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Analysis of Urine to Monitor Exposures to Benzidine, o-Dianisidine, o-Tolidine, and 4,4′-Methylenedianiline

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Aromatic diamines have great potential for dermal absorption as an exposure route. Also, the body exhibits the ability to reductively cleave an azo dye to its corresponding aromatic diamine. These factors have demonstrated the need for biological monitoring to complement environmental monitoring when assessing occupational exposures to these compounds. This is especially important since certain aromatic diamines are known or are potential human carcinogens. Therefore, a sampling and analytical procedure for the measurement of benzidine, o-dianisidine, o-tolidine, and 4,4′-methylenedianiline in urine was developed for use in industrial hygiene investigations. After hydrolysis of urine metabolites, the free diamines are isolated using a C_{18} solid sorbent. The free diamines are eluted, concentrated, and injected into a high-performance liquid chromatography system. Separation is achieved using a C_{18} column with a mobile phase of acetonitrile and water. Identification and quantitation is achieved by monitoring the ultraviolet (UV) absorbance at 280 or 245 nm and the electrochemical response. Using UV detection, the limit of detection (LOD) for these aromatic diamines was less than 2 μg/L while the limit of quantitation (LOQ) was less than 6 μg/L. For electrochemical detection, the LOD was less than 0.3 μg/L and the LOQ was less than 0.9 μg/L. Recoveries range from 87 to 102 percent at 2 μg/L, 10 μg/L, and 20 μg/L levels. **Neumeister, C.E.: Analysis of Urine to Monitor Exposures to Benzidine, o-Dianisidine, o-Tolidine, and 4,4′-Methylenedianiline. Appl. Occup. Environ. Hyg. 6:953–958; 1991.**

Introduction

Evidence of the carcinogenicity of certain aromatic diamines was recognized by Rehn in 1895 when high incidences of bladder cancer were noted among men employed in dye factories.[1] Since 1974, benzidine has been regulated as a human carcinogen without a permissible exposure limit (PEL) by the U.S. Occupational Safety and Health Administration (OSHA).[2] The National Institute for Occupational Safety and Health (NIOSH) actively participated in the formulation of this policy and recognizes benzidine as an occupational carcinogen without a recommended exposure limit (REL). Workers' exposure to benzidine is to be controlled through the required use of engineering controls, work practices, and personal protective equipment, including respirators.[3] The American Conference of Governmental Industrial Hygienists (ACGIH) has assigned benzidine a "skin" notation which refers to the potential contribution to the overall exposure by the cutaneous route, including mucous membranes and eyes, either by airborne or, more particularly, by direct contact with the substance.[4]

Of an absorbed dose, benzidine is excreted in the urine as free benzidine (4%–10%), mono- or diacetyl benzidine (7%–16%), and much of the remainder as salts.[5] The maximum rate of excretion of the benzidine or its metabolites occurs 2 to 3 hours after an exposure, and urinary levels decline with a half-life of 5 to 6 hours.

Dianisidine and tolidine, the dimethoxy and dimethyl congeners of benzidine, are animal carcinogens.[6] ACGIH classifies o-tolidine as a suspected human carcinogen and gives it a "skin" notation.[4]

Sciarini and Meigs[7] investigated the metabolism of dianisidine and tolidine by intraperitoneal injections into dogs. Of an absorbed dose, they estimated that dianisidine is excreted in the urine as free dianisidine (0.4%) and conjugates (5%), and tolidine is excreted in the urine as free tolidine (4%) and conjugates (40%).

