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Analysis of Xenobiotics: A Review

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ABSTRACT

A xenobiotic is a chemical which is found in an organism but which is not normally produced or expected to be present in it. It can also cover substances which are present in much higher concentrations than are usual. This study deals with the analysis of xenobiotics in detail including the concepts like Xenobiotic Metabolism(Xenobiotic Biotransformation), Rapid analysis of pharmaceuticals and excreted xenobiotic and endogenous metabolites with atmospheric pressure infrared MALDI mass spectrometry , Detection of xenobiotic and endogenous metabolites in urine, X-ray Crystallographic Analysis of Xenobiotic Exporter Proteins of Escherichia coli, Determination Of Chiral Xenobiotics By Capillary Electrophoresis, Biomarkers as Biological Indicators of Xenobiotic Exposure, Bioinformatic analysis of xenobiotic reactive metabolite target proteins and their interacting partners.

Key Words: *xenobiotics, biotransformation, chiral xenobiotics, biomarkers.*

INTRODUCTION

Xenobiotic is any chemical that would not normally be found in a living organism or be expected to be produced by it. It can also cover substances which are present in much higher concentrations than are usual. Specifically, drugs such as antibiotics are xenobiotics in humans because the human body does not produce them itself, nor are they part of a normal diet.

Natural compounds can also become xenobiotics if they are taken up by another organism, such as the uptake of natural human hormones by fish found downstream of sewage treatment plant outfalls, or the chemical defenses produced by some organisms as protection against predators.

However, the term xenobiotics is very often used in the context of pollutants such as dioxins and polychlorinated biphenyls and their effect on the biota, because xenobiotics are understood as substances foreign to an entire biological system, i.e. artificial substances, which did not exist in nature before their synthesis by humans. The term xenobiotic is derived from the Greek words ξένος (xenos) = foreigner, stranger and βίος (bios, vios) = life.

Xenobiotics in the environment:

Xenobiotic substances are becoming an increasingly large problem in Sewage Treatment systems, since they are relatively new substances and are very difficult to categorize. Antibiotics, for example, were derived from plants originally, and so mimic naturally occurring substances. This, along with the natural monopoly nature of municipal Waste Water Treatment Plants makes it nearly impossible to remove this new pollutant load.

Some xenobiotics are resistant to degradation. For example, they may be synthetic organochlorides such as plastics and pesticides, or naturally occurring organic chemicals such as polyaromatic hydrocarbons (PAHs) and some fractions of crude oil and coal. However, it is believed that microorganisms are capable of degrading almost all the different complex and resistant xenobiotics found on the earth [1]. Many xenobiotics produce a variety of biological effects, which is used when they are characterized using bioassay.

Xenobiotic metabolism:

The body removes xenobiotics by xenobiotic metabolism [2]. This consists of the deactivation and the excretion of xenobiotics, and happens mostly in the liver. Excretion routes are urine, feces, breath, and sweat. Hepatic enzymes are responsible for the metabolism of xenobiotics by first activating them (oxidation, reduction, hydrolysis and/or hydration of the xenobiotic), and then conjugating the active secondary metabolite with glucuronic or sulphuric acid, or glutathione, followed by excretion in bile or urine. An example of a group of enzymes involved in xenobiotic metabolism is hepatic microsomal cytochrome P_{450} [3]. These enzymes that metabolize xenobiotics are very important for the pharmaceutical industry, because they are responsible for the breakdown of medications.

The Importance of Understanding Xenobiotic Metabolism:

Animals have a number of enzymes capable of undertaking biotransformation,which are concentrated in the liver of vertebrates or the food processing tissues of invertebrates (e.g. the digestive gland of molluscs). These enzymes primarily convert hydrophobic, lipophilic, organic molecules into water-soluble metabolites that can be excreted. Phase I metabolism (oxidation, reduction and peroxidation, mainly by the Cytochrome P450 (CYP450) super family of isozymes) involves the introduction or modification of a functional group including –OH, -COOH, $-NO_2$ into the compound, to which a large polar moiety such as glutathione, glucuronide or sulphate is added by enzymes during phase II metabolism [4]. Biotransformation is known to be a key modulator of the toxicity and bioaccumulation of xenobiotics, since these reactions can, for example, produce metabolites that have different fates to the parent compound, alter elimination rates, or affect the persistence of the chemical in the organism.

