6 Safety Testing of Drug Metabolites: A Practical Approach for the Implementation of the MIST Guidance in PKDM

RAJU SUBRAMANIAN and J. GREG SLATTER
Pharmacokinetics and Drug Metabolism, Amgen Inc, Seattle, WA, USA

It should be emphasized that there is no straightforward or one standardized approach to the evaluation of the safety of drug metabolites. A case-by-case approach should be exercised when the information in the guidance does not apply.

–Aisar Atrakchi, *Chem. Res. Toxicol.*, 2009, 22, 1217–1220

6.1 INTRODUCTION

The Safety Testing of Drug Metabolites Guidance, also known as the *Metabolites in Safety Testing* or *MIST Guidance*, was issued by the FDA Center for Drug Evaluation and Research (CDER) in February 2008. This new guidance defined thresholds for

Encyclopedia of Drug Metabolism and Interactions, 6-Volume Set, First Edition.
Edited by Alexander V. Lyubimov.

metabolite exposure in humans and preclinical species that would assure regulators that the preclinical species employed to define the safety of a drug candidate were in fact exposed adequately to all quantitatively significant human metabolites [1]. The US guidance was followed in June 2009 with the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) M3(2) guidance, which differed in important ways with respect to metabolite exposure thresholds, but sought to achieve the same result. Both guidances acknowledged the complexity of the issue and advocated for "case-by-case" strategies that are developed with Agency consultation [2].

Since July 2009, the pharmaceutical industry focus has shifted from the thresholds and logic of MIST, to practical implementation of bioanalytical aspects of plasma metabolite analysis. For example, a "holistic" strategy for practical implementation of MIST compliance was described [3], bioanalysts have started to address best practices [4], and reviews on the evolution of scientific thinking about MIST are now appearing [5–7]. Collectively, these efforts recently culminated in a special issue of *Bioanalysis* in July 2010 [8]. The number of MIST-related articles has grown from 17 in July of 2009 [7], to approximately 30 in Sept of 2010. Therefore, it is timely to look at what drug metabolism practitioners are likely to do to address MIST, against a plethora of possible approaches that mix some old and standard activities, such as *in vitro* cross species comparisons, with new approaches focused on plasma metabolite analysis.

This chapter outlines the elements of the roll out of a strategy to address the MIST guidances at Amgen. Our objectives were twofold: first, to highlight a strategy for implementing a MIST plan in Translational Sciences [an organization within research and development (R&D) divisions of some pharma and biotechnology companies that oversees activities approximately from the time a candidate is selected for advancement to first-in-human (FIH) through end of phase 2] and second, to illustrate the relative advantages of plasma pooling strategies and cross species semi-quantitative metabolite comparison by liquid chromatography–high resolution mass spectrometry (LC-HR-MS).

6.2 SCOPE OF THE CHAPTER

The history and pharmacokinetics and drug metabolism (PKDM) issues in the pharmaceutical industry's implementation of the MIST guidance were reviewed up to July 2009 by Slatter and several others [4,6,7]. This chapter covers practical implementation and growing experience with the FDA and ICH guidances that occurred across the industry from the summer of 2009 to the end of 2010. Our experience with the development and implementation of a Translational-Sciences-wide MIST strategy will be illustrated with examples of a practical analytical strategy.

6.3 IMPACT OF MIST GUIDANCE ON INDUSTRIAL ADME RESEARCH

The 2008 CDER guidance flowchart is reproduced in Fig. 6.1 with an overlay that shows the numerous organizational codependencies that must be addressed in a MIST plan rollout [7]. The key effect of the MIST guidance was to shift most of the burden of

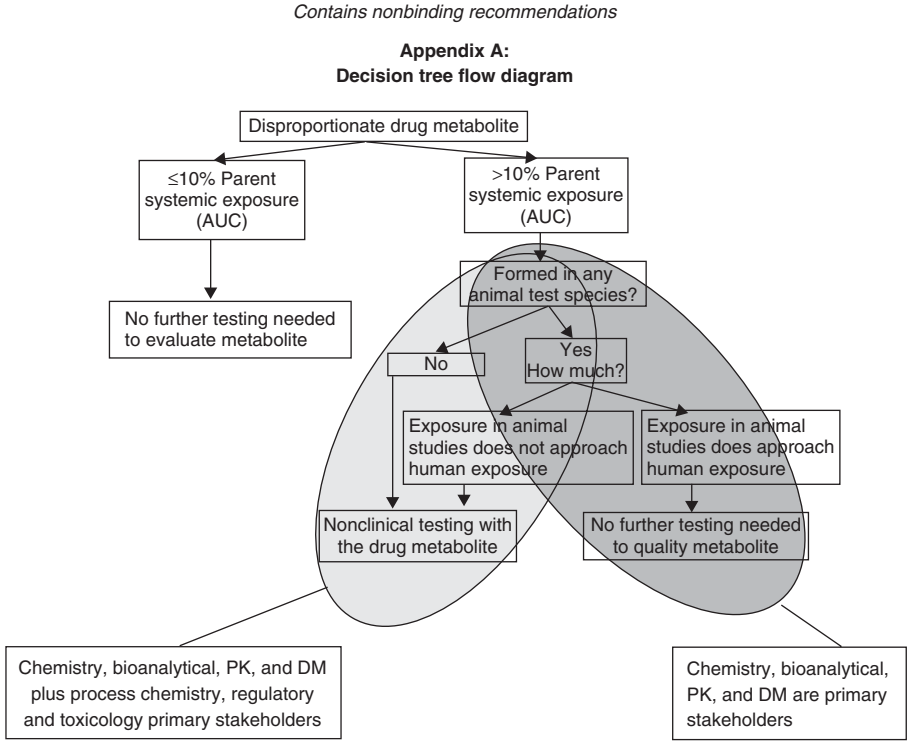

Figure 6.1 Adapted from the MIST guidance decision tree [1], modified with workload codependencies related to metabolite and internal standard synthesis, bioanalysis, regulatory interactions, and safety studies on a disproportionate metabolite. *Source:* Reprinted with permission from Ref. 7.

compliance to absorption, distribution, metabolism, and excretion (ADME) practitioners. It was now PKDM's job to prove that any metabolite characterized would be "not disproportionate." In the less-than-likely event that a metabolite(s) was disproportionate, this had to be known early enough to alert drug safety and get metabolite synthesis, qualification, activity, and a safety testing strategy completed by the end of phase 2.

