Biomarkers of Xenobiotic Exposures

MARGE A. BREWSTER, PH.D.

University of Arkansas for Medical Sciences,
Departments of Pathology and Pediatrics
and
Arkansas Children's Hospital,
Little Rock, AR 72202

ABSTRACT

Direct measurement of xenobiotic (foreign) chemicals is not always feasible as an exposure assessment,—owing to rapid metabolism, sequestration into fatty tissues, or lack of suitable assay methods. Furthermore, suspect exposures often involve complex mixtures of organics. In these circumstances, indirect biomarkers of exposure can be most helpful. This paper reviews four urinary parameters that hold promise as biomarkers of exposure in occupational and environmental settings: glucaric acid (end-product of the glucuronidation pathway), thioethers (end-product of glutathione reaction with electrophilic or alkylating agents), porphyrin pattern (altered with disruption in heme biosynthesis), and the Ames mutagenicity test.

Introduction

Classical approaches to assessing exposure to xenobiotic (foreign) chemicals involve measurement of the suspect agents in biologic fluids or tissues. Numerous organic chemicals are rapidly metabolized to other compounds for which suitable assays may not exist; highly fat-soluble compounds may be undetectable in aqueous biologic fluids; the suspect exposure may involve complex mixtures of organics (often potentially including substances of unknown identity). These circumstances dictate application of indirect assessment of exposure status. This paper reviews several urinary parameters that hold promise as biomarkers of exposure in occupational and environmental medicine.

Glucaric Acid

Many xenobiotics are biotransformed to conjugates of glycine, sulfate, acetic acid, or glucuronic acid; often this biotransformation is accompanied by enhancement of the transformation pathways. Glucuronidation occurs with primary amines (produced by reduction of azides and nitrites or oxidation of alkylamines) and with alkyl and aryl alcohols (produced from oxidation of aromatic hydrocarbons, ethers and aryl and alkyl hydrocarbons or hydrolysis of esters) and thus is a major transformation route for a variety of xenobiotics. Stimulation of the glucuronidation pathway results in increased excretion of glucaric acid, primarily through decreased production of...
glycogen with a possible role of direct induction of pathway enzymes⁶⁷:

UDP Glucose

Glucose

UDP Glucuronic Acid → D-Glucuronic Acid → D-Glucaric Acid

Numerous therapeutic drugs stimulate this pathway and often concomitantly increase hepatic microsomal drug metabolizing enzymes.⁴⁰,⁴¹,⁵¹,⁵²,⁵₅,⁵₈,⁶₆,⁶⁹,⁷₀ Hepatic cytochrome P-450 content correlates significantly with urinary excretion of glucaric acid,⁴₂,⁷₉ but there is evidence that enhanced glucaric acid excretion is not always accompanied by induction of hepatic microsomal enzymes.⁷¹

Pregnancy³⁵ and estrogen therapy⁶² produce a non-significant increase in glucaric acid excretion. Smoking has been reported to have no effect²³ and to have a significant effect.⁷⁶ Intra-individual variation has been reported as high as 50 percent, possibly reflecting variation in daily effects of environmental factors.⁷⁹ Endogenous metabolites known to conjugate with glutathione include dihydroxyphenylalanine (DOPA), estrogens, and prostaglandins.⁸³

Reference ranges reported are summarized by Fiedler et al,²³ and most were obtained with modifications of the Marsh⁵⁸ enzymatic assay (in which glucaric is partially converted by heat to D glucaro-1, 4-lactone which inhibits beta-glucuronidase activity at acid pH). Fiedler et al²³ questioned the inconsistencies in reference ranges and found the enzymatic approach to be highly subject to interference by other urine constituents. Time, pH, and temperature all affect the equilibrium between glucaric acid and its intramolecular esters, the lactones. Fiedler and coworkers, therefore, preferred the ion-exchange chromatography/colorimetric method of Ishidata⁴⁴ and the gas-liquid chromatographic method of Gangolli.²⁶ Disease states known to be accompanied by increased excretion of glucaric acid include alcoholism,⁶⁰ early stage renal disease of children,⁶⁵ and liver diseases.³⁶,⁸₈ Adults with chronic renal insufficiency did not increase excretion.⁴⁶ Decreased values of glucaric acid excretion have been found to occur in patients with congestive heart failure,¹⁰⁰ starvation,⁹⁰ severe burns,¹⁰ favism,⁸ and with dystonic reactions to normal doses of antiemetics.⁹

