A Simple LC/MS/MS Method to Determine Relative Plasma Exposures of Drug Metabolites across Species for Metabolite Safety Assessments (MIST) Part 2: Application to Unstable Metabolites

Hongying Gao and R. Scott Obach

Pharmacokinetics, Dynamics and Metabolism, Pfizer Inc, Groton, CT
Running title: LC-MS/MS for Safety Assessments of Unstable Metabolites

Corresponding author: Hongying Gao

Pharmacokinetics, Dynamics and Metabolism, Pfizer, Inc., Groton, CT 06340

Tel: 860-715-2432

Email: Hongying.Gao@pfizer.com

Text Pages: 15

Tables: 3

Figures: 4

References: 16

Abstract: 221

Introduction: 916

Discussions: 1270

ABBREVIATION: MIST, Metabolites in Safety Testing; LC, liquid chromatography; MS/MS, mass spectrometry; MIM, multiple ion monitoring; EPI, enhanced product ion; MRM, Multiple reaction monitoring; IS, internal standard; ISR: Incurred sample reanalysis.
ABSTRACT

We previously described a simple LC/MS/MS method to determine relative plasma exposures of drug metabolites across species for metabolite safety assessments. It offers time and resource-sparing advantages to ascertain metabolite exposure comparisons between humans and laboratory animal species for stable metabolites with high confidence. In this study, we tested the limitation of the methodology with compounds possessing six substituents found in unstable metabolites. Stabilization procedures were used and compared to untreated samples for structures with established stabilization processes. In most cases, the parent compounds with established stability were used as the intrinsic stability references except in cases where the metabolite was more stable than the parent compound. Long term storage stability of the unstable structures was tested by comparing the response ratio of the metabolite to the stability reference compound for multiple independent analyses covering the storage duration. Auto sampler stability was tested using the same response ratio of the re-injections of the reconstituted solution overnight over the first injections. The results supported that it is possible that an abbreviated LC/MS/MS peak area ratio comparison can be applied to epoxide, amide, catechol, and acyl glucuronides to determine the relative plasma exposure of drug metabolites across species; but it may not be suitable for iminium ions and esters. Stability of suspected unstable metabolites can be tested using the methodology described above.
INTRODUCTION

Toxicology studies conducted in laboratory animals are an essential component to human risk assessment for new drug candidates. Recently, there has been a focus on ensuring the safety not only of the drug itself, but also for circulating metabolites (Luffer-Atlas, 2008; Robison and Jacobs, 2009; Smith and Obach, 2009; Frederick and Obach, 2010; Anderson, et al., 2010) which was triggered by a position offered by scientists from a pharmaceutical industry trade organization (Baillie, et al., 2002). Many papers have been published laying out various strategies for incorporating an assessment of human circulating metabolites (i.e. prediction and identification; Leclercq, et al., 2009; Nedderman, 2009; Walker, et al., 2009; Nedderman and Wright, 2010) that can be used in determining whether further examination of safety attributes of metabolites is warranted. While there has not been perfect agreement among all discussants of this topic, regulatory guidance documents have been issued by the International Conference on Harmonization (ICH) as well as the U.S. Food and Drug Administration in which guidelines have been outlined defining the thresholds for when human circulating metabolites merit further consideration in risk assessment. The ICH guidance states that human circulating metabolites that comprise 10% or more of total drug-related material in circulation should also be present in equivalent or greater concentrations in at least one of the laboratory animal species that are used in toxicology studies. It is important to note that the most reliable means by which it can be determined that a metabolite truly exceeds 10% of total drug-related material is through analysis of data from a carbon-14 (or in some cases tritium) human metabolism study. Such an approach offers the only means by which it can be assured that all drug-related material has been accounted for in a complex biological matrix. Measurements can be made using radiometric approaches, or if a carbon-14 labeled drug is used, then accelerator mass spectrometry can be
used to measure drug-related material. Approaches whereby HPLC-MS is used to locate metabolites in the absence of radio-labeled material, such as high resolution mass spectrometry, have improved our ability to find and identify metabolites. However, these are still not assured of being comprehensive in finding all metabolites and quantitative comparisons cannot be made to determine the percentages of each metabolite, due to differences in ionization efficiencies among the parent drug and various metabolites.