Table No. 1: Biotransformation Enzyme- Containing Cells in Various Organs

Organ	Cell(s)
Liver	Parenchymal cells (hepatocytes)
Kidney	Proximal tubular cells (S3 segment)
Lung	Clara cells, Type II alveolar cells
Intestine	Mucosa lining cells
Skin	Epithelial cells
Testes	Seminiferous tubules, Sertolis cells

The Role of Cytochrome P_{450}s in Metabolism:

The Mechanism of Action of Cytochrome P_{450}s:

Cytochrome P_{450}s (CYPs) are a haem-containing enzyme superfamily that are one of the most important groups involved in catalysing Phase I reactions [5] of both endogenous (fatty acids, prostaglandins, steroids) and exogenous (carcinogens, drugs, chemical pollutants) substrates [6]. The overall catalysis results from the transfer of an oxygen atom from O2 into the substrate, and the enzyme receives electrons from NADPH via electron transfer proteins that are often coupled to CYPs inside the cell membrane.

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$$RH + O2 + 2e\text{-} + 2H+ (NADPH) \longrightarrow ROH + H2O$$

There are at least four distinct steps involved in the reaction:

i) Binding of the substrate with the enzyme (introduces a conformational change)
ii) Electron donation (from NADPH (via the reductase) reduces the iron in the enzyme-substrate complex from the ferric to the ferrous state)
iii) Addition of oxygen and rearrangement
iv) Donation of a second electron from NADPH, the complex rearranges, one atom of oxygen binds to the substrate and the product is released.

The Importance of Isoenzymes of CYPs in the Metabolism of Xenobiotics:

Members of the CYP1, CYP2 and CYP3 subfamilies are of key importance in the biotransformation of xenobiotics [7]. In the context of the marine environment, CYP1A1 is the best-studied member of the CYP superfamily predominantly as it is inducible by dioxins, PAHs and PCBs, all of which are environmentally important contaminants. Importantly, these senzymes are also implicated in the bioactivation of numerous genotoxins including BaP (CYP1A1), 2-AAF (CYP1A2). Forms that are identical or related to members of these groups have been reported in fish, including Rainbow Trout [6] and the Atlantic Salmon Salvo salar and in invertebrates such as molluscs. Further evidence that isoenzymes of these families are present in certain invertebrates include the reported metabolism of PCBs by crustaceans,suggesting that CYP1 and CYP2-like proteins are present , while unique forms have been identified in some organisms, such as CYP2L in the Caribbean spiny lobster P. Argus and CYP10 in the snail Lymnaea stagnalis . Metabolism of compounds including BaP has also been reported in both marine and freshwater algal species, implying that CYP1A-like enzymes are present in these organisms. The presence of CYP1 and CYP2-like proteins in invertebrates and algae is suggestive of the ability of these organisms to bioactivate xenobiotics, which is of great importance when investigating the potential of such species to be used as sentinel species for assessing the impact of genotoxins.

Biotransformation Reactions:

Phase-I Reactions:

- Enzymatic reactions that add or expose functional groups to xenobiotics such as -OH, -SH, -NH_2 or –COOH
- Functional groups are analogous to having a trailer hitch on a vehicle.

Phase II Reactions:

- Enzymatic reactions that result in the conjugation of large water-soluble, charged (polar)biomolecules to xenobiotics.
- For these reactions to occur, a functional group must be present on either the parent compound or its Phase I product.

Factors that Affect Xenobiotic Biotransformation:

Species, strain, and genetic variations-

- Risk assessment is often based on responses observed in animals.
- In this regard, there are often significant differences between species in their abilities to metabolize xenobiotics.
- Likewise, even within a species, including man, there are differences.
- The basis of such differences is often genetic (polymorphisms).

Examples of Factors that Affect Xenobiotic Biotransformation:

Species, strain, and genetic variation-
Exposure to organic solvents [8]

- Hexobarbital
- Aflatoxin B1
- Benzo[a]pyrene 7,8 –dihydrodiol
- Isoniazid
- Age
- Diet
- Exposure to other chemicals

Bioactivation as a Basis for Chemical Toxicity:

One of the possible results of the interaction of a xenobiotic with enzyme systems is the biotransformation of that compound to a chemically reactive intermediate (i.e. Bioactivation).The reaction of either this initial reactive metabolite or secondary reactive products with target molecules brings about changes in cellular function (the Molecular Targets Concept).

Chemical Nature of Reactive Intermediates:

Electrophiles: Form covalent (irreversible) bonds with cellular nucleophiles such as
GSH, proteins and DNA
Free Radicals: Odd or unpaired electron

- Can act as electrophiles
- Can abstract hydrogen from target molecules, such as lipids or nucleic acids
- Can activate molecular oxygen

Redox Cycling of Xenobiotics:

Redox cycling of xenobiotics initially results in the formation of a form of active oxygen called superoxide (O2• –).

Through a series of non-enzymatic often metal catalyzed, reactions other forms of reactive oxygen are formed-

These include hydrogen peroxide (H2 O2), the hydroxyl radical (.OH) and singlet oxygen ($^1O2$).

