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Metabonomics in Toxicology: A Review

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Metabonomics and its many pseudonyms (metabolomics, metabolic profiling, etc.) have exploded onto the scientific scene in the past 2 to 3 years. Nowhere has the impact been more profound than within the toxicology community. Within this community there exists a great deal of uncertainty about whether metabonomics is something to count on or just the most recent technological flash in the pan. Much of the uncertainty is due to unfamiliarity with analytical and chemometric facets of the technology and the attendant fear of any "black-box." With those fears in mind, metabonomics technology is reviewed with particular emphasis on toxicologic applications in preclinical drug development. The jargon, logistics, and applications of the technology are covered in some detail with emphasis on recent work in the field.

Key Words: **metabonomics; metabolomics; biomarkers; mechanisms.**

I. THE NATURE OF THIS REVIEW

Metabonomics and metabolomics have been the subject of numerous reviews in recent years (Bino *et al.*, 2004; Fernie *et al.*, 2004; Fiehn, 2002; Goodacre *et al.*, 2004; Lindon *et al.*, 2003a, 2004b,c; Nicholson *et al.*, 1999; Nicholson and Wilson, 2003; Reo, 2002; Weckwerth, 2003), and a volume on metabolic profiling was published in 2003 (Harrigan and Goodacre, 2003). Even within the specific arena of the toxicological applications of metabonomics a great deal has been written (Griffin, 2003; Lindon, 2003b, 2004d; Nicholson *et al.*, 2002; Robertson *et al.*, 2002; Shockcor and Holmes, 2002). So what is different about this review? This review will focus on toxicology applications of metabonomics technology, as written by a practicing toxicologist for practicing toxicologists. The particular focus is on those who may be considering utilization of the technology either in-house or via purchase of contract research organizations (CRO) services. Specifically, the review spotlights the application of the technology within the pharmaceutical industry, as that is where the technology has had its greatest impact (from a toxicology perspective). The hope is that such a focused review will lend clarity to the discussion and make obvious those issues of greatest concern to the toxicologist.

II. METABONOMICS OR METABOLOMICS?

Before any discussion of metabonomics is initiated, some explanation (though unfortunately not resolution) of a significant source of confusion must be attempted. *What is the difference between metabonomics and metabolomics, and when is the use of either term appropriate?* The answer, unfortunately, is that, depending on whom you read or talk to, both terms may be appropriate in most cases, and the distinctions are more a matter of historical usage than meaningful scientific definition. As the reader may divine by the use of the word "metabonomics" in the title of this review—the author has his own bias. However, it is based on the fact that metabonomics was coined and defined by its Greek roots, rather than by simple concatenation. Metabonomics is defined as "the quantitative measurement of the time-related multi-parametric metabolic response of living systems to pathophysiological stimuli or genetic modification" (Nicholson *et al.*, 1999). The actual term was devised by Jeremy Nicholson, Elaine Holmes, and John Lindon of Imperial College (London) from the Greek roots "meta" (change) and "nomos" (rules or laws) in reference to chemometric models that have the ability to classify changes in metabolism (Lindon *et al.*, 2004a). The origin of the word metabolomics is a bit murky. While the concept of the metabolome has been in existence for some time as part of metabolic control theory and flux analysis (Cornish-Bowden, 1989; Derr, 1985), it was not routinely used in publications until the late 1990s (Tweeddale *et al.*, 1998). While not expressly defined, the term metabolomics was indicated by Fiehn (2001) to be the "comprehensive and quantitative analysis of all metabolites. . ." It will be left to the reader to divine the difference between those definitions. Compounding the naming convention problem is the fact that metabonomics and metabolomics have been described as subsets of each other (Fiehn, 2001; Lindon *et al.*, 2003a). As of now, appropriate

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omic term choice is a bit like the Betamax/VHS argument, both have legitimate claims of superiority but the user community will ultimately decide—at least until the metabonomic equivalent of the DVD comes along, anyway. The term ''metabonomics'' will be employed in this review, with the realization that either term may be acceptable.

III. WHY OMICS?

Perhaps forgotten in our rush to generate novel omics terms is that the progenitor omics—genomics—was derived from the term genome which was derived from a combination of the words gene and chromosome to indicate the complete set of chromosomes and the genes contained in them. What may be surprising was that the term was coined in the 1920s—well before the structure of DNA was even understood (McKusick, 1997). The use of genomics as a term followed much later in the mid-1980s and was, at the time, the subject of much debate. In that light, transcriptome and proteome (and the derivative omics) may be of dubious etymological origins, but they are in common usage. An omics technology has come to mean an approach capable of generating a comprehensive data set of whatever is being measured, be it transcripts (transcriptomics), proteins (proteomics), or metabolites (metabonomics). Why should toxicologists care what terms we use? The understanding one brings to the terms impacts the perception and expectations of the technology. When considering omics in general, an obvious question is when does measurement of a few parameters within a molecular family graduate to the omic level. Biofluid nuclear magnetic resonance spectroscopy (NMR) has been around for about as long as NMR instrumentation has been around (Gadian, 1982; Lindon, 1999). What differentiates metabonomics or metabolomics from biofluid NMR (or biofluid mass spectrometry (MS) for that matter)? The difference lies in what is known about the parameters being measured and the expectation of what the data will reveal. Biofluid NMR analysis is typically done with *a priori* knowledge of what a parameter is and what it reflects. It brings with it the expectation that a measurement or combination of several measurements reveals something about a specific target. In an omic technology, one need not know what every parameter is (think expressed sequence tags (ESTs), surface-enhanced laser desorption-ionization (SELDI) peaks, or NMR resonances) or what it indicates to obtain useful information (not that such parameters aren't important!). The expectation is that the response pattern of numerous analytes is reflective of a change in physiology indicative of efficacy, toxicity, disease, or physiological change, and the comprehensive nature of the data set enables an *in toto* evaluation of systemic response. This pattern may be (and should be) searchable for specific analyte information to provide mechanistic relevance, but the pattern itself is useful. In other words, the omics are tools for studying and understanding systems biology (Goodacre *et al.*, 2004). A systems response would be difficult, if not impossible, to decipher from the results of any one analyte measurement. These distinctions are important for toxicologists attempting to manage expectations as to what can be learned from metabonomics. If one has a specific analyte or a specific target organ in mind, metabonomics is probably not the best approach. On the other hand, if biomarker discovery (not necessarily validation) or hypothesis generation is an endpoint, metabonomics may be an excellent choice.

