Metabolomics for Developing Markers of Chemical Exposure and Distinguishing Toxicity Pathways

Tracy Whitehead, Tim Collette, Drew Ekman, John Kenneke, Wayne Garrison, and Quincy Teng
US EPA ORD/NERL/ERD, Athens, GA

Abstract

Metabolomics involves the application of advanced analytical and statistical tools to profile changes in levels of endogenous metabolites in tissues and biofluids resulting from disease onset, stress, or chemical exposure. Nuclear Magnetic Resonance (NMR) spectroscopy–based metabolomics has proven useful in mammalian systems for distinguishing between sites and mechanisms of toxicity for tissue-specific toxins. Metabolomics has been characterized as the true measure of metabolic outcomes suggested by changes in gene and protein expression; as such, metabolomics provides a connection between these molecular endpoints and whole organism responses. Although used mostly in mammalian studies, metabolomics is now finding utility in a wide variety of other organisms, including aquatic species.

We have developed a research program in metabolomics that involves numerous partners across EPA, other Federal labs, academia, and the private sector. Our goals are to (1) develop metabolite-based markers that can be used by EPA in chemical exposure assessments and (2) develop and test hypotheses about toxicity pathways for risk assessments. We are focusing this program on ecologically relevant species—in particular, small fish toxicological models.

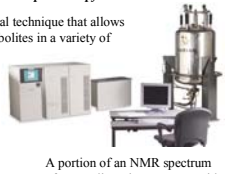
For example, to better understand the mode of action of endocrine-disrupting chemicals (EDCs) in small fish (fathead minnow, zebrafish), we are conducting metabolomic analyses with multiple tissues (brain, blood, liver, and gonad) and urine. Initial metabolomic studies were focused on collection of baseline data for actively spawning male and female fathead minnows. Subsequent work is focusing on animals exposed to potent EDCs, such as the steroid 17α-ethinylestradiol (EE_2). We are developing hypotheses about which tissue- and biofluid-specific metabolite changes will be definitively related to exposure based on the current understanding of modes of action for these chemicals. Results will allow testing of these hypotheses to refine understanding of activity and will help ensure that molecular markers of EDC exposure—another outcome of this research—are meaningful. While certain metabolites are being specifically targeted in these studies, we will also discern changes in the complete metabolic profile using NMR spectroscopic data with statistical approaches that allow capturing subtle changes in less-abundant metabolites. These data will be integrated with genomic, proteomic, and whole organism data from untreated fish and those exposed to known EDCs.

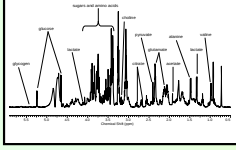
Introduction

The recently developed approach known as metabolomics involves the use of advanced analytical techniques such as NMR (Nuclear Magnetic Resonance) spectroscopy (Figure 1) to characterize changes in the levels of cellular metabolites (e.g. sugars, carbohydrates, amino acids, etc.) that relate to the toxic mechanism(s) involved in responding to the presence of a given chemical. This approach has proven to be a powerful tool in the assessment of toxicity or other physiological alterations in a variety of different organisms (Bailey, 2003; Coen, 2003; Viant, 2003). We have employed NMR-based metabolomics for the determination of changes in metabolite profiles in a variety of tissues obtained from small fish exposed to the endocrine-disrupting compound 17α-ethinylestradiol (EE_2), the active ingredient in oral contraceptives. This compound was chosen because it has been detected in aquatic environments and is considered relevant as an environmental contaminant.

Figure 1 - NMR spectroscopy

NMR spectroscopy is an analytical technique that allows one to observe the levels of metabolites in a variety of tissues and biofluids for metabolomic analyses.





A portion of an NMR spectrum of a trout liver tissue extract with several metabolites labeled. Changes in the levels of these metabolites (e.g. sugars, carbohydrates, amino acids, etc.) can allow one to determine the nature of toxicity induced by exposure to a given compound.

Because changes in an organism's metabolite profile often occur in conjunction with, or as a result of, changes in transcript and protein levels, metabolomics can provide complementary information to genomic and proteomic studies. Moreover, metabolomics (specifically NMR-based) is attractive because it offers some distinct advantages relative to these other techniques that make such a technique attractive for a number of reasons.

NMR-based Metabolomics for Toxicology

Advantages:	Disadvantages:
• no need for sequenced genome	• need for metabolite peak assignment
• open-ended (no preselection of metabolites)	• observed metabolites "preselected" by abundance
• high throughput (< 15 min per sample run in most cases)	* • when metabolites of interest in low abundance (> 1 hr per sample run
• low per sample cost (< $2.00 USD)	• high initial cost for instrumentation (> $750K USD
• high level of structural information	* • "overlapping" information
• non-invasive (i.e., urine)	
• non-destructive	
• little or no sample preparation	
• excellent reproducibility (e.g., cross-instrument)	

* Disadvantages that may be mitigated by complementary use of other analytical techniques and/or chemometrics

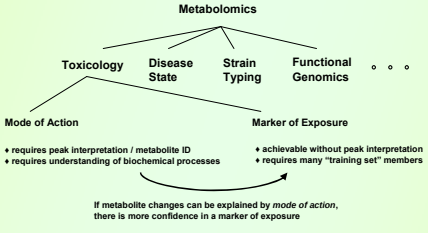
Traditionally, metabolomics has been utilized in many areas of study (e.g. disease, strain typing, etc.). In ORD, our focus is on its application to toxicology. Specifically, we are applying metabolomics to both better understand the mode(s) of action (MOA) of EDCs as well as to develop markers of exposure using small fish models such as fathead minnows and zebrafish (Figure 2).

