Trace Analysis of Potentially Carcinogenic Metabolites of an Azo Dye and Pigment in Hamster And Human Urine as Determined by Two Chromatographic Procedures

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Abstract

Analytical chemical procedures are described for determining traces of possible metabolites of two azo compounds, Direct Black 38 and Pigment Yellow 12, in hamster urine and to monitor the urine from workers who may be occupationally exposed during the manufacture or use of the dye and pigment. These methods were required for metabolism studies designed to assess the hazards that may occur if the two compounds are converted by *in vivo* mechanisms to potential carcinogens. Salient elements of the procedure are: extraction of the free aromatic amines and neutral compounds; alkaline hydrolysis of the aqueous phase and extraction of any hydrolyzed conjugates as free amines, and the analysis of the free amines and acetylated metabolites directly by high pressure liquid chromatography or by electron capture gas chromatography after conversion of the amines to heptafluorobutyryl derivatives. Residues of metabolites in hamster and human urine were determined at levels as low as 1 ppb. Ancillary data concerning hydrolysis of diacetylated metabolites and partition values for possible metabolites in various solvent systems are also presented.

Introduction

Recent epidemiological studies of workers in the dye industry showed an increased incidence of bladder tumors above that of the general population. Early attempts towards solving this problem indicated that many of the azo dyes and pigments used in large quantities contain benzidine or certain congeners such as dichlorobenzidine, dimethylbenzidine, and dimethoxybenzidine. Although benzidine is known to be a carcinogen in humans (1), no similar evidence exists for the three congeners; however, they are known to be mutagenic (2) and to produce tumors in experimental animals. Akiyama (3) reported the release of dichlorobenzidine (DiClBzd) into the urine of exposed workers at a pigment manufacturing plant. Recent work at the National Cancer Institute (NCI) showed that the benzidine dyes, Direct Black 38, Direct Brown 95, and Direct Blue 6 are carcinogenic in rats and hepatotoxic in mice (4). In another study, Rinde and Troll (5) demonstrated the presence of free benzidine (Bzd) and monoacetylbenzidine (MoAcBzd) in the urine of rhesus monkeys fed four benzidine dyes. The colorimetric method they used was based on the reaction of trinitrobenzene sulfonic acid (TNBS) with compounds containing free amino groups, but no provision was made for the assay of diacetylbenzidine (DiAcBzd) and conjugated metabolites. An improvement of this method employing the reagent, fluorescamine (6), increased the sensitivity of aromatic amines into the nanomole range; however, as with the TNBS method, only compounds with free amino groups were detected. Therefore, sensitive and specific analytical chemical methods are required to evaluate properly the results of metabolic studies in animals. Further, assays for trace levels of these metabolites are necessary to monitor the urine from workers in the dye and pigment industries.

Methods for the analysis of free aromatic amines, including Bzd and DiClBzd, in human urine (7-10) are among the most sensitive and specific methods reported in the literature. Although Riggin and Howard (11) had difficulty derivatizing Bzd with fluoroacylating agents no problem was experienced in this work when a catalyst of trimethylamine is used. The electron capture gas chromatographic (EC/GC) methods for DiClBzd (8) and Bzd (10) have minimum detectable levels in urine of about 60 ppt (pg/ml) and 1 ppb, respectively, analyzed as their pentafluoropropionyl derivatives. On the other hand, the procedure of Riggin and Howard (11) employing electrochemical detection is a significant improvement in the assay of wastewater for Bzd and DiClBzd at low levels (ppb) via high pressure liquid chromatography (HPLC). A recent paper by Rice and Kissinger (12) described a specific method involving HPLC with amperometric detection of Bzd, MoAcBzd, and DiAcBzd in human urine. A detection limit of 10 pg of Bzd injected was determined by the criterion of a signal-to-noise ratio of 2. This procedure shows promise for use in assaying urine samples containing Bzd and its acetylated metabolites to levels as low as 10 ppb. Quantitation of levels below 10 ppb would probably require fluoroacylation and EC/GC.

Since methodology sufficiently sensitive and specific for determining possible metabolites of Direct Black 38 and Pigment Yellow 12 in urine could not be found, methods were developed for use in cooperative research between the

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National Center for Toxicological Research (NCTR) and the National Institute for Occupational Safety and Health (NIOSH).

The structural formulas of the dye and pigment, their possible metabolites in urine from hamsters and humans, and their heptafluorobutyryl (HFB) derivatives are presented in Figures 1 and 2. The abbreviations listed for the compounds in both figures will be used throughout the text for the purpose of simplification. The scheme for the extraction, separation, and analysis of Bzd, MoAcBzd, DiAcBzd, and alkaline hydrolyzable conjugates in urine is depicted in Figure 3. Salient elements of the procedure are: (A) extraction of the free aromatic amines and neutral compounds; (B) alkaline hydrolysis of the aqueous phase and extraction of any hydrolyzed conjugates as free amines; and (C) analysis of the free amines and acetylated metabolites directly by HPLC or by EC/GC after conversion to the HFB derivatives.

Experimental

Test Materials and Other Chemicals

The compounds shown in Figures 1 and 2 were obtained from various sources. Direct Black 38 (2,7-naphthalenedisulfonic acid, 4-amino-3-{[4'-[(2,4-diaminophenyl) azo] [1,1'-biphenyl]-4-yl] azo}-5-hydroxy-6-(phenylazo)-, disodium salt) was Lot No. 690 purchased from GAF Corp. (New York, New York). Pigment Yellow 12 (2,2'-[(3,3'-dichloro(1,1'-biphenyl)-4,4'-diyl)-*bis* (azo)]*bis*(3-oxo-N-phenyl)-butanamide) was purchased from the Dry Color Manufacturers' Association (Nutley, New Jersey). Diaminoazobenzene (Basic Orange 2) was purchased from Pfaltz and Bauer Co. (Flushing, New York) and used as a standard as received. Benzidine (Bzd), 4-aminobiphenyl (4-ABP), and 3,3'-dichlorobenzidine (DiClBzd)