IV. WHICH PLATFORM?

A mistake the uninitiated frequently make is to assume metabonomics deals primarily with NMR-derived data, while metabolomics most appropriately describes mass spectrometry (MS)-derived data. As can be gleaned from the definitions given above, this is not the case. This misunderstanding is most likely attributable to the history of the terms, as metabolomics was historically used by plant scientists using MS platforms and metabonomics was used by the Imperial College group and adherents who almost exclusively used NMR for most of their early work. This is currently changing as ''metabonomic studies'' based on the MS platform and ''metabolomic studies'' based on NMR are both present in the literature (Plumb *et al.*, 2002; Reo, 2002). Indeed, specific platforms are not a prerequisite for metabonomic investigations, so in theory, any technique capable of generating comprehensive metabolite measurements can be used for metabonomics. However, while other technologies are now being utilized, mass spectrometry (MS) in its various hyphenated derivations (liquid chromatography-MS, gas chromatography-MS, etc.) and NMR platforms dominate the metabonomic literature. So of these two platforms, which platform is better for metabonomics applications in toxicology? The answer, in true Solomonic fashion, is ''it depends.'' Table 1 provides a comparison of the two analytical platforms toxicologists may find useful when considering their potential for metabonomics analyses. Preparation of such a table is always a risky enterprise, as proponents of a particular technology will dispute every point. Indeed, depending on the specific NMR or MS configuration, almost every line on the table is debatable. However, the intent of the table is not to steer the reader in one direction or another, but rather to guide the discussions toxicologists may have with their local MS or NMR support groups, who will undoubtedly argue the undeniable superiority of their platform. The truth of the matter is, most groups who are seriously pursuing metabonomics are currently or soon will be using both platforms. The sensitivity advantage of MS is undeniable and makes it indispensable for some work, particularly if novel biomarkers are being sought out. On the other hand, the nonselectivity, lack of sample bias, and cross-laboratory/cross-platform reproducibility of NMR (Keun *et al.*, 2002) is extremely important for toxicologists considering screening applications.

TABLE 1
Comparison of NMR Versus Mass Spectrometry for Metabonomics Applications

<table>
<tr><th></th><th>NMR</th><th>Mass Spec</th></tr>
<tr><td>Logistics</td><td></td><td></td></tr>
<tr><td>Capital cost</td><td colspan="2">no advantage</td></tr>
<tr><td>Routine operating costs</td><td colspan="2">no advantage</td></tr>
<tr><td>Maintenance</td><td>advantage</td><td></td></tr>
<tr><td>Per sample cost</td><td colspan="2">no advantage</td></tr>
<tr><td>Footprint</td><td colspan="2">no advantage</td></tr>
<tr><td>Required technical skill[a]</td><td></td><td>advantage</td></tr>
<tr><td>Instrument “up-time”</td><td>advantage</td><td></td></tr>
<tr><td>Instrument life-span</td><td colspan="2">no advantage</td></tr>
<tr><td>Analytical considerations</td><td></td><td></td></tr>
<tr><td>Sensitivity</td><td></td><td>big advantage</td></tr>
<tr><td>Reproducibility (within lab)</td><td>advantage</td><td></td></tr>
<tr><td>Reproducibility (across labs)</td><td>big advantage</td><td></td></tr>
<tr><td>Quantitation</td><td>big advantage</td><td></td></tr>
<tr><td>Average run speed</td><td colspan="2">no advantage</td></tr>
<tr><td>Capacity (samples/day)</td><td colspan="2">no advantage</td></tr>
<tr><td>Sample preparation requirements</td><td>advantage</td><td></td></tr>
<tr><td>Sample analysis automation</td><td>advantage</td><td></td></tr>
<tr><td>Versatility[b]</td><td>advantage</td><td></td></tr>
<tr><td>Selectivity[c]</td><td></td><td>advantage</td></tr>
<tr><td>Nonselectivity[c]</td><td>advantage</td><td></td></tr>
<tr><td>Metabonomics</td><td></td><td></td></tr>
<tr><td>Resolvable metabolites</td><td></td><td>big advantage</td></tr>
<tr><td>Identification of unknowns</td><td></td><td>advantage</td></tr>
<tr><td>Potential for sample bias[d]</td><td>big advantage</td><td></td></tr>
<tr><td>Data analysis automation</td><td></td><td>advantage</td></tr>
</table>

[a]Pool of qualified analysts is much smaller for NMR than MS.
[b]Generally any NMR instrument can be configured for most applications while different MS instrumentation may be required for specific applications.
[c]MS excels at selective identification of a molecular entity, while NMR excels at identification of all proton containing species in a sample. Therefore, selectivity can be an advantage or disadvantage depending on the nature of the application.
[d]Potential for misleading, incomplete or nonreproducible data set due to bias inherent to the technology (e.g., ion suppression in MS).

V. MAGIC ANGLE SPINNING (MAS)

A clear advantage of the NMR platform is that it enables the potential for intact tissue metabonomics. Magic angle spinning (MAS) is an NMR technique in which a small quantity of intact tissue can be placed in the spectrometer and analyzed. It was not included in Table 1 as an advantage for the NMR platform, because it requires specialized equipment and expertise to be conducted and, hence, has received much less attention than traditional biofluid NMR for metabonomics studies. Extracts of tissues can and have been routinely studied using biofluid NMR techniques. However, with any extraction procedure, questions remain about how representative the extract is of the original sample and the potential for secondary (nonbiological) reactions induced by the extraction procedure. MAS spectroscopy provides a path around these limitations. The term “Magic Angle” is derived from the fact that, when samples are spun rapidly at 54.7° relative to the applied magnetic field (the so-called “magic angle”), line-broadening effects that would ordinarily obfuscate a proton spectra of a solid sample, can be reduced (Shockcor and Holmes, 2002). In practice, the technique is carried out by placement of a few milligrams of intact tissue into a specially designed rotor, which is spun at high speed within the bore of the magnet. While MAS will not be a rapid-throughput procedure in the near future, it can and is serving as a tool for linking biofluid changes to mechanism of action in target tissues. This is particularly important when products of intermediate metabolism dominate observed changes in biofluids and are difficult if not impossible to link to any specific target organ. Several nice examples of the application of MAS technology integrated with more traditional biofluid metabonomics have been recently published (Coen *et al.*, 2003, 2004; Garrod *et al.*, 2001; Waters *et al.*, 2000, 2001, 2002). It is clear from these examples that the use of MAS is synergistic with biofluid metabonomics with regard to identifying and understanding basic mechanisms of toxicity.