Demonstrating that a human circulating metabolite is present in larger quantities in toxicology species can pose a large resource burden in obtaining qualified authentic standards of metabolites (which frequently can be challenging to synthesize due to structural complexity) and developing validated bioanalytical methods to measure multiple metabolites simultaneously. To this end, investigators have proposed abbreviated approaches that can yield valuable information regarding relative exposures in animals and humans without the investment in generating an authentic standard or developing a bioanalytical method (Gao and Obach, 2011; Ma and Chowdhury, 2011). Others have proposed using quantitative NMR or use of radiometric calibration standards to make estimates of metabolite concentrations in plasma samples (Espina, et al, 2009; Yi and Luffer-Atlas, 2010; Zguda-Pols, et al., 2010).

Since the guidance documents state that it is important that animals contain higher concentrations of human circulating metabolites, but do not specifically require a particular multiple, we designed and tested an approach whereby it could be determined which species has a higher concentration (Gao, et al., 2010). In this method, human and animal plasma samples containing unknown concentrations of drugs and metabolites are mixed with control plasma from the opposite species to yield samples of identical matrix. These are then processed and injected onto HPLC-MS with monitoring the ion counts for the various metabolites of interest. The peak
responses are compared across the species, and it was shown that if an animal has twice the ion count response as human, then it can be concluded that the animal has an equivalent or greater exposure to the metabolite. Only if the cross-species response ratio is within 2-fold would there be a need to develop a standard analytical method for the metabolite and measure the concentration more precisely. To our knowledge, this is the most resource-sparing approach that can provide assurance that animals receiving the parent drug during toxicology studies were exposed to metabolites relevant to human. However, a potential shortcoming of this approach is that if a metabolite were unstable in plasma or during sample work-up, or if it had differing stability in animal vs. human plasma when stored frozen, then an erroneous conclusion regarding relative exposures in humans vs. animals could be made. In the absence of an authentic standard of a metabolite, quality control samples cannot be prepared and used in assessments of storage and processing stability. It has been shown that stability of metabolites even in the absence of an authentic standard can be assessed using radio-labeled parent drug and biologically generated metabolites (Zgoda-Pols et al., 2010), however, this still has the disadvantage of requiring radio-labeled material.

In this report, we applied our previously described metabolite peak area comparison approach to a set of six pairs of compounds possessing substituents which are also associated with chemical and/or enzymatic stability issues in plasma. The six included acyl glucuronide, catechol, ester, amide, epoxide, and iminium ion substituents. Rat and human plasma were spiked with known concentrations of these metabolites, and the peak area approach was applied in a blinded fashion to determine how closely the measured ratios matched to the nominal values. The relationship between the measured peak area ratio values and storage time was also examined.
MATERIALS AND METHODS

Chemicals and Reagents

HPLC grade water was purchased from J. T. Baker (Phillipsburg, NJ, USA). ACS reagent grade acetonitrile was purchased from Honeywell Burdick & Jackson (Muskegon, MI, USA). Naproxen acyl glucuronide conjugate and N-acetyl sulfadiazine were purchased from Toronto Research Chemicals (Toronto, ON, Canada). Naproxen, carbamazepine, carbamazepine-10,11-epoxide, sulfadiazine, salicylic acid, 2,3-dihydroxybenzoic acid, ascorbic acid, citric acid, cotinine, and methylphenidate were purchased from Sigma/Aldrich (St. Louis, MO, USA). Ritalinic acid was purchased from Isotec International, Inc (Canton, GA, USA). Nicotine iminium ion diperchlorate was synthesized using previously described methods (Perterson, et al). Plastic plasma tubes with glycolytic inhibitor, 5.0 mg NaF and 4.0 mg potassium oxalate were purchased from BD (Franklin Lakes, NJ, USA). Wistar Hanover rat and human K3EDTA plasma were purchased from Bioreclamation, Inc. (Westbury, NY, USA) except that the blank plasmas for the nicotine iminium ion and cotinine analyses were collected in-house.

Sample preparation

To test the limit of our simple LC/MS/MS methodology, a drug and its unstable metabolite pairs were spiked into Wistar Hanover rat and human plasmas. This was done for seven drug/metabolite combinations and each combination was spiked in rat or human plasma to generate 4 different sample sets to cover a range of ratios that may be produced in actual samples. (The concentrations of individual analytes are listed in the Supplemental Table 1). For some drugs, untreated plasma samples and stabilized samples using established stabilization
procedures were generated in parallel. Naproxen and naproxen acyl glucuronide conjugate combinations were treated with 5% 2M citric acid (v:v) to bring down the pH to 3.5; methylphenidate and ritalinic acid combinations were treated with 5.0 mg NaF and 4.0 mg potassium oxalate per 4 mL plasma; salicylic acid and 2,3- dihydroxybenzoic acid combinations were treated with 5% of 500 mg/mL citric acid and 40 mg/ml ascorbic acid in plasma with final pH between 3.5-5. All samples were stored at -20 °C for long term sample storage. Three independent analyses of each combination were performed to evaluate the long term stability during sample storage. The samples and the molecular weights of the drugs and metabolites were provided to the bioanalytical chemist but they were blinded to the parent and metabolite concentrations.