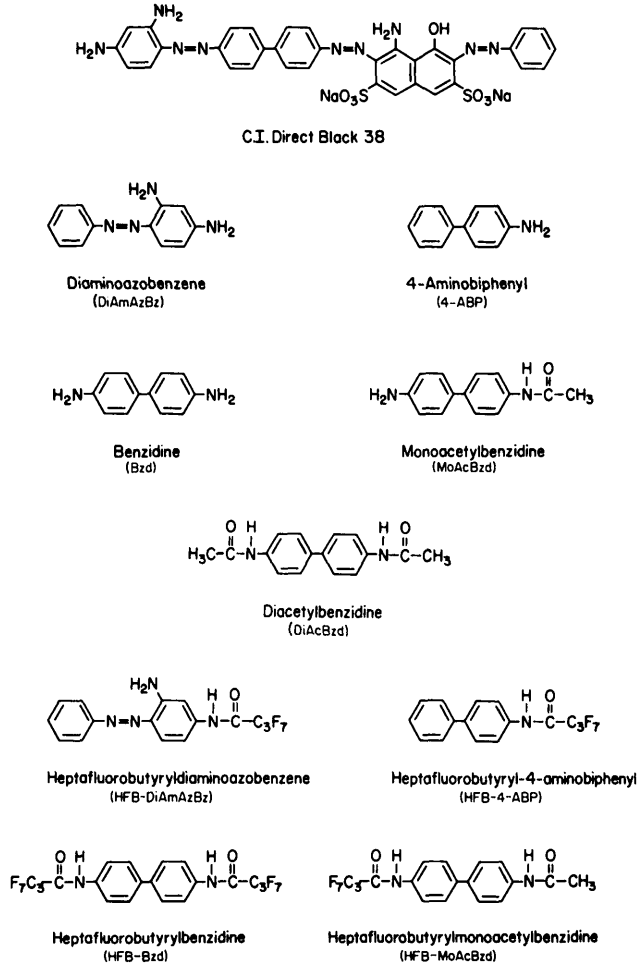

Figure 1. Formulas of Direct Black 38, its metabolites in urine from hamsters, and their heptafluorobutyryl (HFB) derivatives.

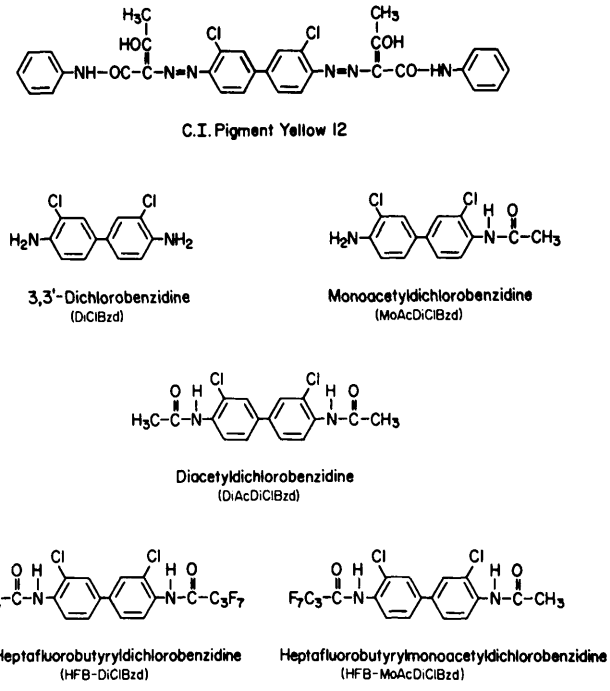

Figure 2. Formulas of Pigment Yellow 12, some of its possible metabolites in urine from hamsters and their heptafluorobutyryl (HFB) derivatives.

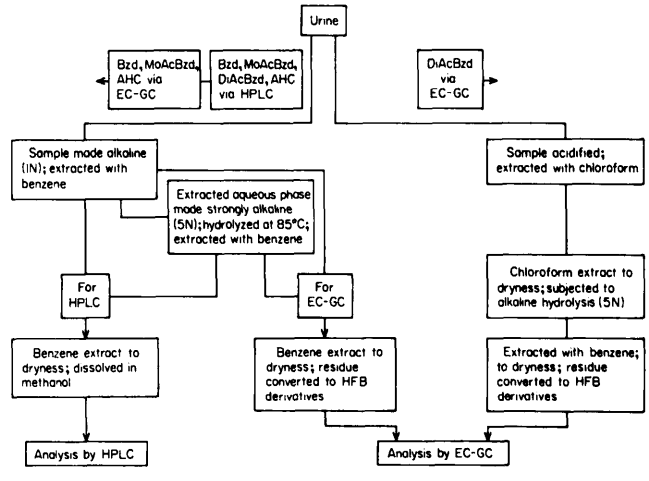

Figure 3. Scheme for extraction, separation, and analysis of benzidine (Bzd), monoacetylbenzidine (MoAcBzd), diacetylbenzidine (DiAcBzd), and alkaline hydrolyzable conjugates in urine.

were obtained from three suppliers as previously reported by Bowman, et al. (7), Holder, et al. (13), and Bowman and Rushing (8), respectively. The monoacetyl- and diacetyl-derivatives of Bzd and DiClBzd were synthesized and purified at the NCTR. The chemical structures of these compounds were confirmed by the electron impact (70 ev) spectra obtained via direct insertion using a Finnigan Instruments (Sunnyvale, California) Model 1015 mass spectrometer. Assays of the derivatives for purity by HPLC indicated MoAcBzd, 97.3%; DiAcBzd, 98.9%; MoAcDiClBzd, 90%; and DiAcDiClBzd, essentially pure. The impurities remaining in MoAcBzd, DiAcBzd, and DiAcDiClBzd were primarily the respective parent compounds and extraneous acetyl derivatives. The MoAcDiClBzd contained about 5% each of DiClBzd and DiAcDiClBzd.

All solvents were distilled-in-glass and all reagents were CP grade. Sodium sulfate and glass wool were extracted with benzene for 40 hr in a Soxhlet apparatus and dried in an oven overnight at 130°C prior to use. All culture tubes were of borosilicate glass equipped with Teflon-lined screw caps. The "keeper" solutions were paraffin oil (20 mg/ml) in benzene for EC/GC assays and diethylene glycol for HPLC assays. (*Note:* The appropriate keeper solution must be used for each assay! Paraffin oil in benzene is not compatible with the HPLC procedure; also, the presence of diethylene glycol in the derivatization procedure for EC/GC depletes the reagents and produces interferences).

Heptafluorobutyric anhydride (HFBA) (No. 63164) was obtained from Pierce Chemical Company (Rockford, Illinois). A 100 µl Hamilton syringe, fitted with a Chaney adapter was used to withdraw the derivatizing agent from the vial and deliver it into the reaction vessel. A fresh vial (1 g) was used for each group of samples. The trimethylamine (TMA) reagent (0.05 M in benzene), buffer solution (potassium monobasic phosphate, pH 6), and sodium hydroxide (10 N) were described by Bowman and Rushing (8) and Nony and Bowman (10).

Preparation of Derivatives for Gas Chromatographic Assays

The heptafluorobutyryl (HFB) derivatives of the metabolites of Direct Black 38 and Pigment Yellow 12 shown in Figures 1 and 2 (except DiAcBzd and DiAcDiClBzd) were prepared by modification of the procedures reported by Walle and Ehrsson (14). A 0.5 ml portion of the TMA solution was added to an 8 ml culture tube containing the compounds (10 µg or less) dissolved in exactly 1.5 ml of benzene (total benzene = 2.0 ml) and followed by the addition of 50 µl of HFBA reagent. The tube was immediately sealed, shaken, heated in a 50°C water bath for 20 min, cooled, and the reaction terminated by adding 2 ml of phosphate buffer (pH 6.0). The tube was shaken for 1 min, and after the phases had separated, the aqueous layer was discarded. The extraction was repeated with an additional 2 ml portion of buffer; the tube was centrifuged for 1 min at 1000 rpm, and the benzene layer was either analyzed directly or appropriately diluted with benzene prior to EC/GC analysis.

