DMD Fast Forward. Published on June 28, 2005 as doi:10.1124/dmd.105.005041

Seeing Through the MIST: Abundance Versus Percentage. Commentary on *Metabolites in Safety Testing*

Dennis A. Smith and R. Scott Obach

Pharmacokinetics, Dynamics, and Metabolism

Pfizer, Inc.

Sandwich, Kent, UK (DAS) and Groton, CT, USA (RSO)

DMD Fast Forward. Published on June 28, 2005 as DOI: 10.1124/dmd.105.005041

Running Title: Metabolites in Safety Testing: Abundance vs. Percentage

Number of Text Pages: 23

Number of Tables: 5

Number of Figures: 3

Number of References: 42

Number of Words in

Abstract: 160

Introduction: 582

Discussion: Not Applicable

Abbreviations: ADME: absorption, distribution, metabolism, and excretion; CNS: central nervous system; GI: gastro-intestinal tract; hERG: human ether a go-go; HPLC: high pressure liquid chromatography; MIST: Metabolites in safety testing; NAPQI; N-acetyl para quinine imine; NSAID: non-steroidal anti-inflammatory drug

Address correspondence to Dennis A. Smith, Pharmacokinetics, Dynamics, and Metabolism, Pfizer, Inc., Sandwich, Kent, UK, CT13 9NJ

Abstract

Recent attention has been given to the potential roles that metabolites could play in safety evaluations of new drugs. In 2002, a proposal was published on “Metabolites in Safety Testing” (“MIST”) (Baillie, et al., (2002) Toxicol. Appl. Pharmacol. 182: 188-196) which suggested some guidelines regarding when it is necessary to provide greater assessment of the safety of metabolites. However this proposal was based on relative abundance values, i.e. the percentage that a metabolite comprises of total exposure to drug-related material. In the present commentary, we propose that absolute abundance criteria be used rather than relative abundance. The absolute abundance of a metabolite in circulation or excreta in humans should be combined with other information regarding the chemical structure of the metabolite (e.g. similarity to the parent drug, presence of chemically reactive substituents) and potential mechanisms of toxicity (e.g. suprapharmacological effects, secondary pharmacological effects, non-specific effects). Decision trees are described that can be used to address human metabolites in safety testing.

Introduction

Much attention has been given to the potential role that metabolites of drugs may contribute to drug-induced toxicity. Since possible mechanisms of toxicity are myriad and in many cases complex, gaining an understanding of the role that drug metabolites can contribute to this is even more challenging than it is for the parent drug. It is not uncommon to speculate that a metabolite(s) could be responsible when toxicity is observed, either in toxicology studies conducted in laboratory animal species or as side effects in clinical trials. This speculation is particularly tempting when the toxicity observed has no apparent link to the target pharmacological mechanism. Such speculations often outnumber and dwarf the number of times that a metabolite is the actual cause. For instance, many publications have linked the teratogenesis associated with phenytoin to metabolites such as epoxides (Martz, et al., 1977; Finnell, et al., 1992; Raymond, et al., 1995). When the pharmacology of the drug is fully considered, it is an I_{Kr} channel blocker (hERG ED_{50}~ 100μM), in addition to its primary activity against the sodium channel (IC_{50}~ 47μM) (Salvati, et al., 1999; Nobile and Vercellino, 1997). I_{Kr} channel blockers, at concentrations not affecting the adult, cause bradycardia, arrhythmia and cardiac arrest in the fetus leading to hypoxia, reoxygenation and alterations in embryonic blood flow. These effects can lead to growth retardation, orofacial clefts and distal digital reduction, and cardiovascular defects (Denielsson et al 2001; Salvati et al, 1999). Thus, phenytoin serves as an example in which it is tempting to propose that a metabolite is responsible for toxicity, but that in actuality the toxicity is caused by the parent drug acting at a non-target receptor.

A drug can yield dozens of metabolites and it is not a common practice to measure exposure to these metabolites in toxicology studies conducted early in the drug development process, and at this early part of the process the identities of most of the metabolites are not even known. In 2002, a group of scientists from the pharmaceutical industry proposed a guideline for assessing the contributions of metabolites to toxicity termed "Metabolites in Safety Testing" or "MIST" for short (Baillie, et al., 2002). This document attempted to define those situations in which metabolites should be further studied to help define a risk assessment for the parent drug. The primary trigger that was proposed for gathering more information on a metabolite is one of relative quantity; that is, if the metabolite is present in humans at 25% or more of the total drug-related material in circulation, it merits further investigation as a potential contributor to safety findings. This figure was based on the need for a defined limit and on pragmatic considerations of the technical feasibility of radiometric methods of quantitation, since this approach can reliably deliver metabolite quantities as a percentage of total, but not in absolute concentration terms. In this commentary, we try to build on MIST and propose a set of criteria to be used to determine if a metabolite should be more extensively studied. The emphasis is to try to base the proposal on a history of metabolite learnings (at least 40 years of work published in the area) and the knowledge we can gain from this. Our criteria focus less on *relative* abundance (proportion, %) as has been suggested in the MIST paper, and more on *absolute* abundance (concentration, mass), and also take into consideration the structure of the metabolite relative to the parent drug and the potential toxic mechanisms of metabolites.

Definitions of Mechanistic Catagories of Toxicity

In order to develop a strategy for metabolites in safety testing, the types of mechanisms of toxicity that could be caused by metabolites, and the chemical structure of the metabolite, relative to parent drug need to be considered. Toxicity can be categorized into four overall types (A, B, C, and D) as defined below:

In Type A, the toxic mechanism has a pharmacological basis. For this commentary, we consider two subtypes of type A: one based on the target pharmacology (A1) and the other based on other non-target pharmacology(s) (A2). For Type A1 toxicity, the parent drug is the most common culprit. This can arise by too much receptor occupancy or enzyme inhibition, or this occurring for too extended a period (“supra-pharmacological effects). It can also occur merely due to concurrent side effects of the pharmacological mechanism. Simple examples include GI bleeding due to cyclooxygenase-1 inhibition by NSAIDs, GI motility decreases due to opioid agonism or extrapyramidal effects of dopamine antagonists. Metabolites, in which structural modifications are minor, occur on substituents not critical for target receptor activity, and do not substantially change the physicochemical properties of the parent drug, are the ones most likely to contribute to pharmacological activity, and hence any supra-pharmacological toxic effects. Thus, any pharmacologically active metabolite can be important if observed toxicity is due to supra-pharmacology.

Type A2 toxicity is that elicited by binding to and altering the activity of a specific receptor or enzyme that is not the primary pharmacological target. In many cases, the binding affinity may be weaker (i.e. have a higher K_d) but slight alterations in the receptor function can have profound physiological consequences. The most well-known example of this would be binding to the I_{Kr} channel that can cause QT interval prolongation and in rare cases result in fatal cardiac arrythmias. Partial block of this channel can have profound influence on cardiac function, therefore the intrinsic potency of the drug or metabolite for the I_{Kr} channel does not have to be as high as that for the primary pharmacology to exert an undesirable side effect. Other examples of type A2 are frequent among neuroleptic agents in which drugs can bind to receptors closely related to the target pharmacological receptor, but which are responsible for other functions.

