Chapter 24

Biomonitoring for Occupational Exposures Using Immunoassays

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Biomonitoring for occupational exposures involves measurement of parent compounds or metabolites in excreta (usually urine), sera or exhaled breath. Classically, biomonitoring involves collection of the matrix, separation and/or isolation of the compounds of interest from the matrix, followed by identification and quantification of the analytes. In most cases this procedure is labor intensive and involves the need for specialized high capital expenditure equipment. Alternative methods for biomonitoring exist that use immunochemical techniques rather than classical chemical techniques for quantification. Immunochemical methods have advantages over classical chemical techniques in speed and cost of analyses, and capital expenditure for equipment, and in most cases are more sensitive than chemical techniques. In the present monograph we review the use of enzyme linked immunosorbent assay (ELISA) immunochemical techniques for the detection and quantitation of pesticides and/or metabolites with comparison to classical analytical techniques. In addition, the use of circulating antibodies developed in response to xenobiotic exposure are also discussed as potential biomarkers. These "legacy biomarkers" of exposure have potentially far reaching medico-legal and other ramifications inherent in their use as they can serve as biomarkers of exposure in the absence of any chemically detectable analyte in excreta or blood.

Exposure to a xenobiotic does not necessarily mean the existence of a body burden of an agent. In order for a xenobiotic to gain entrance it must be absorbed. Occupational absorption can occur via dermal, inhalation, ingestion or a combination of routes. Whether or not absorption occurs depends on the chemical properties of the xenobiotic, in general, related to its lipid solubility. Once absorbed, a chemical is distributed and partitioned into various tissues due to tissue variations in pH, permeability, etc. More

water soluble chemicals are absorbed throughout the total body water, while more lipophilic substances may be distributed totally in body fat. The loss of chemical from the body can loosely be defined as elimination, which depends on metabolism and excretion. Chemicals may be eliminated by numerous routes including fecal, urinary, exhalation, perspiration and lactation. A chemical can be excreted from the body without metabolism, in which case the parent chemical will be detectable in the urine or other excreta. In other cases, the chemical may be metabolized, which is the process of chemical alteration of the xenobiotic in the body. Metabolism may occur in numerous body tissues including liver, kidney, brain, etc. In most cases, metabolism is the result of oxidation, reduction, hydrolysis, or combination of these processes followed by conjugation, however, direct conjugation with an endogenous substrate is also a pathway for excretion. Most important conjugation reactions include glucuronidation, amino acid conjugation, acetylation, sulfate conjugation and methylation. Glucuronidation is the most common metabolic pathway. Metabolism/excretion and the rate of metabolism/excretion can be affected by age, diet, general health status, race, as well as other factors. In general, the metabolized chemical will be more water soluble than the parent. Also, there may be more than one type of metabolite produced from exposure to one parent (e.g., parent-glucuronide, parent-sulfate, etc.). The amount and ratios of parent-metabolites produced are affected by an individual's general health status, diet, nutrition, degree of hydration, time after chemical exposure, etc. In general, the kidney is the major organ of excretion and is the primary route of excretion of water soluble substances.

Biological Monitoring

Biological monitoring has the potential to assess worker exposure to industrial chemicals by all routes including skin absorption and ingestion. However, biological monitoring is not without its limitations. One limitation is the lack of detailed information on the metabolic fate of industrial chemicals in humans. Most of the toxicological/pharmacological (absorption, distribution, metabolism, excretion) information available is from experimental animals and not easily applied to humans. Another concern is the apparent wide variability seen between individuals in response to a toxicant exposure. The human response to the same exposure of a particular chemical may vary widely. This variability has two sources, 1) variability associated with differences in the penetration of the chemical from the environment to the target organ where the enzyme or biochemical system is affected, and 2) variability associated with differences among individuals in the response and delay of the response of the target organ itself. Working conditions in industry are likely to vary considerably from day to day as well as within the shift due to the fluctuation of the exposure concentration. Inhalation exposure and consequently uptake is not constant. The fluctuation of environmental exposure results in fluctuation of concentrations in the target organ(s). The effect of fluctuation of exposure intensity on biological concentrations depends on the kinetic behavior of the chemical in the body. Target organ concentrations of chemicals with short biological half-lives closely follow the