Final residues from the extraction procedures of the basic (free amine) fractions from hamster or human urine were dissolved in benzene (1.5 ml) and derivatized by using TMA solution and HFBA reagent as described. For assays of hamster urine containing amine residues in the order of 0.01, 0.1, 1.0, and not >10 ppm, the entire extract (1 g equivalent) of the sample was derivatized, and 5 µl injections containing 2500, 250, 25, and 2.5 µg equivalents, respectively, were assayed by EC/GC. For assays of hamster urine containing amine residues in excess of 10 ppm, e.g., MoAcBzd, the final extract must be diluted with benzene to a known volume in a calibrated tube or flask and a suitable aliquot taken for derivatization. For assays of human urine potentially containing amine residues in the order of 0.001, 0.01, 0.1, and 1.0 ppm, the entire extract (10 g equivalents) of the treated sample was derivatized, and 5 µl injections containing 25,000, 2500, 250, 25 µg equivalents respectively, were assayed by EG/GC.

Gas Chromatographic Assays

A Hewlett Packard (Palo Alto, California) Model 5750 B instrument equipped with a ^{63}Ni electron capture detector (Tracor, Inc., Austin, Texas) and a 6 ft glass column (4 mm i.d.) containing 5% Dexsil 300 on Anakrom Q (90/100 mesh) conditioned at 300°C for 72 hr prior to use, was operated isothermally at 220°, 260°, and 280°C with nitrogen as the carrier gas flowing at 160 ml/min. The detector, operated in the DC mode, was set at 330°C and the injection port temperature was 20°C higher than the column oven. Heptachlor epoxide and mirex were used as reference standards to monitor the performance of the EC/GC system; all injections were made in 5 µl of benzene. Because of the wide differences in retention time (t_R) values for the various HFB derivatives (Table I), MoAcBzd and MoAcDiClBzd were analyzed at 280°C; then Bzd, DiClBzd, and DiAmAzBz were analyzed at 260°C and 4-ABP at 220°C. DiAcBzd and DiAcDiClBzd must be hydrolyzed and converted to their corresponding free amines prior to derivatization with HFBA; these compounds are then analyzed at 260°C as HFB-Bzd and -DiClBzd. Hydrolyzed samples of unknown DiAcBzd and DiAcDiClBzd content that had been derivatized were quantified by relating the peak height of the HFB derivatives to known amounts of HFB-Bzd or HFB-DiClBzd and the results were expressed as DiAcBzd (Bzd × 1.456 = DiAcBzd) or DiAcDiClBzd (DiClBzd × 1.332 = DiAcDiClBzd). All other derivatized samples of unknown residue content were quantified by relating their peak heights to known amounts of the corresponding HFB derivatives.

High Pressure Liquid Chromatographic Assays

A Waters Associates, Inc. (Milford, Massachusetts) liquid chromatograph equipped with a Model 6000A solvent delivery system, a Model U6K septumless injector, a Tracor Model 970A variable wavelength detector operated at 295 nm, and a 3.9 mm i.d. × 30 cm µBondapak C_{18} (reverse phase) column (Waters No. 27324) was used. The mobile phase, 50% methanol-50% potassium phosphate (0.01 M, pH 6.0), flowed at the rate of 1 ml/min with a pressure of 1600 psi. These conditions were used for assays of the metabolites of Direct Black 38 and their t_R values are reported in Table I.

For assays of possible metabolites of Pigment Yellow 12, the mobile phase was adjusted to 60% methanol-40% potassium phosphate (0.01M, pH 6.0) with a flow rate of 1 ml/min and a pressure of 1500 psi; the detector was operated at 292 nm. The t_R values for possible metabolites of Pigment Yellow 12 obtained under these conditions are also reported in Table I. All injections were made in 5 µl of methanol.

Table I. Retention Times of Possible Metabolites of Direct Black 38 and Pigment Yellow 12 (HPLC) and Their Heptafluorobutyryl Derivatives (EC/GC).

	Retention Time (t_R, min) by Method Indicated			
	EC/GC at Temp. (°C) indicated			
Compound	220	260	280	HPLC
Benzidine (Bzd)	8.15	2.30	1.15	4.40
Monoacetylbenzidine (MoAcBzd)	*a*	10.6	3.95	5.50
Diacetylbenzidine (DiAcBzd)				7.65
2,4-Diaminoazobenzene (DiAmAzBz)	5.95	1.85	0.95	13.6
4-Aminobiphenyl (4-ABP)	1.95	0.85	0.55	14.4
3,3'-Dichlorobenzidine (DiClBzd)	10.7	2.95	1.45	9.10
Monoacetyldichlorobenzidine (MoAcDiClBzd)	*a*	10.0	3.75	7.70
Diacetyldichlorobenzidine (DiAcDiClBzd)				6.30
Heptachlor epoxide (ref. std.)	3.95	1.65	1.00	—
Mirex (ref. std.)	15.0	5.20	2.75	—

[a]Low response precluded determination.

For samples of unknown residue content, the residue was dissolved in 1 ml of methanol for analysis as described. Quantification was achieved by relating the peak height to known amounts of the corresponding metabolites.

Hydrolysis Studies of Diacetyl Compounds

Five microgram amounts of the diacetyl derivatives of Bzd and DiClBzd in 1 ml of methanol contained in 20 ml culture tubes were subjected to various concentrations of NaOH for 2 hr at 85°C in a tube heater (Kontes No. 720,000, Vineland, New Jersey) to determine the optimum alkalinity required for complete cleavage of the compounds and recovery of the free amines. One milliliter portions of NaOH (0.5, 1.0, 2.0, 4.0, 6.0, 8.0, or 10 N) were added just prior to heating the compounds. After cooling to room temperature, 6 ml of deionized water was added, and the aqueous phase was extracted with three 8 ml portions of benzene which were successively percolated through a plug of anhydrous sodium sulfate (18 mm diameter × 20 mm thick) and collected in a 100 ml round-bottom flask containing 1 boiling bead and 1 drop of keeper. The contents were evaporated just to dryness at 60°C by using water pump (aspirator) vacuum; then, 2 ml of methanol was added and again evaporated to dryness to remove any traces of benzene that could interfere with the HPLC assay. Finally, the dry residue was dissolved in 1 ml of methanol and 5 μl was injected for assay by HPLC.