Types B, C, and D toxicities tend to be more related to mechanisms that are not for specific enzymes or receptors, but rather for non-selective effects. In many cases, the structural elements of metabolites that are associated with types B, C, and D toxicity involve the introduction of reactive electrophilic groups, or structural entities that can cause oxidation (e.g. quinones), and in most cases it is the observation of metabolites downstream from these reactive intermediates that arise via reaction with nucleophiles (e.g. mercapturic acids, diols, etc) that are actually observed in vivo. Type B refers to idiosyncratic toxicities, such as drug-induced allergy, that do not necessarily exhibit classic dose-response relationships, and are observed in very low numbers of patients. Mechanisms of type B toxicity are not well-established but the first pivotal event is considered to be activation of the drug to a reactive metabolite that non-selectively covalently bonds to proteins. Some of the haptenized proteins can trigger an immune response that could either target only haptenized proteins (resulting in toxicity only when the drug is administered) or could begin to also recognize native proteins (resulting in autoimmune toxicity that does not require continued drug administration). Type B toxicity can occur in a variety of tissues, and can even occur in different tissues in different patients for the same drug. Normally, though, the three prime sites of toxicity are the liver, blood cells and skin. Many of the drugs causing type B toxicity exhibit effects on all three. These organs and tissues may be uniquely sensitive due to their high intrinsic activity in terms of oxidising systems (e.g. activated neutrophils and the release of hypochlorous acid) or the presence of a highly developed and active immunological defence system. (Park et al., 2000). It should also be noted that toxicity in these organs and tissues are amongst those most easily detected.

In Type C toxicity, the effect of the drug is due to a chemical reaction between drug or metabolite and tissue macromolecules resulting in a rapidly ensuing response. It is rare that drugs themselves elicit this type of toxicity (an exception is direct alkylating agents used in cancer chemotherapy) but some drugs can be bioactivated to chemically reactive entities that can act directly by covalently binding to proteins. It is also possible that drugs can be bioactivated to metabolites that undergo redox cycling (e.g. quinones/hydroquinones), deplete intracellular stores of reduction potential, such as reduced glutathione, and cause oxidative stress. This can lead to cell death and tissue necrosis. Liver toxicity elicited by high doses of acetaminophen via generation of the N-acetyl paraquinoneimine metabolite is a good example of type C toxicity.

Type D is similar in underlying mechanism as types B and C, however the response is delayed, even for years. Examples of type D toxicity include carcinogenesis and teratogenesis. For carcinogenesis, mechanisms could be due to genotoxins or possess an endocrinological basis (which is actually more related mechanistically to Type A toxicity). Genotoxins can also cause teratogenesis, but teratogenesis can have other underlying mechanisms often similar to type A1 and A2. It is important to note that for types B, C, and D, metabolites that could be responsible for toxicity can be reactive enough that they are not detected *per se* in circulation or excreta.

When assessing the potential toxicity of a drug, what is actually being examined is a complex mixture of chemicals: drug plus all metabolites and impurities. Developed policies have been described for assessing the safety of impurities in drugs (FDA, 1997). However, these policies are not directly applicable for metabolites. Chemical structures of impurities can be substantially different from the parent drug, since they may derive from the chemical process employed to synthesize the drug. As completely unrelated structures, impurities can elicit very different toxic responses than the parent drug, so the examination of the safety of impurities can have stringent criteria. Metabolites are generated from the parent drug, so in most cases they bear structural similarity to the parent drug. Also, as described later, determination of the levels of metabolites in biological matrices has a practical limit; most publications describe the lowest abundant metabolites as 5% of dose or higher. This is in contrast to synthetic process impurities that can be detected in bulk drug substances to much lower percentages. Therefore, it is not practical or necessary to apply the same criteria to metabolites as is applied to impurities for safety assessment.

In this commentary, we propose that the following three criteria be considered in formulating a MIST strategy:

(1) Structure of the metabolite, relative to parent drug, and the resulting physicochemical properties;

(2) Absolute abundance of the metabolite (not relative abundance); and

(3)Types of toxicity observed in laboratory animals and humans.

Support for this position is offered in the discussion below, along with a detailed proposal for a metabolites in safety testing (MIST) strategy based on these criteria.

Practical Aspects of Metabolite Identification and Quantitation and Their Limitations

The MIST paper advocates a percentage-based cutoff criteria for determination of which metabolites should be considered major, and hence further considered in safety assessments (Baillie, et al., 2002). It was proposed that those metabolites comprising 25% of the total radioactivity in circulation, as determined in a radiolabel ADME study in humans, are considered major. This has a basis in the practical manner in which metabolites are quantitated in radiolabel studies by radiometric methods. In order to understand the limitations of this, the design and conduct of human radiolabel ADME studies must be briefly discussed.

In human radiolabel studies, there are several objectives:

(1)Determination of the material balance of total drug-related material,

(2)Determination of the routes of excretion (i.e. renal, fecal) of total drug-related material,

(3)Determination of the pharmacokinetics of total drug-related material in circulation, relative to parent drug,

(4)Structure elucidation of metabolites in excreta and circulation, and

(5) Quantitation of relative abundances of metabolites in excreta (to definitively identify all routes of clearance) and circulation (to determine all metabolites to which tissues are exposed).

In the human ADME study, healthy subjects (commonly males) are administered a single dose of study drug of which a portion contains one or more radionuclides at a position within the molecule selected on the basis of expectations that the radionuclide will not be converted to a metabolite that could be incorporated into endogenous metabolism (i.e. CO_2, acetic acid, water, etc). Carbon-14 is the most frequently employed radionuclide, due to there typically being a variety of positions in the molecule into which it can be incorporated and also due to the fact that it is a relatively safe, low energy radiation emitter. Tritium is also sometimes employed, provided it is not readily exchangeable or metabolically labile. If a substantial portion of the drug is expected to be cleaved into two significant portions, then radionuclides may be incorporated on each side. The total dose used in human radiolabel studies is typically one that is pharmacologically active and well-tolerated, or is anticipated to be close to the pharmacologically relevant dose if this has not yet been established. The dose of radioactivity is usually 100 μCi, or less, depending on predictions of exposures of specific tissues from tissue distribution studies conducted in laboratory animals (usually rats) not exceeding radiation limits. Thus, depending on the pharmacologically relevant dose, the specific activity of the dose will vary, and this is an important factor in describing one of the main concerns in the MIST strategy that advocates a percentage-based approach to defining major metabolites (see below).

After administration of the single dose of radiolabelled drug, the study subjects are maintained in an in-patient clinical study site while excreta are quantitatively collected, and blood samples are collected for determination of pharmacokinetics of parent drug and total radioactivity. Subjects are usually kept at the study site until predefined criteria are met regarding a threshold value for total mass balance and/or drop in the rate of daily excretion, or both.

The samples obtained from this study are used in metabolite profiling of the excreta and circulation. Each of the matrices (urine, fecal homogenates, and plasma extracts) are analyzed by radiometric HPLC using radiometric flow detectors, or if the total amount of radioactivity in the sample is too low, fractions are collected and subjected to liquid scintillation counting. In quantitative radiometric profiling, there are practical lower and upper limits as to the total quantity of radioactivity that can be injected. If too much is injected, radioactive peaks will blend together, the detector can become saturated, and baseline drift can confound quantitation. With too little injected, the signal-to-noise ratio becomes too low to permit reliable integration of peaks. *Thus, irrespective of the total dose, the limits of quantitation of metabolites by radiometric methods is percentage-based, and not quantity based.* Herein lies the practical limitation of radiometric quantitation in assessing which metabolites are important and which are not as proposed in MIST (Baillie, et al, 2002). Radiometric HPLC can reliably quantitate metabolites comprising 10% of the total, can adequately quantitate metabolites comprising 5% of the total, and struggles to reliably quantitate metabolites below 5%. It is on the background of these practical considerations, that it was reasonably proposed in the MIST paper that metabolites comprising 25% or more in circulation are to be considered major and worthy of further consideration in safety assessments. It is noticeable that the way that we think about these studies leads us to a percentage-based rationale: that is, mass balance is measured as percentage of the administered dose, routes of excretion as percentage of the administered dose, which lead ultimately to metabolites and their importance expressed as percentage of the dose or percentage of total circulating drug-related material. This is not a sound basis for risk assessment, where we normally use units of mass or concentration.