environmental concentration and therefore have a larger variability. On the other hand, levels of chemicals with long biological half-lives fluctuate very little in the target organs in the majority of biological monitoring data (*1*). Proper knowledge of the fate of a chemical, its pharmacokinetic properties, specific methodologies and other factors described later can control such variability; however, specific methodology and data interpretation remain an obstacle to widespread use of biological monitoring (*2*).

Biological Monitoring for Occupational Exposures. Biological monitoring for occupational exposures usually involves the detection of analytes in matrices such as blood, urine or exhaled air. The classical procedure used to identify and quantitate the analyte of interest includes isolation of the analyte, separation of the analyte from other potentially interferring substances and quantitation by instrumental or other methods. These classical methods have many shortcomings including being highly labor intensive, requiring capital expenditures for expensive equipment, (e.g., gas chromatographs [GC], liquid chromatographs [LC], mass spectrometers [MS] or combinations of these instruments [e.g., GC-MS, LC-tandem-MS]). In addition, recoveries during the separation and isolation phases of the procedure may not be constant, and in some cases be associated with the level of analyte in the original sample, potentially yielding confounding systematic errors. Despite these shortcomings, when adequately controlled, classical chemical biological monitoring has the capacity to quantitate the body burden of substances to the sub-ppb level.

Immunoassays for Urinary Biomarkers

Numerous investigators have described urinary immunoassay as a screen for occupational exposure to a variety of compounds including pesticides (*3-4*). Screening assays are generally quite sensitive, specific and accurate. However, unique patterns of sensitivity and cross-reactivity appear to be assay dependent and a detailed knowledge of assay performance characteristics is necessary for accurate interpretation of urine testing data (*15*). Screening immunoassays have been shown to have high concordance with instrumental methods for the analysis of clinical specimens (*6*). Immunoassays have also been proposed for use in the screening of large numbers of water samples for the analysis of pesticide residues, as they are cost effective and highly efficient (*7*).

Although many examples of the use of immunoassay for biological monitoring could be cited, (e.g., other investigators (*4*) have also shown the usefulness of ELISA techniques as biomarkers of pesticide (atrazine) exposure), a detailed description of one such use in our laboratory will be reviewed comprehensively to give the reader an appreciation of the some of the inherent difficulties and advantages of these techniques. We recently reported on the use of a commercial enzyme linked immunosorbent assay (ELISA) to qualitatively evaluate the body burdens of alachlor or alachlor metabolites in urines collected from pesticide applicators (*8-10*). Twenty pesticide applicators and seven hauler/mixers participated in the study. Also, eight employees of the pesticide application companies, who were thought to have limited exposure to pesticides, submitted urine samples for estimates of alachlor dose. All study participants were male. Participants in the study were asked to provide three urine voids over a 24-hr period: one on the morning of the exposure survey before they began work; one at the end of the

application period; and one as the first-void sample the morning following the exposure survey. Each void was collected separately in a wide-mouthed 500-mL polyethylene bottle, and the time and volume of the void were noted. Two 25- to 50-mL aliquots of each void were transferred to 60-mL high density polyethylene bottles and immediately frozen on dry ice. To estimate possible contamination of urine samples during voiding, a second uncapped 500 mL high density polyethylene bottle containing 50 mL of distilled water was taped to the side of the urine collection bottle (N=4). The urine samples were analyzed by both high-performance liquid chromotography (HPLC) and ELISA techniques.