All samples were thawed at room temperature, and then kept on wet ice during sample preparation. Samples were mixed with an equivalent volume of blank plasma of the opposite species and diluted in mixed blank plasma as in previous work (Gao, et al, 2010). All samples were then prepared for LC/MS/MS analysis by the addition of 4 volumes of 100 ng/mL sulfadimethoxine in acetonitrile as an internal standard (IS) and protein precipitation reagent except for the naproxen/naproxen-acyl glucuronide conjugate plasma samples where 0.1% formic acid was added to the IS solution to stabilize the glucuronide conjugate. All samples were centrifuged and the supernatants were dried under nitrogen and reconstituted with 20 µL of acetonitrile with 0.1% formic acid followed by 180 µL of water with 0.1% formic acid for sample analysis except that methylphenidate/ritalinic acid were reconstituted using acetonitrile and water without formic acid and cotinine/nicotine iminium ion were reconstituted in 100% water. Reinjection of the reconstituted solution was performed to evaluate the auto-sampler stability.
**Liquid Chromatographic and Mass Spectrometric Methods**

All sample analysis was carried out on an API-5500 QTRAP™ triple quadrupole-linear ion trap mass spectrometer equipped with a Turbo V Ionspray ionization source and all instrumentation control and quantitation was through the mass spectrometer Analyst™ software package (Applied Biosystems, Foster City, CA, USA). The HPLC system consisted of two Shimadzu Prominence LC-AD10 components (Columbia, MD, USA). The autosampler was a CTC Analytics PAL autosampler (CTC Analytics AG, Zwingen, Switzerland) with temperature controlled at 11°C. Chromatographic separation was accomplished on a Phenomenex Kinetex C18 column (2.6µm, 75×3.0 mm, Phenomenex USA, Torrance, CA), and the mobile phase consisted of two solvents, solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in acetonitrile) for all samples except the nicotine iminium ion/cotinine combination. The gradient conditions (LC1 in Table 1) were used for naproxen/naproxen acyl glucuronide, carbamazepine/carbamazepine-10,11-epoxide, sulfadiazine/N-acetyl sulfadiazine, methylphenidate/ritalinicacid as the following: 0 to 3 min, 2%B; 3 to 33 min, 2% to 80%B; 33 to 36 min, 80% B to 98%B; 36 to 38 min, 98%; 38 to 41 min, 98 to 2%B, 41 to 45 min, 2%B. The gradient conditions (LC2 in Table 1) were modified for salicylic acid/2,3-dihydroxybenzoic acid as the following: 0 to 3 min, 2%B; 3 to 23 min, 2% to 40% B; 23 to 36 min, 40%B to 98%B; gradient conditions from 36 to 45 min were the same as listed above in LC1. The LC separation (LC3 in Table 1) for nicotine iminium ion/cotinine samples was achieved on a Synergi polar RP C18 column (4µm, 75×3.0 mm, Phenomenex USA, Torrance, CA), and mobile phase A was 10 mM ammonium format with 0.1% formic acid and mobile phase B was 100% acetonitrile. The LC gradients were the following: 0 to 5 min, 2% B; 5 to 23 min, 2% to 30% B; 23 to 28 min, 30% to 90% B; 28 to 32 min, 90% B; 32 to 36 min, 90% to 2% B; 36 to 45 min,
2% B. The total run time of each injection for all LC methods was 45 minutes. A Valco VICI valve (Valco Instruments Co., Houston, TX) was used to divert the first 0.5 min and the last 7 minutes of HPLC effluent to waste. The injection volume was 10 µL.

For each analyte/metabolite combination, the analyte ionization parameters were optimized by infusing solutions of the parent drugs and internal standard in 50:50 0.1% formic acid in water: acetonitrile. Ionization and mass dependent parameters for the parent drugs were used for their metabolites, the source temperature was set at 500°C except for the naproxen acyl glucuronide conjugate where the source temperature was lowered to 400°C. The internal standard was monitored at the MRM transition of \( m/z \) 311 → \( m/z \) 155 in positive ion mode and \( m/z \) 309 → \( m/z \) 122 in negative ion mode.