Extraction and Preparation of Hamster Urine for Analysis of Metabolites of Direct Black 38

Benzidine (Bzd) monoacetylbenzidine (MoAcBzd) and diacetylbenzidine (DiAcBzd). One milliliter of sample, 4 ml of deionized water, 0.5 ml of methanol, 0.5 g of NaCl, and 0.5 ml of 10 N NaOH were added to a 20 ml culture tube, shaken, and allowed to stand for 5 min. A 5 ml portion of benzene was added to the tube and the contents shaken gently for 1 min; the tube was centrifuged at 1500 rpm for 5 min. The benzene layer was carefully withdrawn by using a syringe and cannula and percolated through a plug of anhydrous sodium sulfate (ca. 18 mm diameter × 20 mm thick) into a 50 ml graduated cylinder. The extraction was repeated with three additional 5 ml portions of benzene. The combined extracts were then brought to exactly 30 ml and divided into two equal fractions: one for assay by HPLC and the other for assay by EC/GC. (The extracted aqueous phase was reserved for determining alkaline hydrolyzable conjugates.) A glass bead and the respective keeper solutions were added to each fraction contained in 100 ml round-bottom flasks, and the contents evaporated to near dryness by using water pump vacuum and a 60°C water bath. The residue for HPLC assay was dissolved in 2 ml of methanol and again evaporated to dryness as described to remove any traces of benzene that could interfere with the assay. The residue was either assayed immediately or stored at 5°C for subsequent assay by HPLC.

The residue for EC/GC assay was transferred to an 8 ml culture tube by using three 1 ml portions of benzene and evaporated to dryness in a tube heater at 50°C with a stream of dry nitrogen. The residue was reserved for subsequent derivatization with HFBA and assay by EC/GC. (*Note:* Any DiAcBzd that may be present in this residue must be alkaline hydrolyzed prior to derivatization and assay by EC/GC. This procedure is discussed under *Preparation of Sample for Analysis of Diacetylbenzidine via EC/GC.*)

Alkaline hydrolyzable conjugates (AHC) of benzidine (Bzd). The extracted aqueous phase reserved for alkaline hydrolysis of conjugates of Bzd was made strongly alkaline (5 N) by carefully adding 2 g of NaOH to the contents of the tube. Five milliliters of methanol were then added and the sealed tube was heated in a tube heater at 85°C for 2 hr with occasional shaking. After the tube had cooled, any Bzd or other aromatic amine freed from the conjugates by the hydrolysis was extracted by using three 8 ml portions of benzene. The extracts were combined and evaporated to dryness as previously described. The residue was reserved for analysis by HPLC or subsequent derivatization with HFBA and assay by EC/GC.

Preparation of sample for analysis of diacetylbenzidine (DiAcBzd) via EC/GC. One milliliter of sample, 4 ml of water, 0.5 ml of methanol, and 0.5 ml of 1.2 N HCl were added to a 15 ml culture tube, shaken, and allowed to stand for 5 min. A 5 ml portion of chloroform was then added, the contents shaken gently for 2 min, and the tube centrifuged at

1500 rpm for 5 min. The chloroform layer was carefully withdrawn by using a syringe and cannula, percolated through a plug of anhydrous sodium sulfate (18 mm diameter × 20 mm thick), and collected in a 50 ml round-bottom flask containing a glass bead and 0.5 ml of keeper solution. The extraction was repeated by using two additional 5 ml portions of chloroform, and the combined extracts containing DiAcBzd were evaporated just to dryness by using water pump vacuum and a 50°C water bath. The residue was transferred to a 20 ml culture tube using three 1 ml portions of chloroform, and the solvent in the tube was then evaporated to dryness in a tube heater set at 50°C by using a gentle stream of dry nitrogen. The residue was hydrolyzed by heating the sealed tube containing 1 ml of methanol and 1 ml of 10 N NaOH in a tube heater set at 85°C for 2 hr. After the tube had cooled, 6 ml of deionized water was added and the contents extracted three times with 8 ml portions of benzene. Each extract was successively percolated through a plug of sodium sulfate (18 mm diameter × 20 mm thick) and collected in a 50 ml round-bottom flask containing a boiling bead and 0.5 ml of keeper solution. The contents were evaporated just to dryness in the usual manner; the residue was transferred to an 8 ml culture tube by using three 1 ml portions of benzene which were also evaporated to dryness in a tube heater at 50°C by using a stream of dry nitrogen. The residue containing benzidine was reserved for subsequent derivatization with HFBA and assay by EC/GC.

Extraction and Preparation of Hamster Urine for Analysis of Possible Metabolites of Pigment Yellow 12

3,3'-Dichlorobenzidine (DiClBzd), monoacetyldichlorobenzidine (MoAcDiClBzd), and diacetyldichlorobenzidine (DiAcDiClBzd). One milliliter samples were treated exactly as described for Bzd, MoAcBzd, and DiAcBzd.

Alkaline hydrolyzable conjugates (AHC) of dichlorobenzidine (DiClBzd). The extracted aqueous phase reserved for alkaline hydrolysis of conjugates of DiClBzd was made strongly alkaline (3 N) by adding 1.1 g of NaOH to the contents of the tube and treated exactly as described for *Alkaline Hydrolyzable Conjugates of Bzd.*

Preparation of sample for analysis of diacetyldichlorobenzidine (DiAcDiClBzd) via EC/GC. One milliliter of the sample, 0.5 ml of water, 0.5 ml of methanol, 4.0 ml of concentrated HCl, and 5 ml of benzene were added to a 15 ml culture tube and gently shaken for 5 min. The tube was centrifuged at 1000 rpm for 5 min and the benzene layer was carefully withdrawn by using a syringe and cannula, percolated through a plug of anhydrous sodium sulfate (18 mm diameter x 20 mm thick), and collected in a 50 ml round-bottom flask containing a glass bead and 0.5 ml of keeper solution. The extraction was repeated by using two additional 5 ml portions of benzene and the combined extracts containing DiAcDiClBzd were evaporated just to dryness in the usual manner. The residue was transferred to a 20 ml culture tube by using three 1 ml portions of benzene, and the solvent in the tube was then evaporated to dryness in a tube heater set at 50°C by using a gentle stream of dry nitrogen. The dry residue was hydrolyzed by heating the sealed tube containing 1 ml of methanol and 1 ml of 6 N NaOH in a tube heater set at 85°C for 2 hr. The contents of the tube were treated exactly as described for DiAcBzd via EG/GC. The final residue containing DiClBzd was reserved for subsequent derivatization with HFBA and assay via EC/GC.

Extraction and Preparation of Human Urine for Analysis of Metabolites of Direct Black 38 via EC/GC

Benzidine (Bzd) and monoacetylbenzidine (MoAcBzd). Ten milliliters of sample, 1 ml of methanol, 1 g of NaCl, and 1.0 ml of 10 N NaOH were added to a 30 ml culture tube, shaken, and allowed to stand for 5 min. A 10 ml portion of benzene was added to the tube, and the contents shaken gently for 1 min; the tube was centrifuged at 1500 rpm for 5 min. The benzene layer was carefully withdrawn by using a syringe and cannula and percolated through a plug of anhydrous sodium sulfate (ca. 25 mm diameter x 20 mm thick) and collected in a 100 ml round-bottom flask containing a glass bead and 0.5 ml of keeper solution. The extraction was repeated with three additional 10 ml portions of benzene, and the combined extracts were evaporated just to dryness in the usual manner. (The extracted aqueous phase was reserved for determining alkaline hydrolyzable conjugates.) The residue in the flask was transferred to an 8 ml culture tube by using three 1 ml portions of benzene and evaporated to dryness in a tube heater at 50°C with a stream of dry nitrogen. The residue was reserved for subsequent derivatization with HFBA and assay by EC/GC. [*Note:* Any DiAcBzd in this residue must be alkaline hydrolyzed prior to derivatization and assay by EC/GC. This procedure is discussed under *Diacetylbenzidine (DiAcBzd).*]

Alkaline hydrolyzable conjugates (AHC) of benzidine (Bzd). The extracted aqueous phase reserved for alkaline hydrolysis of conjugates of Bzd was made strongly alkaline (5 N) by carefully adding 4 g of NaOH to the contents of the tube. Ten milliliters of methanol were then added and the sealed tube heated in a water bath at 85°C for 2 hr with occasional shaking. After the tube had cooled, any Bzd or other aromatic amine freed from the conjugates by the hydrolysis was extracted by using three 8 ml portions of benzene. The extracts were combined and evaporated to dryness as previously described. The residue was reserved for subsequent derivatization with HFBA and assay by EC/GC.