High-performance Liquid Chromatography (HPLC). Briefly, putative alachlor metabolites present in the urine are alkaline-hydrolyzed at 150°C, and the resultant diethylaniline (DEA) produced is quantitated by HPLC. The urine samples were hydrolyzed in methanol/ sodium hydroxide, and hydrolysis performed for 1 hr in a bath consisting of sand and aluminum oxide which was fluidized by compressed air and maintained at 150°C. In order to control for hydrolyses and systematic losses, normal volunteer control urine samples were spiked with a DEA-yielding pseudo-metabolite of alachlor [(2-[2,6-diethylphenyl)-(methoxymethyl)-amino]-2-oxo-ethane acid, pseudo-DEA] and analyzed by HPLC as above.

Enzyme Linked Immunosorbent Assay (ELISA). A commercially available immunoassay kit (EnviroGard™, ImmunoSystems, a subsidiary of Millipore Corp, Scarborough, ME), designed for the analysis of alachlor in water, was also modified for the urinary analyses. The immunoassay format is a competitive solid phase ELISA method which is based on the inhibition of the reaction of enzyme-labelled (horseradish peroxidase) alachlor with immobilized polyclonal anti-alachlor antibodies by free alachlor present in the standard or test sample. Briefly, 80 μL standardized sample or a diluted urine sample were added (in triplicate) to each of the wells of the pre-coated 96-well microtiter plates. Eighty (80) μL of alachlor-enzyme-conjugate were then added to each well and the plates were covered and mixed on an orbital shaker (200 rpm) for 2 hr at room temperature. Plates were then thoroughly washed, substrate (hydrogen peroxide)added followed by chromogen (tetramethylbenzidine) followed by incubation at room temperature for 1 hr, again, with shaking. Forty (40) μL of stop-solution (H_2SO_4) were then added and the plate solutions were agitated again (200 rpm). The absorbance of the solutions in the wells were read on an automatic microplate reader at 450 nm against an air blank.

The ELISA and HPLC analytical methods gave statistically significantly ($P<0.0001$, one way ANOVA) different results when applied to the 82 specimens that were above the analytical LOD for both methods. The mean result for the samples, analyzed by ELISA (N=82) with results above the analytical LOD was 22.6 ± 1.79 μmole/L as alachlor equivalents (± standard error, [SE]), while the HPLC method gave a mean result of 3.23 ± 0.38 μmole/L DEA (Figure 1). When correlation between the two methods was investigated using simple orthogonal regression techniques, a highly significant ($P<0.0001$; $r=0.89$) linear association was observed. The relationship between the two methods was ELISA results (as alachlor equivalents [μmole/L]) = 4.12 HPLC (as DEA, [μmole/L]) + 9.25 (Figure 2). These results demonstrate a positive bias

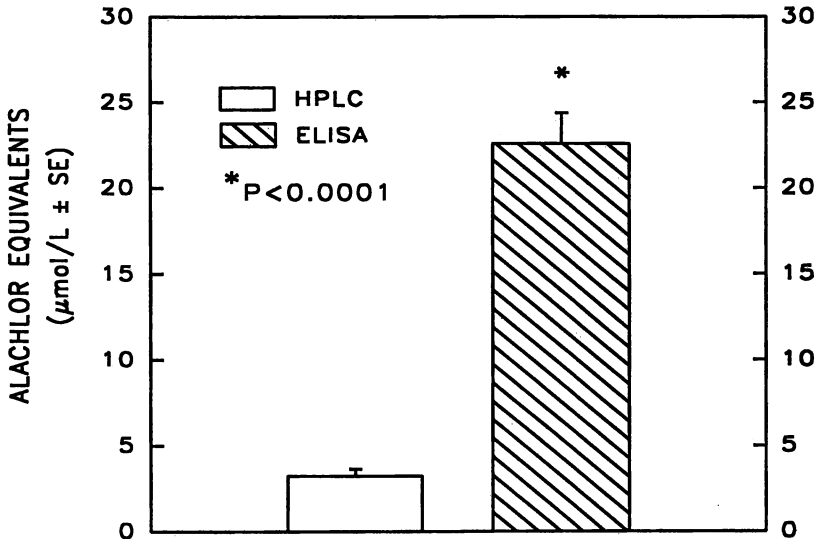

Figure 1. Results for alachlor equivalents determinations by HPLC (μmole/L DEA and ELISA μmole/L), N=82. Adapted from ref. 8.