This results in an oxidative stress in cells and the subsequent modification of critical biomolecules leading to cellular toxicity.

Rapid analysis of pharmaceuticals and excreted xenobiotic and endogenous metabolites with atmospheric pressure infrared MALDI mass spectrometry:

Atmospheric pressure (AP) infrared (IR) matrix-assisted laser desorption/ionization (MALDI) mass spectrometry (MS) was demonstrated for the rapid direct analysis of pharmaceuticals, and excreted human metabolites [9]. More than 50 metabolites and excreted xenobiotics were directly identified in urine samples with high throughput. As the water content of the sample was serving as the matrix, AP IR-MALDI showed no background interference in the low mass range. The structure of targeted ions was elucidated from their fragmentation pattern using collision activated dissociation. The detection limit for pseudoephedrine was found to be in the sub-femtomole range and the semi-quantitative nature of the technique was tentatively demonstrated for a metabolite, fructose, by using a homologous internal standard, sucrose. A potential application of AP IR-MALDI for intestinal permeability studies was also explored using polyethylene glycol.

Detection of xenobiotic and endogenous metabolites in urine:

Urine is an easily and noninvasively collected biofluid rich in both endogenous and xenobiotic metabolites. The rapid detection of drug metabolites in urine can be challenging due to their low concentrations and interferences from endogenous biomolecules. Two of the major components in urine, water and urea, exhibit strong absorption at the 2.94 lm laser wavelength and thus can act as a matrix for AP IR-MALDI. To determine the role of water in the ionization process of urine samples, it was eliminated through drying and reintroduced. The air-dried urine sample failed to produce any mass spectrum while the sample re-wetted with a few microliters of water produced nearly identical mass spectra to the original. This simple experiment indicated that water acted as a matrix in the mid-infrared laser desorption ionization of the molecular components in urine.The pharmacokinetics of absorption, distribution,metabolism and excretion (ADME) of a drug molecule is pivotal in drug development [10].

To follow the excretion of xenobiotic compounds, an over-the counter cough medicine, containing pseudoephedrine (10mg) and three other active ingredients, was orally administered to a healthy volunteer.Urine samples were collected before and 2.5 h and 24 h after the ingestion of the medication [11]. Pseudoephedrine is a common nasal decongestant used for the treatment of common cold and other related aliments. It is known that more than eighty percent of the administered pseudoephedrine is excreted unchanged in the urine within 24 h, peaking at 4 h following its oral administration. Only a small fraction of the pseudoephedrine is metabolized to norpseudoephedrine through N-demethylation [12]. Small volumes (3 µl) of the unprocessed urine sample were analyzed directly without drying, extraction or any other sample preparation steps. For the investigation of xenobiotics and endogenous metabolites in the urine, an average of more than 200 mass spectra was collected. The target was continuously rastered to ensure even sampling of the target surface. The mass spectra were further validated by multiple analyses of the same sample as well as by a parallel study on the same individual within two weeks. In the urine collected after 2.5 h, the protonated pseudoephedrine was detected at m/z 166.1239 along with its fragment after OH loss at m/z(Mass-to-charge ratio) 148.1124. The metabolites for dextromethorphan at m/z 272.208, guaifenesin at m/z 199.1037 and acetaminophen at m/z 152.0696, as well as the excipients such as polyethylene glycol and mannitol/sorbitol (m/z 183.0795) were also detected in the urine spectra.

Compared to the 2.5 h level, the urine sample collected at 24 h showed a relative ion intensity reduced by *84% for pseudoephedrine. Along with the xenobiotic drug metabolites, many ions related to endogenous compounds were also observed in the urine mass spectrum. The identification of the endogenous metabolites was based on matching the observed monoisotopic mass (within ± Dm 35 mDa), and isotope distribution with those of known metabolites for normal human urine listed in the Human Metabolome Database [13]. The most abundant

components in the urine samples were detected both as protonated and as sodiated species. For example, urea, which accounts for half of the total urinary solid, was detected both as m/z 61.0318 ($M + H^+$) and m/z 83.0161 ($M + Na^+$). The positive ion AP IRMALDI spectrum of urine showed peaks for diverse chemical species such as amino acids, organic acids, amines, ketones, carbohydrates, steroids, etc. Some metabolites have close m/z values and similar chemical composition. Accurate m/z values and isotopic distributions alone might not be sufficient to identify them. For example, the m/z 166.1239 ion might correspond to either unmetabolized pseudoephedrine or to hordenine, a phenylethylamine alkaloid found in human urine after consumption of beer brewed using barley. Both have the same chemical formula and thus the same monoistopic mass of 165.1154. The m/z 166.1239 ion was identified as pseudoephedrine after it had produced a CAD fragmentation pattern identical to pseudoephedrine from the formulated medicine. This example shows that AP IRMALDI combined with tandem mass spectrometry can be used to unambiguously identify detected metabolites.