Benzidine, dianisidine, and tolidine (Figure 1) have been used extensively in the manufacture of synthetic dyes and pigments. Evidence of the metabolic conversion of benzidine-based dyes to free benzidine increased the potential number of occupationally exposed workers in a variety of industries (Figure 2).[8] Also, it repeatedly has

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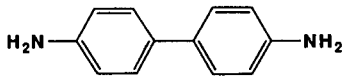
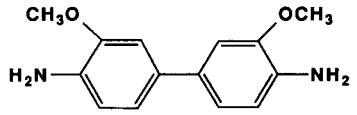
Benzidine



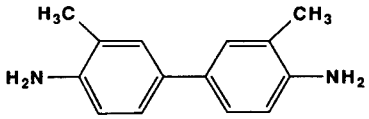
(4,4'-Diaminobiphenyl, 4,4'-Biphenyldiamine)

o-Dianisidine



(3,3'-Dimethoxybenzidine, 3,3'-Dimethoxy-4,4'-Diamoinobiphenyl)

o-Tolidine



(3,3'-Dimethylbenzidine, 3,3-Dimethyl-4,4'-Diaminobiphenyl)

FIGURE 1. Chemical structure of benzidine, o-dianisidine, and o-tolidine.

been demonstrated that intestinal and many hepatic systems reductively cleave the azo bond to liberate free benzidine.[6] The impact of this metabolic fate was reflected by the issuance of NIOSH/NCI Current Intelligence Bulletin 24 for Direct Blue 6, Direct Black 38, and Direct Brown 95.[9] In this document, NIOSH recommends that it would be prudent to handle these dyes in the workplace as if they were human carcinogens. This reductive cleavage of the azo bond to generate the parent diamine is cause for similar concern with dyes based on dianisidine and tolidine. Using sodium hydrosulfite, Kennedy and Seymour[10] demonstrated the ability to reductively cleave benzidine-, dianisidine-, and tolidine-based dyes to generate the free diamine. Although each of the benzidine congener-based dyes have not been specifically tested for health effects, they have several important similarities. These similarities led NIOSH to conclude, after extensive review of the data, that exposure to tolidine- or dianisidine-based dyes in the workplace may increase the risk of cancer to exposed workers and recommends that exposure be minimized.[11]

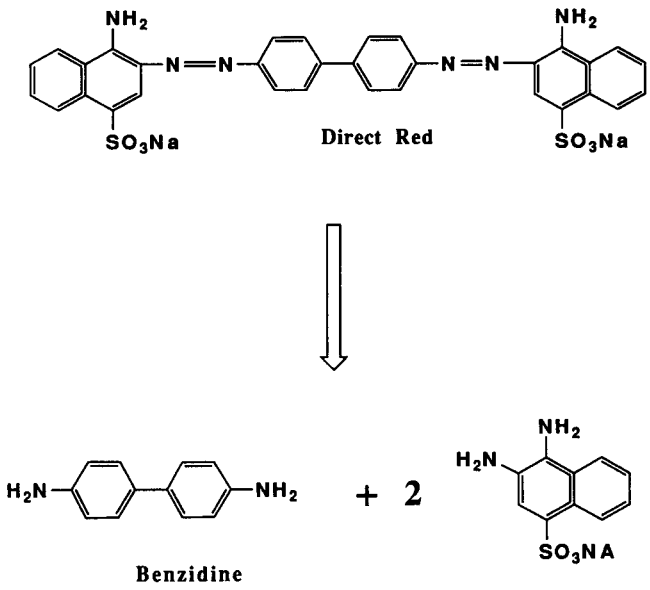
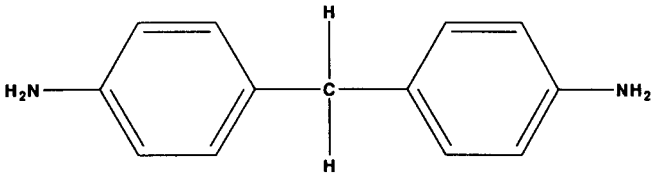


FIGURE 2. Reduction of the azo-linkage in a benzidine-based azo dye.



(MDA, 4,4"-Diaminodiphenylmethane)

FIGURE 3. Chemical structure of 4,4'-methylenedianiline.