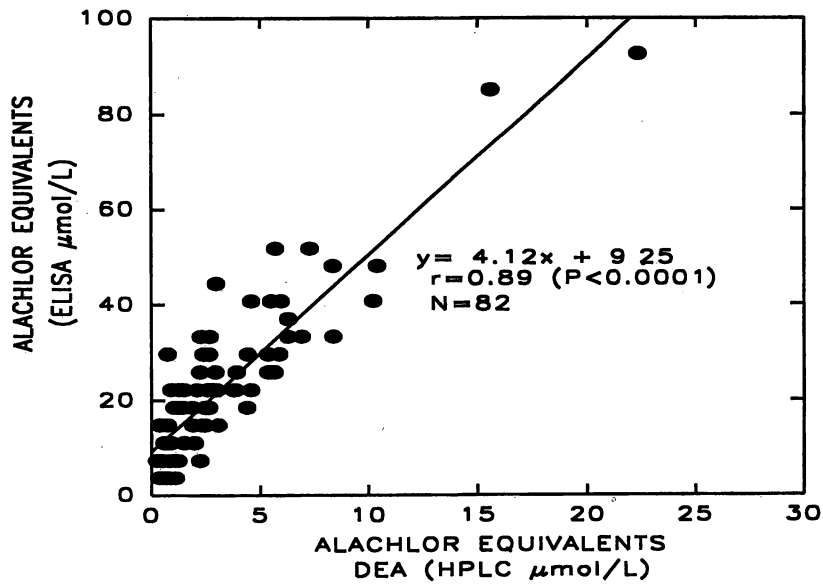

Figure 2. Orthogonal regression analysis of alachlor equivalents measured by HPLC and ELISA. The equation for the regression line is given on the Figure. Adapted from ref. 8.

by the ELISA method when compared with the hydrolysis/HPLC method (8-10). The basis of this systematic bias is unknown, but probably is related to similarities in structure between putative human alachlor metabolites excreted in the urine and the primary immunogen used to produce the polyclonal antibodies used as the basis of commercial kits.

Alachlor, MW 270, is too small to be immunogenic in its own right. To overcome this, most antibodies for alachlor and other chloroacetanilide herbicides are raised against a derivatized chloroacetanilide that is coupled to a carrier macromolecule (usually a protein) with a thioether linkage (7,15). Polyclonal antisera to these alachlor-protein-thioethers would be expected to contain antibodies to numerous antigenic determinants on the immunogen molecule, including the thioether region, probably with differing affinities and avidities for each antigenic determinant. We hypothesized that higher affinity of the putative thiolated human urinary metabolites of alachlor present in the operators' urine was the reason for the discrepancy between our observed HPLC and ELISA results. In order to test this hypothesis, alachlor mercapturate (a known human metabolite of alachlor metabolism in humans, personal communication, Jack Driscoll, CDC/CEH) was synthesized. Briefly, alachlor was reacted with N-acetyl cysteine in the presence of sodium methoxide as a base. The resulting reaction mixture is adjusted to pH ~ 7 with aqueous sodium phosphate followed by continuous liquid/liquid extraction with methylene chloride. The existence of the mercapturate was verified by fast atom bombardment mass spectrum (FAB-MS) MH+ peak at 397 atomic mass units (amu) and the electron impact mass spectrum (EI-MS) M+/. peak at 396 amu.

When alachlor mercapturate was evaluated for binding in the Millipore kit, it was found that essentially parallel standard curves were observed, with the alachlor mercapturate curve shifted to lower concentrations yielding greater (lower optical density) responses. Linear interpolation of the two curves suggests that that if alachlor mercapturate concentrations were interpolated from a standard curve produced with alachlor parent, an approximate 5X overestimation in concentration would occur (see Figure 3).