Detecting xenobiotic metabolites originating from pharmaceuticals can help to assess the pharmacological and toxicological effects of drug candidates, while identifying endogenous metabolites might have essential diagnostic applications in the biomedical sciences and in clinical applications. Mass spectrometers have already been successfully used in the diagnosis of many metabolic disorders, e.g., in screening for inborn metabolic disorders in infants [14, 15]. The detection of amino acids by AP IR-MALDI is faster than any of the classical methods of analysis by specialized assays for each analyte or the simultaneous detection afforded by hyphenated mass spectrometric techniques. With an appropriate internal standard, AP IR-MALDI tandem mass spectrometry might be used as a screening tool for the diagnosis and prognosis of disease states. Even with tandem mass spectrometric capability, AP IR-MALDI cannot distinguish between stereoisomers. Online coupling of AP IR-MALDI with chiral separation techniques can be helpful in these cases [16].

Table No. 2: Comparison of measured and calculated monoisotopic m/z values for the analyzed active ingredients

Active ingredient	Chemical formula	Monoisotopic mass	Measured m/z	Δm (mDa)	Studied formulations
Acetaminophen	C8H9NO2	152.0712	152.0705(+H)	-0.70	Gelatin capsule*, pure analyte, syrup, tablet
Aspirin	C9H8O4	181.0501	181.0489 (+H)	-1.20	Powder*
Caffeine	C8H10N4O2	195.0882	195.0926 (+H)	4.40	Powder*, tablet
Dextromethorphan	C18H25NO	272.2014	272.2036 (+H)	2.20	Gelatin capsule*, syrup
Doxylamine	C17H22N2O	271.1810	271.1737 (+H)	-7.30	Syrup*
Guaifenesin	C10H14O4	199.0970	199.0952 (+H)	-1.80	Gelatin capsule*, syrup
Ibuprofen	C13H18O2	205.1229	205.1271 (-H)	4.20	Tablet*

* Formulation of the active ingredient used to obtain the measured m/z value; Symbols in parentheses: (+H) indicate protonated and (-H) indicate deprotonated species.

Concluding remarks:

Analysis of biofluids is extensively used to follow metabolic changes. In this communication we demonstrated how the water content of these fluids enables the rapid direct analysis of endogenous metabolites and xenobiotics with AP IR-MALDI mass spectrometry. The wide range of metabolites detected in urine points to diverse applications in metabolism research and to potential clinical uses. For example, the rapid mass spectrometric detection lends itself to large scale stable isotope studies. The minimal sample preparation requirement and extremely fast analysis time are ideal for high throughput studies and population screening. The analysis at atmospheric pressure outside of the vacuum of the mass spectrometer also provides an opportunity to work with non-traditional samples, e.g., to conduct in vivo pharmacokinetic and diagnostic studies. By coupling the AP IR-MALDI ion source with a translation stage, the analysis method can be used to detect the spatial distributions of metabolites in a tissue [17]. Although the preliminary results presented in this study are encouraging, further improvement is necessary to expand the range of metabolites, and increase both the sensitivity and selectivity. Enhancements in the AP ionization and in the ion collection/transport will increase the sensitivity and possibly the diversity of the collected biomolecules. An automated AP IR-MALDI system with a higher repetition rate laser and the integration of online separation techniques, e.g., ion mobility spectrometry, should enable the swifter detection of a wider variety of biomolecules.

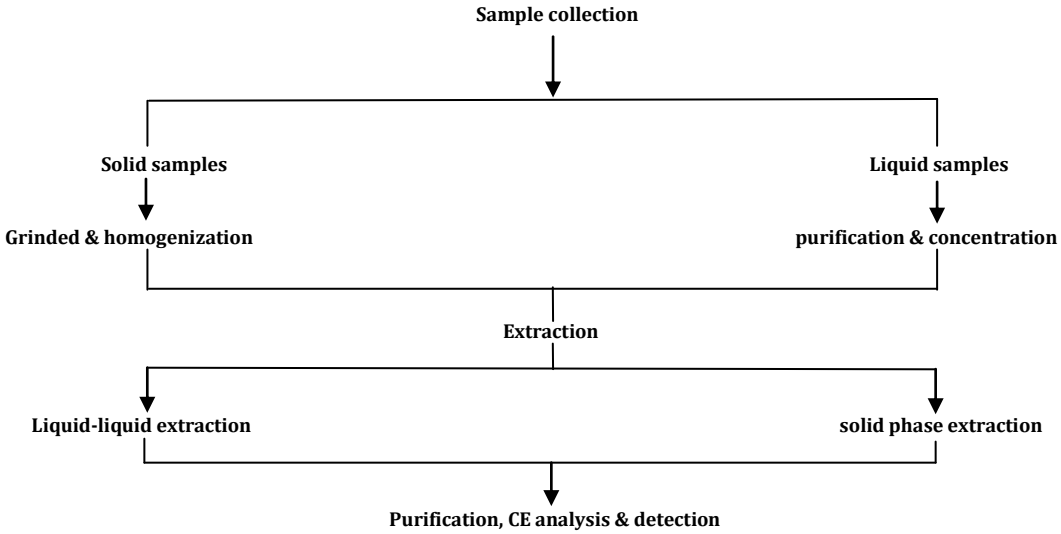
X-ray Crystallographic Analysis of Xenobiotic Exporter Proteins of Escherichia coli:

The xenobiotic exporter proteins, also known as multidrug efflux transporters, pump a wide variety of noxious compounds out of a cell across the cell membrane. They play an important role in multidrug resistance of pathogenic bacteria and cancer cells [18]. Genomic sequence analysis has revealed the presence of many putative xenobiotic exporter genes in chromosomes from microorganisms to mammalian cells. There are 19 transporter genes in Escherichia coli, which actually mediate the efflux of some drugs and toxic compounds. Among them, AcrB is constitutively expressed and is a major contributor for intrinsic drug resistance of E.coli [19]. AcrB cooperates with a membrane fusion protein, AcrA, and an outer membrane channel, TolC. This AcrAB-TolC system exports an unusually wide variety of noxious compounds, e. g. dyes, detergents and most lipophilic antibiotics directly out of the cell, bypassing the periplasmic space driven by proton motive force. AcrB is the most important component of this system; it mediates energy coupling and determines substrate specificity. First of all we cloned a histidine-tagged AcrB into a multicopy plasmid and overproduced it in E. Coli. Then, AcrB was solubilized and purified in the presence of a non-ionic detergent, n-dodecyl-bD maltopyranoside. Using the vapor diffusion method, we obtained crystals of AcrB and we employed the multiple isomorphous replacement method to solve the phase problem. A molecular model was built and refined at a resolution of 3.5 Å. All the diffraction data used for structure determination were collected at beamline BL44XU [20]. AcrB comprises a trimer of 1,049-residue protomers, and its appearance resembles that of a jellyfish with a three-fold symmetry axis perpendicular to the membrane plane. It comprises an extra-membrane (periplasmic) headpiece approximately 50 Å × 100 Å, and a transmembrane region of dimensions 70 Å ×80 Å. The headpiece is divided into two stacked parts: the upper and lower parts are 30-Å and 40-Å thick, respectively. The side view of the upper part has a trapezoidal appearance that is ~ 70-Å wide at the bottom and ~ 40-Å wide at the top. Viewed from above, the upper part is open like a funnel, with an internal diameter of 30 Å. This funnel connected by a pore, located between the headpieces of the three protomers to a large central cavity at the interface of the headpiece and transmembrane region of the protomers. Within the membrane, the protomers are arranged like a ring with a 30-Å hole between them, which may be filled with phospholipids.The protomers appear to be interlocked by three hairpin-like structures 35 Å long, each protruding from one protomer into the next protomer. To achieve this mutual insertion, the protomers are tightly packed as a trimer. Between every protomer in the periplasmic domain, there are three vestibules that open wide into the periplasm and lead to the central cavity inside the headpiece, and a substrate located in the periplasmic space or on the membrane plane might gain access to the cavity through any of these vestibules. The funnel opened at the top of the AcrB trimer seems to be a perfect fit for the proximal end of the TolC protein.The crystal structure of TolC protrudes 100 Å into the periplasm [21], thus, the sum of the periplasmic length of AcrB and TolC is ~170 Å, which is just enough to cross the periplasmic space, indicating that AcrB and TolC might direct dock with each other. Xenobiotic substrates from the cytoplasm or inner leaflet of the membrane are transported through every protomer across the membrane and collect in the central cavity. Substrates from the periplasmic surface of the inner membrane or the outer leaflet of the membrane also gain access in the cavity through the vestibules. It appears, then, that substrates in the cavity are actively exported through the pore into the funnel and then on into the TolC tunnel. The energy for active transport is captured in the transmembrane region and transmitted to the extra-membrane pore by a remote conformational coupling. This beautiful AcrB structure provides a great deal of important insight into the function of multidrug resistance mediated by xenobiotic exporters. This structure is not only the first structure of a multidrug exporter, but also the first atomic-level structure of a membrane transporter that couples with proton translocation across the membrane. Therefore, we believe that this work is a real milestone for understanding active membrane transport mechanisms based on molecular structure.