Methylenedianiline (MDA) (Figure 3) is a commercially important industrial aromatic diamine used in the production of diisocyanates and as a curing agent for epoxy resins. Evaluation of animal dose data led to the issuance of NIOSH Current Intelligence Bulletin 47 for MDA.[12] In this document, NIOSH recommends that MDA be considered a potential human carcinogen in the workplace. As prudent public health policy, employers should assess the conditions under which workers may be exposed to MDA and take reasonable precautions to reduce exposures to the lowest feasible limit. During the OSHA informal public hearing on MDA on March 20, 1990,[13] NIOSH supported a proposed rule for MDA that establishes a PEL for air of 10 ppb and a short-term exposure limit (STEL) of 100 ppb. However, NIOSH continues to recommend that occupational exposures be reduced below the PEL to the lowest feasible level. At this hearing, NIOSH further recognized that the primary route of exposure was dermal and recommended the use of urine monitoring of workers be utilized to assess the degree of exposure where the likelihood of dermal exposure exists.

Cocker, Gristwood, and Wilson[14] have observed that MDA is excreted in the urine as free MDA (17%), mono- or diacetyl benzidine (31%), and much of the remainder as simple conjugates. Maximum elimination occurs about 2 to 3 hours after the end of the workshift.

Industrial hygienists and epidemiologists continue to conduct studies to evaluate worker exposure to these aromatic diamines. In order to conduct such studies, an analytical procedure is needed for biological and air sam-

ples. This article reports the development of a sampling and analytical procedure for these aromatic diamines in urine.

Methods found in the literature pertained to either benzidine or MDA and utilized liquid–liquid extraction, derivatization, and gas chromatography/electron capture (GC/EC), gas chromatography/mass spectrometry (GC/MS), or UV/thin-layer chromatography (UV/TLC) analysis.[14–17] The reported LOD for these methods ranged from 1 to 10 μg/L. The choice of liquid–solid extraction allows selective extraction, analyte enrichment, greater accuracy due to less cross contamination, faster sample preparation, and greater recoveries due to minimal sample transfer. Liquid chromatography with UV and EC detection provided a sensitive measurement of the compound of interest without derivatization.

Experimental

Equipment

Reverse phase high-performance liquid chromatography (HPLC) followed by UV and electrochemical detection was used to separate and quantitate the free diamines. The HPLC system consisted of a Waters 720 System Controller, Waters 6000 pump, Waters 720B Automatic Injector, Applied Biosystems 783A Programmable Absorbance Detector, Bioanalytical Systems LC-4A Amperometric Detector, Waters Nova C_{18} column in a Radial Compression Unit, Soltec strip chart recorder, and a Hewlett–Packard Laboratory Automation System. The UV detector was set to monitor 280 nm when analyzing for benzidine, tolidine, or dianisidine. For MDA, the UV detector was set at a wavelength of 245 nm. The electrochemical detector (EC) was operated in the oxidative mode and contained a glassy carbon working electrode held at a potential of 0.85 V versus a silver/silver chloride reference electrode. The mobile phase consisted of 0.01 *M* sodium acetate in 30 percent acetonitrile and 70 percent water isocratic. The flow rate was 1 ml/min and the injection volume was 25 μL.

The solid sorbent used during sample preparation was a Waters C_{18} Sep Pak. It consisted of a silica bed whose surface was treated with octadecylsilane held in a self-contained cartridge with a void volume of 0.5 ml.[18]

Reagents

Acetonitrile, methanol, and benzene were Burdick and Jackson High Purity Solvents. The water was from an in-house Pec Water Purification System. Benzidine (95%), o-dianisidine (Practical), and o-tolidine (Practical) were purchased from Sigma Chemical Company. MDA (99%) was purchased from Aldrich Chemical Company.