An information dependent acquisition (IDA) method was employed to trigger the collection of enhanced product ion scans for method development and qualitative identification of the metabolites. The first experiment of the mass spectrometer was set in the MRM (parent) or MIM (metabolites) mode for test sets. Detection of an ion signal greater than 5000 cps then triggered collisionally activated enhanced product ion (EPI) scans to generate structurally specific dissociation of the ion. EPI spectra were collected for three consecutive scans followed by an EPI scan mode exclusion time of 1 second. The parent ion for the IS was listed in the IDA exclusion list across the full LC run. The EPI scan was operated at a scan rate of 10000 amu/s using the dynamic fill option in the linear ion trap. The collision energy was set at 50 eV (-50 eV for naproxen samples) with a spread of 40 eV. The dwell time for each MIM/MRM transition was 20 ms and the total cycle time was approximately 0.2 s.
MIM_EPI and MRM_EPI for Qualitative Identification and Quantitation of Metabolites. The methodology for structural confirmation of the metabolites and selecting suitable daughter ions of the metabolites to achieve optimal sensitivity and selectivity for quantitation has been described in previous work (Gao, et al, 2010).

Chromatographic peaks were integrated and peak area ratios of metabolites vs. internal standard at the MRM transitions were calculated. Direct comparisons of metabolites in rat vs. human plasma were achieved by calculating the rat: human ratio of these peak area ratios. To monitor the long-term storage and auto-sampler stability, a known stable compound was selected and served as the “intrinsic stability reference” compound in the plasma samples. The parent drug served this purpose for all the drug/metabolite combinations except that ritalinic acid (a stable hydrolyzed metabolite) was used for methylphenidate (an ester drug). Peak area ratio of the unstable metabolites vs. the intrinsic stability reference compound was calculated, and the ratios at the testing date were normalized by the first injection or by day 1 measurements for auto-sampler stability and long term storage stability evaluations, respectively. Ratio measurements of the drug and metabolite in rat vs. human plasma were compared to the nominal ratios, and the errors were stabilized using the geometric mean fold error.

RESULTS

Auto-sampler stability Six unstable substituents and the stable counterparts (Fig. 1) were selected to test the limitation of the simplified LC/MS/MS methodology described previously. All unstable compounds have been detected using the LC/MS/MS methodology described in the Methods section. The response ratio of the unstable compound vs. the intrinsic stable reference compound collected after overnight injection was compared to the response ratio
of the first injection at day 1. If the test metabolite is stable, the ratio of the metabolite vs. the intrinsic stability reference compound should be constant and remain as 1 when the ratio is normalized by the response ratio at day 1. Table 2 showed the auto sampler stability test results for the six unstable structure substituents after the sample preparations. Both untreated and treated naproxen/naproxen acyl glucuronide conjugate were stable during the overnight injection once the samples were prepared using the acidic work-up solution and then reconstituted in acidic solution. Both treated and untreated salicylic acid/2,3 dihydroxybenzoic acid samples, sulfadiazine/N-acetyl sulfadiazine, and nicotine iminium ion/cotinine samples were stable for overnight injection in the temperature controlled auto sampler. Carbamazepine/carbamazepine-10,11-epoxide degraded slightly during the overnight injection. The untreated methylphenidate/ritalinicacid combination samples degraded significantly while the treated samples degraded slower than the untreated samples during the overnight injection.

**Long-term storage stability** The long term storage stability was tested using three independent analyses of the samples covering the storage duration. Without using the synthetic standards, the long term storage stability of the metabolites was evaluated by comparing the response of unstable structure vs. the intrinsic stability reference compound at any test day normalized by the response ratio at day 1. Similar to the auto sampler stability test, the response ratio of the metabolite vs. the intrinsic stability reference compound should remain constant and will be 1 when normalized by the response ratio at day 1 if the metabolite is stable. The untreated naproxen acyl glucuronide conjugate went through migration while the acidified naproxen glucuronide conjugate were stable during sample storage and repeated analyses as shown in Figure 2. The trend of degradation can be revealed by plotting the response ratio vs. the storage time. For example, the treated naproxen/naproxen acyl glucuronide conjugate
samples was stable over 130 days stored at -20 °C while the untreated naproxen acyl glucuronide conjugate degraded over time as shown in Figure 3. Table 3 listed the test results for five parent/metabolite combinations. Nicotine iminium ion/cotinine combination was not tested due to measurement errors in exposure comparison. Both treated and untreated salicylic acid/2,3-dihydroxybenzoic acid, carbamazepine was stable over the tested period. N-acetyl sulfadiazine were degraded on the 2nd test then stabilized after the initial degradation. The treated methylphenidate/ritalinic acid samples were more stable than the untreated samples, however, the intra-assay variability was larger for treated samples, indicating that the treated samples may undergo degradation during the sample preparation.