This can be illustrated in the following example of two drugs. The pharmacologically relevant dose for drug A is 1 gm per day and the pharmacologically relevant dose for drug B is 1 mg/day. In human radiolabel studies, drug A would be dosed at 1 gm containing 100 μCi (0.1 μCi/mg) and drug B dosed at 1 mg containing 100 μCi (100 μCi/mg). For total dose in excreta, a 10%-prevalent metabolite for drug A represents 100 mg of the dose while for drug B this represents 0.1 mg, yet these two metabolites will be equally detectable and quantifiable, and in a percentage-based system of assessing which metabolites are major and which are minor, these two metabolites are considered equal in safety considerations, despite a 1000-fold difference in abundance. Likewise, in order to do the same diligence for drug A as is done for drug B, one would need to be able to identify and quantitate all metabolites comprising 0.001% of the dose, which is a practical impossibility with presently available methods.

This situation becomes even more complex when considering metabolites in circulation. First, the amount of total drug-related material (as total radioactivity) present in circulation is far less than that present in excreta, and the amount of sample that can be obtained is substantially lower than that obtained for excreta. Thus, plasma samples must frequently be subjected to extraction and concentration processes prior to radiometric HPLC, throughout which careful attention must be paid to ensure nearly quantitative recovery of radioactivity so as to not bias the metabolite profile by selective extraction of some metabolites and not others.

On this background of practical challenges, there are biological aspects that can confound an assessment of which circulating metabolites may be truly important in safety assessments. After biotransformation, the physicochemical properties of metabolites may differ substantially from the parent drug, which will have a profound influence on their relative distribution properties. Metabolites tend to be more hydrophilic than their parent drugs, and therefore less able to distribute from the plasma into tissues, which can magnify their relative abundance and importance. Also, metabolites can be more or less extensively bound to plasma proteins (e.g. albumin, α1-acid glycoprotein) which can provide misleading assessments of relative importance in circulation. A common example of this is when an amine drug gets oxidatively deaminated to a carboxylic acid (Figure 1). Carboxylic acids tend to be highly bound to albumin, while amines generally readily partition into tissues by virtue of association with phospholipids membranes. Thus the parent amine will appear to be low in circulation and the carboxylic acid metabolite will be high, deceiving one into believing that the carboxylic acid metabolite is ‘major” and the amine of little importance.

When Are Metabolites Toxic and What Considerations Apply to Their Measurement?

When formed, metabolites clearly have the potential to be both beneficial and toxic. Three types of effect can be identified, all with different outcomes and requirements.

Target Mechanism-Related Leading to Type A1 Toxicity: Metabolites may possess similar pharmacology to the parent. Analysis of a wide variety of drugs indicates that active metabolites tend to mirror the pharmacology of the parent rather than introduce novel de novo pharmacology. In certain specific cases the parent drug acts as a prodrug (e.g. losartan and its major metabolite EXP3179 or bopindolol and its major metabolite 18-502; Kraemer, et al., 2002; Harron, et al., 1991). In these cases the structure activity relationships of the receptor make it highly predictable that the metabolites will show increased intrinsic potency. In many drugs the presence of a tertiary amine function leads to metabolism along a sequential pathway of N-dealkylation reactions through the secondary amine to the primary amine. Due to the increased metabolic stability of metabolites it is often found that they have greater duration in the circulation than the parent and thus exert a longer lasting effect if active. Moreover, more accumulation of the metabolite will occur leading to a greater influence of activity with repeated dosing of the parent drug.. Fluoxetine and norfluoxetine provide an excellent example of this phenomenon (Rudorfer and Potter, 1997). A limitation of radiolabel ADME study designs is that they are routinely done as single dose studies, thus the extent of metabolite accumulation that could occur after multiple dose administration is not directly measured. However, if the half-life of the total radioactivity is long when compared to the half-life of the parent drug, the possibility of accumulation of a metabolite of interest is raised and will need to be considered in the understanding of the potential contribution of the metabolite to toxicity.

When the pharmacological activity of the metabolite is understood and dosage regimens adjusted accordingly the effects are beneficial (e.g. losartan, Kraemer et. al., 2002). Where the contribution of the metabolite is ignored and accumulation of pharmacological effects occurs toxicity can be witnessed. This is illustrated by risperidone, an antipsychotic drug, with activity at D_2 and 5-HT_2 receptors. Its 9-hydroxy metabolite is equipotent at these receptors, but has a greater unbound fraction in plasma (10% vs. 23%). Moreover the metabolite has a longer elimination half-life (Yamada et al, 2002). After a 2.5 mg dose of risperidone, the parent drug would occupy approximately 15% of D2 receptors and 40% of $5HT_2$. When the metabolite is also included the effect of a 2.5 mg dose of risperidone is 50% occupancy of D2 receptors and 95% occupancy of $5HT_2$. Extra pyramidal side effects may occur at higher dopamine receptor occupancies and the metabolite will be a major factor.

9-Hydroxyrisperidone, like most active metabolites only shows a small molecular change from the parent, such as hydroxylation, demethylation and desaturation. Examples of larger changes such as the active metabolite of morphine (morphine-6-glucuronide) are extremely rare. As shown in Table 1, the intrinsic potency of metabolites is similar or less than the parent molecule. This reflects the frequently observed relationship between lipophilicity and target receptor affinity.

Since circulating metabolites are in the vast majority of cases equally or less active than parent, unbound concentrations of circulatory material is a major guide. Again the overall guiding principal is that the metabolites show only small structural

DMD Fast Forward. Published on June 28, 2005 as DOI: 10.1124/dmd.105.005041
This article has not been copyedited and formatted. The final version may differ from this version.

changes and are usually the result of Phase 1 oxidative metabolism. Ideally, during the pre-clinical phase, potential metabolites produced in human microsome systems should be synthesized and screened for pharmacological activity. When activity approaches that of 25% parent based on *in vitro* pharmacological assays, specific bioanalytical assays should be developed prior to toxicological and human testing. If the activity in man is less than 25% of parent after human variation and disease is taken into account (e.g. renal function), then the need for such assays is attenuated. In human radiolabelled ADME studies, metabolites identified in circulation should be considered using the unbound free material as a guide to estimate pharmacological activity, with contributions of greater than 25% of parent considered relevant. The figure appears similar to the MIST figure, but is now based on the contribution to pharmacological activity that a metabolite of similar pharmacological potency to the parent would make. If contribution of the metabolite to pharmacological effect were 25% or below, it could be considered negligible. This figure is based on the rationale that regardless of which pharmacokinetic/pharmacodynamic model is used, a metabolite with pharmacology matching that of the parent will not make a significant contribution to the effect of the drug until its unbound concentration/invitro potency ratio approaches 25% of the parent. For instance, many antagonists achieve around 75% receptor occupancy at steady state. If a metabolite (with 25% of the in vitro potency of the parent) was present in some individuals at the same unbound concentration as the parent it would raise receptor occupancy to approximately 79%. Therefore, below 25% the changes in receptor occupancy are minimal.

Loss of Pharmacological Selectivity Due to Metabolism Leading to Type A2 Toxicity: Another case in which metabolism may play a role in the beneficial or side effects of drugs is when the metabolites of a drug show changes in selectivity compared to humans. Examples of beneficial change include the gain in selectivity for various antihistamine compounds. Terfenadine and hydroxyzine are lipophilic bases with high affinity for the I_{Kr} channel, and have been found to cause prolongation of the QT-interval in volunteers and patients. Their major metabolites, fexofenadine and cetirizine, are much more selective at H1 antagonism vs I_{Kr} blocking (Anthes, et al., 2002; Chiu, et al., 2004; Carmeliet, 1998; Taglialatela, et al., 1998) due to their zwitterionic nature (both are carboxylic acid metabolites). The structure activity relationships of the I_{Kr} channel render zwitterionic compounds extremely unlikely to have affinity and activity (Paakkari, 2002). The majority of cases indicate that pharmacological selectivity of parent drug does not change upon metabolism: the decreases in lipophilicity due to metabolism lowering affinity for both primary and secondary pharmacology in parallel. For instance the metabolite of halofantrine (log D $_{7.0}$ 6.5), N-desbutylhalofantrine (log D $_{7.0}$ 3.8) has an IC_{50} against hERG K+ channels of 72 nM compared with an IC_{50} of 22 nM for the parent (Mbai et al, 2002).