Biological Specimens

All experiments were conducted with pooled, fortified urine collected from laboratory personnel. The aromatic amines were dissolved in methanol and used to fortify the urine. Urine samples were obtained by NIOSH industrial hygienists involved in surveys of manufacturers using dyes or MDA. The sampling protocol was determined by the field industrial hygienist. Sample collection at a dye manufacturing facility was pre- and postshift. Sample collection involving MDA was first morning void which included a complete 8-hour collection, a 4-hour sample at the end of the work shift, and a final sample 4 hours after the end of the work shift.

Urine was collected in 100-ml, high-density, polyethylene bottles and immediately frozen until time of analysis. When freezing was not possible within 40 hours of sample collection, the urine was adjusted to pH 3 with citric acid.

Cleanup Procedure

At the time of analysis, the urine was thawed, and a representative 50 ml aliquot was transferred to a polypropylene centrifuge tube. The sample pH was adjusted to approximately 12 by adding five pellets of potassium hydroxide (KOH) to the urine. The sample was then heated in an oven at 80°C for 2 hours to hydrolyze the samples to the free diamine. After cooling to room temperature, the samples were centrifuged to remove suspended particulate.

A C_{18} Sep Pak was attached to a 50-ml disposable syringe, activated with 3 ml of methanol, followed by 5 ml of water. The urine sample was transferred into the syringe taking care not to include the settled particulates. Addition of particulates will block the C_{18} Sep Pak. The urine was eluted through the C_{18} Sep Pak at a flow of approximately 1 ml/min. The water and more polar compounds were eliminated while the less polar aromatic diamines were retained on the sorbent. A 10-ml water wash of the centrifuge tube was added to the syringe and eluted through the C_{18} Sep Pak.

The aromatic diamines were eluted from the C_{18} Sep Pak with 10 ml of benzene at a flow rate of approximately 1 ml/min and collected in a graduated glass centrifuge tube. Approximately 0.2–0.4 ml of water remaining on the bed from the wash was eluted from the solid sorbent and was allowed to remain in the tube while the benzene was evaporated with a stream of nitrogen and gentle heat. The samples were diluted to 1 ml with 0.1 *N* methanolic KOH and allowed to stand overnight. Following filtration through a 0.45-μm PTFE filter, the samples were loaded into vials for analysis by HPLC.

Measurements and Calculations

A series of standards for each diamine was prepared from commercially procured compounds. Stock solutions were made by weighing 0.01000 g of each aromatic diamine on a Mettler five-place electronic balance and dissolving in 10 ml of 0.1 *N* methanolic KOH. Standards were made by serial dilution of the stock solutions with 0.1 *N* methanolic KOH.

A recovery study was performed for each aromatic diamine. Five liters of urine were collected from laboratory personnel, stirred for homogeneity, and transferred to 1-liter polyethylene bottles. Each diamine was dissolved in methanol, and the urine was fortified at concentrations of 2, 10,

TABLE I. Method Sensitivity

	Benzidine	Tolidine	Dianisidine	MDA
LOD[A] (EC)[B]				
ng/ml[C]	12	15	8	6
µg/L[D]	0.24	0.30	0.16	0.12
LOQ[E] (EC)				
ng/ml	37	44	35	20
µg/L	0.74	0.88	0.70	0.40
LOD (UV)[F]				
ng/ml	73	68	47	88
µg/L	1.5	1.4	0.9	1.8
LOQ (UV)				
ng/ml	245	227	156	293
µg/L	4.9	4.6	3.1	5.9

[A]LOD = limit of detection.
[B]EC = electrochemical detector.
[C]ng/ml based on analytical standards.
[D]µg/L based on analytical standards calculated to reflect 50 ml urine.
[E]LOQ = limit of quantitation.
[F]UV = ultraviolet detector.

and 20 µg/L. All urine analyses for studies or samples used the cleanup procedure and analytical method described in this article.