Comparison of measured peak area ratios vs. nominal ratios values across species for unstable metabolites  The ratio of peak areas for each analyte in rat vs. human were calculated and compared to the rat vs. human concentration ratio calculated from the nominal concentrations. The geometric mean fold errors between measured and nominal ratios assessed in three separate occasions were shown in Figure 4. A value of unity reflects a perfect agreement between the measured and nominal values. It was shown in our previous work that if the cross-species response ratio is equal or more than 2, then it can be concluded that the animal has an equivalent or greater exposure to the metabolite (Gao, et al., 2010). Naproxen and its acyl glucuronide were measured both with and without the addition acid to stabilize the glucuronide. The peak area ratios for acid-treated samples were lower than two in all cases. For untreated samples, the error in the ratios increased over time such that the error value exceeded two on the third analysis (after 130 days stored frozen). This showed that the peak area ratio method was applicable for acyl glucuronides but only following stabilization with acid.
The analyses of salicylic acid/2,3-dihydroxy benzoic acid, carbamazepine/carbamazepine-10,11-epoxide, sulfadiazine/N-acetyl sulfadiazine combinations were reproducible with errors less than 2.0 in all instances (Figure 4). Among them, salicylic acid/2,3-dihydroxy benzoic acid combination measurements were the most accurate with all three analyses less than 1.17. Measurement errors for ritalinic acid in both treated and untreated plasma samples sets were close to 2.0 while the measurement errors for methylphenidate were smaller than the stable metabolite. The errors in peak area ratios for nicotine iminium ion and cotinine were the largest among the compounds with error more than 3.5.

DISCUSSION

Without synthetic standards or radio-labeled material, the stability of the metabolites can be tested and established using an intrinsic stability reference. The concept of the stability reference is similar to the internal standard in bioanalysis, in which the reference should be in the sample and stored under the same condition as the test metabolites, and its stability should have been demonstrated or well known. In most cases, the parent drug can be used as the stability reference since its stability usually has been tested and established as a part of the validation of its bioanalytical assay. In those cases that the parent is potentially unstable (e.g. ester) or not suitable as a reference (e.g. the concentration is too high, or is changed significantly due to degradation of major metabolites), a stable metabolite (e.g. carboxylic acid for ester) can be used as the stability reference. The parent or a known stable metabolite was in the same samples as the unstable metabolites when the samples were collected, thus these stability references are truly intrinsic. The response ratio of the test metabolite vs. the stability reference should remain
constant and the ratio of the response ratio at any test day vs. the day 1 response ratio should be 1, if the metabolite is stable during the storage and sample analysis process.

In recent EMEA guideline on bioanalytical method validation (http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2011/08/WC500109686.pdf), stability is evaluated using QC samples and the mean concentration of each level should be ±15% from the nominal concentrations. For discovery bioanalysis where the bioanalytical method is not validated, ±20% from the nominal concentrations is deemed accurate (Gao and Obach, 2011). Therefore, for the peak area ratio approach without synthetic standard, if the ratio of response ratio at any test day vs. the day 1 response ratio is within ±0.2 (or 20%) from 1 (e.g. 0.8-1.2), the metabolite can be deemed stable. This cut-off can be applied to auto sampler stability and long term storage stability results in Tables 2 and 3 for unstable metabolites. A time profile of the ratio of the response ratio at the test day vs. day 1 response can also uncover if the test compound is stable or not; an example is the treated vs. untreated naproxen acyl glucuronide conjugate. Much attention should be paid to the trend of the data set to determine if the degradation occurred during the storage time. Similarly, the auto-sampler stability can also be tested using the same methodology. In some instances, the unstable metabolite can degrade to the parent drug (e.g. acyl glucuronide conjugate of a carboxylic acid drug hydrolyzing back to the carboxylic acid) and thus not only is the measurement of the peak area ratio for the metabolite inaccurate but the ratio for the parent drug can be inaccurate as well, with the degree of inaccuracy dependent on the rate of degradation and the relative concentration of the two entities.