6.4 GUIDANCE HARMONIZATION BETWEEN THE FDA AND ICH

One key aspect of MIST compliance is the clarification in the past year of the quantitative threshold for metabolite scrutiny. The CDER threshold for a disproportionate metabolite was set in the guidance at 10% of parent drug area under the plasma concentration–time curve (AUC) in human plasma at steady state. This threshold had the potential to increase the number of metabolites warranting scrutiny. ICH M3R2 defined the disproportionate metabolite threshold at 10% of plasma drug-related material in drugs that were dosed at 10 mg or higher [2]. The latter threshold was more aligned with conventional ADME practice, as it was anchored to absolute dose mass, while the former threshold created a sliding scale relative to parent drug in circulation. As the relative AUC of circulating parent drug decreased relative to metabolites, more metabolites were above the 10% of parent threshold [7,9,10]. Recently, FDA authors

Robison and Jacobs [11] clearly indicated, with the usual disclaimers, that when the CDER guidance threshold resulted in more metabolites warranting scrutiny than the ICH M3R2 guidance, threshold set by the latter could be used. This was scientifically rational and further enabled the industry to focus on metabolites that were quantitatively significant, relative to the mass of drug material administered.

The ICH S9 draft guidance "Draft Consensus Guideline Nonclinical Evaluation For Anticancer Pharmaceuticals S9" [12], like the CDER guidance, had an exemption for the evaluation of drug metabolites of cancer drugs. The ICH S9 guidance states "for disproportionate metabolites of anticancer agents, a separate general preclinical safety evaluation might not be warranted for patients with late stage or advanced cancer, as human safety of the metabolite would have been assessed in phase 1 clinical trials. If the parent compound is positive for embryofetal toxicity or genotoxicity then separate studies for the disproportionate metabolite might not be warranted. Unless there is a specific cause for concern, nonclinical testing of the metabolite is not warranted."

6.5 A MIST STRATEGY

In the time following the availability of the CDER guidance in February 2008, content experts across the industry monitored the implementation of the guidance. At the PhRMA Drug Metabolism Technical Group's (DMTG) Annual Roundtable with FDA in 2008, both the positive aspects and some of the shortcomings of the guidance were discussed. PKDM program representatives conducted individual candidate risk analysis and immediate course corrections for their current clinical programs. Over the ensuing year, as they monitored the rapid evolution of MIST guidance thinking in the public domain and tested key technologies internally, companies developed MIST plans by assessing and selecting best practices for metabolite analysis. The last step was soliciting input on the draft plan inside and outside the PKDM unit before a roll out to partner organizations (such as toxicology, clinical development, and regulatory).

6.5.1 Modifications of the ADME Development Plan

Several key departures from conventional ADME development were added to small molecule drug candidate development plans in order to enable the shift from an *in vitro* and excreta focused approach to a plasma focused plan. As shown in Fig. 6.2, pre-FIH activities, such as cross species metabolite profiling and metabolite identification in microsomes and hepatocytes, were performed to discern the second species that would have metabolism most similar to humans. Significant human metabolites in microsomes and differences in profiles between human microsomes and hepatocytes were used to predict the most likely human metabolites [13,14]. Overall, the predictive value of *in vitro* screening using human hepatic microsomes and hepatocytes is best for single oxidations and decreases for sequential metabolism and for phase II metabolites. *In vitro* metabolism data can be used to reassure stakeholders that PKDM's efforts will generate a prognosis of "not disproportionate" in the majority of cases and provide lead time in those rare cases where more data are needed. The most common complication at this stage comes as a result of lead enhancement to reduce metabolism, and some leads with good pharmacokinetics (PK) are slowly or negligibly metabolized *in vitro*. Nonetheless, with a pending focus on plasma analysis, consultation with chemists and

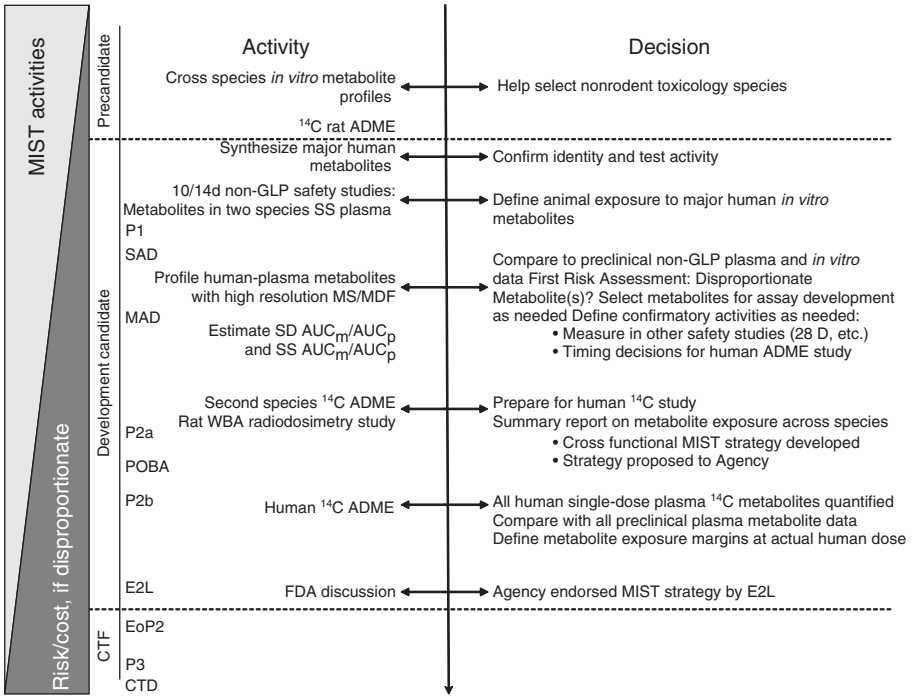

Figure 6.2 Strategy: build a data-driven proactive MIST development plan for each small molecule candidate. Timescale and order of operations are approximate; to be adjusted based on program needs. P1, phase 1; SAD, single ascending dose; MAD, multiple ascending dose; P2a, phase 2a; POBA, proof of biological activity; P2b, phase 2b; E2L, early to late stage development; EoP2, end of phase 2; P3, phase 3; CTD, common technical document; CTF, commit to file.

pharmacologists on the feasibility of synthesis and activity testing of the most probable human metabolites begins after candidate selection for advancement to FIH.