VI. CHEMOMETRICS

No review of metabonomics technology can be complete without at least some mention of chemometrics. Any toxicologist considering metabonomics needs to give adequate thought to chemometrics support. It is a frequently overlooked resource requirement for those just starting out in the technology. Chemometrics support is second only to analytical support for the toxicologist when considering what resources he or she needs to initiate research in metabonomics. Frequently chemometrics support is most available in analytical chemistry groups, because that is where the field is derived from. This review can only touch on the subject, because the arcana of neural networks, k-nearest neighbor clustering, and the like is a surefire cure for insomnia. As with many aspects of the omic sciences, we must first dispense with some terminology. What is the difference between chemometrics and bioinformatics? Chemometrics can be broadly thought of as the application of mathematical and statistical methods to chemistry (Deming, 1986; Lavine and Workman, 2004). In the context of NMR- or MS-based metabonomics, it would include any mathematical or statistical tool used for spectral processing, peak alignment, outlier detection, normalization, etc. (Duran *et al.*, 2003; Farrant *et al.*, 1992). The science of bioinformatics, on the other hand, involves the storage, retrieval, and analysis of computer-stored information in biological research (Bains, 1996). This has come to include everything from molecular biologists who can interpret the meaningfulness of basic local alignment search tool (BLAST) searches to code writers developing search algorithms (Gershon *et al.*, 1997). There is of course some overlap, particularly

when databases are being generated from processed spectra rather than annotated metabolite lists. Much of the early chemometric work within metabonomics was done with the goal of doing just that—using the entire spectrum as a data source rather than limiting analysis to just those peaks that could be positively identified (Anthony *et al.*, 1995b; Holmes and Antti, 2002; Holmes *et al.*, 1992a,b, 1994, 1998, 2000; Lenz *et al.*, 2004b; Spraul *et al.*, 1994; Stoyanova *et al.*, 2004). This work uses and expands on well-known pattern recognition techniques, greatly amplifying the power of the individual spectrum, since the entire data spectrum can be utilized and is not limited to only those small areas of the spectrum that are readily annotated (Ebbels *et al.*, 2003; el-Deredy, 1997; Keun *et al.*, 2004). It is this pattern recognition approach that drove the establishment of the COMET consortium (Lindon *et al.*, 2003b). The beauty of the approach is that individual metabolite identification is not sacrificed to pursue patterns. Like other omic data, the patterns provide one level of information, but the spectra can be drilled down into to obtain component identification, which, in most cases, if done in sufficient detail, will be as important as, or more important than the pattern data. However, the cost of those identifications can be large in terms of instrument time and man-hours. A survey of all multivariate statistical methods that have been used in metabonomics studies could be the subject of a review all by itself. Therefore, individual approaches will not be discussed in detail with one exception. Principal component analysis (PCA) has become a routine statistical method for use in many types of omic data, and hence a brief description is warranted.

A typical NMR metabonomics study can generate hundreds of biofluid samples and, hence, hundreds of NMR spectra. Examining each spectrum individually can be a daunting exercise even for the trained spectroscopist. Tools have been developed or borrowed from other fields for assessing large numbers of NMR spectra in a relatively rapid fashion. PCA is one such tool that has been borrowed for metabonomics to such a point that PCA cluster plots, also known as ‘‘scores plots,’’ have become iconic of the metabonomics publication or presentation. Masses of spectral data, such as that generated by NMR, can be thought of in terms of a multivariate statistical problem. The true variables are the metabolite concentrations. In practice, pseudo-variables are generated by integrating the spectral data into discrete regions about the width of spectral peaks associated with metabolites. The integrated area under the curve of each of these regions (referred to as ‘‘bins’’ or ‘‘buckets’’) is calculated, and these values serve as variables. A 0.04 ppm-wide region is a typical bin width which will produce 200 to 250 ‘‘buckets’’ of data from the typical 10 PPM NMR spectrum. Certain regions of the spectrum, such as those containing water and urea resonances (for urine), are typically excluded from the binning process. In the case of NMR spectra of biofluids, it can be expected that subsets of variables will be highly correlated with each other, because molecules may have more than one spectral peak and, hence, contribute to more than one bucket or variable. Principal component analysis (PCA) is a statistical method that reduces a great number of variables (usually correlated) into a smaller number of uncorrelated variables, which are called principal components. In short, PCA is a decomposition of the raw data into ‘‘scores’’ that indicate the relationship between samples and ‘‘loadings,’’ which indicates the relationships (correlations) between the variables. The first principal component explains the greatest variability in the data, the second principal component is independent of (orthogonal to) the first component and second best explains the variability of the data and so on. The goal of the exercise is dimensional reduction, while sacrificing as little accuracy as possible. The analysis itself can be conducted using commercial multivariate statistical software available from several vendors. As used in a typical metabonomics study, a 200- to 250-variable set representing one spectrum is reduced to two or three variables, which can be represented as a single point in two- or three-dimensional plots, respectively. It is these plots that one typically sees in publications or presentations of metabonomics data. Whether the preceding paragraph is clear or not, the important point for the toxicologist to remember is that each point on a metabonomics PCA scores plot represents all the data contained in one spectrum. Sample points that cluster together have more similar spectra (and hence more similar biochemical makeup) than things that cluster apart. PCA plots are extremely powerful for rapid identification of inherent clusters in the data (which may be suggestive of a common effect or mechanism), assessment of dose-related and time-related changes, and the identification of individual outliers. However, by themselves, the scores plots add little to biomarker identification, provide little mechanistic insight on a molecular basis, and say nothing about the toxicological significance of the clusters. However, the PCA data can be examined in more detail by examining the loadings to find out which variable relationships are responsible for the loadings. After identifying the variables that are primarily responsible for separation, spectral regions can be identified and specific molecules postulated. PCA is only one of many statistical tools that can be used for metabonomics study. The scope and nature of these tools is beyond the extent of this review, and the reader is referred to any of the above-cited references for guidance on these techniques.