Immunoassays for Circulating Antibodies

The measurement of circulating antibodies for biomonitoring of exposure is not new, and is commonly used in clinical medicine for assistance in the diagnosis of diseases where exposure to a pathogen has caused an antibody response. The use of anti-xenobiotic antibodies as biomarkers of exposure, has also been reported (*11-13*). And in some cases, such as immediate hypersensitivity diseases, the biomarker (IgE) serves as both a marker of exposure and is pathognomic in diagnosis. Again, to describe these phenomena, a detailed example from our laboratory will be reviewed. An interesting concept regarding the use of circulating antibodies for detecting exposure is that the half-life of the antibody may be longer than the half-life for elimination of the parent compound or metabolite. In this situation, a specific biomarker of exposure is present in the absence of chemically detectable parent or metabolite in serum or excreta. This is more of a "legacy" or history of exposure rather than a direct biomonitoring technique. Also, "legacy biomonitoring" is subject to false negatives, but not to false

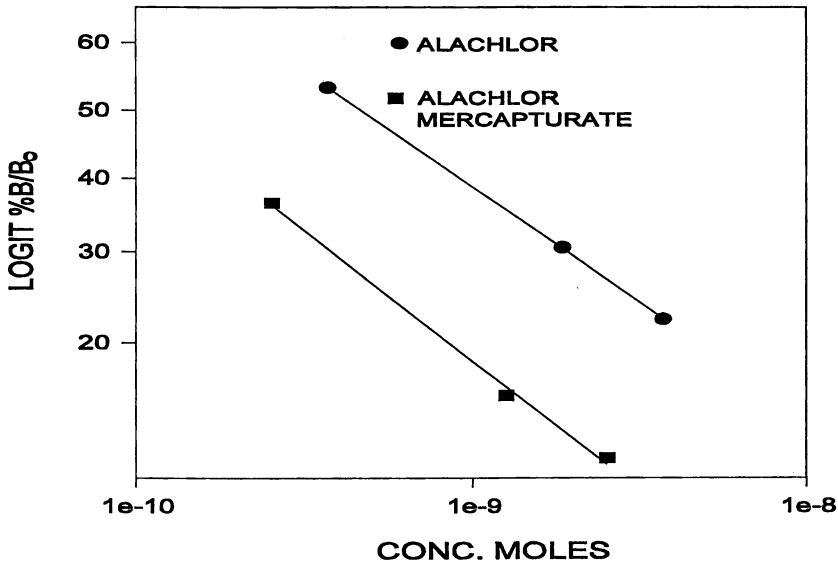

Figure 3. Comparison of standard curves of alachlor vs. alachlor mercapturate. For clarity in direct comparisons, concentrations are given in moles. Adapted from ref. 8.

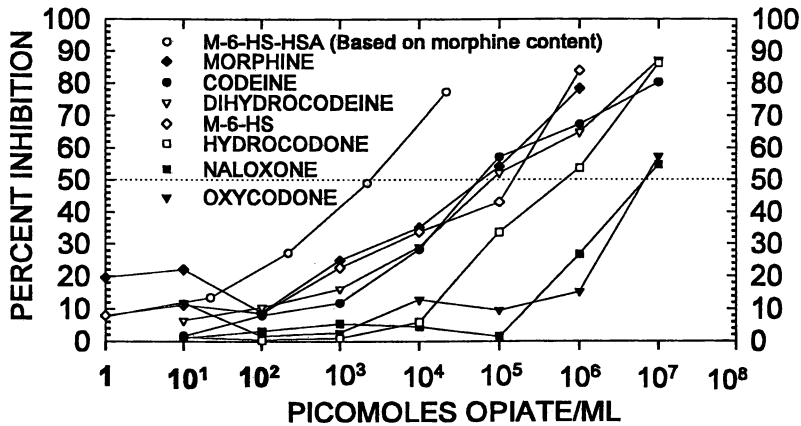

Figure 4. ELISA inhibition studies with opiate nucleus containing compounds. Fifty percent inhibition is indexed by a dotted line. Adapted from ref. 16.