The ratio measurement of metabolites in rat vs. human demonstrated that the three independent analyses should be reproducible with good accuracy if the metabolite is stable or is
stabilized through established procedures. The measurement error may not increase significantly even if the metabolite is not stable during storage and sample analysis, but only if the rate of degradation is similar in rat and human plasma and the samples were stored at the same time and prepared back to back for LC/MS/MS analysis. This is the case for N-acetyl sulfadiazine and the untreated naproxen glucuronide conjugate where degradation of the conjugate metabolite during long term storage was observed, however, the measurement errors for the unstable metabolite didn’t increase significantly. Interestingly, the measurement errors for untreated naproxen increased more than that for naproxen acyl glucuronide conjugate, especially when the concentrations of the metabolite were much more than the parent. This was due to the fact that the unstable metabolite degraded to the parent and increased the concentrations for the parent in the samples. This is also confirmed in the methylphenidate/ritalinic acid combination where the measurement errors for ritalinic acid were larger than methylphenidate. This impact is most significant when the concentrations of the unstable metabolite are much more than the downstream stable structures. The measurement error for sulfadiazine was larger than N-acetyl sulfadiazine when the metabolite concentrations were much higher than the parent, although the error for the average cross-species ratio measurements didn’t increase significantly. Thus it is important to simultaneously monitor the unstable compound and the downstream stable structure as more measurement errors would be introduced to the stable structure if the conversion takes place during sample storage and analysis. In the case where the metabolites convert to the parent drug, e.g. N-Acetyl, glucuronide conjugates, incurred sample reanalysis (ISR, http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2691460/pdf/12248_2009_Article_9100.pdf, http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2011/08/WC500109686.pdf) would uncover the issue and the impact on the parent drug concentration
measurements from the metabolite degradation. If the unstable metabolites convert to
downstream metabolites without synthetic standards, multiple repetitive analyses of the samples
should be executed as described above to assess the impact of degradation, a stable metabolite
other than the degraded product can be employed as the “intrinsic stability reference”. In
practice, if the animal samples have been stored longer than the human samples under the same
storage condition, and the ratio of the metabolites in animal vs. human is still larger than 2-fold,
that would preclude the false positive error for the unstable metabolites due to stability issue.
However, multiple repetitive analyses of the samples will still be needed in the later case.

The simplified LC/MS/MS peak area methodology for relative exposure comparison
across species can be applied to most unstable metabolites. Stabilization should be undertaken
when the samples are collected, e.g. acidify the acyl glucuronide. The unstable metabolite and
the downstream metabolite or structure should be monitored and the impact of the degradation of
the unstable metabolite should be evaluated through multiple analyses. In the example of the
ester and carboxylic acid pair (i.e. methylphenidate and ritalinic acid) greater error was observed
for the carboxylic acid due to hydrolysis of the ester. The error was greater than two-fold, which
was the cutoff value previously demonstrated to offer 99% confidence that the actual ratio was
greater than unity. Thus, we conclude that it is unlikely that this method can be used for ester
drugs and their carboxylic acid metabolites. However it should be noted that most carboxylic
acid metabolites of ester drugs are accessible by standard synthetic methods, thus the need to
determine relative animal vs. human exposures using the peak area approach can be set aside in
favor of a more conventional bioanalytical approach. For the other structural types for which
examples were tested in this study, it is likely that the approach will be acceptable. Nevertheless,
even in those cases attention must be paid to the consistency of the ratio measured on separate
casions to ensure the validity of the measurement.

In this study, we have described the application of our previously described LC/MS/MS
peak area approach that addresses relative cross-species metabolite exposures to compounds
possessing structures prone to chemical or enzyme catalyzed instability. Compounds possessing
six substituents found in unstable metabolites were selected and tested to establish the limitation
of the simple LC/MS/MS methodology for relative exposure comparison across species. The
results indicated that the abbreviated LC/MS/MS peak area ratio comparison could be applied to
metabolites possessing epoxide, amide, catechol, and acyl glucuronide groups, but it is not
suitable for esters and iminium ions. Stability of the suspected unstable metabolites and the
degradation impact on downstream metabolites can be assessed using the methodology described
in this work. It will be important to further test this approach with more examples of potentially
unstable metabolites.
Authorship Contributions:

*Participated in research design:* Gao and Obach.

*Conducted Experiments:* Gao and Obach.

*Contributed new reagents or analytical tools:* Not Applicable

*Performed data analysis:* Gao and Obach

*Wrote or contributing to the writing of the manuscript:* Gao and Obach.
References:


across species for metabolite safety assessments. *Drug Metabolism and Disposition* 38(12): 2147-2156.