Hunter et al⁴¹ studied glucaric acid excretion of factory workers involved in production of organochlorine pesticides (Aldrin, Dieldrin, Endrin and DDT). An increase was shown in some of the exposed employees and glucaric acid was correlated quantitatively with DDT metabolism. Two studies of Endrin manufacturing plant workers have been performed, showing higher values after seven working days than after three leave days¹⁰⁹ and higher values correlated to exposure days and to excretion of the major Endrin metabolite.⁷₄ Following a six week shutdown of the Endrin manufacturing plant for maintenance, excretion of glucaric acid in workers returned to normal. Notten and Henderson⁷₂ studied glucaric acid excretion of employees from various metal and chemical factories. They again showed increase in some of the potentially exposed employees. Styrene exposure, as documented by urine metabolites measurement (mandelic and phenylglyoxylic acids), did not increase glucaric
acids excretion in workers.\textsuperscript{39} Persons identified as being at high risk for exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin in Times Beach, MO were not significantly different in glucaric acid excretion compared to those at low risk for that exposure.\textsuperscript{93} A significant increase in glucaric acid excretion was observed in a survey of electrical workers exposed to polychlorinated biphenyls (PCBs).\textsuperscript{57} Notten and Henderson\textsuperscript{71} studied excretion of glucaric acid in guinea pigs chronically exposed (eight to 35 days) to low doses of 26 compounds, including organic solvents and pesticides. Eighteen of these compounds resulted in significant elevation above controls, leading Notten and Henderson to state that “it seems justified to assume that an enhanced urinary excretion of D-glucaric acid is useful as a nonspecific parameter for exposure to xenobiotic compounds.”

**Thioethers**

Electrophilic (alkylating) agents can be inactivated by reaction with glutathione or other sulfhydryl compounds. These conjugates often appear in urine as mercapturic acids or other thioether (R-S-R) products:

\[
\begin{align*}
\text{Xenobiotic (alkylating)} & \quad \text{Glutathione} \\
\gamma-\text{Glu-Cys-Gly} & \\
S & \\
\text{Xenobiotic alkyl group} & \\
N-\text{acetyl-Cysteine} & S \\
& \text{Alkyl}
\end{align*}
\]

Xenobiotics known to undergo this detoxification sequence include aromatic hydrocarbons, arylamines, arylhalides, halogenonitrobenzones, aralkylhalides, aralkylesters, alkyl phenols, alkyl halides, nitroalkanes, cycloalkanes, halogenocy-cloalkanes, carboxyl acids, esters, sulphonamides, sulphur mustards, and \(\alpha-\beta\) unsaturates.\textsuperscript{55} Most genotoxic compounds (or their metabolites) are in this electrophilic class.\textsuperscript{34} Although studies of this detoxification pathway began in 1879, measurement of urinary thioether excretion as a biological parameter of human exposure to potentially alkylating agents was first proposed in 1977 by Seutter-Berlage et al.\textsuperscript{84} The urinary products of reactive metabolites combined with glutathione (collectively measured as thioethers), are considered by Mitchell\textsuperscript{81} to be an index of the formation of chemically active drug metabolites in the organism.