VII. OTHER APPLICATIONS OF METABONOMICS

While this review will focus on the preclinical application of metabonomics, it would do the technology a great disservice to ignore all applications outside the preclinical arena. In particular, three broad application areas need be mentioned: (A) environmental applications, (B) clinical applications, and (C) biomedical applications (other than clinical and toxicologic applications).

A. Environmental Applications

Some of the most significant efforts in the area of metabolic profiling have been made in the area of the botanical sciences (Fiehn *et al.*, 2000a,b, 2001; Fiehn and Weckwerth, 2003; Roberts, 2000; Roberts and Jardetzky, 1981; Roberts and Xia, 1995; Trethewey *et al.*, 1999; Weckwerth *et al.*, 2004). Though this may seem an odd literature for toxicologists to spend their time perusing, the tools and techniques used by these researchers are quite powerful and applicable to any biological investigation. Of particular note are the efforts of these investigators to bring metabolic control and flux analysis into their experimental design and interpretation. Their experiments serve as a harbinger of where mammalian metabonomics efforts will soon be headed.

Beyond plants, metabonomics technology has made significant inroads into the environmental research community. The environmental applications of metabonomics have been recently briefly reviewed (Viant *et al.*, 2003), and the diversity of work is fascinating. Some of the most interesting work in this area has been conducted in earthworms, where metabonomics has been shown to be useful for speciating worms by phenotype, a typically difficult task (Bundy *et al.*, 2002c), and for monitoring exposure to environmental chemicals or other physiologic disruption (Bundy *et al.*, 2002a,b, 2001, 2002c, 2003; Warne *et al.*, 1999).

In the marine world, metabonomics has been shown to differentiate between normal, stunted, and diseased abalone (Viant *et al.*, 2003) and the embryonic stages of the Japanese *Medaka* (Viant, 2003). Mammals other than laboratory species have not been ignored. Renal MAS and urine biofluid assessment of wood mouse, white-toothed shrew, and bank vole have been recently compared to the laboratory rat and inferences made with regard to the findings and varied metabolic processes between the species (Griffin *et al.*, 2000).

B. Clinical Applications

For the toxicologist, clinical application of metabonomic technology may be as important as, if not more important than any preclinical work in which he or she may become involved. After all, the human population is the intended target for almost all their efforts (we can't forget about the veterinary market!). Ideally, techniques developed to identify safety concerns preclinically, would be readily transferable to the clinic. One of the great strengths of metabonomics technology is that the use of urine as a primary sample enables noninvasive monitoring of both efficacy and toxicity endpoints.

Clinical application of NMR technology has a long and storied history (Andrew, 1984). Lindon (Lindon *et al.*, 1999) reviews a series of approximately 40 human inborn errors of metabolism that have been studied using NMR techniques over the past 20 years. To some extent, the questions raised earlier now become apparent. Is there a difference between biofluid NMR and metabonomics, and when does the former graduate to the latter? Lindon *et al.* (1999) reports on work conducted by Foxall (Foxall *et al.*, 1993) and Le Moyec (Le Moyec *et al.*, 1993) on an interesting case that may serve as a transition between traditional clinical NMR and clearly ascribed clinical metabonomics applications (of course, it is debatable whether any clinical application of NMR can be called traditional, but that is another story). A significant clinical problem with transplant patients is differentiating between patients undergoing graft rejection and those suffering from cyclosporin toxicity. Cyclosporin is an immunosuppressive drug frequently given to transplant patients, and the clinical presentation of the toxicity can look very similar to graft rejection. To address this problem, urine was collected from patients undergoing kidney transplants that were given cyclosporin and monitored by NMR spectroscopy. The use of NMR coupled with pattern recognition techniques and trajectory analysis could clearly differentiate when a patient was undergoing graft rejection versus succumbing to cyclosporin. This may be considered a true clinical metabonomic application, because the spectral pattern and trajectory change were what was used to differentiate the toxicity, not any specific biochemical marker. This is not to say that, had a unique biomolecule been associated with either graft rejection or cyclosporin toxicity, it couldn't be used in isolation, but that isolation and identification of such a unique biomarker was not necessary to gain important and clinically relevant information.

Several recent publications demonstrate quite convincingly the power of clinical metabonomics. While earlier studies on inborn errors of metabolism focused on molecular identification of the relevant metabolic pathways (Holmes *et al.*, 1997), a more recent effort details methods for rapid identification of inborn errors of metabolism using pattern recognition techniques (Constantinou *et al.*, 2004). Even more compelling was recent work demonstrating that metabonomics could be used to rapidly and noninvasively assess the severity of coronary heart disease in a clinical population (Brindle *et al.*, 2002). The same group demonstrated a relationship between serum metabolic profiles and hypertension (Brindle *et al.*, 2003). Metabonomic patterns have also been shown to be useful in the diagnosis of interstitial cystitis (IC), having the ability to differentiate IC from bacterial cystitis in a clinical population with a success rate of approximately 84% (Van *et al.*, 2003). Beyond disease diagnoses, metabonomics has also been shown to be an effective tool for assessing lifestyle markers of health, particularly related to nutritional variation (German *et al.*, 2003a,b; Noguchi *et al.*, 2003; Teague *et al.*, 2004).

An oft-raised concern about the use of metabonomics in clinical trials is the inherent variability of the clinical population. Given that metabonomics can identify even the slightest variation in animal studies, what might we expect from the clinical population where genetic and environmental factors (including dietary) can only be minimally controlled? An answer to that question was recently published by Lenz

et al. (2003), who demonstrated that both urine and plasma could be reliably collected for metabonomic analyses in well-controlled clinical studies.

It can be anticipated that the rate of expansion of clinical applications of metabonomic technology will probably increase at an even greater pace than preclinical applications. The costs and difficulties associated with conducting clinical studies demand that more efficient, comprehensive tools be made available, so that greater levels of information can be gained from costly clinical trials without increasing the level of discomfort to the patient or decreasing the practicality to the physician. Metabonomics can meet both those requirements.