The most significant departure from excreta-based development is the need to obtain plasma for metabolite analysis and compare metabolite profiles semi-quantitatively across species. The two preclinical options are a separate dose escalation PK study in the rodent and nonrodent species, or alternatively, to obtain unused plasma from toxicokinetic (TK) analysis in non-GLP or good laboratory practice (GLP) studies. The latter option has only recently become feasible due to the recent availability of sensitive and selective high resolution mass spectrometry (HR-MS) techniques. As such, retasking of unused TK and clinical study plasma was discussed with Drug Safety and Clinical opinion leaders. Factors in implementation were study timelines, maintaining GLP compliance, protocol revisions, sample archiving, metabolite stability, reporting results, and verification of the new HR-MS techniques (Section 6.9). New protocol/informed consent language and plasma sample handling processes for unused FIH single ascending dose (SAD) and multiple ascending dose (MAD) plasma were developed.

Agreement on the need for the new plasma metabolite analyses developed quickly, since the consequence of doing metabolism "the old way" could include a clinical hold at the end of phase 2. It was recognized that a proactive approach gained time for metabolite synthesis, qualification, and safety testing, in the unlikely event that a

disproportionate metabolite was observed. Initial plasma metabolite profiling focused on identifying human steady-state metabolites in MAD samples that exceed 10 % of drug-related materials (DRMs). If no metabolites were above this quantitative threshold, subsequent comparisons to preclinical species would become unnecessary. Proximity of metabolites to the 10 % threshold would determine how prudent it would be to rely on similar observations in the definitive human ^{14}C ADME study. According to the guidance, the human ^{14}C ADME study is still the definitive proof for human metabolite exposure, albeit from a design that is typically single dose. We anticipate that HR-MS will supplant the human ^{14}C study as the definitive determination of circulating human metabolites because most researchers in the industry are moving toward HR-MS techniques to profile nonradioactive metabolites earlier in clinical development. These techniques were not as well established when the guidance was developed. As such, case-by-case arguments to regulatory authorities for the absence of disproportionate metabolites will probably be advanced before the completion of the human ^{14}C ADME study. Suitable protocol language to enable these analyses was developed proactively for clinical and preclinical safety protocol templates.

The observation in MAD samples of human plasma metabolites that exceed the 10 % of DRM threshold would trigger the analysis of unused TK samples from preclinical safety studies, or samples from a separate PK dose-ranging study, conducted to address this issue.

PKDM development team representatives are now expected to provide an ongoing MIST guidance risk assessment to their management, educate their development team, and refine their development plans to allow time to react after pivotal MIST data from the human MAD study become available. When there are no unique human metabolites, or the human metabolites greater than 10 % of DRM are unambiguously "not disproportionate," these data can be included in a dedicated metabolism report that integrates all the available metabolism data and makes a clear case that further metabolite monitoring or metabolite assessments are not needed. This final report can be submitted in the routine annual update to the common technical application (CTA). In the alternate scenario, a disproportionate metabolite triggers dialogue with drug safety, clinical, regulatory, and pharmaceutical sciences to address the issue in a case-by-case way. The actual *in vivo* testing of disproportionate metabolites is complicated, as the ADME of separately administered metabolites may not be equivalent to the ADME of the metabolite generated *in vivo* [15,16]. There are also concerns over the potential toxicological irrelevance of any study on a separately administered metabolite [17].

The main values of the phase 1 centric plasma analysis approach are the lead time garnered for dealing with either an unique or disproportionate metabolite(s) and the ability to defer the human ADME study to after clinical proof of concept, with minimal risk of a surprise.

6.6 NEW APPROACHES TO NONRADIOACTIVE METABOLITE QUANTITATION: FOCUS ON PLASMA SAMPLE POOLING, HIGH RESOLUTION MS, AND MASS DEFECT FILTER MASS SPECTROMETRY

6.6.1 Plasma Availability and Plasma Pooling Strategy

Preclinical TK and SAD/MAD PK plasma bioanalysis do not consume all the available sample. It is therefore possible to use unused plasma for exploratory metabolite

profiling and avoid dedicated preclinical PK studies or additional human subject cohorts. The plasma volumes that are typically unused from different PK and TK studies after routine parent drug are shown in Table 6.1. Plasma from no observed adverse effect level (NOAEL) doses in animals, or maximum feasible dose and the best estimate of the effective dose in human SAD/MAD studies (if this can be predicted by target coverage) are appropriate to show metabolite coverage across species.

A semi-quantitative estimate of all the drug-related components that contribute to the total DRM AUC is obtained from time-proportional pooling of left over plasma volumes from different time-points across the sample collection period. This method is referred to as the *Hamilton-pool* or the *AUC-pool* [18] and is well documented [19]. An example of the necessary pooled sample volumes for a hypothetical time course of sample collection is shown in Table 6.2. A plasma aliquot volume from each time-point is selected based on the interval between time-points and the pooled sample is created volumetrically or gravimetrically.

The AUC-pool approach has several advantages. The metabolite profile from an AUC-pool provides a valid semi-quantitative estimate of each metabolite's contribution to total drug-related exposure (AUC) or to a separately measured parent drug exposure, as required by the guidances. The approach requires only a single liquid chromatography–mass spectrometry (LC-MS) injection from a single pooled sample, rather than the separate analysis of individual points in the concentration versus time

TABLE 6.1 An Example of Unused Plasma Sample Volumes from PK and TK Analysis

Species and Study Type	*n*/Dose-group	# Time-points	Plasma Volume (μL)	Volume for Metabolite Profile (μL)/Animal
Rat PK	3	9–12	100–120	50
Dog/monkey PK	3	10–12	225	175
Rat 4-d TK	3	5	80	50
Rat 10-d to 6-mo TK	4–5	5–6	120–160	70
Dog/monkey 10-d to 6-mo TK	3–4	6	200–400	70
Human PK	>4	9–12	700–1000	500–900

The remaining volumes can be used for a relevant metabolite profiling, avoiding the expense, time, and additional animal usage involved in separate studies. Unused plasma can be pooled across time and/or across animals with no changes to current PK and TK sampling approaches. If dried blood spot analysis is required or becomes common, separate plasma samples from animals or subjects may be needed to enable metabolite analyses.