Procedures for the measurement of thioethers (R-S-R) generally involve their conversion to thiols (R-SH) by alkaline hydrolysis, followed by thiol quantitation according to Ellman\textsuperscript{18} (RSH + dithiobisnitrobenzoic acid \(\rightarrow\) 2-nitrobenzoic acid). Seutter-Berlage et al\textsuperscript{83} modified this method to correct for free SH groups and disulfides in urine by a pre-hydrolysis and post-hydrolysis subtraction. The method also included a volatilization step to reduce the contribution of volatile sulfur compounds originating in food substances (onions, cabbage species, pineapples, and beans).\textsuperscript{85} As cysteine is a major contributor to high background values, van Doorn et al\textsuperscript{104} added an ethyl acetate extraction step to reduce the cysteine contribution; this step also introduced the factor of variable recovery of cysteine conjugates. Correction for the presence of disulfides has been performed by sodium borohydride reduction prior to measurement of “background” thiols.\textsuperscript{83,104} Buffoni et al\textsuperscript{7} developed a highly specific method involving removal of free SH and SS groups by reduction and affinity chroma-
tography, partial removal of cysteine and yellow pigments by cation-exchange, and followed by application of the Ellman reagent.

All these methodologies show that smoking results in approximately two-fold increases in excretion. Van Doorn found a good correlation between daily cigarette consumption and thioether excretion.

Measurement of urinary thioethers in workers of chemical and metal industries revealed higher mean excretion by chemical workers (possible exposures were acrylonitrile and biphenyl) compared to metal workers. Studies of chemical plant employees showed that rubber and tire workers had concentrations twice that of clerks, mixers of plastic monomers, and shoe-makers.

It took workers new to the rubber industry three to six months to induce the thioether-producing enzymes. Pesticide packaging workers showed elevation in thioether excretion. Morning (pre-work) and afternoon (post-work) urine samples of persons working near waste incinerators of a chemical plant differed almost two-fold (controls showed no morning-afternoon differences), with 24 percent of the values exceeding the 95 percentile of a (smoking + non-smoking) reference range. Workers at a waste water treatment plant receiving pesticide wastes (spills of chlorinated cyclodiene pesticides and of flame retardants, — primarily hexachlorocyclopentadiene and hexachlorobicycloheptadiene) did not as a group exceed the thioether excretion of workers at another waste water facility lacking such potential exposure. Four of the 10 "exposed" workers had values above any found in the controls, but thioethers did not correlate with urinary concentrations of these two chemicals. Exposure to carbon disulfide by spinners in the viscose-rayon industry was studied by twice daily urinary thioether excretion across a work week, showing repeatedly higher values in the post-work samples (twice the pre-work value). Intra-individual pre-work samples were not elevated, but they did increase slightly across the week, indicating a slight accumulation and also emphasizing that exposure to rapidly metabolized compounds would not be detectable after a few hours. Methylchloride exposure (used as a chemical intermediate for the production of silicone and tetramethyl lead and as a blowing agent in molding polystyrene and polyurethane foams) to 90 ppm in air was not detectable by thioether assay. It was detected by assay of methyl mercaptan, indicating that methylmercapturic acid is not a major metabolite of methyl chloride. Nurses handling cytotoxic drugs (mainly cyclophosphamide, vincristine, and cytoxan) for five days showed a seven-fold increase vs. control nurses; values returned to control range three days post-exposure. Another study of oncology nurses handling and administering adriamycin and cyclophosphamide found only a few "exposed" nurses to have elevated thioethers; cancer patients receiving this chemotherapy were also studied. At the high doses used, an inverse dose response was interpreted as being indicative of a change in detoxification efficiency. Six women employed in dry cleaning workshops (with tetrachloroethylene air concentrations 15 to 50 ppm) were studied over one week for evidence of thioether formation. Excretion of thioethers from five individuals increased each day; the one individual studied across three non-working days showed decreased excretion after the rest days. Despite this daily increase, all values were within the range established for non-smokers not known to have exposure. The exposure of petroleum retailers was studied midweek in employees (greater than one year) of
self-service stations, attendant-served stations, and garage mechanics. Post-shift values were significantly higher than pre-shift, with greater differences in attendant-operated (mean = three fold) than in self-service (mean = 1.5 fold) correlating with exposure risk. There was a significant positive interaction between smoking and work-related thioether output.\textsuperscript{92}