When a monoamine transporter or receptor is the pharmacological target, substitution on the amine is critical and changes in selectivity and potency occur upon modification. These changes however occur within the pharmacology of the parent molecule and do not introduce de-novo pharmacology into the molecule. Table 2 illustrates this, comparing the potency ratio of metabolites of various CNS drugs against the three monamine transporters.

The same guidance applies as that outlined for type A1. Although unlikely, metabolites could possess novel pharmacology from parent. Testing of these metabolites should be extended to include determination of binding to a broad array of receptors that are routinely examined in in vitro safety pharmacology studies for binding by the parent drug, to address the possibility of side effects not associated with the primary pharmacology (Table 3). As a specific case, metabolites produced by N-dealkylation reactions from drugs acting on neurotransmitter targets (receptors and transporters) should be further emphasized and as such should be synthesized and tested. This is due to the critical pivotal nature of the basic nitrogen (presuming one is present) and the high likelihood of metabolism by this route. It is very likely, outside of these drug classes above, that the guidance of 25% of parent drug-free concentration would be a diligent rule for characterizing metabolites.

Circulating metabolites that represent a substantial structural departure from the parent drug should be considered only on the basis of an absolute concentration. As judged from the human radiolabel study, if such a metabolite were present at 1 μM or more, then the free fraction should be determined. If the unbound concentration exceeds 1 μM, then non-selective types of binding interactions at other receptors becomes possible. Such metabolites should be subject to testing at an array of non-target receptors in an analogous manner as is ordinarily done for the parent drug in in vitro safety pharmacology studies to determine if they possess other non-target pharmacological activities. Assessment of whether the receptor activity could elicit an in vivo effect can be made from calculations of receptor occupancy by the metabolite, which are made from the receptor potency, circulating concentration, and free fraction values, and

DMD Fast Forward. Published on June 28, 2005 as DOI: 10.1124/dmd.105.005041
This article has not been copyedited and formatted. The final version may differ from this version.

demonstration of penetration to the tissue of interest. If occupancy is projected to be significant, then routine monitoring in animals and humans may be warranted to provide an understanding of safety. Such an approach emphasizes the absolute concentrations rather than amounts relative to the total circulating drug-related material. A concentration cutoff value of 1 μM is proposed based off of general knowledge around potency of small molecule ligands for receptor targets generally residing between 1 nM and 1 μM (Williams, et al., 1995). The likelihood that metabolism will suddenly impart high potency toward a receptor where there previously was none possessed by the parent drug is highly unlikely. However, if the metabolite circulates at a free concentration greater than 1 μM, even relatively low affinity interactions could occur and be responsible for toxic effects.

Toxicity Arising via Non-Selective Effects (Type B C & D): Most cases of non-selective toxicity, caused by metabolites, are triggered by irreversible binding of the metabolite to a macromolecule or oxidative stress via redox recycling. These types of interactions can lead to immunoallergenic toxicity, direct organ toxicity, mutagenicity and carcinogenicity. Certain cases are observed where there is a weak reversible affinity and a metabolite is implicated (e.g. phospholipidosis via the generation of desacetylketoconazole which intercalates into membranes better than the parent drug ketoconazole; Brasseur, et al., 1983; Whitehouse, et al., 1994). In many cases such chemically reactive metabolites are not readily detected in circulation or excreta, although sometimes a downstream metabolite of a reactive metabolite such as a mercapturic acid conjugate may be observed in the excreta. Although there is much concern on this type of toxicity, in general it is observed most frequently in compounds administered at high clinical doses (i.e. >200 mg/day). To illustrate this table 4 lists drugs withdrawn due to idiosyncratic type B toxicity and their daily clinical dose. It is noteworthy that the clinical doses are high and greater than 200mg day in over 80% of the cases. The two lowest dose drugs, mebanazine and nialamide, contain hydrazine functions, which are typically viewed as very undesirable moieties to include in drug molecules. Even though reactive metabolites are probably involved in most cases, the need for a high clinical dose is striking.

A case example of the importance of dose size or mass is acetaminophen (paracetamol), often implicated in overdose hepatotoxicity. Dose recommendations normally state that less than 4 g/day is safe and even 10 g/day is considered a modest overdose. Very rare idiosyncratic hepatotoxicity has been observed at doses of 500-1500 mg/day (Vitols, 2003) accompanied in one case with skin rash. Sufficient quantities of the N-acetyl-p-benzoquinone (NAPQI) metabolite have to be generated to first deplete glutathione prior to reaction with macromolecules, triggering oxidative stress etc (James et al, 2003). Toxic doses of acetaminophen also saturate the glucuronidation and sulfation pathways of drug metabolism. The proportion of NAPQI formed, as judged by the excretion of glutathione-derived conjugates (an underestimate) approximates to 8% at doses of 0.5 and 3.0 gm (Slattery et al, 1987). Under the MIST proposal this is a minor or trace metabolite. The large mass of NAPQI formed at toxic doses is not referred to often, however simple calculation suggests it represents over 1 gm total body burden per day in a human overdose situation . The significance of this expressed in this way is striking. Even in the very rare idiosyncratic occasion, around 50 mg of NAPQI was formed per day. Similar observations can be made for felbamate, another well studied drug causing

DMD Fast Forward. Published on June 28, 2005 as DOI: 10.1124/dmd.105.005041
This article has not been copyedited and formatted. The final version may differ from this version.

type B toxicity. Felbamate forms 2-phenylpropenal, a reactive Michael acceptor. This metabolite is implicated in the aplastic anemia and hepatotoxicity observed in some patients receiving felbamate. Although again recognized as a minor metabolite (Dieckhaus, et al, 2000), as judged by its excretion as a glutathione–derived conjugate (6.3% of the dose), the material is of major significance (150 mg/day) when the high dose of felbamate (2.4 g/day) is taken into account. Clearly the definition of minor (based on proportion or %) does not in any way capture the biological significance of the large amounts of an obviously reactive material being formed.

<u>MIST and Toxicity from Non-Selective Effects (B C and D):</u> The importance of dose or mass is clearly important and should help guide what metabolites are important. For these types of toxicities, the excreted metabolites normally provide the most information as in the examples above. The reactive metabolites or their de-activated forms seldom can be detected in the circulation. It then seems appropriate that mass, rather than % should guide identification and characterization. For a drug dosed to humans at less than 50 mg, excreted metabolites are of little concern except to guide and help understand the clearance pathways of active circulating metabolites and thereby be able to predict inter-subject variation. We propose the value of 50 mg based off of knowledge that very few drugs dosed below this level are associated with these types of toxicities and also based on the data cited above for acetaminophen and the estimate of the amount of formation of its reactive metabolite. At higher doses the excreted metabolites may suggest reactive metabolite generation and greater diligence should be performed. It would seem prudent that this was based on mass and not proportion, so that the higher the dose of drug, the more characterized the metabolites are. This guidance

would support identifying all metabolites with an approximate mass of 10 mg or greater in total excretion (urine and feces) of humans. This corresponds to 10% at 100 mg but would be 1% at a dose of 1 g.

The figure of 10 mg represents a conservative amount based on general consideration of metabolic pathways and excreted metabolites which may be of significance in the safety of drugs, and a pragmatic reconsideration of proportion based quantitation (eg 10% of excreted dose as cut-off for identification of metabolites. An example to clarify these considerations is tolcapone in which the nitrocatechol is reduced to a number of amine metabolites which can be oxidatively bioactivated to reactive species. The total mass produced by this pathway can be as much as 40 mg at a dose of 200 mg if the glucuronide metabolites are included, but if the amine metabolite itself is considered the amount falls to 20 mg. This pathway is a possible distinguishing feature from the better tolerated entacapone (Smith et al, 2003; Jorga et al, 1999).