TABLE 6.2 The Time-Proportional Plasma Pool

Time (h)	0	1	2	4	8	24	Sum
Δt - pool (μL)	5	10	15	30	100	80	230

The "AUC-pool" measures metabolite contribution to total AUC. These principles are well established in the literature and enable the use of unused plasma from TK analysis to afford a reasonable estimate of exposure in one LC-HR-MS run. Pooling across animals/subjects further enables measurement of an "average" exposure to the full slate of plasma metabolites. The plasma volumes would be available from either a PK or TK study.

curve. The plasma volume requirements are well within the range of unused volumes from completed quantitative GLP analyses of parent drug (Table 6.1). The AUC-pool may be built for each individual animal or subject in the study or the plasma from different animals/subjects may be pooled to comprise a single average exposure estimate for all the individuals in the study. If plasma volumes are too limited or time-points are sparse, individual time-points could be analyzed or a separate study or sample cohort would be necessary.

6.6.2 High Resolution Mass Spectrometry (HR-MS) Methods

New HR-MS methods now provide a unique ability to discern all metabolites in biofluids with high resolution and sensitivity without dosing a radiolabeled drug. High resolution mass spectrometers with time-of-flight and Orbitrap [20] architectures have the analytical and data analysis capabilities to conduct HR-MS screening of plasma samples. These mass spectrometers allow an accurate determination of mass/charge ratio (m/z) of both the base mass spectrometry (MS) peak and its collision-induced dissociation (MS^n) fragments down to millimass or higher accuracy. After acquisition of LC-HR-MS data, techniques such as mass defect filtering, background subtraction, and isotope ratio filtering can discern both parent drug and drug-related metabolite peaks in the chromatogram.

The mass defect of a pseudomolecular ion is the difference between its high resolution m/z and its nominal m/z (e.g., if an analyte's high resolution m/z was 500.1234, its mass defect would be 0.1234 Da). Mass defects of a drug and its metabolites are similar, within predictable ranges, and importantly, are different from most endogenous interferences [21,22]. A suitable range above and below the mass defect is selected (e.g., $\pm$100 mmu) based on the parent drug accurate mass and logical biotransformations and ions are "mass defect filtered" to distinguish the drug and its metabolites from endogenous interference ions [23,24]. When control sample such as predose plasma is available, comparing its high resolution liquid chromatography–mass spectrometry (HR-LC-MS) profile with a dosed sample allows for every metabolite to be found based on the mass defect filter (MDF) alone without use of any biotransformation templates. Figure 6.3 illustrates the sensitivity and selectivity of the approach with plasma collected after a single dose of a [^{14}C]-labeled compound. The top and middle panels are an ultra performance liquid chromatography (UPLC) unfiltered total-ion chromatogram and corresponding MDF chromatogram, respectively. The bottom panel is the radiochromatogram obtained on a conventional high performance liquid chromatography (HPLC) with an in-line radiometric detector. The HR-MS-MDF profile recapitulates the radio-profile.

Background subtraction is another way to process the LC-HR-MS data. A difference chromatogram is obtained by subtracting the dosed plasma profile from a predose profile. Historically, this method has been sensitive to retention time shifts that can occur between the two LC runs. A new algorithm tolerant of retention time shift and that has additional noise filtering has been described recently [25]. Isotope ratio filtering can also be applied if the parent drug and metabolites have atoms with high abundance +2 isotope ions (e.g., Cl and S) [26].

Metabolite related information evolves throughout development and there is a sequential accumulation of both qualitative and quantitative data that eventually are integrated to discern any disproportionate metabolite. The plasma MDF data can be

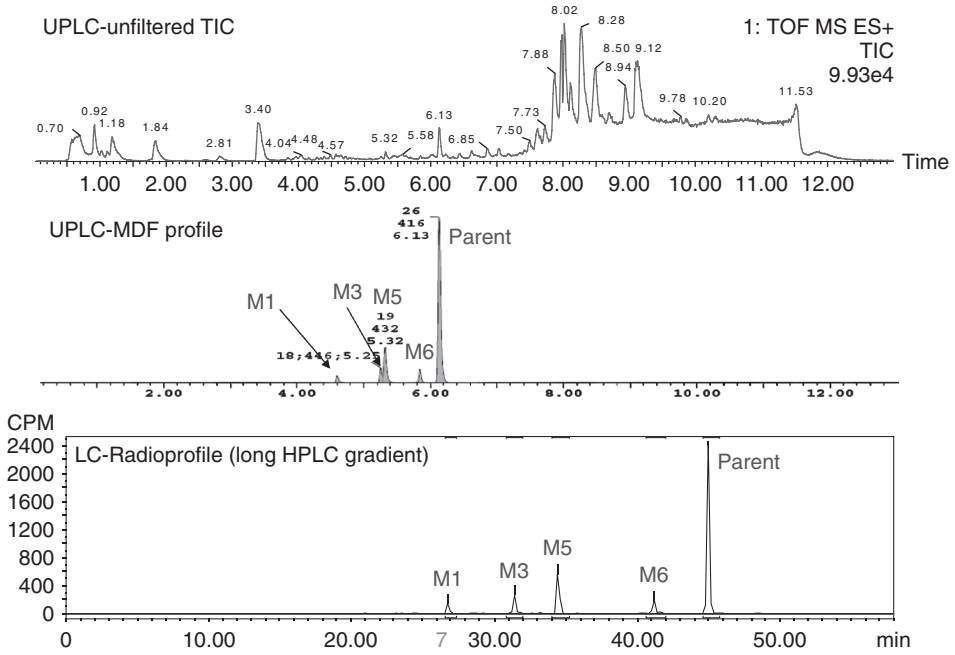

Figure 6.3 Metabolite observation by LC-HR-MS-MDF profiling. TIC, total ion chromatogram. (See color insert.)

compared to human and preclinical *in vitro* metabolite profiles and to the available preclinical *in vivo* ^{14}C ADME data.