Van Doorn et al\textsuperscript{104} have calculated that an eight hour exposure to an air contaminant at one ppm will at most result in thioether excretion increase by 25 mmoles SH per mole creatinine (complete absorption, complete conjugation to glutathione and excretion as thioether in urine) above the non-exposed upper limit. Van Doorn and coworkers further predict, using animal data on conversion to thioethers, that exposure to maximum allowable concentration (MAC) of biphenyl (0.2 ppm) "should not lead to significant thioether excretion, that exposure to benzylchloride at the MAC value of 1 ppm would result in an increase of about 4 mmoles/mole creatinine, naphthalene (10 ppm) about 8 mmoles/mole creatinine and of chlorobenzene (75 ppm) about 200 mmoles/mole creatinine." However, as only traces of mercapturic acids were found in the urine of humans receiving a single 500 mg dose of naphthalene, Van Doorn et al advise caution in the extrapolation to human of animal metabolic data for such predictions.

All these studies are considered as qualitative-only indicators of exposure, since metabolite identities are unknown, losses are variable during extraction and hydrolysis, and dose-response is unknown. Henderson et al\textsuperscript{34} and Van Doorn et al\textsuperscript{107} have critically reviewed the suitability of the urinary thioether assay as a method for detecting exposure to electrophilic agents or their precursors. They recommend its use as a signal, i.e., for workers involved in chemical waste incineration, in that increased excretion likely indicates exposure, but they caution that normal values should not be concluded to represent no, or negligible, exposure. They urge development of more selective assays of thio compounds in urine for use in specific biologic monitoring applications and also combination of the thioether assay with other non-specific exposure assays.

**Porphyrians**

In the process of heme synthesis in liver, uroporphyrinogen, a tetrapyrrole compound with eight carboxylic groups, is stepwise decarboxylated to hepta-, hexa-, penta-porphyrinogen and then to coproporphyrinogen. Each of these porphyrinogens can auto-oxidize to the corresponding porphyrin. All of these porphyrinogens and porphyrins are normally present in plasma and are excreted in small amounts in urine and feces. Chronic disturbances in hepatic synthesis of porphyrins leads to excess porphyrin excretion and skin symptoms in the final stage. Evidence summarized by Strik\textsuperscript{96} supports the progression sequence:

Liver damage \[\rightarrow\] secondary coproporphyrinuria

Chronic hepatic porphyria \[\rightarrow\] latent porphyria

(Type A \[\rightarrow\] B \[\rightarrow\] C)

Overt porphyria cutanea tarda
Doss\textsuperscript{16} discusses the chronic hepatic porphyrias resulting from liver diseases, alcohol or estrogens, and from an enzymatic defect in uroporphyrinogen decarboxylase. This latter enzymatic defect can be genetic (autosomal dominant) or secondary to certain xenobiotic compounds. The secondary chronic hepatic porphyrias can occur in toxin exposures without an underlying genetic defect; however, this defect is required for manifestations of porphyria induced by liver disease, alcohol, or estrogens.

Each of these stages can be recognized chemically by the urinary excretion of total porphyrins combined with the pattern of urinary porphyrins. The progression of porphyrias is accompanied by gradually increasing total porphyrin excretion, with later stages accompanied by alteration in pattern of the porphyrins excreted (greater uro- and heptaporphyrins, lesser coproporphyrins).\textsuperscript{16}

Increase in total urine porphyrins is known to follow intoxication with heavy metals,\textsuperscript{28} hormones,\textsuperscript{31} and drugs,\textsuperscript{31,81} as well as with halogenated aromatic hydrocarbons.\textsuperscript{29,30,73,94} Halogenated hydrocarbons known to produce chronic hepatic porphyria in man include hexachlorobenzene, octachlorostyrene, 1,4-dichlorobenzene, hexabromobenzene, polychlorinated biphenyls (PCBs), polybrominated biphenyls (PBBs), lindane, 2,3,7,8-tetrachlorodibenzodioxin (TCDD), methyl chloride, vinylchloride, and possibly allylchloride.\textsuperscript{96} In experimental studies, a chronic exposure is usually needed to evoke porphyria, especially in mammals.\textsuperscript{95}