Leclercq, Laurent; Cuyckens, Filip; Mannens, Geert S. J.; de Vries, Ronald; Timmerman, Philip; Evans, David C. (2009) Which Human Metabolites Have We MIST? Retrospective Analysis, Practical Aspects, and Perspectives For Metabolite Identification and Quantification in Pharmaceutical Development. *Chemical Research in Toxicology* 22(2): 280-293.


Walker, Don; Brady, Joe; Dalvie, Deepak; Davis, John; Dowty, Martin; Duncan, J. Neil; Nedderman, Angus; Obach, R. Scott; Wright, Pat. (2009) A Holistic Strategy for Characterizing the Safety of Metabolites through Drug Discovery and Development. *Chemical Research in Toxicology* 22(10): 1653-1662.


Zgoda-Pols, Joanna; Chowdhury, Swapan; Alton, Kevin. Method Development for Sample Processing and Metabolite Profiling by LC-MS during Drug Development. *58th ASMS conference proceedings* (2010), Salt Lake City, Utah.
Figure Legend

Figure 1. Structures of parents and metabolites.

Figure 2. TICs of untreated vs. stabilized naproxen acyl glucuronide conjugate at day 1 vs. day 130 stored at -20 °C freezer. a. TIC of untreated human sample 2 at day 1, b. TIC of untreated human sample 2 at day 130, c. TIC of treated human sample 2 at day 130.

Figure 3. Storage stability test for naproxen acyl glucuronide conjugate. ■ Response ratio of stabilized naproxen acyl glucuronide conjugate vs. naproxen normalized by day 1 response ratio; ♦ Response ratio of untreated naproxen acyl glucuronide conjugate vs. naproxen normalized by day 1 response ratio. Error bars were standard deviation of measurements in 4 rat and 4 human plasma samples.

Figure 4. Ratio measurement errors of three independent analysis for drug and metabolites (singlet analysis for nicotine iminium ion and cotinine combination) in rat vs. human plasma, n=16. ■ Test 1 at day 1, ■ Test 2 at day 30-90, ■ Test 3 at day 100-180.
Table 1. Mass spectrometer settings and LC conditions used in the analysis of parent compounds and metabolites

<table>
<thead>
<tr>
<th>Parent/Metabolite</th>
<th>Biotransformation Reaction(s)</th>
<th>LC conditions</th>
<th>Q1</th>
<th>Q3</th>
<th>CE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naproxen</td>
<td>--</td>
<td>LC1</td>
<td>229</td>
<td>169</td>
<td>-40</td>
</tr>
<tr>
<td>Naproxen acyl glucuronide</td>
<td>Glucuronidation</td>
<td></td>
<td>405</td>
<td>229, 185, 169†</td>
<td>-40</td>
</tr>
<tr>
<td>Carbamazepine</td>
<td>--</td>
<td>LC1</td>
<td>237</td>
<td>194†, 117</td>
<td>27,43</td>
</tr>
<tr>
<td>Carbamazepine-10,11-epoxide</td>
<td>Oxidation</td>
<td></td>
<td>253</td>
<td>210†, 180</td>
<td>27, 43</td>
</tr>
<tr>
<td>Salicylic acid</td>
<td>--</td>
<td>LC2</td>
<td>137</td>
<td>109, 93†</td>
<td>-26</td>
</tr>
<tr>
<td>2,3-Dihydroxybenzoic acid</td>
<td>Oxidation</td>
<td></td>
<td>153</td>
<td>109†</td>
<td>-26</td>
</tr>
<tr>
<td>Sulfadiazine</td>
<td>--</td>
<td>LC1</td>
<td>251</td>
<td>156, 108†</td>
<td>27, 35</td>
</tr>
<tr>
<td>N-Acetyl sulfadiazine</td>
<td>N-Acetylation</td>
<td></td>
<td>293</td>
<td>185, 134†, 108</td>
<td>27, 35, 35</td>
</tr>
<tr>
<td>Methylphenidate</td>
<td></td>
<td>LC1</td>
<td>234</td>
<td>128†, 115</td>
<td>65, 69</td>
</tr>
<tr>
<td>Ritalinic acid</td>
<td>Hydrolysis</td>
<td></td>
<td>220</td>
<td>128†, 115</td>
<td>65, 69</td>
</tr>
<tr>
<td>Nicotine Δ1’,5’-iminium ion</td>
<td>--</td>
<td>LC3</td>
<td>161</td>
<td>130†, 118</td>
<td>35</td>
</tr>
<tr>
<td>Cotinine</td>
<td>Oxidation</td>
<td></td>
<td>177</td>
<td>118, 98, 80†</td>
<td>35</td>
</tr>
</tbody>
</table>