Diacetylbenzidine (DiAcBzd). Ten milliliters of sample, 1 ml of methanol, and 1 ml of 1.2 N HCl were added to a 30 ml culture tube, shaken, and allowed to stand for 5 min. A 10 ml portion of chloroform was then added, the contents shaken gently for 2 min, and the tube centrifuged at 1500 rpm for 5 min. The chloroform layer was carefully withdrawn by using a syringe and cannula, percolated through a plug of anhydrous sodium sulfate (18 mm diameter x 20 mm thick), and collected in a 100 ml round-bottom flask containing a glass bead and 0.5 ml of keeper solution. The extraction was repeated by using two additional 10 ml portions of chloroform, and the combined extracts containing DiAcBzd were evaporated just to dryness by using water pump vacuum and a 50°C water bath. The residue was treated exactly as described for hamster urine under *Preparation of Sample for Analysis of DiAcBzd via EC/GC.*

Recovery Experiments

Hamster urine. Control urine from hamsters (300 ml) was diluted with 1200 ml of deionized water and sonicated for 5 min to disrupt sedimentary particles so that a sufficient supply of representative samples would be available for the complete development of analytical methodology. The diluted urine composite was stored at -20°C when not in use.

Triplicate 5 ml samples of diluted urine composite each containing a 1 g equivalent of urine were placed in 12 ml

culture tubes and separately spiked with 50 µl of methanol containing the appropriate amount of Bzd, MoAcBzd, DiAcBzd, and 4-ABP to produce residues of 0, 0.01, 0.1, 1.0, and 5.0 ppm. In addition, MoAcBzd was spiked at the 200 ppm level. The tubes were sealed, mixed, and allowed to stand overnight at -20°C prior to extraction and analysis by EC/GC.

In a similar manner, triplicate 5 ml samples of diluted urine composite were separately spiked as described with the appropriate amount of Bzd, MoAcBzd, DiAcBzd, and 4-ABP to produce residues of 0, 0.5, 1.0, and 5.0 ppm. In addition, MoAcBzd was spiked at the 200 ppm level. The tubes were sealed, mixed, and allowed to stand overnight at -20°C prior to extraction and analysis by HPLC.

Quadruplicate 5 ml samples of the diluted urine composite were separately spiked with the appropriate amount of Bzd, MoAcBzd, DiAcBzd, and 4-ABP to prepare residues of 1.0 ppm. One control sample (unspiked) was prepared. The tubes were sealed, mixed, and allowed to stand overnight at -20°C prior to extraction and analysis by HPLC. Two of the extracted aqueous phases were again spiked in an identical manner and hydrolyzed (5 N NaOH) along with the other two extracted aqueous phases that had not been spiked a second time. After hydrolysis and extraction with benzene, all samples were analyzed by HPLC.

Triplicate 5 ml samples of the diluted urine composite were separately spiked with the appropriate amounts of DiClBzd, MoAcDiClBzd, and DiAcDiClBzd to produce residues of 0 and 1.0 ppm. The tubes were sealed, mixed, and allowed to stand overnight at -20°C prior to extraction and analysis by EC/GC and HPLC. In addition, 5 ml samples of the diluted urine composite were spiked with the appropriate amount of DiAcDiClBzd to produce residues of 0.01 and 0.1 ppm for assay only by EC/GC.

Human urine. Control human urine was collected and stored at -20°C for use in the development of trace analytical methodology based on EC/GC analysis. Triplicate 10 ml samples of urine were placed in 30 ml culture tubes and separately spiked with 100 µl of methanol containing the appropriate amount of Bzd, MoAcBzd, DiAcBzd, DiAmAzBz, and 4-ABP to produce residues of 0, 0.001, 0.01, and 0.1 ppm. In addition, MoAcBzd was spiked at the 1.0 ppm level. The tubes were sealed, mixed, and allowed to stand overnight at -20°C prior to extraction and analysis by EC/GC as described.

Quadruplicate 2.5 ml samples of control human urine were separately spiked with the appropriate amount of Bzd, MoAcBzd, DiAcBzd, and DiAmAzBz to prepare residues of 1.0 ppm. One control sample (unspiked) was prepared. The tubes were sealed, mixed, and allowed to stand overnight at -20°C prior to extraction and analysis by HPLC as described. Two of the extracted aqueous phases were again spiked in an identical manner and hydrolyzed (5 N NaOH) along with the other two extracted aqueous phases that had not been spiked a second time. After hydrolysis and extraction with benzene, all samples were analyzed by HPLC as described.

Results and Discussion

EC/gas chromatograms of derivatized standards of appropriate aromatic amines related to possible metabolites of Direct Black 38 (Figure 1) and Pigment Yellow 12 (Figure 2) are presented in Figures 4 and 5, respectively. Work reported previously (10) made use of pentafluoropropionic anhydride (PFPA) as the reagent of choice for derivatizing aromatic amines; however, the presence of a large amount of an impurity, 2,4-diaminoazobenzene (DiAmAzBz), also known as Basic Orange 2, in Direct Black 38 prevented the use of PFPA because of poor resolution between the PFP derivatives of DiAmAzBz and Bzd. Therefore, heptafluorobutyric anhydride (HFBA) was substituted for PFPA, and since an acceptable degree of resolution was achieved (Figure 4, 260°C), HFBA was chosen for exclusive use in these methods where EC/GC was involved. The inclusion of 4-aminobiphenyl (4-ABP) in this project, as depicted in Figures 4 and 6 (220°C), was necessary because of its presence as an impurity in Direct Black 38 and also its generation during metabolism of the dye as determined by preliminary feeding studies in hamsters. Although 4-ABP does not appear to be a metabolite of Direct Black 38 in humans, EC/gas chromatograms of this compound in human urine (Figure 7, 220°C) have been included as guides in the event that it is detected as the free amine before or after alkaline hydrolysis of possible conjugates.