The mechanisms proposed to account for the porphyrogenicity of the poly-halogenated hydrocarbons include early induction of liver microsomal enzymes\textsuperscript{14,29} and later decrease in uroporphyrinogen decarboxylase activity, stimulating the initial step in heme synthesis and leading to liver accumulation and urinary excretion of excessive amounts of uro- and heptaporphyrins. Earlier stages of chronic hepatic porphyria appear to be non-symptomatic and potentially reversible once the damaging agent is removed. Presumably, all xenobiotics known to elevate total porphyrins would show the urinary pattern of milder stages at an earlier time, but the porphyrin pattern has not been studied in every porphyrogenic exposure.

The original method of pattern determination was quite laborious, involving multiple extractions and thin-layer chromatography,\textsuperscript{98} limiting the number of samples assessed in exposure situations. A recent method of separating and quantitating individual porphyrins by high pressure liquid chromatography\textsuperscript{25} appears to greatly simplify this assessment and lends itself to automation. Normal ranges of porphyrins and porphyrin precursors in human urine have been reported by Doss\textsuperscript{16} employing the original method of thin-layer chromatography and by Ford et al\textsuperscript{25} with the high pressure liquid chromatographic method. Effects of age upon these values have apparently not been determined.

A 1978 study of 115 urine samples from persons exposed to dioxin (TCDD) in the 1976 plant explosion in Seveso, Italy revealed 84 percent of samples with abnormal porphyrin pattern, and 23 percent also with increased total porphyrins.\textsuperscript{97} A 1977 study of exposure to PBBs (polybrominated biphenyls; Firemaster BP-6) by Michigan farm families via cattle feed (accident beginning in 1973) showed 47 percent of 142 samples with abnormal porphyrin pattern, none of which had increased total porphyrins.\textsuperscript{97} These two studies detected abnormalities greater than two years post exposure to substances known to store in body fat. In contrast, a 1978 study of 16 persons exposed in 1968 to PCBs (polychlorinated biphenyls) via contaminated rice oils did not reveal an abnormal porphyrin pattern, — a 10 year study lag.\textsuperscript{99}
Workers exposed to PCBs via production of electrical transformers and condensers and having blood PCBs concentrations averaging ten-fold the non-exposed values showed higher excretion (average two-fold) of each of the porphyrin homologues, but not alteration in pattern.11 Strik96 studied 22 samples of persons with occupational exposure to 2,4,5-T (including dioxin), finding 64 percent with abnormal porphyrin pattern, and 50 percent also with increased total porphyrins. Of 40 cases with vinylchloride disease, 75 percent had abnormal prophyrin patterns.50 Residents of Times Beach, MO in the dioxin exposure risk group had significantly higher mean level of urinary uroporphyrins and an increased prevalence of higher urinary values.37 Of 39 samples of persons occupationally "exposed" to hexachlorocyclopentadiene, allylchloride, epichlorohydrin, and Endrin, no abnormal porphyrin patterns were seen.64

Mutagenic Activity in Bacteria

Durston and Ames17 developed a Salmonella assay that has been widely used to test for presence of mutagenic agents or mutagenic metabolites. Sensitivity is often enhanced by the presence of a bioactivation system (e.g., liver microsomes), by testing several bacterial strains, by cleavage of inactive conjugates with B-glucuronidase or arylsulfatase, and by concentration or extraction of the urine. An extensive description of the method has been published.96 Test data on over 5000 chemicals (tested directly) have been compiled,19 and an automated assay system has been developed which monitors increasing turbidity in up to 200 wells simultaneously. Endogenous growth factors (i.e., histidine) can be reliably detected as greater auxotrophic growth, allowing those samples to be retested along with controls correcting for that factor.51 A similar approach is the bacterial fluctuation test developed by Green et al.32