Unfortunately, MDF metabolite profiles are not quantitative, and due to a number of factors, the MS response of the parent drug and metabolites can be different. These include electrospray ionization differences, ionization quenching by co-eluting endogenous compounds, or differences in solvent gradient composition at different elution times. When synthetic standards are available, the MDF profile peaks can be semi-quantitatively compared to metabolite mass by calculating response factors. MS response can be compared with response factors calculated from the diode-array detector (DAD) response across a wavelength range [19], with a radiolabeled metabolite peak area [27,28], or based on NMR quantitation of an isolated metabolite [29]. Each type of MS response factor calculation has its own limitations [30,31]. MS ionization methods employing lower flow rates appear to mitigate the ionization differences and new methods such as captive-spray ionization are being developed [31,32].

The HR-MS-MDF approach described here uses parent drug concentrations that were separately determined by validated LC-MS methods to benchmark the MDF response of each metabolite. Benchmarking to parent drug concentration enables semi-quantitative comparison of every human metabolite to comparably benchmarked preclinical plasma data. This approach to the MDF metabolite profile is illustrated in Fig. 6.4. The top panel shows a hypothetical HR-MS-MDF profile from an AUC-pool of plasma obtained from preclinical safety species at steady state. The bottom panel shows a human circulating metabolite profile obtained from an AUC-pool of plasma collected following either a single or a multiple oral dose. A qualitative examination

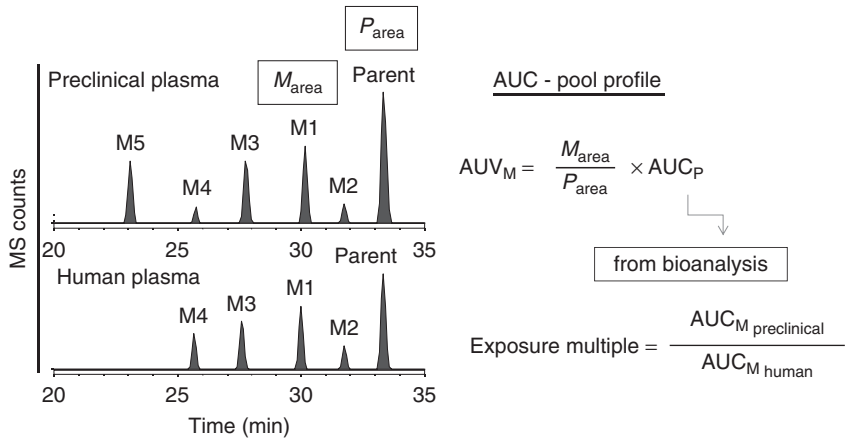

Figure 6.4 Estimation of circulating human metabolite coverage by obtaining HR-MS-MDF profiles of AUC-pooled plasma from human and safety species; and compute and compare the relative "AUC_M" from each profile.

reveals whether the metabolites observed in human were also found in preclinical safety species for the program. For a given metabolite, relative coverage can be estimated from the same data set without determining its actual concentration. Because the profile was obtained from an AUC-pool, the relative AUC of metabolite ("AUC_M") can be computed:

$$AUC_M = \frac{M_{area}}{P_{area}} \times AUC_P$$

$$\text{Exposure multiple} = \frac{AUC_{M_{preclinical}}}{AUC_{M_{human}}}$$

where M_{area} and P_{area} are the peak areas of the metabolite and parent from HR-MS-MDF profile and AUC_P is the parent AUC, a parameter estimated already available from prior analysis using a qualified or validated bioanalytical method. Exposure multiple is then computed for each metabolite using the equation shown above. Metabolite coverage is satisfied if exposure multiple is equal to or greater than unity. The principal advantage of this method is that there is no requirement for any metabolite reference standard (synthetic, radiolabeled, or isolated). Furthermore, any interspecies metabolite abundance comparisons are focused only on metabolites that are quantitatively above the 10 % of DRM threshold in humans.

An analogous approach of estimating exposure multiple by simply computing the ratio of metabolite area in preclinical versus human from their HR-MS profiles was published recently [33]. In contrast to the approach advocated here, these authors do not benchmark metabolite abundance to the parent AUC in their exposure multiple calculations. In another variation, Gao *et al*. [34] have proposed measurement of relative plasma exposures. After qualitative metabolite profiling of AUC-pooled plasma discerns useful metabolite multiple reaction monitoring (MRM) transitions, a ratio of each metabolite and parent drug to an internal standard is measured by MRM on a triple-quadrupole mass spectrometer. In both methods [34,33], the dosed plasma is

mixed with the undosed comparator plasma, for example, dosed human plasma would be mixed with equal volume of undosed preclinical safety species plasma (or vice versa) to account for any potential endogenous interference in peak intensity that may arise from the mass spectrometric analysis. The drawback of this approach is unknown sample stability because addition of the undosed plasma could potentially alter metabolite levels due to species differences in plasma borne enzyme activities (e.g., esterases and amidases).

For any human metabolite that is at or near the 10% of DRM threshold, the higher the AUC_M in the preclinical safety species relative to human, the greater the confidence that the preclinical safety species covered the human metabolite. If the animal to human AUC_M ratio is close to 1 or lower, a synthetic standard and qualified bioanalytical method for the metabolite in question will probably be applied to enable a decision of whether the metabolite is disproportionate. A decision tree has been published that determines when a more rigorous analytical determination of a potentially disproportionate metabolite is needed [34].

Best practices incorporating a tiered analytical validation approach to metabolite quantification has been proposed by the European Bioanalysis Forum [4] and others [30,31]. Semi-quantitative estimation of metabolites by the HR-MS-MDF methods described above represents a screening method that will discern obvious and borderline disproportionate metabolites, but these methods do not constitute either qualified or validated assays that would be needed for further investigation of borderline cases.