C. Biomedical Applications

There are a number of biomedical metabonomic applications that fall outside the realm of clinical and toxicology applications. For example, a recent review documents the significance of metabolic profiles of cancer cells as a tool for understanding tumor development and progression (Griffin and Shockcor, 2004). Since metabonomics, by definition, will describe a biochemical phenotype of whatever living system is being evaluated, an obvious application of the technology is generating strain phenotypes from either experimentally altered genotypes (e.g., transgenic models) or those derived by selective breeding (congenic, coisogenic, consomic, etc.). Early work demonstrated the biochemical differences of two albino rat strains commonly used in the pharmaceutical industry, the Han Wistar and Sprague–Dawley rat (Holmes *et al.*, 2001). The technology also answered the age-old question of how you tell a white mouse (AlpK:ApfCD) from a black mouse (C57BL/10J). So important was the question that it was answered using both NMR (Gavaghan *et al.*, 2000) and MS (Plumb *et al.*, 2003) based platforms. More recent work demonstrated that metabonomic assessment of brain extracts was able to distinguish phenotypic differences in a transgenic mouse model of spinocerebellar ataxia as compared to the background C57BL/6J strain (Griffin *et al.*, 2004).

The role of the gut microflora in metabolism and toxicity, though well recognized (Boxenbaum *et al.*, 1979; Coates, 1975; Eyssen, 1973; Gibson, 1998; Gonthier *et al.*, 2003; Rowland, 1981, 1988; Upreti *et al.*, 2004; van der Waaij, 1991) has not been seriously evaluated at the omic level. Recently Nicholson and Wilson emphasized the role of the gut microflora in understanding systemic response to drug or toxins at both the level of metabolism and pathophysiology (Nicholson and Wilson, 2003). This is particularly true for anyone trying to understand a metabolic response via metabonomic technology. An interesting amplification of this concept was conducted by Nicholls *et al.*, who followed the urinary metabolic profiles as axenic rats adapted to normal gut microflora in the laboratory environment (Nicholls *et al.*, 2003).

Other recently reported applications of metabonomics include the identification of a unique biomolecular signature associate with a parasite infection in mice (Wang *et al.*, 2004) and metabonomic assessment of adrenal lipids in the hypoxic neonatal rat (Bruder *et al.*, 2004).

VIII. METABONOMICS AND TOXICOLOGY

To date, metabonomics has had perhaps its greatest impact in the area of toxicology, particularly in preclinical toxicology. This is not to say that will always be the case. As indicated above, use of the technology for clinical endpoints is rapidly expanding and may eventually surpass preclinical applications in overall impact. The visibility of toxicological applications should not be surprising. As has been noticed by other omics practitioners, toxicity studies typically generate clearly definable endpoints (mortality, clinical signs, clinical chemistry abnormalities, and/or histopathology) that are usually quite obvious and readily understood. Additionally, in many situations (but certainly not all) these endpoints can be arrived at fairly quickly (7 days or less) using available techniques and equipment. This makes them ideal for the correlative work attempting to associate metabolites (or genes and proteins, for that matter) with endpoints. From these correlations, hypotheses can be generated and, in some cases, be fairly easily tested. In contrast, *in vivo* pharmacology models (for efficacy) seldom have as many robust (and dramatic) endpoints for correlation, are frequently long term (2 weeks or more), and in most cases, require some additional pharmacologic or surgical modifications that would cloud any interpretation of omic data. This is not to say omic technologies are being any less utilized by pharmacologists, but that, in many people's eyes, toxicity studies are easier for demonstrating impact.

The disadvantage of toxicity models is that toxicities seldom are as "clean" as some literature may suggest. Classical target organ toxins frequently are only classic because most of the literature has focused on one target organ (e.g., CCl_4 and hepatic toxicity). While the literature usually correctly identifies the primary target of a toxin (i.e., dose-limiting toxicity), what are frequently missed are all the other systemic effects that are produced by the compound. The omic technologies, and metabonomics is no exception, will not let you forget about other targets. Too often we attempt to associate omic findings with what we know of a compound, which may or may not be indicative of all that is happening. For example if we know a compound is hepatotoxic, we make inferences that the gene, protein or metabolite changes we are seeing must in some way be reflective of the hepatic effect. However, what if the drug is also producing inappetence? As mentioned earlier, that effect may be more profound from an omic standpoint (number of genes, proteins and metabolites affected) than is the hepatic toxicity.

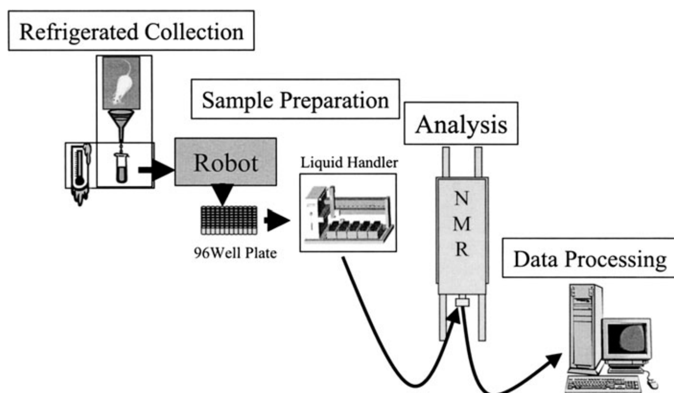

FIG. 1. Schematic diagram of typical metabonomics set—up for preclinical toxicity studies in rats.

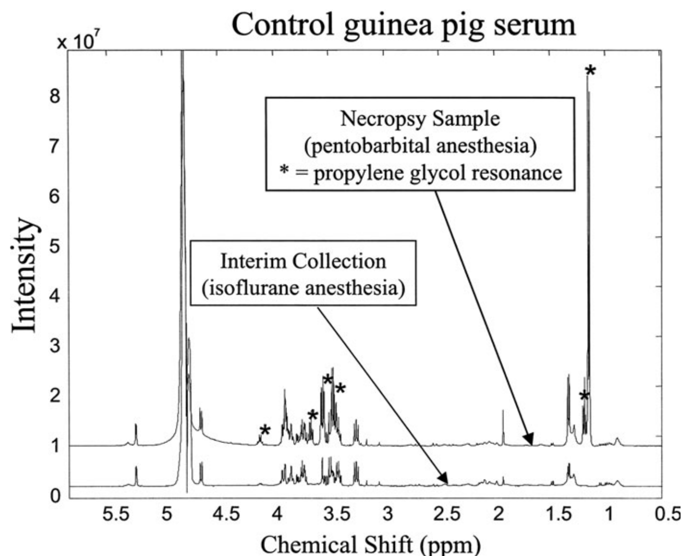

FIG. 2. Comparative 600 MHz ^{1}H-NMR spectra from serum samples from guinea pigs anesthetized with isoflurane (bottom trace) or parenteral pentobarbital containing 40% propylene glycol by volume (top trace). Note the numerous resonances attributable to the propylene glycol in the injected anesthetic.