positives, i.e., one can only make antibodies to substances to which they are exposed (with the caveat of antibody cross-reactivity to similar substances due to the polyclonal nature of the human antibody response) . In the case of environmental exposures, numerous individuals are exposed to numerous environmental immunogens daily, (e.g., molds and pollen). Not all individuals will make the same type, amount or specificity of antibody from these exposures as there are individual genetic and other factors which control antibody production. However, in the case of an occupational or environmental agent to which there are few confounding environmental exposures (e.g., soluble platinum halide salts, opiates), the existence of antibodies indicates, with some exceptions, a positive exposure history to the compound or class of compounds.

Some reactive small molecular weight molecules, while not immunogenic in their own right because of size and other limitations, may bind to constitutive polymers (such as host proteins) and become immunogenic, causing the production of specific antibodies. Alternatively or in addition, exposure to some small molecular weight proteins may cause the production of new antigenic determinants (NADs) formed by interaction of these relatively reactive small molecular weight compounds with selected protein carrier molecules. Antibodies can be made to these NADs of constitutive proteins or to the parent hapten-conjugate.

In an investigation of factory workers who extract morphine and other related alkaloids from opium gum or related opium poppy (Papaver somniferum) concentrates, antibodies to opiates were observed. Morphine-6-hemisuccinate (M-6-HS) was prepared by heating morphine (Morphine Alkaloid Powder U.S.P.) with succinic anhydride. The M-6-HS was then conjugated to human serum albumin, dialyzed under reduced pressure and purified by gel filtration. Specific IgG antibodies to M-6-HS-HSA were measured by a modified indirect microtitre plate ELISA method. In order to determine the specificity of human antimorphine antibodies, inhibition studies with morphine nucleus containing pharmaceuticals were performed (see Figure 4). Varying concentrations of M-6-HS-HSA, morphine sulfate, codeine phosphate, dihydrocodeine bitartrate, oxycodone HCl, hydrocodone and naloxone HCl solutions were incubated with a positive serum known to have high levels of specific IgG antibodies to M-6-HS-HSA, for two hours at 37°C. Following this 2 hr preincubation, the sera were analyzed by ELISA as above. The amount of specific IgG binding (in triplicate, represented by optical density) contained in the inhibited serum was compared to that in the uninhibited serum, and the percent inhibition calculated. As can be seen, there was cross- reactivity between the different opiate compounds tested, indicating, as would be expected, a polyclonal antibody response from opiate exposure. These findings have been essentially corroborated by other investigators studying heroin addicts (*14*).

The usefulness of "legacy biomonitoring" becomes most apparent when one wants to evaluate the results of engineering controls for reducing exposures. It would be impractical or impossible to daily monitor the urine or other excreta of someone exposed to a xenobiotic in order to evaluate if an engineering control methodology was effective. However, within certain limitations, antibody levels are related to exposure levels. In general, if antibody levels are reduced, exposures have been reduced. For example, in the study of workers exposed to opiates previously described, engineering controls were initiated to control exposures in February, 1988, and sera were collected

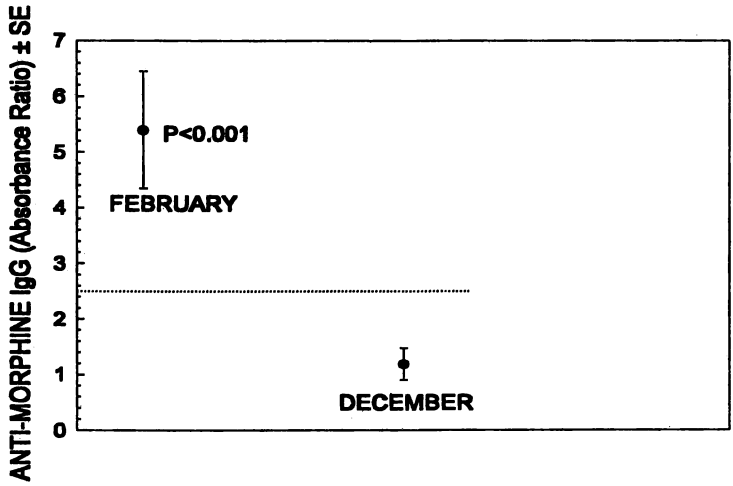

Figure 5. Morphine specific antibodies in workers' sera in February and December of the same year after implementation of an improved respiratory protection program. The dotted line indicates the mean absorbance ratio value of normal non-exposed controls. See text for details. Adapted from ref. 16.