An Abundance-Based Rather Than Percentage-Based MIST Strategy

Circulating Metabolites: Ideally during the pre-clinical phase, potential metabolites produced in human in vitro systems that are not substantially different from parent drug with regard to structure and physicochemical properties should be synthesized and screened for pharmacological activity. When activity approaches that of 25% parent based on *in vitro* pharmacological assays, specific bioanalytical assays should be developed for use in determination of plasma protein binding and circulating concentrations in toxicological and human testing (Table 5). If the contribution of the metabolite to pharmacological activity in human is less than 25% of parent, considering receptor affinity, free fraction, and target tissue penetrability as well as the potential for interpatient variability (e.g. genetic polymorphism in metabolism, disease, age, etc), then the need for such assays is attenuated. Additionally, metabolites identified in human radiolabelled ADME studies that are present at concentrations of 1 μM or higher (preferably based on C_{avg}) should also be considered for the potential to have pharmacological effects different from the target pharmacological receptor. Authentic standards should be synthesized, free fraction should be determined, and if free concentrations are ≥ 1 μM, then an assessment of the potential to bind to a broad array of receptors, as is routinely done for the parent drug in standard in vitro safety pharmacology studies should be undertaken. If active at an alternate receptor or enzyme, then the metabolite should be appropriately monitored in preclinical safety and clinical studies, such that an adequate risk assessment can be made for the metabolite. This should include measuring metabolite exposure parameters in all animal species used in safety assessments following multiple dose administration and in clinical studies following multiple dose administration (if applicable) to a wide enough variety of study subjects and/or patients to ensure that extremes of low and high metabolite exposures have been reasonably demonstrated. A flowchart illustrating this process is offered in Figure 2.

Excreted Metabolites: For a drug dosed to humans at less than 50 mg, excreted metabolites are likely of little concern except to guide and help understand the clearance pathways of the parent and thereby be able to predict inter-subject variation in exposure and potentially effect. This should be the guidance and the rationale for the work regarding excretory metabolites. At higher doses, when the excreted metabolites may suggest reactive metabolite generation then greater diligence should be performed. It would seem prudent that this was based on absolute mass and not proportion of drug-related material, so that the higher the dose of drug, the more characterized the metabolites will need to be. A guidance would support identifying all metabolites with an approximate mass of 10 mg/day total body burden or greater (Table 5). This corresponds to metabolites present at 10% of dose or more at a 100 mg dose but only ≥1% at a dose of 1 g. A decision tree for excretory metabolites is depicted in Figure 3.

Conclusions

These arguments favor replacing the simple percentage-based criteria described in the MIST paper with an approach that considers abundance, structure, mass of amount formed and toxic effect.

Acknowledgement: The authors extend their gratitude for thoughtful discussions and input on this topic with Don Walker and Dr. Fatima Matos.

References:

Anthes JC, Gilchrest H, Richard C, Eckel S, Hesk D, West RE, Williams SM, Greenfeder S, Billah M, Kreutner W, Egan RW. (2002) Biochemical characterization of desloratadine, a potent antagonist of the human histamine H1 receptor. Eur. J. Pharmacol. 449: 229-237.

Baillie TA, Cayen MN, Fouda H, Gerson RJ, Green JD, Grossman SJ, Klunk LJ, LeBlanc B, Perkins DG, Shipley LA. (2002) Drug metabolites in safety testing. Toxicol. Appl. Pharmacol. 182: 188-196.

Bakke OM, Wardell WM, Lasagna L. (1984) Drug discontinuations in the United Kingdom and the United States, 1964 to 1983: issues of safety. Clin. Pharmacol Ther. 35: 559-567

Bakke OM, Manocchia M, de Abajo F, Kaitin KI, Lasagna L. (1995) Drug safety discontinuations in the United Kingdom, the United States and Spain from 1974 through 1993: a regulatory perspective. Clin. Pharmacol Ther. 58: 108-17

Brasseur R, Vandenbosch C, Van den Bossche H, Ruysschaert JM (1983) Mode of insertion of miconazole, ketoconazole and deacylated ketoconazole in lipid layers. A conformational analysis. Biochem. Pharmacol. 32: 2175-2180.

Cahill SA and Gross GJ. (2004) Propafenone and its metabolites preferentially inhibit 1Kr in rabbit ventricular myocytes. Journal of Pharmcology and Experimental Therapeutics 308: 59-65.

Carmeliet E (1998) Effects of cetirizine on the delayed K^+ currents in cardiac cells: comparison with terfenadine Br. J. Pharmacol. 124: 663-668.

Chiu PJS, Marcoe KF, Bounds SE, Lin CH, Feng JJ, Lin A, Cheng FC, Crumb WJ, Mitchell R. (2004) Validation of a [3H]astemizole binding assay in HEK293 cells expressing HERG K+ channels. J. Pharmacol. Sci. 95: 311-319.

Cohen ML, Bloomquist W, Schmid CR, Robertson DW, Wong DT. (1993) Zatosetron, a selective 5-HT3 receptor antagonist: pharmacological activities of human and animal metabolites. Drug Development Research 29: 209-215.

Danielsson BR, Skold, AC, Azarbayjani F. (2001) Class III antiarrhythmics and phenytoin: teratogenicity due to embryonic cardiac dysrhythmia and reoxygenation damage. Curr. Pharm. Design 7: 787-802.

Dieckhaus CM, Miller TA, Sofia RD, Macdonald TL. (2000) A mechanistic approach to understanding species differences in felbamate bioactivation: relevance to drug-induced idiosyncratic reactions. Drug Metab. Dispos. (2000), 28: 814-822.

Finnell RH, Buehler BA, Kerr BM, Ager PL, Levy RH (1992) Clinical and experimental studies linking oxidative metabolism to phenytoin-induced teratogenesis. Neurology 42: (Suppl 5), 25-31.

Food and Drug Administration (1997) Guidance for Industry Q3B Impurities in New Drug Products http://www.fda.gov/cder/guidance/1317fnl.htm#ATTACHMENT%20II (accessed March 28, 2005)

Harron DW, Goa KL, Langtry HD (1991) Bopindolol. A review of its pharmacodynamic and pharmacokinetic properties and therapeutic efficacy. Drugs 41: 130-149.

Heptner W, Hornke I, Cavagna F, Fehlhaber HW, RuppW, Neubauer HP. (1978) Metabolism of nomifensine in man and animal species. Arz. Forsch. 28: 58-64.

Harrower ABD. (1996) Pharmacokinetics of oral antihyperglycermic agents in patients with renal insufficiency. Clinical Pharmacokintics 31: 111-119.

James LP, Mayeux PR, Hinson JA. (2003) Acetaminophen-induced hepatotoxicity. Drug Metab. Dispos. 31: 1499-1506.

Jorga K, Fotteler B, Heizmann P, Gasser R. (1999) Metabolism and excretion of tolcapone, a novel inhibitor of catechol-o-methyltransferase. Br. J. Clin. Pharmacol. 48:513-520.

Kerbusch T, Milligan PA, Karlsson MO. (2004) Assessment of the relative invivo potency of the hydroxylated metabolite of darifenacin in pharmacokinetic – pharmacodynamic data. British Journal of Clinical Pharmacology 57: 176-180.

Lasser KE, Allen PD, Woolhander SJ, Himmelstein DU, wolfe SM, Bor DH. (2002). Timing of new black box warnings and withdrawals for prescription medications. JAMA. 287: 2215-2220

Kraemer C, Sunkomat J, Witte J, Luchtefeld M, Walden M, Schmidt B, Boeger RH, Forssmann WG, Drexler H, Schieffer, B. (2002) Angiotensin II receptor-independent antiinflammatory and antiaggregatory properties of losartan. Role of the active metabolite EXP3179. Circ. Res. 90: 770-776.