Assays of this type have been widely used to detect effects of smoking,112 diet (various cooked foodstuffs),3,12,63,91 and medications.53,87,101,110,111 These tests have also been used to estimate total content of mutagens excreted in urine of occupationally exposed workers.33 Rubber industry workers had greater mutagenic activity than controls, both smoking and non-smoking; a synergism was suggested between smoking and occupation.13,22 Mutagenic activity was significantly higher in urine of non-smoking chemical and coke oven workers than in urine from non-smoking controls; among smokers, there was no difference.49 Three studies of nurses handling cytostatic drugs gave different results: mutagenic activity with smoking, not correlated with age, coffee, alcohol or antineoplastic drug handling,4 a cytostatic drug effect,20 and a cytostatic drug effect influenced by smoking.5 Similarly, studies of the exposure of pharmacy personnel to antineoplastics report increased mutagenic activity reducible with precautionary measures.2,66 Anesthesiologists exposed to halogenated anesthetic gases had mutagenic urine.59

Mutagenic activity has been present43 and not present108 in urine of workers exposed to coal tar and pitch, present in urine of epichlorohydrin exposed workers47 and foundry workers,82 higher in chemical workers before leave than after,15 and present in carbon electrode industry employees.75 Lowering of occupational exposure decreases average mutagenic activity.89 Most studies have shown wide individual variation in mutagenic activity.103

Simultaneous Urine Parameter Studies

Seutter-Berlage et al86 reported increased excretion of glucaric acid and
thioethers of production workers in a pesticide packaging factory (as compared to administration). Workers with a low risk for exposure to mineral oils in a steel plant did not show elevation in glucaric acid excretion, but they did show mutagenic activity, with suggestion of a smoking synergism.\(^7\)\(^6\) Workers in a chemical plant producing a large variety of pharmaceuticals and explosives were studied in 12 exposure groups by urine samples taken pre- and post-shift and post-vacation. No differences were found in any of the exposure subgroups for thioethers excretion; the subgroup exposed to 2,4,6-trinitrotoluene (TNT) had a significant increase in urinary mutagenic activity and TNT was detected in urine of the workers with highest mutagenic activity, although air concentrations did not exceed allowable limits.\(^1\)

### Exposure Biomarkers

Hogue and Brewster\(^3\)\(^8\) suggest that an "urinary exposure screen" is feasible and desirable as an initial approach to potential exposure situations. Although the initial suggestion included only thioethers, porphyrin pattern, and glucaric acid as components of such a screen, mutagenic activity would also be a candidate for inclusion.

Lohman et al\(^5\)\(^4\) emphasize that "none of the biomonitoring methods presently available is calibrated in terms of human disease (i.e., risk). Consequently, all present biological methods should be considered as indicative of exposure only." Foa\(^2\)\(^4\) emphasizes the advantages of non-specific biomarkers, "they express with a single value the global response of the organism to exposures to occupational and environmental chemicals also taking into account factors related to individual metabolism and lifestyle." Opportunity for multiple concomitant low-level exposures is increasing, situations where dose assessment is not feasible.

Wider application of these tests should lead to better standardization of methodologies, greater knowledge of reference values, effects of dietary factors, disease states, life style influences, and more understanding of chemical sensitivities and specificities.

### References

11. COLORMY, A., MARONI, M., FERIOLIO, A., CASTOLDI, M., JUN, L. K., VALLA, C., and FOA, V.: Increase in urinary porphyrin excretion in


66. NGUYEN, T. V., THEISS, J. C., and MATNEY, T. S.: Exposure of pharmacy personnel to
91. SOUSA, J., NATH, J., TUCKER, J. D., and ONG, T.: Dietary factors affecting the mutagenicity assay system. I. Detection of mutagenic activity