Figure 2 – Small fish models used in ecometabolomics studies

Zebrafish (*Danio rerio*)

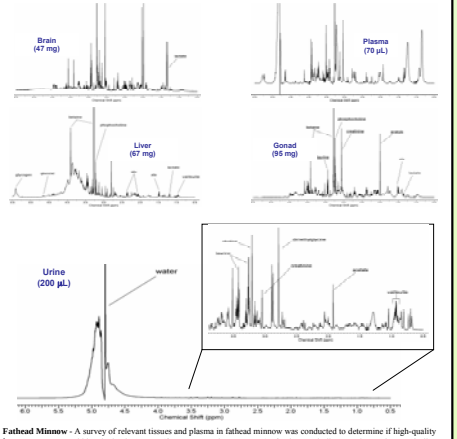
Fathead Minnow (*Pimephales promelas*)

While determining a given MOA requires a greater understanding of biochemical processes, we believe that this understanding is important for establishing reliable and meaningful biomarkers.



Results

Figure 3 – NMR spectra of fathead minnow tissue extracts and biofluids



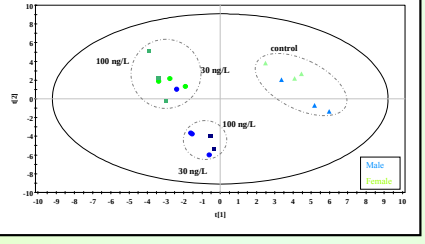
Fathead Minnow - A survey of relevant tissues and plasma in fathead minnow was conducted to determine if high-quality ^{1}H-NMR spectra could be obtained. Spectra (from aqueous tissue extracts) of polar metabolites are shown above. Similar analyses have been conducted for zebrafish (not shown). A comprehensive assignment of metabolite resonances for these tissues and biofluids is in progress. Although metabolites in fathead minnow urine are dilute, high-field NMR (i.e. ≥600 MHz) instrumentation coupled to a cryogenic probe allows for acquisition of information-rich spectra. As urine is a physiological endpoint, this will provide a means to more fully assess the effects of exposure.

Data collected for aqueous extracts from fathead minnow (Figure 3) and zebrafish tissues have shown that it is possible to identify metabolites that are tissue specific.

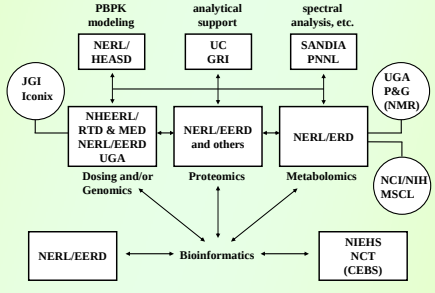
After collecting spectra from a statistically-relevant number of organisms, the number of metabolites changing in response to a given toxic insult from a large sample population can be overwhelming without the use of statistical tools that can quickly identify changes.

Various computational methods designed to simplify the analysis of large amounts of data (e.g., principal components analysis (PCA) and partial-least squares discriminant analysis (PLS-DA, Figure 4)) provide the ability to quickly determine metabolite changes as opposed to assessing changes simply by eye. While these methods are effective, more- advanced methods are needed to observe very subtle changes that may carry information crucial to more accurately defining organism responses.

Figure 4 – PLS-DA analysis showing separation between control zebrafish and EE_2-exposed zebrafish (liver tissue extract)



Support from researchers in academia, industry, and various State and Federal agencies has been crucial for the early success of the metabolomics program in ORD. At present, there are several collaborations that have been established and that have active projects that are underway.



Conclusions

The potential for metabolomics to supply rapid and accurate information on the toxicity of compounds of interest to the EPA has been shown here through the analysis of the effects of endocrine-disrupting compounds (EDCs) on aquatic species. One goal of the ORD NMR facility is to use metabolomics, in conjunction with genomic, proteomic, and whole organism data, to develop markers of EDC exposure in small fish models. To ensure that these markers are meaningful, we also seek to better understand the modes-of-action of EDCs within various biological contexts. Prior to achieving this, we have sought to determine that metabolites can be readily observed in relevant tissues and biofluids from single animals and that exposure to environmentally-relevant compounds lead to observable changes for dosed fish. As illustrated here, we have now made these determinations and are moving forward to determine toxic modes-of-action and develop biomarkers indicative of exposure. By collaborating with other EPA researchers (NERL and NHEERL), academic institutions and other agencies, it is our intention to extend NMR-based metabolomics into the ecotoxicology realm. As a result of Agency support and collaborative efforts with the entities mentioned above, this initial study serves as a major step in the establishment of an integrated metabolomics program at EPA.

References

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