Martz F, Failinger III C, Blake DA. (1977) Phenytoin teratogenesis: correlation between embryopathic effect and covalent binding of putative arene oxide metabolite in gestational tissue. J. Pharmacol. Exp. Ther. 203: 231-239.

Mbai M, Rajamani, S. January CT. (2002) The anti-malarial drug halofantrine and its metabolite N-desbutylhalofantrine block HERG potassium channels. Cardiovascular Res. 55: 799-805.

Nilvebrant, L. (2002) Tolterodine and its active 5-hydroxylmethyl metabolite: pure muscarinic receptor antagonists. Pharmacology and Toxicology 90: 260-267.

Nobile M and Vercellino P. (1997) Inhibition of delayed rectifier K+ channels by phenytoin in rat neuroblastoma cells. Br. J. Pharmacol 120: 647-652.

Oatis JE, Russell MP, Knapp DR, Walle T. (1981) Ring hydroxylated propranolol: synthesis and beta-receptor antagonist and vasodilating activites of the seven isomers. Journal of Medicinal Chemistry 24: 309-314.

Owens MJ. (1997) Neurotransmitter receptor and transporter binding profile of antidepressants and their metabolites. Journal of Pharmacology and Experimental Therapeutics 283: 1305-1322.

Paakkari I. (2002) Cardiotoxicity of new antihistamines and cisapride. Toxicol. Lett. 127: 279-284.

Park BK, Kitteringham NR, Powell H, Pirmohamed M. (2000) Advances in molecular toxicology – towards understanding idiosyncratic drug toxicity. Toxicology 153:39-60.

Raymond G V, Buehler B A, Finnell R H, Holmes L B. (1995) Anticonvulsant teratogenesis: 3. Possible metabolic basis. Teratology 51: 55-56.

Rudorfer MV and Potter WZ. (1997) The role of metabolites of antidepressants in the treatment of depression. CNS Drugs 7: 273-312.

Salvati P, Maj R, Caccia C, Cervini M A, Fornaretto M G, Lamberti E, Pevarello P, Skeen G A, White H S, Wolf H H, Faravelli L, Mazzanti M, Mancinelli E, Varasi M, Fariello R G. (1999) Biochemical and electrophysiological studies on the mechanism of action of PNU-151774E, a novel antiepileptic compound. J. Pharmacol. Exp. Ther. 288: 1151-1159.

Sanchez C and Hyttel J. (1999) Comparison of the effects of antidepressants and their metabolites on reuptake of biogenic amines and receptor binding. Cellular and Molecular Neurobiology 19: 467-489.

Slattery, JT, Wilson JM, Kalhorn TF, Nelson SD. (1987) Dose-dependent pharmacokinetics of acetaminophen: evidence of glutathione depletion in humans. Clin. Pharmacol.Ther. 41: 413-418.

Smith KS, Smith PL, Heady, TN, Trugman JM, Harman WD, MacDonald TL. 2003 Invitro metabolism of tolcapone to reactive intermediates: relevance to tolcapone liver toxicity. Chem. Res. Toxicol. 16:123-128

Svartengren J. (1997) Interaction of the novel antipsychotic drug amperozide and its metabolite FG5620 with central nervous system receptors and monamine uptake sites: relation to behavioral and clinical effects. Biological Psychiatry 42: 247-259.

Taglialatela M, Pannaccione A, Castaldo P, Giorgio G, Zhou Z, January C T, Genovese A, Marone G, Annunziato L. (1998) Molecular basis for the lack of HERG K+ channel block-related cardiotoxicity by the H1 receptor blocker cetirizine compared with other second-generation antihistamines. Molec. Pharmacol. 54: 113-121.

Vitols, S. (2003) Paracetamol hepatotoxicity at therapeutic doses. J. Inter. Med. 253: 95-98.

Watanabe K, Kawamura K, Yamashita S, Fujita S, Ishihara T, Yabuuchi Y. (1989) Effects on β-adrenoceptor of 8-hydroxycarteolol, main metabolite of β-adrenoceptor blocking agent carteolol. Oyo Yakuri 38: 271-277.

Whitehouse L W, Menzies A, Mueller R, Pontefract R. (1994) Ketoconazole-induced hepatic phospholipidosis in the mouse and its association with de-N-acetyl ketoconazole. Toxicology 94: 81-95.

Williams M, Deecher DC, Sullivan JP (1995) Drug Receptors. In: Burger's Medicinal Chemistry and Drug Discovery, 5th Edition, Volume 1 (ed. Wolff ME) John Wiley & Sons, p353.

Yamada Y, Ohno Y, Nakashima Y, Fukuda M, Takayanagi R, Sato H, Tsuchiya F, Sawada Y, Iga, T. (2002) Prediction and assessment of extrapyramidal side effects induced by risperidone based on dopamine D2 receptor occupancy. Synapse 46: 32-37.

Figure Legend

Figure 1 Hypothetical example illustrating the ambiguity of the term major metabolite. The acid (assuming no alcohol product) and the amine must be formed in equal amounts and yet depending on which criteria is used either can be termed minor or major.

Figure 2. Decision tree concerning metabolite monitoring for human circulating metabolites.

Figure 3. Decision tree concerning metabolite monitoring for human excretory metabolites.

Table 1 - Examples of Active Metabolites Formed by Hydroxylation

Drug	**Metabolite**	**In Vitro Activity Ratio with Parent**[a]	**Reference**
Acetohexamide	hydroxyacetohexamide	2.5	Harrower, et al, 1996
Carteolol	8-hydroxycarteolol	1.0	Watanabe, et al, 1989
Darifenacin	hydroxydarifenacin	0.1	Kerbush, et al, 2004
Propranolol	4-hydroxypropranolol	0.8	Oatis, et al, 1981
Proprafenone	5-hydroxypropafenone	0.25	Cahill and Goss, 2004
Risperidone	9-hydroxyrisperidone	1.0	Yamada, et al, 2002
Tolazamide	hydroxytolazamide	0.4	Harrower, et al, 1996
Tolbutamide	5-hydroxytolbutamide	0.25	Nilvebrant, 2002
Zatosetron	3-hydroxyzatosetron	0.1	Cohen, et al, 1993

[a] This value is calculated as in vitro potency (eg. K_i) parent drug/in vitro potency metabolite for the target receptor

DMD Fast Forward. Published on June 28, 2005 as DOI: 10.1124/dmd.105.005041
This article has not been copyedited and formatted. The final version may differ from this version.

DMD Fast Forward. Published on June 28, 2005 as DOI: 10.1124/dmd.105.005041
This article has not been copyedited and formatted. The final version may differ from this version.

Table 2 - Ratio of Pharmacological Affinity/Activity for Drug and Metabolite Against Monoamine Transporters. Compiled from from Sanchez and Hyttel, 1999; Owens, 1997 and Svartengren, 1997.

		Ratio of Activity of Metabolite to Parent Drug[a]		
Drug	**Metabolite**	**5-HT**	**NA**	**DA**
Amitryptiline	Nortryptiline	0.07	7	inactive
Dothiepin	Northiaden	0.4	2.8	inactive
Dothiepin	Sulfoxide	inactive	inactive	inactive
Imipramine	Desipramine	0.06	31	--
Imipramine	2-hydroxyimapramine	1	1	--
Clomipramine	Norcloipramine	0.01	15	--
Fluoxetine	Norfluoxetine	1.8	0.64	inactive
Sibutramine	Demethylsibutramine	100	7	--
Sibitramine	Di-demethylsubatramine	100	6	--
Sertraline	Demethylsertraline	inactive	inactive	inactive
Citaprolam	Demethylcitaprolam	0.12	inactive	inactive
Amperozide	FG5620	inactive	--	--

[a] This value is calculated as in vitro potency (eg. K_i) parent drug/in vitro potency metabolite for the target receptor. The entry “inactive” means that the metabolite lacks appreciable affinity for the receptor.