A storage study for MDA in urine investigated immediate freezing, the addition of citric acid, or the addition of potassium hydroxide as MDA sample stabilizers. Eighteen 50-ml aliquots of pooled urine were placed into high-density polyethylene bottles and fortified with 10.6 µg (212 µg/L) of MDA. Six fortified urine samples were adjusted to pH 3 with citric acid. Another six samples were adjusted to pH 12 with potassium hydroxide. The remaining six samples were untreated and had a pH of 6. All samples were frozen immediately, and one sample at each pH level was analyzed weekly.

A second study compared the stability of urine fortified with MDA when stored at room temperature. Ten 50-ml aliquots of pooled urine were placed into high-density polyethylene bottles and fortified with 10.4 µg (208 µg/L) of MDA. One sample set was adjusted with citric acid (pH 3) while the other was untreated (pH 6).

Results

Reproducibility and Sensitivity

The wavelength selection for UV monitoring was determined using a Waters 990+ Photodiode Array Detector. Standards containing these diamines were analyzed according to the described HPLC procedure. The photodiode array detector monitored the wavelength range from 190 to 360 nm. The absorption maximum for benzidine, o-tolidine, and dianiside was determined to be 280 nm. The absorption maximum for MDA was determined to be 245 nm.

Using a least squares linear regression program, a calibration curve for each aromatic diamine was established for both detectors using standards in methanolic KOH ranging from 1 to 1000 ng/ml. The LOD was defined as the amount of analyte that can be distinguished from background and estimated as three times the standard error (S_e) of the calibration graph. The Limit of Quantitation (LOQ) was defined as approximately the mass of analyte for which relative standard deviation (S_r) equals 0.10.[16] The analytical LODs and LOQs are given in Table I as ng/ml based on external standards in methanolic KOH and calculated to µg/L assuming a 50-ml urine volume.

Data from the recovery study are shown in Figure 4. Pooled urine was spiked at levels of 2, 10, and 20 µg/L. Each level consisted of three samples containing all four diamines. The samples were treated and analyzed by the