Determination of Chiral Xenobiotics by Capillary Electrophoresis:

The determination of enantiomeric composition of chiral xenobiotics and pollutants is a very difficult job due to their low amounts and poor detection by commonly used UV detector. The presence of chiral pollutants and xenobiotics in our environment is a serious issue due to the different toxicities of their enantiomers [22]. Moreover, these molecules degradate stereo-specifically, leading to more toxic products.

Besides, bio-transformation of the chiral pollutants may be stereo-specific making different uptake, metabolism and excretion of enantiomers. Normally, metabolites of the chiral xenobiotics are chiral and the enantiomeric composition of the chiral pollutants may be changed in these processes. Among various analytical techniques, chromatographic and capillary electrophoretic methods have achieved a good reputation in the area of chiral analyses [23, 24]. Of course chromatographic modalities are the ideal methods but the low amounts of chiral xenobiotics in the environment compel scientists to work with capillary electrophoretic methods. Capillary electrophoresis (CE) is a suitable technique for such type of analyses due to its requirement of low amount of samples and low detection limits. The determination of chiral pollutants involves two steps i.e. sample preparation and analyses by CE. Both steps are integral parts of the complete work and sample preparation is carried out by liquid-liquid extraction and solid phase extraction (SPE). But nowadays, sample preparation unit is hyphenated with mail analytical unit, which provide the more sensitivity, reproducibility and time saving.



Analyses by Capillary Electrophoresis:

In spite of good advancement in CE still chiral resolution is achieved by adding chiral selectors in back ground electrolyte (BGE). However, few chiral capillaries are available in market but, unfortunately, only few publications available that deal with the chiral resolution on a capillary coated with the chiral selector in CE [25]. The most commonly used chiral BGE additives are polysaccharids, cyclodextrins, macrocyclic glycopeptide antibiotics, proteins, crown ethers, ligand exchangers, and alkaloids [26, 27].

Detection:

Of course, a sensitive and of low limit method of detection is required as chiral xenobiotics are found at low concentrations in the environment. Most commonly used detectors in the chiral CE are UV, electrochemical, fluorescence, and mass spectrometry. Normally, the detection of the chiral drugs and pharmaceuticals in CE has been achieved by a UV mode [28, 29] but the detection of the chiral pollutants is difficult due to the transparent nature of mostly chiral xenobiotics. Besides, few chiral selectors,such as proteins and macrocyclic glycopeptide antibiotics are UV absorbing in nature. Therefore, the detection of enantiomers becomes poor. Hence, other detection methods are required, which include electrochemical and mass spectrometric detectors. Only few papers are available in the literature dealing with the limits of the detection for the chiral resolution of environmental pollutants by CE, indicating mg/L to µg/L as the limits of the detection. Mechref and EI Rassi [29] described good detection limits for herbicides in the derivatized mode, in comparison to the underivatized mode. The limit of the detection was enhanced by almost 1 order of magnitude from 1×10^{-4} M (10 pmol) to 3×10^{-5} M (0.36 pmol). In the same study, 2.5×10^{-6}M and 1×10^{-9}M as the detection limits of the herbicides by fluorescence and laser induced fluorescence detectors respectively were reported. An extensive study was carried out on the determination of the limits of the detection for the chiral resolution of herbicides [30]. 230 nm wavelength was used for the detection and the minimum limit of the detection reported was 4.7×10^{-3}M for 2, 4-dichlorophenoxy acetic acid. Asami and Imura [31] reported trace enantiomeric compositions of glufosinate (D, L-GLUF); a phosphorus-containing amino acid-type herbicide; in a river. The chiral separation and detection (10^{-9}M) were achieved by solid phase extraction and γ-cyclodextrin based CE. Experience on the CE and the properties of chiral xenobiotics dictates us that mass spectrometric detection is the best choice for this purpose.

Conclusions:

Analysis at trace level is a very important and challenging issue especially in the environmental matrices and CE can accept this challenge by the above cited hyphenation. To make the CE applications more reproducible, the background electrolyte should be developed in such a way ensuring its physical and chemical properties remain unchanged during the experimental run.

Besides, other aspects should also be developed so that CE can be used as a routine method in this field. The most important points related to this include the development of new and better chiral selectors, detector devices. In addition,chiral capillaries should be developed and CE device should be hyphenated with sample preparation units, mass spectrometer, polarimetric, and circular dichroism detectors,which may result into good reproducibility and improved limits of detection. All the capabilities and possibilities of CE as analytical technique have not been explored till today and are underway. However, CE will be realized as a widely recognized method of choice in analytical science. Briefly, there are a lot of to be developed for the advancement of CE and, definitely, it will prove itself as one of the best analytical technique within the coming few years.