DMD Fast Forward. Published on June 28, 2005 as DOI: 10.1124/dmd.105.005041
This article has not been copyedited and formatted. The final version may differ from this version.

Table 3- A List of Pharmacological Receptors at Which Human Circulating Metabolites Could Be Tested. If a parent drug possesses activity at one of the receptors/enzymes listed below, then human circulating metabolites that bear close structural and physicochemical similarity (e.g. demethylation, hydroxylation, deasturation, etc) should be tested for potency at the same receptor(s). Also, metabolites present in human circulation at a free concentration of ≥ 1 μM should be tested across a broad range of receptors and enzymes. The list below shows the general breadth of such screening.

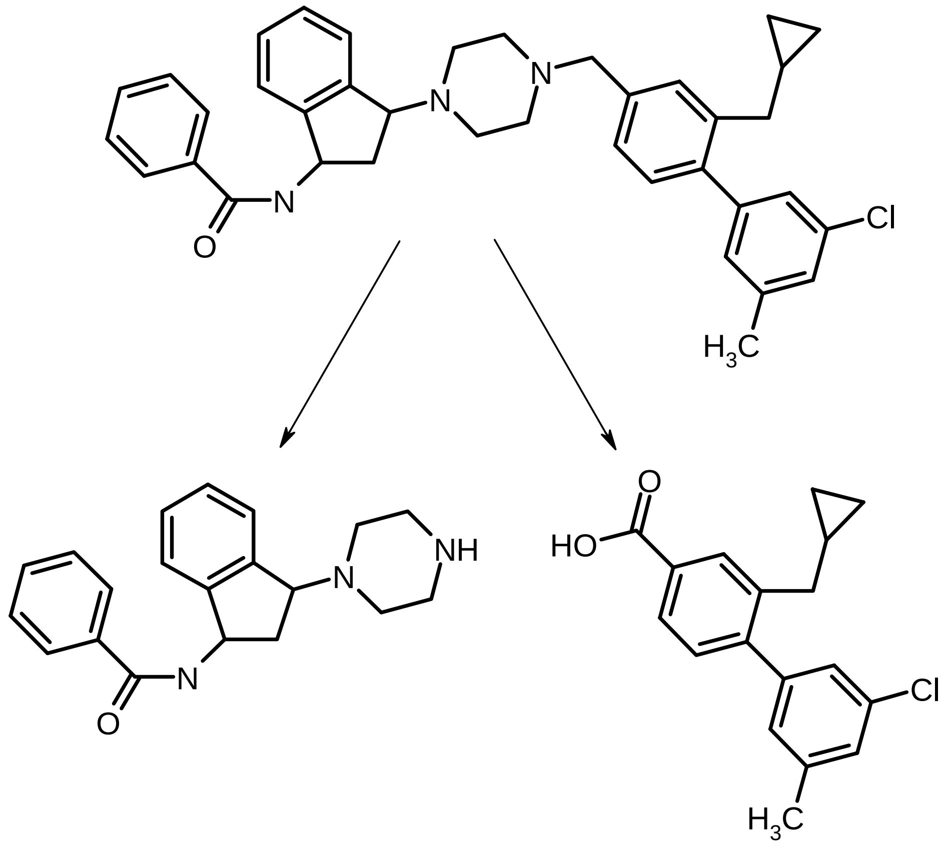
Acetylcholinesterase	Dopamine D_1	5-HT_6
Adenosine A_1	Dopamine D_{2s}	5-HT_7
Adenosine A_{2A}	Dopamine D_3	IK_r (hERG)
Adenosine A_{2B}	Dopamine D_4	K^+_{ATP} channel
Adenosine A_3	Dopamine Transporter	K^+_V channel
α_{1A} Adrenergic	Endopeptidase	SK^+_{Ca} channel
α_{2A} Adrenergic	$Endothelin_A$	MAO-A
α_{2B} Adrenergic	$Endothelin_B$	MAO-B
α_{2C} Adrenergic	GABA Transporter	Muscarinic M_1
β_1 Adrenergic	Glutamate (AMPA)	Muscarinic M_2
β_2 Adrenergic	Glutamate (Kainate)	Muscarinic M_3
β_3 Adrenergic	Glutamate (NMDA)	Na^+ channel
Angiotensin-II (AT_1)	Glycine (strychnine sensitive)	Na^+/K^+ ATPase
Angiotensin-II (AT_2)	Glycine (strychnine insensitive)	Nicotinic (neuronal)
Angiotensin Converting Enzyme	Histamine H1	Nicotinic (muscular)
Benzodiazepine	Histamine H2	Norepeinephrine Transporter
Bombesin Subtype 3	Histamine H3	δ2-Opioid
Bradykinin B2	5-HT_{1A}	κ-Opioid
Ca^{2+} channel (Ca_V1) (L)	5-HT_{1B}	μ-Opioid
Ca^{2+} channel ($Ca_V2.2$) (N)	5-HT_{1D}	Serotonin Transporter
Ca^{2+} channel (RY3)	5-HT_{2A}	Somatostatin-4
Carbonic anhydrase II	5-HT_{2B}	Somatostatin-5
Catechol O-Methyl Transferase	5-HT_{2C}	Urotensin-1
Choline Transporter	5-HT_3	Vasopressin V_{1a}
Cl^- channel	5-$HT_{4(e)}$	Vasopressin V_{1b}

Table 4 - Drugs Withdrawn since 1960 Due to Type B hepatotoxicity and their Clinical Doses (compiled from Bakke, et al., 1984; Bakke , et al., 1995, Lasser ,et al. 2002)

Drug	Daily Clinical Dose (mg)
Isaxonine	1500
Fenclofenac	1200
Nitrefazole	1200
Ebrotidine	800
Piprofen	800
Benoxaprofen	600
Chlormezanon	600
Flipexide	600
Ibufenac	600
Suloctidyl	600
Bendazac	500
Moxisylyte	480
Clometacin	450
Tienilic acid	400
Troglitazone	400
Tolrestat	400
Fenclozic acid	300
Perhexiline	300
Tolcapone	300
Zimeldine	300
Cyclofenil	200
Dilevalol	200
Trovafloxicin	200
Exifone	180
Pemoline	150
Nomifensine	125
Nialamide	100
Mebanazine	30

Table 5. A Summary of Recommended Actions for Metabolites in Safety Testing

Matrix	Structure of Metabolite	Cutoff	Action
Circulation	Minor Change from Parent Drug (e.g. demethylation, deethylation, hydroxylation, desaturation)	In vitro affinity ≥25% of that of the parent drug	Determine free fraction; if free concentration can contribute 25% of target receptor occupancy, then routinely measure in safety and clinical studies; understand clearance mechanism of the metabolite; understand target organ penetrability
Circulation	Major Change from Parent Drug (e.g. conjugation, cleavage into two major portions, N-deamination)	Total concentrations exceed 1 µM	Determine free fraction, if free concentration is ≥ 1 uM then test for broad ligand activities; routinely measure in safety and clinical studies if active at a secondary receptor
Excreta	Any structure	>10 mg total body burden per day	Assess activity of the metabolite, determine if it could be downstream of a reactive metabolite; ensure that species used in safety assessments generate the metabolite



High volume, moderate ppb-
low circulating concentrations,
moderate unbound concentrations
penetrates cells

Low volume, high ppb-
high circulating total concentrations,
low unbound concentrations
little penetration of cells