† Daughter ions were used in MRM transitions for peak area ratio measurements
Table 2. Auto Sampler Stability Test of Unstable Compounds

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Unstable substituents</th>
<th>Untreated</th>
<th>Treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naproxen acyl glucuronide conjugate</td>
<td>Acyl glucuronide</td>
<td>0.988 ± 0.158</td>
<td>0.982 ± 0.189</td>
</tr>
<tr>
<td>2,3-dihydroxybenzoic acid</td>
<td>Catechol</td>
<td>1.07 ± 0.08</td>
<td>1.07 ± 0.13</td>
</tr>
<tr>
<td>N-Acetyl sulfadiazine Amide</td>
<td>Amide</td>
<td>0.827 ± 0.176</td>
<td>NA</td>
</tr>
<tr>
<td>Carbamazepine-10,11-epoxide</td>
<td>Epoxide</td>
<td>0.771 ± 0.198</td>
<td>NA</td>
</tr>
<tr>
<td>Nicotine iminium ion Iminium ion</td>
<td>Iminium ion</td>
<td>0.95 ± 0.20</td>
<td>NA</td>
</tr>
<tr>
<td>Methylphenidate</td>
<td>Ester</td>
<td>0.303 ± 0.132</td>
<td>0.683 ± 0.123</td>
</tr>
</tbody>
</table>

Average ratio of (unstable structure response /stable reference) overnight/day 1 response ratio ± Standard deviation, n=8. ND: not detected, NA: not applicable. A ratio between 0.8-1.2 is deemed stable.
Table 3. Long Term Storage Stability Test for Unstable Compounds

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Test 1 (Day 1)</th>
<th>Test 2 (Day 30-90)</th>
<th>Test 3 (Day 100-180)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naproxen acyl glucuronide (Treated)</td>
<td>1</td>
<td>1.2 ± 0.25</td>
<td>0.84 ± 0.28</td>
</tr>
<tr>
<td>Naproxen acyl glucuronide (Untreated)</td>
<td>1</td>
<td>0.766 ± 0.13</td>
<td>0.531 ± 0.25</td>
</tr>
<tr>
<td>2,3-dihydroxybenzoic acid (Treated)</td>
<td>1</td>
<td>1.00 ± 0.06</td>
<td>1.02 ± 0.06</td>
</tr>
<tr>
<td>2,3-dihydroxybenzoic acid (Untreated)</td>
<td>1</td>
<td>1.09 ± 0.07</td>
<td>0.88 ± 0.05</td>
</tr>
<tr>
<td>Carbamazepine-10,11-epoxide</td>
<td>1</td>
<td>1.14 ± 0.04</td>
<td>1.00 ± 0.39</td>
</tr>
<tr>
<td>N-Acetyl sulfadiazine</td>
<td>1</td>
<td>0.54 ± 0.095</td>
<td>0.599 ± 0.117</td>
</tr>
<tr>
<td>Methylphenidate (Treated)</td>
<td>1</td>
<td>1.24 ± 0.40</td>
<td>1.33 ± 0.55</td>
</tr>
<tr>
<td>Methylphenidate (Untreated)</td>
<td>1</td>
<td>0.893 ± 0.145</td>
<td>0.466 ± 0.171</td>
</tr>
<tr>
<td>Nicotine Iminium ion</td>
<td>1</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

Average ratio of (unstable structure response /stable reference) at test day/day 1 response ratio ± standard deviation, n=8, NA: not applicable. A ratio between 0.8-1.2 is deemed stable.
<table>
<thead>
<tr>
<th>Parent Metabolite Potential Instability</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Parent</strong></td>
</tr>
<tr>
<td>Naproxen</td>
</tr>
<tr>
<td>Carbamazepine</td>
</tr>
<tr>
<td>Salicylic Acid</td>
</tr>
<tr>
<td>Sulfadiazine</td>
</tr>
<tr>
<td>Methylphenidate</td>
</tr>
<tr>
<td>Nicotine</td>
</tr>
</tbody>
</table>

**Figure 1**
Figure 2

(a) Naproxen acyl glucuronidate

(b) Migrated naproxen acyl glucuronidate

(c) Naproxen