A. Logistical Considerations

A schematic representation of the logistical steps necessary for conducting metabonomics studies is given in Figure 1. It is beyond the scope of this review to detail the physical and logistical requirements for the analytical instrumentation and support necessary for metabonomics technology. It is hoped that the toxicologist will seek out appropriate support and expertise for these functions, because trying to start them from scratch would be a daunting and expensive proposition. A summary of the NMR requirements for metabonomic studies can be found in several places (Lindon *et al.*, 2004a,d; Robertson *et al.*, 2002). Regardless of the specific details, three general requirements, common to both the MS and NMR platforms that any toxicologist needs to keep in mind when pursuing metabonomic technology are (1) capital cost (if existing equipment can not be utilized), (2) space (these are not small instruments and require significant space), and (3) trained personnel (the most critical requirement). Fortunately, for most large industrial and academic institutions, appropriate instrumentation, space, and personnel are typically already available. The biggest need then becomes instrument time and the time of the trained personnel.

Though far less complex and expensive, those exploring the biology side of metabonomics technology also have to keep in mind several logistical considerations. These also have been previously reviewed (Robertson *et al.*, 2002), so will only be dealt with briefly. Two key principles should be kept in mind when considering animal studies. Firstly, metabonomics is exquisitely sensitive to any environmental or physiologic change the animal may undergo during the course of the study, regardless of whether it is part of the protocol design or not. Correspondingly, even minor details within a protocol, like vehicle selection, can have a dramatic effect on metabonomic studies. For example, it was noted early on that even a 4-week difference in age (8 week old vs. 13 weeks old) can have a marked effect on metabonomic profile (Robertson *et al.*, 2000). This should not be terribly surprising, as the older rats are roughly 50% heavier than their younger counterparts. Diurnal and estrus cycles also cause measurable difference in urine NMR spectral profiles (Bollard *et al.*, 2001). Vehicles used to deliver test compound can have a profound effect on metabonomic profiles, not only because they may present as interfering signals in urine or serum NMR spectra, but they frequently have physiologic effects suggesting they may not be as innocuous as we would like to believe (Beckwith-Hall *et al.*, 2002; Robertson *et al.*, 2000). Even anticoagulants such as ethylenediaminetetraacetic acid (EDTA) can have an adverse effect on serum NMR spectra, markedly complicating them. An interesting example of how protocol details can complicate metabonomic data analysis in unexpected ways is presented in Figure 2. An overdose of barbiturate, followed by exsanguination is a common method for euthanizing animals. In this case, guinea pigs were sacrificed by exsanguination immediately after succumbing to an overdose of pentobarbital. What may not be obvious is that many barbiturate formulations contain high concentrations of propylene glycol, which produces prominent resonance peaks in the serum NMR spectra obtained from animals exsanguinated immediately after succumbing to the barbiturate (Fig. 2). Even more surprising (and confounding) is that urine obtained from these animals also contains widely varying concentrations propylene glycol (millimolar in some cases) even though the time from barbiturate injection to exsanguination may be only a few minutes (unpublished observations). This finding highlights not only the caution one must exercise when conducting metabonomics studies, but also the power the analysis has for identifying factors that might otherwise be missed. It seems likely that these

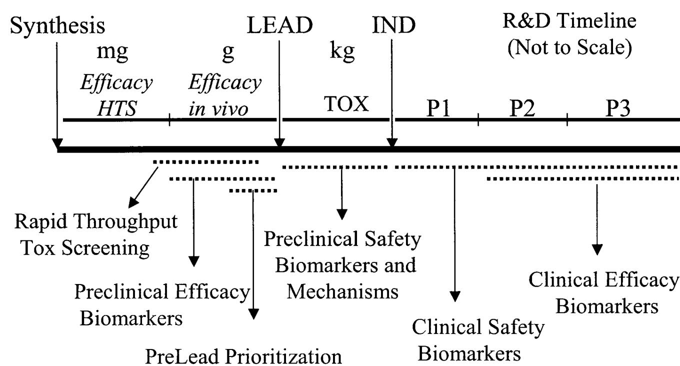

FIG. 3. Schematic of typical drug-development timeline overlaid with points of entry and applications of metabonomics technology. Relative availability of bulk drug is indicated by mass designations (mg, g, or kg quantities).

TABLE 2
The "Usual Suspects"[a]

2-oxoglutarate
acetate
citrate
creatine
creatinine
glucose
hippurate
lactate
succinate
taurine
trimethyl amine/trimethyl amine oxide (TMA/TMAO)

[a]This is not intended to be a comprehensive list and there are several other metabolites that are often seen changing in metabonomic studies but not often enough to consider them "usual suspects."

millimolar concentrations of propylene glycol have confounded more than one type of analytical assay without the investigator ever understanding the cause of the problem.

B. Toxicology Applications

Metabonomics has potential applications across the spectrum of pharmaceutical development (Fig. 3). Of particular interest to this review are those applications that directly relate to safety assessment of candidate therapeutic agents. Within preclinical toxicology there are three broad areas in which metabonomics is having and will continue to have significant impact. Those areas are screening, biomarkers of safety, and mechanism of action.