Figure 1

DMD Fast Forward. Published on June 28, 2005 as DOI: 10.1124/dmd.105.005041

Proposed MIST Algorithm for Human Circulating Metabolites

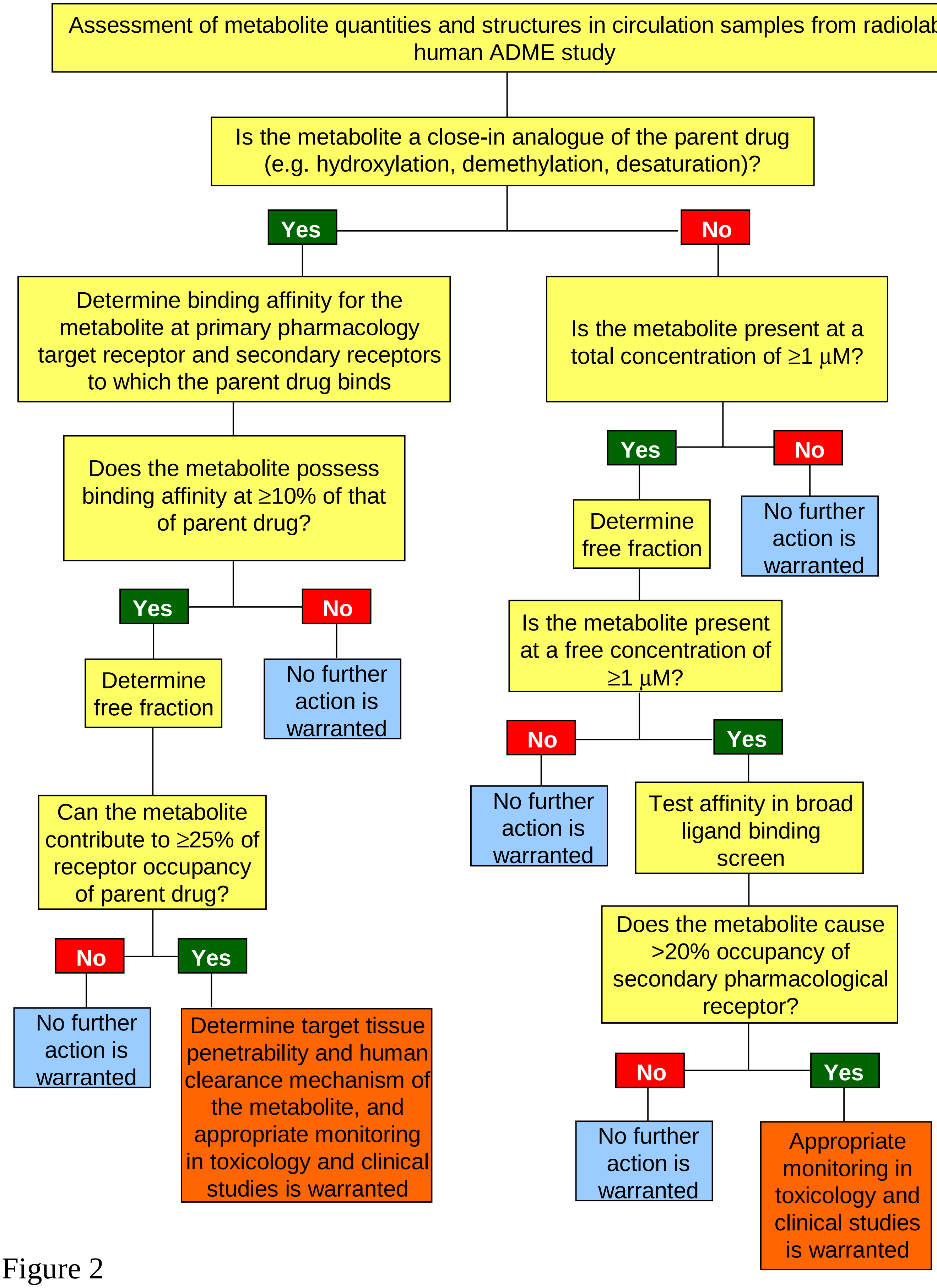


Figure 2

DMD Fast Forward. Published on June 28, 2005 as DOI: 10.1124/dmd.105.005041

Proposed MIST Algorithm for Human Excretory Metabolites

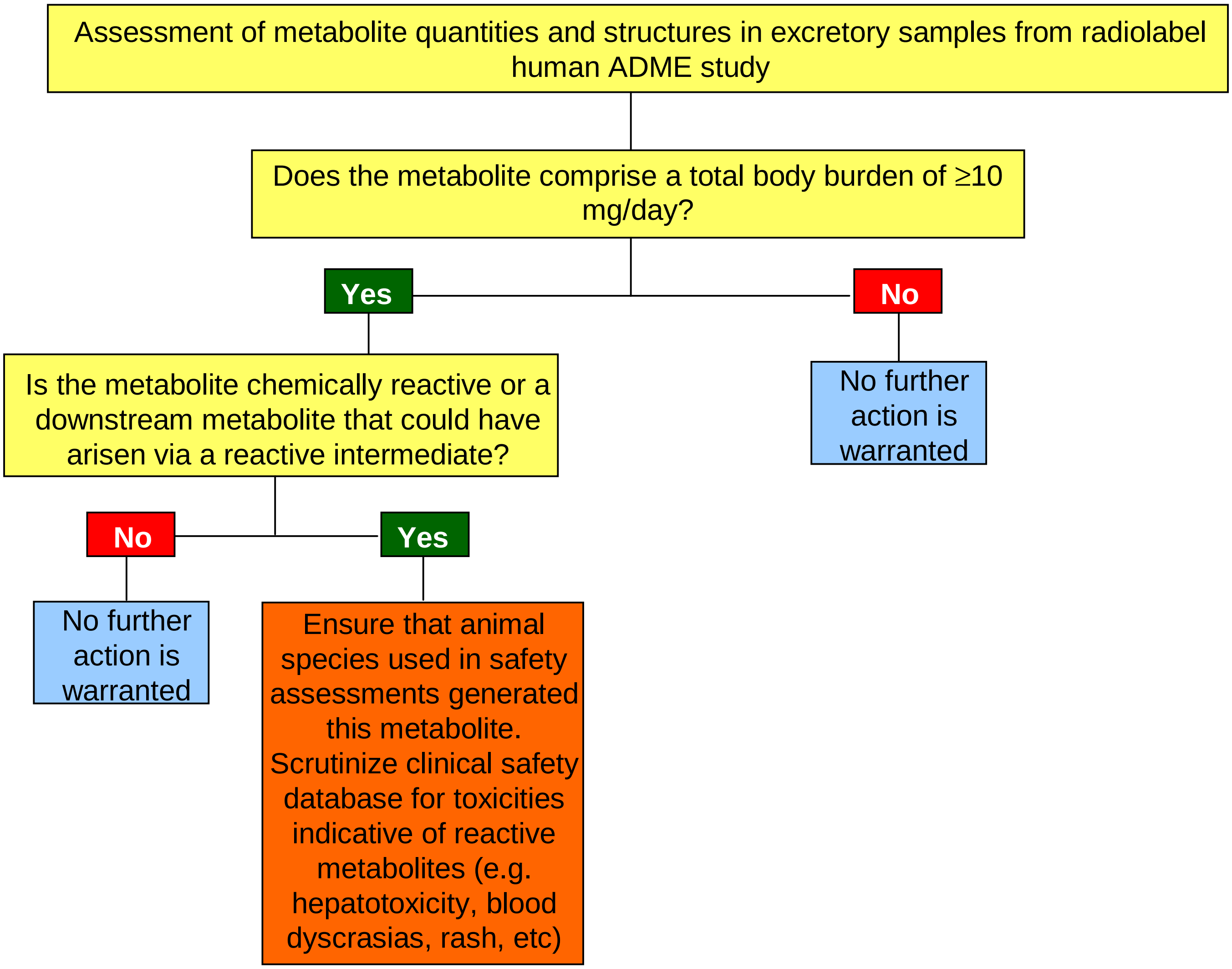


Figure 3