Biomarkers as Biological Indicators of Xenobiotic Exposure:

The presence of a xenobiotic in the environment always represents a risk for living organisms. However, to talk about impregnation there is a need to detect toxicity in the organism, and the concept of intoxication is related to specific organ alterations and clinical symptoms. Moreover, the relationship between the toxic levels within the organism and the toxic response is rather complex and has a difficult forecast because it depends on several factors, namely toxicokinetic and genetic factors. One of the methods to quantify the interaction with xenobiotics and its potential impact on living organisms, including the human being, is monitoring by the use of the so-called biomarkers [32, 33]. They can provide measures of the exposure, toxic effect and individual susceptibility to environmental chemical compounds and may be very useful to assess and control the risk of long-term outcomes associated with exposure to xenobiotic (i.e. heavy metals, halogenated hydrocarbons, pesticides).The use of biological markers in the evaluation of disease risk has increased markedly in the last decade. Biomarkers are observable end points that indicate events in the processes leading to disease. They are particularly useful in the evaluation of progressive diseases that manifest their symptoms long after exposure to initiating factors. In such cases, traditional early warning symptoms of developing disease may be lacking. At the same time, the disease, once clinically apparent, may be essentially irreversible [34].

Specificity of biomarkers:

The ideal biomarker should have the following characteristics: [35]
(i) Sample collection and analysis is simple and reliable.
(ii) The biomarker is specific for a particular type of exposure.
(iii) The biomarker only reflects a subclinical and reversible change.
(iv) Relevant intervention or other preventive effort can be considered.
(v) Use of the biomarker is regarded as ethically acceptable.

Classification of Biomarkers:

Biomarkers are generally classified into three groups: exposure, effects and susceptibility [33, 36-38] .

Biomarkers of exposure:

These allow measurement of the internal dose by chemical analysis of the toxic compound or metabolite in body fluids or excreta such as blood, urine and exhaled air.

Biomarkers of susceptibility:

These serve as indicators of a particular sensitivity of individuals to the effect of a xenobiotic or to the effects of a group of such compounds.

Response or effect biomarkers:

Response or effect biomarkers are indicative of biochemical changes within an organism as a result of xenobiotic exposure.

Bioinformatic Analysis of Xenobiotic Reactive Metabolite Target Proteins and their Interacting Partners:

Background:

Protein covalent binding by reactive metabolites of drugs, chemicals and natural products can lead to acute cytotoxicity. Reactive electrophilic metabolites derived from xenobiotic agents covalently modify endogenous cellular proteins [39, 40]. Such covalent binding by reactive metabolites has been strongly correlated with, and is widely believed to be responsible for, the acute organ-damaging effects of a wide range of xenobiotic agents including drugs and natural products [41, 42]. Despite extensive investigation, the mechanisms by which covalent binding events trigger cytotoxic outcomes remain largely unclear [43, 44]. A major reason for this gap is that only recently has it become technically feasible to identify numbers of individual proteins targeted by xenobiotic reactive metabolites. By 1997, only 28 proteins targeted by xenobiotic reactive metabolites had been isolated and identified, largely by N-terminal sequencing [43]. In 1998, however, the coupling of 2D gel electrophoresis with mass spectrometric methods of protein identification literally revolutionized the field. To help keep track of this information and to facilitate its analysis, we recently built the Reactive Metabolite Target Protein Database (TPDB) [45, 46].

Methods:

The identities of the target proteins used in this study, and the structures and names of the protoxins whose metabolites bind to them, are freely available from the reactive metabolite target protein database [45, 46]. For the Gene Ontology and KEGG Pathway analyses we used all 171 proteins listed in the TPDB as of February 2008. For the protein-protein interaction analysis we used the "rank by number of hits" function within the TPDB to select the 28 proteins most commonly targeted by different reactive metabolites. For the machine learning study we used 37 proteins targeted by thiobenzamide metabolites, 20 proteins targeted by bromobenzene metabolites, and 25 proteins targeted by both (82 proteins total). To create a negative learning dataset, all rat proteins in UniProtKB [47] were downloaded on May 5, 2008. A software program CD-HIT [48] was used to filter redundant sequences in the dataset at the level of 80% identity. We also eliminated all sequences with lengths less than 40 residues and subtracted all known target proteins to arrive at a set of 11482 proteins in the negative (non-target) learning dataset. Although the list of target proteins is incomplete, the percentage of all proteins in living cells that actually become detectably adducted by reactive metabolites is relatively small (< 10% based on comparisons of 2D gels vs. their autoradiograms [44, 49]; thus the vast majority of proteins in the negative dataset are considered as non-target proteins. In addition, the Random Forest algorithm used to analyze target vs. non-target proteins is relatively tolerant of small amounts of "noise" in the data.