1. Screening. The ultimate safety screen has long been the Holy Grail for preclinical toxicologists. Ideally, it would be fast, require minimal compound, and be absolutely reflective of potential human effects. Unfortunately, the search for such a screen has been about as successful as the search for the aforementioned chalice. *In vitro* approaches have been heavily researched because they typically meet the first two requirements. It may seem that human cells would be more reflective of human effects, but immortalized cell lines (of any species) frequently respond quite differently from primary cells, and primary cells are frequently difficult to obtain (particularly human), culture, and maintain (in their differentiated state) for any reasonable period of time. The bottom line is that the majority of preclinical toxicologists view *in vitro* approaches as extremely useful when the *in vivo* target link has been established; however they fall well short as a generic safety screen. It might seem that metabonomics would also aid evaluation and interpretation of *in vitro* toxicity data. However the literature in this area is limited (Anthony *et al.*, 1995a; Bailey *et al.*, 2003; Griffin *et al.*, 2003; Hassel *et al.*, 1994), probably because of the sample requirements and sensitivity limitations for NMR analyses. As MS-based metabonomics becomes more prevalent, it can be anticipated that *in vitro* metabonomics applications will increase

For the foreseeable future, *in vivo* assessment in animal models will remain the primary method for identifying safety issues. With this in mind, one of the early hopes was that metabonomics would provide a generic safety screen for rapid throughput toxicity assessment (Robertson *et al.*, 2000). While that hope has not been abandoned, it has been tempered by the reality of systemic responses to toxins and the complexity of differentiating "off-target" effects from target-organ-specific effects. However, generic screens are not the only type of screening for which metabonomics has application. In some instances, early identification and characterization of a known or presumed toxicity within a pharmacological or chemical class is extremely valuable, particularly when there are no available peripheral biomarkers of that toxicity. Drug-induced vascular injury (vasculitis) is one such application, where there is a lack of definitive peripheral biomarkers, and histopathology of affected tissues is still required to make the diagnosis. Metabonomics has been shown to be quite useful for noninvasive detection of the lesion in rats (Robertson *et al.*, 2001).

2. Biomarkers. It has been recognized for some time that metabonomics had enormous potential to identify novel biomarkers of toxicity, with early work focused primarily on biomarkers of renal and hepatic toxicity (Anthony *et al.*, 1994a,b; Holmes *et al.*, 1992a,b, 1995, 1996; Nicholls *et al.*, 2001; Robertson *et al.*, 2000). This work was eye opening with regard to systemic response to hepatic and renal toxins that has been overlooked for years. However, one need only run a quick comparison of these papers and others like them to recognize one of the most oft-cited criticisms of metabonomics as a tool in toxicology. That is the problem of "usual suspects." Table 2 is a list of urine metabolites that frequently change in response to toxicant administration, regardless of the nature of the toxicant, its mechanism of action, or its target. Importantly, not

all these molecules change in response to every toxicant, nor do they necessarily follow the same trajectory (temporal response), but changes in some or most of them frequently drive pattern separation using unsupervised pattern recognition techniques like the now ubiquitous PCA (Beckwith-Hall *et al.*, 1998; Gartland *et al.*, 1991; Jansen *et al.*, 2004; Scholz *et al.*, 2004; Waters *et al.*, 2001). This has led to a jaded view of the technology by some observers, with one toxicologist wag calling a high-field magnet nothing more than a big "citrate-ometer," emphasizing the fact that citrate changes are a frequent response to toxicant administration. A comprehensive evaluation of this phenomenon attributed changes to many of the usual suspects to altered diet and/or bodyweight changes which are a frequent consequence of toxicity (Connor *et al.*, 2004), As the authors observe though, even among the usual suspects the magnitude, direction, and temporal response of the changes may still be useful in providing mechanistic or biomarker data, as long as the changes are evaluated in the context of the systemic effect. It is important to note that metabolites driving pattern separations within PCA does not mean that these are the only metabolites changing within an NMR (or MS) spectrum, nor does it imply that the molecules are necessarily the most interesting, from a biomarker or mechanistic perspective. Weight loss is a quite profound physiological disruption (at least from the animal's perspective), so it should not be surprising that it is responsible for acute biochemical perturbations that dominate the systemic biochemistry. The trick, of course, is separating specific biomarkers of the target of interest from the numerous changes caused by diet and other secondary factors. Rather than fulminate over metabonomics-derived usual suspects, it might be better to ask "where are the usual suspects for the other omic technologies?" After all, no one is suggesting that these components are in any way artifactual; therefore they must be derived from the actions of proteins and the genes that code them.

Despite these perceived limitations, metabonomics-derived biomarkers are not restricted to the usual suspects. Early metabonomic work suggested the potential of urinary D-β-hydroxybutyrate, in the absence of other ketone bodies (proximal tubules), or trimethylamine (TMA) and dimethylamine (renal papilla) as potential region-specific biomarkers of nephrotoxicity (Anthony *et al.*, 1994b; Gartland *et al.*, 1989a). Phenyl-acetyl glycine was identified as a candidate biomarker for drug-induced phospholipidosis (Nicholls *et al.*, 2000), and 2-aminoadipate was proposed as a mechanism-associated biomarker of hydrazine-induced neurotoxicity (Nicholls *et al.*, 2001). Recently, urinary dicarboxylic aciduria was implicated as a mechanistic marker of impaired fatty acid metabolism in rats (Mortishire-Smith *et al.*, 2004).

Depending on how you define a biomarker, these biomolecular components may or may not fit the bill. Certainly most, if not all, of these biomarkers are unlikely to be specific only to the target of interest. While lack of specificity may hamper use of these markers in the general population, many will probably be adequate for preclinical safety studies or even controlled clinical trials where absence of an effect is of most critical interest.

3. Mechanisms of toxicity. Arguably, the most important endpoint for an omics investigation would be in elucidating a mechanism of toxicity. An understood mechanism of toxicity will always deliver a biomarker (whether that biomarker is analytically feasible or practical is another story). While biomarkers can be identified without an understood mechanism, there would be little argument that a mechanistically linked biomarker is far more saleable. Metabonomics has proven to be a powerful tool for gaining insights on mechanisms of toxicity. The fact that the usual suspects are usual, by itself, is an interesting mechanistic finding. Most mechanistic metabonomic work has focused on renal and hepatic toxins, associating temporal biofluid biochemical correlations with toxicity endpoints. In most cases, these data were accompanied by speculative inferences of the biological significance of the various metabolic changes (Anthony *et al.*, 1992, 1994b, 1995b; Gartland *et al.*, 1989a,b; Halligan *et al.*, 1995; Holmes *et al.*, 1992b, 1995, 1996, 1998; Lenz *et al.*, 2004a,b; Lindon *et al.*, 2004d; Nicholson *et al.*, 2002; Robertson *et al.*, 2000; Shockcor and Holmes, 2002; Warne *et al.*, 1999; Waters *et al.*, 2001).