6.7 THE HUMAN ^{14}C ADME STUDY

According to the CDER guidance, the human ^{14}C ADME is still the definitive study for human metabolite exposure and the impact of the guidance on the timing of the human ADME study has been discussed by Anderson *et al*. [30]. However, the Guidance did not foresee the technological advance of HR-MS-based mass defect filtering, which enables the visualization and semi-quantitative measurement of a slate of metabolites with minimal interference from endogenous peaks. The case-by-case provisions in the guidance enable a data-based argument to maintain the status quo for timing the human ADME study after clinical proof of concept and to use HR-MS-MDF analysis of plasma to drive earlier decisions on potentially disproportionate metabolites. There are specific cost advantages here, given the unfortunately high probability of compound termination at or before clinical proof of concept. There is still value in the human ADME study for discerning the rate and routes of excretion of radioactive metabolites and the complete recovery of the DRM. The non-ambiguous, quantitative measurement of radioactive metabolites and parent drug in plasma after a single radioactive dose in half a dozen healthy volunteers or patients can be used to corroborate the risk-based decisions on human metabolites that were built on the human MAD plasma profiles and by cross species comparison. The advantage of using the human ADME study for MIST strategy validation rather than MIST strategy development is completely avoiding the inferred need for a steady-state answer from a type of study that under almost all circumstances should be run as a single dose.

6.8 ACCELERATOR MASS SPECTROMETRY (AMS)-BASED HUMAN ADME STUDIES

Most companies conduct the human ^{14}C ADME study using a conventional radiochemical (ca. 100 μCi) dose and apply a liquid scintillation counting (LSC)-based detection whenever sensitivity allows. In cases where circulating radiochemical concentrations are low, accelerator mass spectrometry (AMS) will be necessary to obtain the requisite quantitative plasma metabolite profile. Accelerator mass spectrometers are essentially ^{14}C atom counters that enable plasma drug or metabolite quantitation many orders of magnitude below the limit of detection of LSC-based measurement [35]. Plasma obtained from a conventional 100 μCi dose of a potent drug that affords very few radiocounts in plasma can be analyzed by AMS to obtain quantitative metabolite profiles. Alternatively, clinical human ADME studies can be conducted using radiochemical doses in the hundred nanocurie range, without a prerequisite rodent whole-body autoradiography-based radiodosimetry or the need for good manufacturing practice (GMP) radioformulation [36].

One drawback of AMS for quantitative metabolite profiling is that identification of isolated radioactive HPLC peaks is done by retention time only, as graphitization destroys the sample and affords only the quantitative data on ^{14}C atom concentration. Second, the analysis must be outsourced by most companies and AMS can be expensive when metabolism is extensive. The AUC-pool strategy can be used to decrease the cost of metabolite profiling by AMS. AMS vendors are all versed in the implications of the MIST guidance and have a unique ability to generate quantitative plasma metabolite profiles at plasma concentrations of radioactivity that are not measurable by any other method.

AMS vendors have proposed that AMS-based clinical designs could accelerate the human ADME study into phase 1 to address MIST. With the availability of the HR-MS techniques discussed above, this may be unnecessary except under unusual circumstances that might include exploratory investigational new drug (eIND) approaches for lead selection [37]. In the eIND approach, human metabolite profiles could be obtained by AMS from select superior leads being advanced to a full CTA. As such, AMS approaches within the eIND approach may enable earlier human plasma metabolite quantitation in select programs.

6.9 IMPLEMENTATION

After the final MIST guidance issued in February 2008, at Amgen, we monitored and contributed to the discussions occurring throughout the industry [7,38]. Some aspects of the guidance elicited extensive discussion in the 17 MIST-related papers published in 2009. Many of these publications focused on different analytical approaches to metabolite assessment and also made data-driven comparisons of our ability to predict the structure and relative importance of human metabolites in plasma (reviewed in Ref. 7). Some literature and company database meta-analyses on the probability that a metabolite will be disproportionate were developed [9,39]. An opinion paper from a CDER representative reiterated the case-by-case aspects of guidance compliance [40]. By the end of 2009, PKDM groups were testing and implementing strategies to comply with the FDA guidance when the December 2009 ICH M3(R2) guidance

became effective. It set a different and less conservative threshold of 10 % of DRM and dose greater than 10 mg. In papers and seminars from 2010, the focus shifted to the role of bioanalysts in MIST strategy and the industry converged on MS-based approaches [31].

Our initial strategy within PKDM was to triage discovery and development of small molecule programs and work with PKDM scientists to identify programs at risk for disproportionate metabolites. Development plans of affected programs were then modified to address the MIST guidances and team PKDM scientists embarked on case-by-case development team education and data generation. Meanwhile extramural and intramural experience grew and consistent internal procedures were developed. Opinion leaders in drug safety, early clinical development, regulatory affairs, and pharmaceutical development previewed the PKDM MIST strategy and helped develop best practices for plasma procurement and metabolite profiling. Discussions with chemists and pharmaceutical development revolved mainly around responsibility for an earlier synthesis of the major *in vitro* human metabolites to enable activity testing and use as analytical standards. In some cases, depending on synthetic complexity, biogeneration of the metabolite(s) [41] may be required and purification and determination of analytical purity can be done by NMR [29] or a variety of MS techniques [31].

Good laboratory practice/good clinical practice (GLP/GCP) compliance and metabolite stability aspects of procuring unused plasma from preclinical safety and human SAD/MAD studies were worked out. The use of unused plasma and sample pooling is efficient and avoids the time involved in conducting separate studies. When clinical plasma becomes available, unused plasma could be obtained from GLP studies by amendment or from a separate PK study. Owing to uncertainties regarding metabolite stability, protracted cold storage of plasma for metabolite analysis is less desirable than the use of more recently procured samples. Non-GLP analysis of the plasma samples was preferred, with a bioanalytical report as the deliverable. After conducting the initial human plasma screening of the SAD and MAD samples, steady-state human metabolites at or near the 10 % of DRM threshold would be the reason for, and focus of, subsequent analysis of preclinical plasma samples.

Similar discussions with clinical development centered on mechanisms for the transfer of samples after PK analysis, suitable protocol, and informed consent language.

As in the analysis of preclinical safety samples, non-GLP analysis would be pursued and a bioanalytical report would be generated. Ultimately, a MIST summary report encompassing all the *in vitro* and *in vivo* metabolism data would be assembled along with a recommendation for any necessary subsequent activities. With adequate preclinical coverage, the recommendation would be no further metabolite profiling is necessary until the human ADME study. The report could be submitted in late phase 1, coincident with the next annual report to the CTA. Regulatory endorsement that all MIST issues are resolved would be requested. Clearly disproportionate metabolites or metabolites at or near the threshold would entail more interactive discussions on the path forward with teammates and regulators.