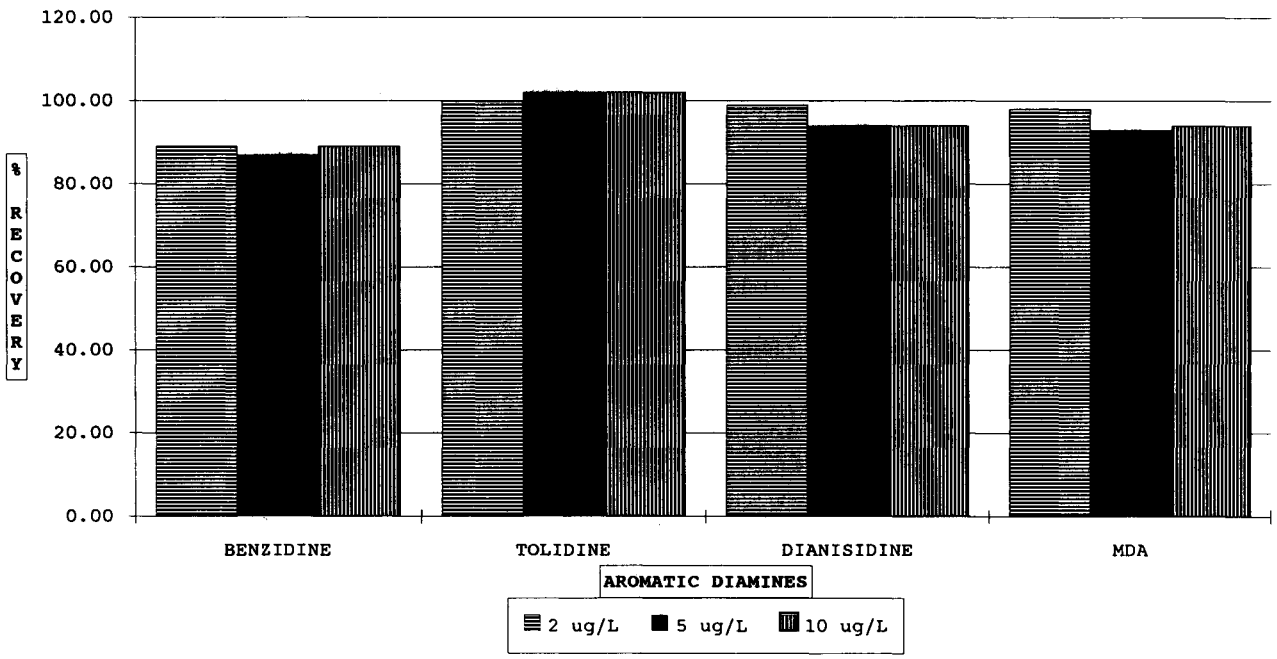


FIGURE 4. Recovery study for benzidine, tolidine, dianisidine, and MDA.

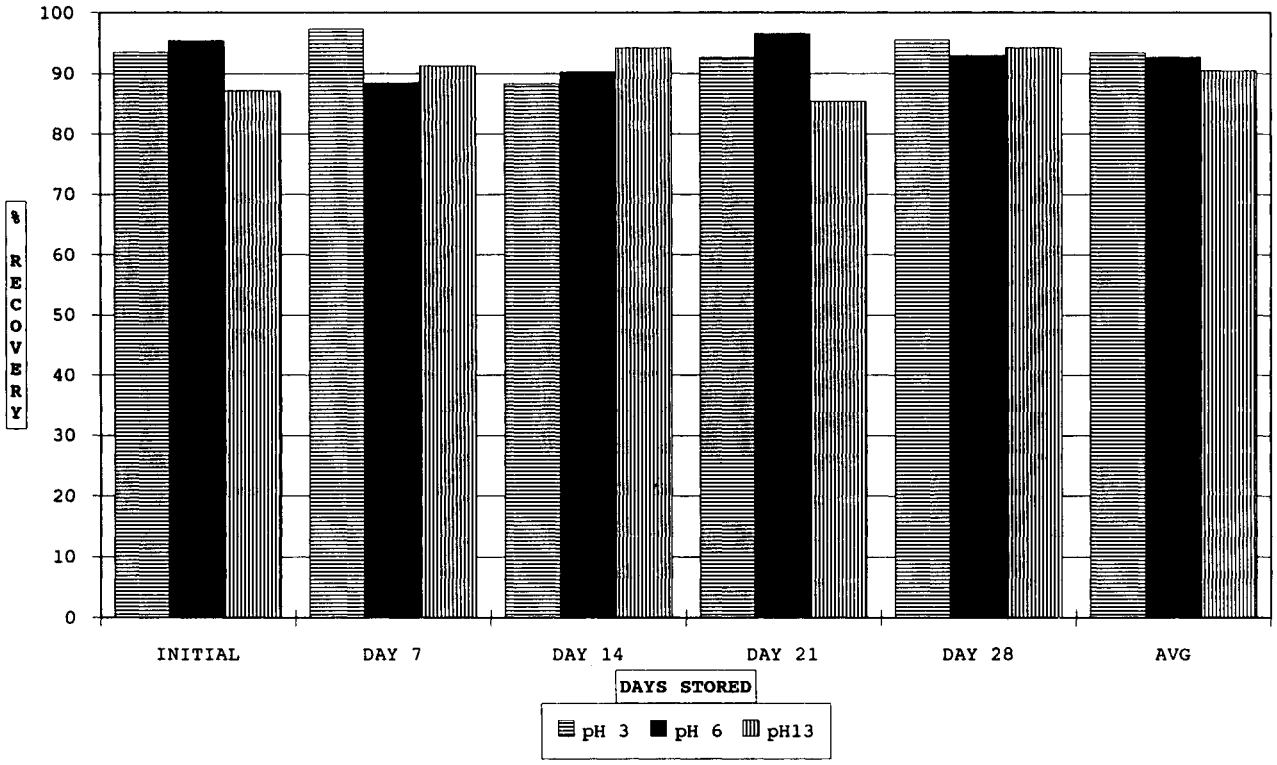


FIGURE 5. Storage stability study for MDA.

described procedure. Recoveries for each diamine were greater than 89 percent. Blank urine samples showed no detection for any of the diamines.