Preliminary experiments with the HFB derivatives of MoAcBzd and MoAcDiClBzd indicated the need for GC column temperatures of 315°C or higher to obtain chromatographic peaks with good symmetry. However, this condition, used with injector and detector temperatures of 340° and 360°C, resulted in unpredictable responses, and early failure of the detector resulted. It therefore became necessary to lower the column temperature to 280°C and the injector and detector temperatures to 300° and 330°C, respectively. Under these conditions, the HFB derivatives of MoAcBzd and MoAcDiClBzd emerged from the GC column in a reasonably short time, but some tailing of the peaks was noted (Figures 4 and 5). Also, with the column temperature at 280°C, the t_R values increased as the amount of injected compound was decreased. Responses of unknown amounts of the two derivatives were adjusted to compensate for changes in t_R values by using the ratio of their t_R values to those of a fixed amount of their respective standards. The validity of these corrections is evidenced by comparisons between parallel EC/GC and HPLC assays. Although considerable tailing of the peaks was obtained at 280°C, no attempt was made to find an alternate column since the quantitative results were satisfactory and the thermal stability of Dexsil 300 is high. A summary of the EC/GC retention times of HFB derivatives of possible metabolites is presented in Table I with two reference standards, heptachlor eposide and mirex. Since the t_R values of the MoAcBzd and MoAcDiClBzd are dependent upon the amount injected, it should be noted that 250 pg amounts were used to obtain these values. The t_R values for the various metabolites as determined by HPLC are also presented in Table I, and corresponding chromatograms are shown in Figures 8 and 9.

Before assays of hamster or human urine samples could be conducted, levels of background interference for each compound to be assayed were determined. Chromatograms of the various compounds are illustrated in Figures 4, 5, 6, and 7 (EC/GC) and Figures 8 and 9 (HPLC). The chromatograms depicted by solid lines are tracings of derivatized standards or derivatized extracts of urine from untreated hamsters or human controls. Broken lines (superimposed) generally represent responses from derivatized standards spiked into extracts of urine from untreated hamsters and human con-

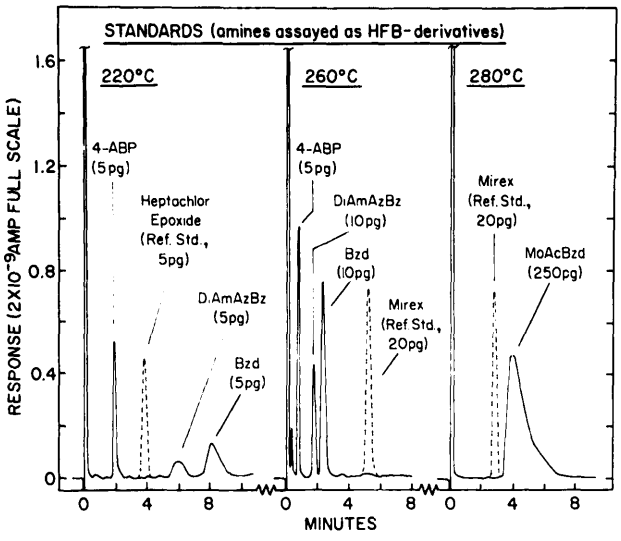

Figure 4. Electron capture/gas chromatograms of HFB-derivatives of metabolites of Direct Black 38 and reference standards of heptachlor epoxide and mirex. All injections are in 5 μl of benzene.

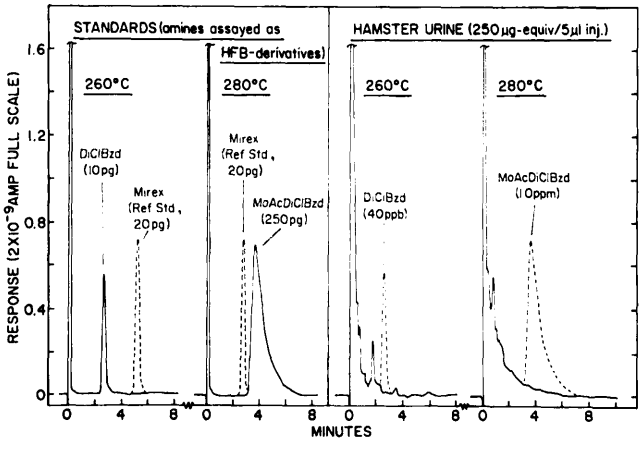

Figure 5. Electron capture/gas chromatograms. Left: Solid lines are HFB-derivatives of possible metabolites of Pigment Yellow 12 in urine from hamsters; broken lines are mirex reference standards. Right: Solid lines are derivatized extracts of urine from untreated hamsters; broken lines (superimposed) illustrate responses from the possible metabolites analyzed as HFB-derivatives. All injections are in 5 μl of benzene.

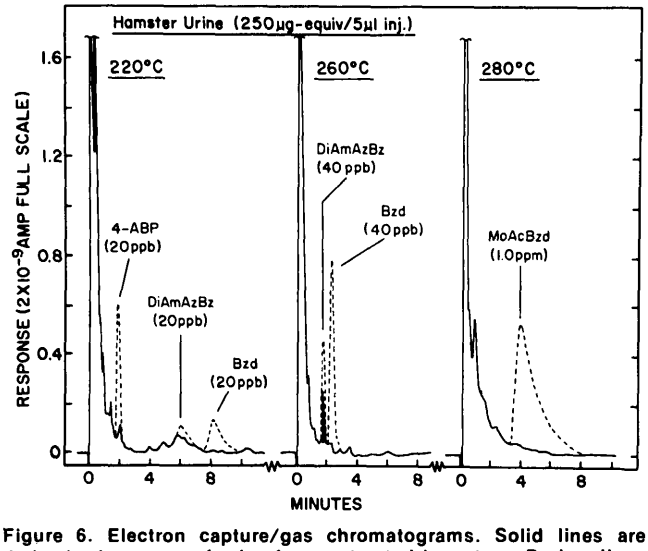

Figure 6. Electron capture/gas chromatograms. Solid lines are derivatized extracts of urine from untreated hamsters. Broken lines (superimposed) illustrate responses from metabolites of Direct Black 38 analyzed as HFB derivatives. All injections are in 5 μl of benzene.

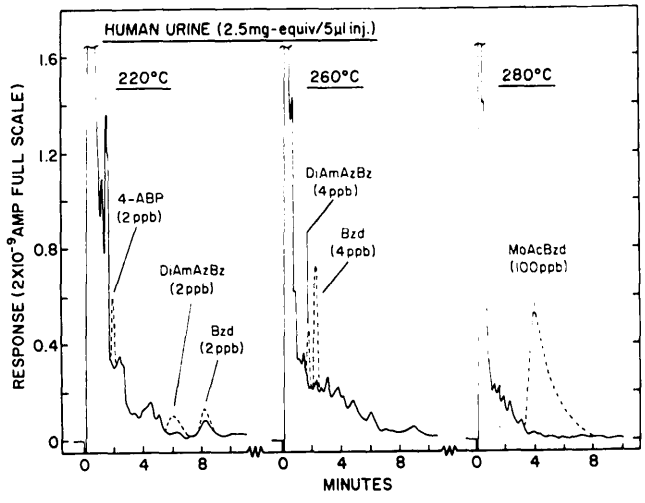

Figure 7. Electron capture/gas chromatograms. Solid lines are derivatized extracts of control human urine. Broken lines (superimposed) illustrate responses from metabolites of Direct Black 38 analyzed as HFB derivatives. All injections are in 5 μl of benzene.