Beyond these studies, a few notable metabonomic investigations stand out with regard to their mechanistic insights. In 2001 Nicholls *et al.* published data on hydrazine toxicity that mechanistically linked the neurotoxic effects of hydrazine to markedly increased levels of 2-aminoadipate (2AA), which is known to affect kynurenic acid levels in the brain, thus providing a plausible hypothesis for the heretofore unexplained neurotoxic effects of the compound (Nicholls *et al.*, 2001). Slim *et al.*, demonstrated that the urinary metabolite changes induced by Type 4 phosphodiesterase (PDE4) inhibitors were not the indirect result of concurrent inflammation but were directly associated with vascular pathology (Slim *et al.*, 2002). Clayton *et al.*, mechanistically linked the "usual suspect" creatine to hepatotoxicity via effects on cysteine synthesis. They later related elevated creatine levels in serum and urine with hepatotoxicity and nutritional effects (Clayton *et al.*, 2003, 2004). Mortishire-Smith linked urinary dicarboxylic aciduria to impaired fatty acid metabolism, which may be common to some hepatotoxic mechanisms (Mortishire-Smith *et al.*, 2004).

While these reports highlight individual mechanisms, novel approaches to metabonomic studies have also served to enhance the utility of the technology for mechanistic purposes. Integrated metabonomics makes use of combined serum and urine biofluid metabonomics coupled with tissue MAS to present a much more comprehensive mechanistic picture of toxicity. These efforts have been successfully employed looking at cadmium, α-naphthlyisothiocyanate (ANIT), and

acetaminophen toxicity (Coen *et al.*, 2003; Griffin *et al.*, 2001; Waters *et al.*, 2001). Even more exciting are recent efforts to combine metabonomics with other omic technologies, including proteomics and transcriptomics (Coen *et al.*, 2004; Kleno, 2004; Verhoeckx *et al.*, 2004).

IX. STATE OF THE TECHNOLOGY

Where is metabonomics now, and where is it going? Metabonomics has moved beyond the status of emerging technology. It is now in that critical realm of value determination. It would be an overstatement to say that metabonomics has reached the point of standard practice in toxicology. The reasons for this are numerous, and many of them are not unique to metabonomics. Because toxicological applications have largely been reported using the NMR platform, there is a built-in discomfort level, as most toxicologists are unfamiliar with NMR, and hence the technique has a bit of a ''black box'' feel. MS has wider utilization within the tox community, and as MS-supported tox applications grow in acceptance, it can be hoped that some of this fear will dissipate. Beyond this perceived problem, there are the real problems of poor NMR sensitivity, which once again MS will help alleviate. Other issues currently facing metabonomics have a great deal in common with problems facing the other omic technologies. Standardization (or lack thereof), concerns about regulatory implications of metabonomics data, and the all-too-frequent conclusion to ''let someone else see if it works before we waste any money on it'' are all challenges metabonomics faces. Perhaps the greatest challenge for metabonomics, though again the same challenge is facing the other omics, is that our ability to generate masses of data far outstrips are capacity to understand it. A real need is to have bioinformatics tools that will be able to link genes, proteins, and metabolites. This means more than an annotated pathway chart. An interactive tool that indicates what up-regulation or down-regulation of a gene or protein means to the upstream or downstream metabolites in those pathways would be very helpful, particularly if we could link the secondary effects of up- and down-regulation of those metabolites back on the genes and proteins. Clearly, much of that biochemical information is not currently known, but we have to start somewhere. Many companies and vendors are attempting to attack this bioinformatics issue. However, the current frenetic pace of development of statistical and bioinformatics approaches has only compounded the problem for the toxicologist as he or she is faced with a mind numbing number of ways to look at information, all employing different techniques and criteria and presentation formats. While these approaches can make for impressively glitzy presentations, we all too frequently are left asking the same question—''but what does it mean''?

What to do? While significant challenges face metabonomics, the technology clearly has much promise, and none of the problems are insoluble. Success breeds acceptance. Increased presentation and publication of clear demonstrations of impact on real world toxicology issues (not CCl_4 or bromoethylamine examples!) will go a long way in moving the technology forward. The catch, of course, is that the biggest success stories are probably the most valuable from an intellectual property perspective, diminishing the likelihood that they will appear in the literature anytime soon. Still, when possible, the metabonomics community needs to push these examples out to the toxicology community, or the technology may never get the acceptance it deserves.

How will metabonomics be employed in toxicology departments 10 years from now? Who knows? However, if properly researched and developed, metabonomics can take its place as a standard tool in experimental toxicology. The ability to assess samples noninvasively makes it ideal for deployment in early discovery studies at the time sufficient bulk compound becomes available for *in vivo* studies. Metabonomics can easily be piggybacked on existing *in vivo* studies, requiring little or no additional technical resources to gather the data. One can envision safety endpoints being moved very early into the discovery process, enabling early attrition of toxicologically problematic compounds. Moreover, the search for biomarkers of efficacy and safety can start with the first dose in whole animals, and derived putative biomarkers will be readily transferable to the clinical setting. The ability to gather comprehensive metabolic profiles will prove invaluable for elucidating mechanisms of action not readily apparent using traditional endpoints such as histology and clinical pathology. Metabonomics clearly has a lot of promise, but just as clearly still a long way to go.

X. CONCLUSION

Like most technologies, metabonomics will probably not live up to all expectations, but it will certainly add value in many areas of biology. In particular, the technology has shown significant promise in numerous applications in toxicologic sciences. Unlike many technologies, metabonomics has been most readily adopted by industry, with the academic community just now starting to catch up. It can be anticipated that metabonomics will be extremely useful in completing the omics circle from gene (genomics) to protein (proteomics) to metabolite (metabonomics). Given that the phenotype is an endpoint, in many cases it will be easier to work backwards from the phenotype to the genotype than to try and guess the significance of thousands of changes in transcript expression. Clearly, the technology has already demonstrated significant potential, and now that potential needs to be realized. Only when the value added can be measured in terms of decreased morbidity and mortality or dollars-and-cents impact will the technology be proven. We are close in some applications, but not yet there. It should be an exciting ride.

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