The responsibilities of the PKDM project leader were as follows: Develop a MIST action plan for their candidate, include a MIST risk analysis statement and related activities in monthly highlights, and engage development team partners. PKDM project leaders were to leverage available pre-IND samples for preliminary method development and analysis, arrange synthesis of key metabolite(s), and arrange determination of their pharmacological on target activity, with a team dialogue on how much activity data

were appropriate. Protocols were to be checked for appropriate sample retention, storage, and transfer. The PKDM project leader would discern human metabolites greater than 10 % of DRM first, compare other species as necessary, and then write MIST bioanalytical reports to compile into the consensus metabolism report and action plan. Recommendations might include subsequent metabolite(s) monitoring in TK studies and/or in larger clinical studies. When this is necessary, bioanalysts could be engaged in GLP method development, and regulatory and early development partners would then help define the more complicated, case-by-case path forward by discussion with regulators.

The rollout was conducted at unit meetings in preclinical safety and regulatory and clinical safety units. All the planning and rollout of these activities took about one year from initial discussions to endorsement and routine application. The plan evolved significantly over time as many new publications became available that discerned how the industry as a whole would deal with MIST guidance compliance.

6.10 CONCLUSION

This chapter overviewed the development and rollout of a plan to comply with new regulatory guidances on metabolites. A proactive, data-driven, best-practice approach was developed by consensus with key development partners. Changes to traditional metabolism approaches included nonradiometric profiling of plasma metabolites across species and the development of a data-driven plan to preclude the existence of disproportionate metabolites. When potentially disproportionate metabolites are observed, under this plan, there is still time to conduct subsequent metabolite assessments, well in advance of the end of phase 2.

Suddenly a mist fell from my eyes and I knew the way I had to take.
–Edvard Grieg, Composer (b1843–d1907) [42]

ACKNOWLEDGMENTS

We acknowledge valuable discussions with scientists in the Drug Metabolism Technical Group (formerly the PhRMA DMTG) and Amgen. We acknowledge with thanks the critical review of the manuscript by Drs. Bradley Wong, Larry Wienkers, Benny Amore, and Dean Hickman.

ABBREVIATIONS

AMS	accelerator mass spectrometry
ADME	absorption, distribution, metabolism, excretion
AUC	area under the plasma concentration versus time curve
CDER	Center for Drug Evaluation and Research
CTD	common technical document

DDI	drug–drug interaction
DRM	drug-related material
IND	investigational new drug
eIND	exploratory investigational new drug
FIH	first-in-human, the development phase when the drug candidate is first dosed in human subjects
HR-MS	high resolution mass spectrometry
ICH	International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use
MDF	mass defect filter
MIST	metabolites in safety testing (safety testing of metabolites)
MAD	multiple ascending dose
m/z	mass/charge ratio
NOAEL	no observed adverse effect level
SAD	single ascending dose
PhRMA	DMTG Drug Metabolism Technical Discussion group of the Pharmaceutical Research and Manufacturers Association
R&D	research and development
PKDM	pharmacokinetics and drug metabolism organization (DMPK in some organizations)
POBA	proof of biological activity
PK	pharmacokinetic
TK	toxicokinetic

REFERENCES

1. CDER. Guidance for industry: safety testing of drug metabolites. Available at http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm079266.pdf. 2008. Accessed 2011 Nov 14.
2. ICH. 2009. ICH guidance on nonclinical safety studies for the conduct of human clinical trials and marketing authorization for pharmaceuticals M3 (Revision 2) Current Step 4 version dated June 11 2009. Available at http://www.emea.europa.eu/docs/en_GB/document_library/Scientific_guideline/2009/09/WC500002720.pdf. Accessed 2011 Nov 14.
3. Walker D, Brady J, Dalvie D, *et al*. A holistic strategy for characterizing the safety of metabolites through drug discovery and development. Chem Res Toxicol 2009;22(10):1653–1662.
4. Timmerman P, Anders-Kall M, Gordon B, *et al*. Best practices in a tiered approach to metabolite quantification: views and recommendations of the European Bioanalysis Forum. Bioanalysis 2010;2(7):1185–1194.
5. Tonelli A. Why I love metabolites in safety testing (MIST): a light-hearted historical perspective. Bioanalysis 2009;1(4):683–685.
6. Nedderman ANR, Wright P. Looking back through the MIST: a perspective of evolving strategies and key focus areas for metabolite safety analysis. Bioanalysis 2010; 2(7):1235–1248.
7. Slatter JG. Safety testing of drug metabolites: MIST guidance impact on the practice of industrial drug metabolism. In: Ala Nassar AF, editor. Biotransformation and metabolite elucidation of xenobiotics. New York (NY): Wiley; 2010. pp. 295–312. Chapter 8.
8. Chaudhary A. Welcome to MIST and bioanalysis. Bioanalysis 2010;2(7):1169–1170.