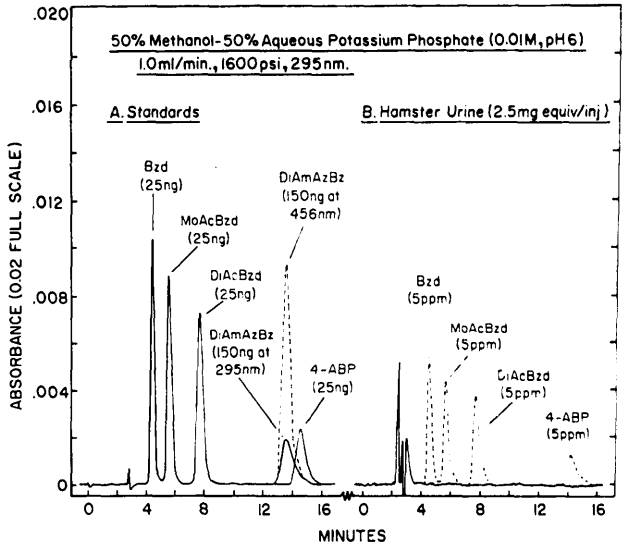

Figure 8. High pressure liquid chromatograms. A: Solid lines are standards of metabolites of Direct Black 38 assayed at 295 nm; broken line illustrates the response of DiAmAzBz at its absorption maximum (456 nm). B: Solid line is an extract of urine from untreated hamsters; broken lines (superimposed) illustrate responses from the specified levels of metabolites in the urine extract. All injections are in 5 μl of methanol.

trols. These data were used to calculate the minimum detectable levels for both methods as reported in Table II.

The purity of the dye and pigment, as received, was determined by assaying for the presence of free aromatic amines by EC/GC (15). Both compounds contained excessive amounts of impurities, and each one was purified manually by a liquid-liquid extraction procedure (15).

The stability of the purified dye was tested in hamster and human urine at 5°, 25°, and 37.5°C and found to be stable at 5°C for at least 96 hr and at 25°C for up to 24 hr. Based on these findings, essentially no degradation of the dye occurs in the analytical procedure. Nevertheless, it is recommended that samples of urine from hamsters or humans be collected and stored under dry ice initially, then at -20°C prior to analysis to

Table II. Minimum Detectable Levels of Possible Metabolites of Direct Black 38 and Pigment Yellow 12 in Hamster and Human Urine

	Minimum Detectable Level (ppb) by Method Indicated (twice background)		
	EC/GC[a]		HPLC
Compound	Hamster	Human	Hamster
Benzidine (Bzd)	3	1	180
Monoacetylbenzidine (MoAcBzd)	70	2	210
Diacetylbenzidine (DiAcBzd)[b]	2	0.2	260
2,4-Diaminoazobenzene (DiAmAzBz)	5	1	850
4-Aminobiphenyl (4-ABP)	4	2	870
3,3'-Dichlorobenzidine (DiClBzd)	8	c	525
Monoacetyldichlorobenzidine (MoAcDiClBzd)	48	c	660
Diacetyldichlorobenzidine (DiAcDiClBzd)[b]	7	c	600

[a]Assayed as the HFB derivatives.
[b]Assayed as the HFB derivative after alkaline hydrolysis to the free amine.
[c]Not determined.

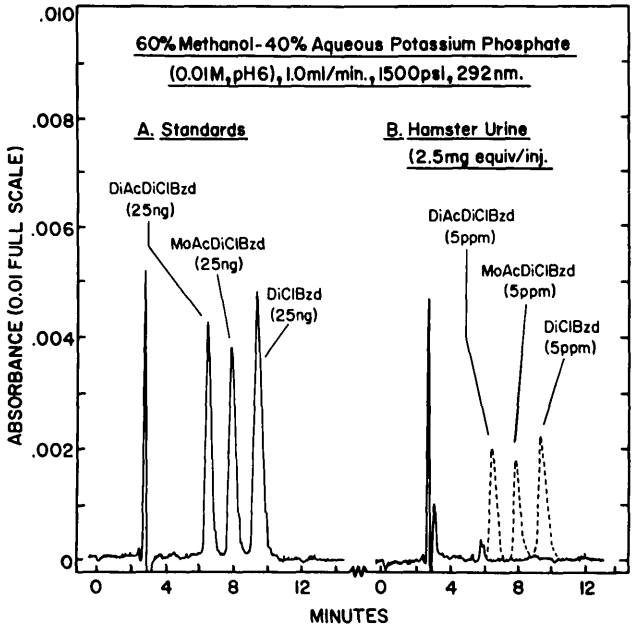

Figure 9. High pressure liquid chromatograms. A: Standards of proposed metabolites of Pigment Yellow 12. B: Solid line is an extract of urine from untreated hamsters; broken lines (superimposed) illustrate responses from the specified levels of proposed metabolites in the urine extract. All injections are in 5 μl of methanol.

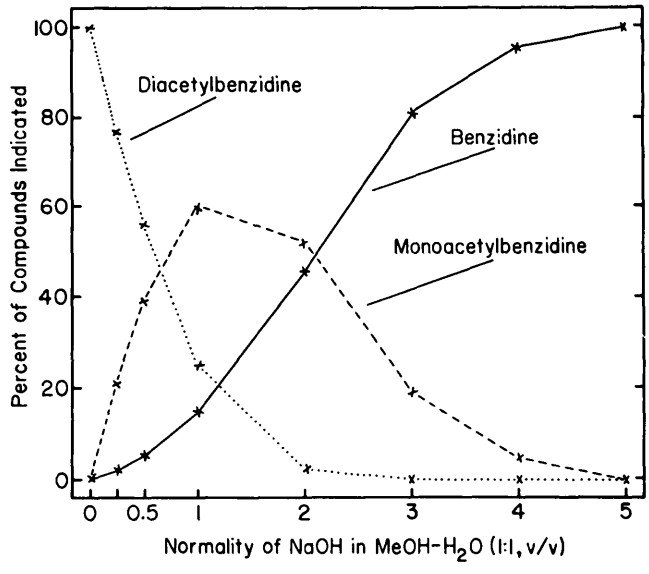

Figure 10. Alkaline hydrolysis of diacetylbenzidine. Dotted line illustrates the de-acetylation of DiAcBzd; broken line illustrates the generation of MoAcBzd from DiAcBzd and eventual de-acetylation of MoAcBzd; solid line illustrates the gradual generation of Bzd from concurrent de-acetylations of DiAcBzd and MoAcBzd. Each data point represents a result from a 2 hr reaction at 85°C at the indicated normality of NaOH.

prevent possible degradation of any dye that might be present in the urine.