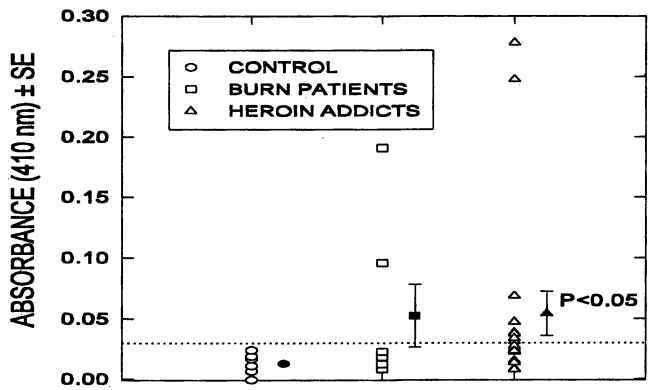

Figure 6. Morphine antibody levels in heroin abusers, burn patients, and non-opiate exposed controls. Individual absorbance values as well as group mean absorbance values (filled symbols ± standard error; variance of the control patient means is within the symbol) are given on the Figure. The dotted line indicates 2.5 X the mean absorbance value of normal non-exposed controls. Adapted from ref. 16.

for antibody levels. Ten months later in December, 1988, sera were again collected and evaluated for antibody levels. As can be seen from Figure 5, results from workers who provided sera at both the February and December, 1988, testing periods showed statistically significant reductions in antibody levels at the December 1988 testing period, coinciding with the improved engineering controls and putative reductions in opiate exposures. Data are presented as mean $\pm$ SE (standard error) absorbance ratios (5.4 ± 0.95 in February, 1988 and 1.19 ± 0.95 in December, 1988). ELISA results were normalized by calculating an absorbance ratio (absorbance of experimental sera/absorbance of volunteer negative control sera [N=6]). The negative control sera were obtained from individuals in the Cincinnati, OH, area with no known chronic or abusive opiate exposure except for possible sporadic therapeutic exposure to low levels of codeine-containing analgesics and antitussives.

It seemed apparent that anti-morphine antibodies could be used to determine morphine exposure in the absence of identifiable parent or metabolite in excreta. We obtained 28 samples from the National Institute on Drug Abuse (NIDA) for use in a double-blind study. Eighteen of the samples were obtained from 8 healthy male, HIV negative, individuals admitted to NIDA for treatment. These subjects identified heroin as their drug of choice, had been drug-free for a minimum of three days, and all had non-detectable levels of heroin or morphine (opiates) in their urine. Seven other samples were obtained from burn patients prior to surgery, who were considered drug-free except for perhaps sporadic use of morphine for analgesia. Three control samples were obtained from NIDA staff members. Two of 8 of the burn patient samples and 7/18 of the heroin abuser samples were positive ($\geq$2.5 times mean control absorbance). Mean absorbance values for the heroin abusers were significantly greater than control values ($P<0.05$). Sera from burn patients, while having positive evidence of antibodies in 2 individuals, was statistically indistinguishable from control sera (Figure 6).

Summary

In the present monograph, we reviewed some of the methods we have used with immunoassays to detect exposure to xenobiotics in occupationallly and other exposed individuals. We have divided our discussion into two distinct parts, immunochemical measurements of parent or metabolite in excreta (urine) or measurements of specific antibodies produced from exposure to selected xenobiotics (legacy biomonitoring). Examples were given from our past work and describe the benefits and disadvantages of both methods when applied to real field study paradigms.

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