of fenfluramine and norfenfluramine on the change in 5-HT in the rat cerebral cortex (Campbell et al., 1991). This analysis is derived from the data published by Zaczek et al. (1990). At the low pharmacological dose of 2 mg/kg administered in divided doses over 4 d, there is an increase in brain 5-HT levels, but as the dose increases up to a maximum of 48 mg/kg, the 5-HT levels progressively decrease. In an attempt to relate these changes to the brain drug concentrations, combined peak levels of fenfluramine and the active metabolite norfen-

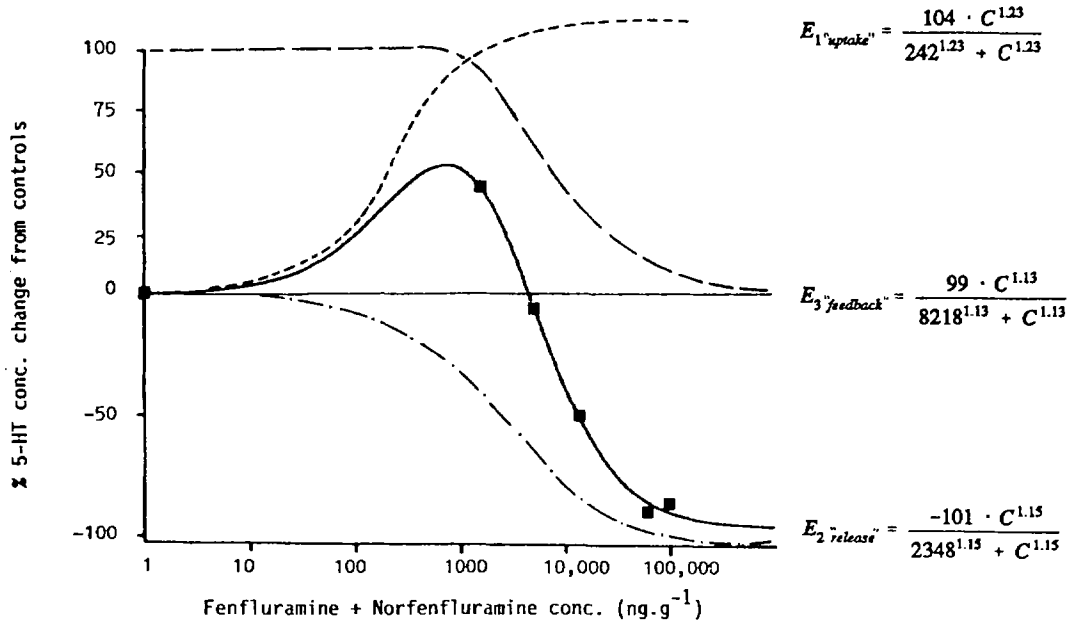


Fig. 7. Brain-dynamic modeling showing computed line of best fit (—) through data points (■) from three hypothesized "receptor" activities, E_1 (uptake 1--), E_2 (release –·–), E_3 (feedback – –), where C = brain concentration of fenfluramine + norfenfluramine.

fluramine were related to the percentage change in 5-HT from controls at the same time (0.5 h), and a three-receptor mathematical model was found to describe best the resultant curve using the following formula for each receptor interaction:

$$Effect(E) = (E_{max} \cdot C^{\gamma}/EC_{50}^{\gamma} + C^{\gamma}) \quad (3)$$

where E_{max} is the maximum effect, C the concentration of drugs in the brain, EC_{50} the concentration at 50%, and E_{max} and γ are the slope of the relationship or Hill coefficient. In vitro receptor binding studies in the rat indicate that, at low levels, fenfluramine blocks the reuptake of serotonin, whereas at higher levels, both fenfluramine, and to a greater extent norfenfluramine, release 5-HT from serotonergic terminals (Mennini et al., 1991). In addition, at higher doses, paroxetine binding, a measure of the functional ability of uptake sites, is decreased, suggesting a reduced number, function, or a feedback downregulation in the presence of high initial synaptosomal 5-HT levels (Gobbi et al., 1993). Assuming that these processes may be involved in the observed 5-HT changes, it is possible to obtain measures of the activity at half-maximum effect (potency), and these can be compared to the dissociation or binding constants found in in vitro studies (Fig. 7). Although only six points were used for this modeling, interestingly, the computed EC_{50} from the assumed uptake inhibition receptor model (242 ng/mL), and that for release (2348 ng/mL) are indeed comparable with those found directly from in vitro binding studies, using rat synaptosomes and combined fenfluramine and norfenfluramine values of approx 200 and 1000 ng/mL, respectively (Garattini et al., 1988). Such multi-E_{max} models have also been used to explain the complex action of other CNS drugs, such as clonidine, on central blood pressure control (Paalzow, 1984). Using this integrated mechanistic approach, it is possible to investigate more confidently the exposure and the possible underlying mechanism that may occur in humans at therapeutic doses from data obtained in animals.

The steady-state levels of more than 300 patients taking dexfenfluramine for up to 12 mo (30 mg/d) are 20 and 12 ng/mL for dexfenfluramine and dexnorfenfluramine, respectively (Gordon, 1991). Using these combined drug levels to calculate brain levels in humans assuming a brain/plasma ratio of 9 (Fleisher and Campbell, 1969; Holmes and Gordon, 1981), it can be seen from Fig. 7 at a combined human brain level of 300 ng/mL that the action of the drug in humans at therapeutic doses is mainly uptake blockade with little release, and that the levels are approx 20-fold lower than the crossover point where there is no change in 5-HT levels (≈6000 ng/mL) and 350 times less than those that produce the maximum reduction in 5-HT (100,000 ng/mL). This approach makes many assumptions, including that the response in the rat and humans is similar, but this is still a better measure of comparative exposure than other techniques, and the results can be updated when comparative in vitro binding constants in humans are obtained.

Conclusion

It is hoped that this discussion has highlighted the problems of obtaining toxicological data from one experiment and attempting to extrapolate the findings to another dose, species, or experimental condition without some measure of drug and or metabolite exposure. The new worldwide harmonized discussion document on toxicokinetics (ICH2, 1993), which when used with the draft guideline on the assessment of neurotoxicity (Federal Register, 1993), may provide a more meaningful framework for future studies. It would be nice to think that once the compound is measured in the appropriate body tissue, the kinetic aspect can be forgotten, but as suggested in the text, toxicokinetics is still in its infancy, and there is still much to be discovered. Most important is what should be measured, and for MDMA, which has undergone considerable research, we are still far from understanding which chemical moiety is responsible for the apparent toxicity, unchanged drug, an active metabolite, a free radical, or a catabolic product of 5-HT or dopamine. However, without some attempt to measure these entities and then to relate their concentrations in some meaningful mathematical mechanistic method, attempts to understand the underlying processes fully will come to naught.

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