9. Leclercq L, Cuyckens F, Mannens GS, *et al*. Which human metabolites have we MIST? Retrospective analysis, practical aspects, and perspectives for metabolite identification and quantification in pharmaceutical development. Chem Res Toxicol 2009;22(2):280–293.
10. Smith DA, Obach RS, Williams DP, *et al*. Clearing the MIST (metabolites in safety testing) of time: the impact of duration of administration on drug metabolite toxicity. Chem Biol Interact 2009;179(1):60–67.
11. Robison TW, Jacobs A. Metabolites in safety testing. Bioanalysis 2010;1(7):1193–1200.
12. ICH. 2009. Consensus guideline nonclinical evaluation for anticancer pharmaceuticals S9 current Step 4 version dated 29 October 2009. Available at http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Safety/S9/Step4/S9_Step4_Guideline.pdf. Accessed 2011 Nov 14.
13. Anderson S, Luffer-Atlas D, Knadler MP, *et al*. Predicting circulating human metabolites: How good are we. Chem Res Toxicol 2009;22(2):243–256.
14. Dalvie D, Obach RS, Kang P, *et al*. Assessment of three human *in vitro* systems in the generation of major human excretory and circulating metabolites. Chem Res Toxicol 2009; 22(2):357–368.
15. Prueksaritanont T, Lin JH, Baillie TA, *et al*. Complicating factors in safety testing of drug metabolites: kinetic differences between generated and preformed metabolites. Toxicol Appl Pharmacol 2006;217(2):143–152.
16. Pang KS. Safety testing of metabolites: expectations and outcomes. Chem Biol Interact 2009;179(1):45–59.
17. Powley MW, Frederick CB, Sistare FD, *et al*. Safety assessment of drug metabolites: implications of regulatory guidance and potential application of genetically engineered mouse models that express human P450s. Chem Res Toxicol 2009;22(2):257–262.
18. Hamilton RA, Garnett WR, Kline BJ, *et al*. Determination of mean valproic acid serum level by assay of a single pooled sample. Clin Pharmacol Ther 1981;29(3):408–413.
19. Vishwanathan K, Babalola K, Wang J, *et al*. Obtaining exposures of metabolites in preclinical species through plasma pooling and quantitative NMR: addressing metabolites in safety testing (MIST) guidance without using radiolabeled compounds and chemically synthesized metabolite standards. Chem Res Toxicol 2009;22(2):311–322.
20. Hu Q, Noll RJ, Li H, *et al*. The orbitrap: a new mass spectrometer. J Mass Spectrom 2005; 40(4):430–443.
21. Zhang H, Zhang D, Ray K, *et al*. A software filter to remove interference ions from drug metabolites in accurate mass liquid chromatography/mass spectrometric analyses. J Mass Spectrom 2003;38(10):1110–1112.
22. Mortishire-Smith RJ, O'Connor D, Castro-Perez JM, *et al*. Accelerated throughput metabolic route screening in early drug discovery using high-resolution liquid chromatography/quadrupole time-of-flight mass spectrometry and automated data analysis. Rapid Commun Mass Spectrom 2005;19(18):2659–2670.
23. Zhu M, Ma L, Zhang D, *et al*. Detection and characterization of metabolites in biological matrices using mass defect filtering of liquid chromatography/high resolution mass spectrometry data. Drug Metab Dispos 2006;34(10):1722–1733.
24. Tiller PR, Yu S, Bateman KP, *et al*. Fractional mass filtering as a means to assess circulating metabolites in early human clinical studies. Rapid Commun Mass Spectrom 2008;22(22):3510–3516.
25. Zhu P, Ding W, Tong W, *et al*. A retention-time-shift-tolerant background subtraction and noise reduction algorithm (BgS-NoRA) for extraction of drug metabolites in liquid chromatography/mass spectrometry data from biological matrices. Rapid Commun Mass Spectrom 2009;23(11):1563–1572.
26. Zhu P, Tong W, Alton K, *et al*. An accurate-mass-based spectral-averaging isotope-pattern-filtering algorithm for extraction of drug metabolites possessing a distinct isotope pattern from LC-MS data. Anal Chem 2009;81(14):5910–5917.

27. Yu C, Chen CL, Gorycki FL, *et al*. A rapid method for quantitatively estimating metabolites in human plasma in the absence of synthetic standards using a combination of liquid chromatography/mass spectrometry and radiometric detection. Rapid Commun Mass Spectrom 2007;21(4):497–502.
28. Yi P, Luffer-Atlas D. A radiocalibration method with pseudo internal standard to estimate circulating metabolite concentrations. Bioanalysis 2010;2(7):1195–1210.
29. Espina R, Yu L, Wang J, *et al*. Nuclear magnetic resonance spectroscopy as a quantitative tool to determine the concentrations of biologically produced metabolites: Implications in metabolites in safety testing. Chem Res Toxicol 2009;22(2):299–310.
30. Anderson S, Knadler MP, Luffer-Atlas D, *et al*. Overview of metabolite safety testing from an industry perspective. Bioanalysis 2010;2(7):1249–1261.
31. Ramanathan R, Josephs JL, Jemal M, *et al*. Novel MS solutions inspired by MIST. Bioanalysis 2010;2(7):1291–1313.
32. Ramanathan R, Raghavan N, Comezoglu SN, *et al*. A low flow ionization technique to integrate quantitative and qualitative small molecule bioanalysis. Int J Mass Spectrom 2011;301(1–3):127–135.
33. Ma S, Li Z, Lee KJ, *et al*. Determination of exposure multiples of human metabolites for MIST assessment in preclinical safety species without using reference standards or radiolabeled compounds. Chem Res Toxicol 2010;23(12):1871–1873.
34. Gao H, Deng S, Obach RS, *et al*. A simple LC/MS/MS method to determine relative plasma exposures of drug metabolites across species for metabolite safety assessments (MIST). Drug Metab Dispos 2010;38(12):2147–2156.
35. Lappin G, Seymour M. Addressing metabolite safety during first-in-man studies using ^{14}C-labeled drug and accelerator mass. Bioanalysis 2010;2(7):1315–1324.
36. Xceleron. 2010. Want faster human mass balance/ADME data? Reference: Xceleron launches solution to FDA MIST. Available at http://www.xceleron.com/news/xceleron-solution-fda-mist/. 28 April 2009. Accessed 2011 Nov 14.
37. CDER. 2006. Exploratory IND studies. Available at http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm078933.pdf. Accessed 2011 Nov 14.
38. Slatter JG. Overview of the MIST guidance and implications for DM/PK. 12th Annual Land O' Lakes Conference on Drug Metabolism and Applied Pharmacokinetics. Meerimac (WI); 2009.
39. Smith DA, Obach RS. Metabolites: have we MIST out the importance of structure and physicochemistry. Bioanalysis 2010;2(7):1223–1233.
40. Atrakchi AH. Interpretation and considerations on the safety evaluation of human drug metabolites. Chem Res Toxicol 2009;22(7):1217–1220.
41. Zoellner A, Buchheit D, Meyer MR, *et al*. Production of human phase 1 and 2 metabolites by whole-cell biotransformation with recombinant microbes. Bioanalysis 2010;2(7):1277–1290.
42. Brainyquote. 2010. Grieg, Edvard. Available at http://www.brainyquote.com/quotes/keywords/mist.html. Accessed 2011 Nov 14.