Storage Stability

Two storage stability studies for MDA were performed. These studies were initiated by the concern over the possible biodegradability of MDA in urine and the effect of pH on stability. The first was a 28-day storage study of MDA-fortified urine stabilized with acid (pH 3), base (pH 12), and untreated (pH 6) before freezing. Analysis of the fortified urine showed acceptable recoveries ranging from 87 to 97 percent for storage up to 28 days as shown in Figure 5.

The second study compared the stability of MDA-fortified urine samples stored at pH 3 and pH 6 when stored at room temperature. Analysis of samples under both conditions after 17 and 41 hours produced recoveries of approximately 90 percent. After 65 hours, urine stabilized at pH 3 retained a 90 percent recovery, while recoveries for urine at pH 6 degraded to approximately 75 percent.

Discussion and Conclusion

Aromatic diamines exhibit a high dermal absorptivity as shown by the ACGIH "skin" notation for benzidine, tolidine, and MDA.[4] They also have a low vapor pressure with that of MDA being 1.5×10^{-7} torr at 25°C.[12] These two factors coupled with the biotransformation of benzidine-, dianisidine-, and tolidine-based dyes to the parent free amine indicate a need to supplement environmental monitoring with biological monitoring when assessing exposure to these aromatic diamines or their associated dyes.

Urine was the natural choice as a biological monitor for these diamines. Urine represents a noninvasive source of a biological sample that may contain the compound of interest. Although incomplete, knowledge of the metabolic fate of these compounds is available.

The known metabolites of these compounds are the free diamine, the acetyl-metabolites, or conjugates. The addition of base to pH 12–13 converts most conjugates to the free diamine. Upon heating the pH-adjusted urine to 80°C for 2 hours, the acetyl-metabolites hydrolyze to the free diamine. Nony and Bowman[17] reported the hydrolysis of the acetylated benzidine to free benzidine to be 100 percent. Cocker, Gristwood, and Wilson[14] reported the hydrolysis of acetylated MDA to free MDA to be 100 percent. Although the acetyl-metabolites can be separated chromatographically, their conversion allows measurement of total recoverable diamine in urine samples. This conversion will allow quantifiable results when the amount of aromatic diamines is near the LOQ of the procedure.

The use of a C_{18} solid sorbent for isolation of these aromatic diamines from 50-ml urine samples enables trace enrichment of these species into 1 ml and eliminates the need for liquid–liquid extraction. The choice of a C_{18} solid sorbent was based on the limited solubility of the diamines in water and a greater solubility in an organic phase. This phase equilibrium allowed the retention of the diamine on the solid sorbent and elution of the more polar compounds. The use of a nonpolar solvent (benzene) offers selective elution of the free amine from the solid sorbent. After concentration and redissolving in methanolic KOH,

the free diamine is injected into a HPLC system without derivatization. Using UV detection, the analytical LODs for all these diamines were less than 2 μg/L, and the LOQs were less than 5 μg/L. Inclusion of the electrochemical detector increases the sensitivity and specificity. The analytical LODs were less than 0.3 μg/L, and the LOQs were less than 0.9 μg/L. The use of UV and EC detectors in series gave greater confidence in the identification and quantification of the compounds when response ratioing is used. Agreement of data from two detectors measuring different compound characteristics gave more confidence in proper peak identification, single-peak elution, and data accuracy.

Use of this method will allow practical evaluation of biological exposure. Using these measurements to supplement environmental and medical data, a more complete assessment of worker exposure can be made. It will allow measurement of exposure to these aromatic diamines from various routes that would not be detected during environmental sampling and to test the efficacy of personal protective equipment.

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