Software programs and databases used in the study:

The Gene Ontology project [50] is a collaborative effort to develop standard vocabularies (ontologies) that describe gene products in terms of their associated biological processes, cellular components and molecular functions in a species-independent manner. The controlled vocabularies are hierarchically structured (multiple parent levels are permitted) so that they can be queried at different levels. KEGG is a widely used database of biological pathways. The binary protein-protein interactions (8919 proteins in 34364 distinct PPIs) were then imported into Cytoscape, an open source bioinformatics software platform for visualizing and analyzing biological interaction networks. The Cytoscape plugin BiNGO was used to determine and visualize statistically overpopulated GO categories in a set of genes [51].

Random forest models and feature extraction:

We used a random forest package [43] implemented in the "R" environment for this study. Since random forest models are usually insensitive to the model parameters, we used the default parameters. Values for a set of 211 protein features were calculated using various software programs or in-house scripts. These features include protein composition in terms of amino acid residues, predicted secondary structures, solvent accessibility, and others. Although the predictive model might have benefited from incorporating elements of protein three-dimensional structure, such information is not available for the vast majority of target proteins. Therefore we used only sequence information and predicted secondary structures. Since the sequence of a protein is a major determinant of its structure, it is expected that features calculated from sequence information alone may be sufficient to distinguish target proteins from other proteins. Software programs for predicting a broad spectrum of other protein properties such as secondary structure, solvent exposure, instability index and others are mature and have found many applications.The dipeptide or tripeptide composition of proteins has been found to correlate to a number of protein properties. For example, Guruprasad *et al.* found a correlation between the dipeptide composition and the stability of proteins.More recently, tripeptide composition was applied in a machine learning model to predict protein-protein interactions.In this study, the 20 amino acids were sorted into just five groups according to their physicochemical properties: hydrophobic, GAVLIMPFW; polar, YTSNQ; positive, RKH; negative, DE; the fifth group contained only cysteine because of its unique ability to form disulfide bonds. Therefore the number of possible tripeptide features according to this classification scheme is 5*5*5 or 125.

Ranking feature importance:

The random forest approach offers several methods to assess the importance of features based on their contributions to the correctness of the resulting classification. These methods include

i) The mean decrease in accuracy for each class,

ii) The mean decrease in accuracy over all classes, and

iii) The mean decrease in the Gini impurity criterion [44].

In this study the mean decease in accuracy was used for the minor class (target proteins) because in previous work we found it to give more accurate results than other measures for ranking feature importance.

Performance evaluation:

For imbalanced data (i.e., comparisons of small vs. large data sets), the overall classification accuracy is not an appropriate measure of performance because very high accuracy can be achieved simply by predicting every case as the majority class (i.e. the control proteins) as opposed to predicting the minority class (i.e. target proteins). In this study, we used three indicators of performance: sensitivity, specificity, and area under the curve (AUC) for the receiver operating characteristic (ROC) curve. We ranked all cases in the dataset according to the predicted likelihood of positive status and then employed that rank order to identify correctly classified true positives. We then used these results to generate an ROC curve (i.e., a plot of the true positive rate (sensitivity) against the false positive rate (1 – specificity)). The area under the ROC curve represents the trade-off between sensitivity and specificity over the whole range of data. An AUC of 1 represents a perfect prediction model while an AUC ≥ 0.9 is considered excellent, an AUC between 0.8 and 0.9 is considered good, and an AUC in the range of 0.7–0.8 is fair.

CONCLUSION

The covalent binding of reactive metabolites to cellular proteins has long been associated with the production of acutely cytotoxic effects. The past several years have witnessed much progress toward identifying their reactive metabolites and the specific intracellular proteins that become adducted, often highly selectively, by reactive metabolites from a number of different protoxicants. For the better-studied protoxicants, much is known about the structures and reactivities of their reactive metabolite(s), the structures of the adducts they form on proteins, and the identities of many of their protein targets. Bioinformatic analysis of proteins that interact with greater numbers of target proteins may be able to point toward more fruitful areas for generating and testing hypotheses about mechanisms of toxicity. Finally, given the chemical and structural diversity of cellular proteins, it seems unlikely that simple principles of chemical reactivity will in themselves play an important role in differentiating target from non-target proteins. As shown by studies, it seems equally likely that among adducts generated from toxic compounds, only some but not all will have toxic consequences. It will take time and much more detailed information

about protein adduction in living cells to sort this out, and the outcome is likely to point to a number of mechanistic paths from protein modification to cellular impairment or death.

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