The assay of DiAcBzd and DiAcDiClBzd was straightforward when conducted by HPLC as evidenced by the chromatograms shown in Figures 8 and 9; however, the presence of the two acetyl groups (Figures 1 and 2) on each molecule and the absence of derivatizable functional groups prevented their assay by EC/GC. It therefore became necessary to develop a hydrolysis procedure to cleave the acetyl bonds and thereby release the free amines, Bzd and DiClBzd, for derivatization with HFBA. The results of the hydrolysis studies with DiAcBzd and DiAcDiClBzd are depicted in Figures 10 and 11. The curves clearly indicate the progression of the hydrolysis reactions, i.e., the gradual decrease of the diacetyl (DiAc) compounds, the generation and eventual decrease of the monoacetyl (MoAc) compounds, and finally, the complete conversion to Bzd or DiClBzd. Because 4 N NaOH appeared to be just sufficient for complete conversion to DiClBzd, 6 N NaOH was used for subsequent hydrolyses of DiAcDiClBzd to ensure that the reaction was complete.

Partition values (p values), which are defined as the fraction of solute partitioning into the nonpolar phase of an equivolume immiscible binary solvent system, are useful in

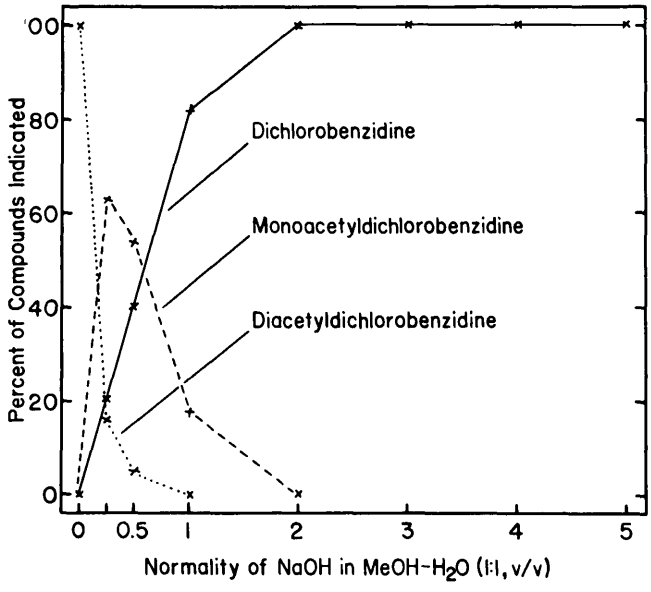

Figure 11. Alkaline hydrolysis of diacetyldichlorobenzidine. Dotted line illustrates the de-acetylation of DiAcDiClBzd; broken line illustrates the generation of MoAcDiClBzd from DiAcDiClBzd and eventual de-acetylation of MoAcDiClBzd; solid line illustrates the gradual generation of DiClBzd from the concurrent de-acetylations of DiAcDiClBzd and MoAcDiClBzd. Each data point represents a result from a 2 hr reaction at 85°C at the indicated normality of NaOH.

Table III. Partition Values of Metabolites of Direct Black 38 as Determined by HPLC

	p-value		
Solvent System	Bzd	MoAcBzd	DiAcBzd
Benzene-aqueous HCl (0.1 N)	0.00	0.00	0.24
Benzene-aqueous buffer (pH 7)	1.00	0.78	0.28
Benzene-aqueous NaOH (0.1 N)	0.95	0.69	0.25
Benzene-aqueous NaOH (1 N)	0.99	0.92	0.60
Chloroform-aqueous HCl (0.1 N)	0.00	0.031	1.00
Chloroform-aqueous buffer (pH 7)	1.00	1.00	1.00
Chloroform-aqueous NaOH (0.1 N)	1.00	0.98	0.93
Chloroform-aqueous NaOH (1 N)	1.00	1.00	0.97
Ethyl Acetate-aqueous HCl (0.1 N)	0.00	0.045	1.00
Ethyl Acetate-aqueous buffer (pH 7)	0.99	0.99	0.93
Ethyl Acetate-aqueous NaOH (0.1 N)	1.00	0.99	0.94

developing extraction and cleanup methods and in confirming identity (16,17). Hence, such values which were determined for Bzd, DiClBzd, and the respective MoAc and DiAc metabolites in several solvent systems by using HPLC are reported in Tables III and IV. This information was then used to devise the scheme for extraction and separation shown in Figure 3. Salient elements of the procedures were as follows: extraction of free aromatic amines and neutral compounds with benzene (pH 14); dividing the extract into two known volumes; evaporating each to dryness and analyzing one residue dissolved in methanol by HPLC; and derivatizing the other residue with HFBA for analysis by EC/GC. The aqueous phase may still contain conjugated metabolites after this extraction; therefore, it is hydrolyzed under strongly alkaline conditions, extracted again with benzene, and any free amines are analyzed either directly by HPLC or after derivatization by EC/GC. For the trace analysis of the neutral DiAc compounds via EC/GC, the residues must be separated from any free amines by extracting the highly acidified sample with chloroform. The residue from the chloroform extract is then subjected to hydrolysis, under strongly alkaline conditions, extracted with benzene, and the resulting free amine derivatized and analyzed by EC/GC. It should be noted that no laborious cleanup steps utilizing adsorbents such as silica gel or alumina are required even for trace analysis by EC/GC. Thus, the method is greatly simplified and amenable to use with large numbers of samples. This scheme for the analysis of hamster urine, with slight modifications, is also applicable to assays of samples containing possible metabolites of Pigment Yellow 12. Although other types of animal urine (e.g., rat or mouse) have not been tried, human urine is assayed by using this scheme with only slight adjustments of the volumes of sample and the amounts of reagents and solvents employed.

Results from triplicate assays of hamster urine unspiked and spiked with 0.01, 0.1, 0.5, 1.0, and 5.0 ppm of the metabolites of Direct Black 38 and also spiked only with MoAcBzd at the 200 ppm level are presented in Table V. Recoveries determined by EC/GC and HPLC were generally good at levels of 0.1 ppm and above for all compounds except Bzd which were 53.9 and 10.8% at the 0.5 and 0.1 ppm levels, respectively. Similar data were previously reported for Bzd in human urine (10), however, the recovery did not decline appreciably until the 1 ppb level was assayed. It should be noted that hamster urine is

Table IV. Partition Values of Possible Metabolites of Pigment Yellow 12 as Determined by HPLC

	p-Value		
Solvent System	DiClBzd	MoAcDiClBzd	DiAcDiClBzd
Benzene-aqueous HCl (8 N)	<0.003	<0.003	0.65
Benzene-aqueous HCl (6 N)	<0.003	0.01	0.92
Benzene-aqueous HCl (4 N)	<0.003	0.12	1.00
Benzene-aqueous buffer (pH 7)	1.00	1.00	1.00
Benzene-aqueous NaOH (1 N)	1.00	1.00	0.99

Table V. Analyses of Hamster Urine Unspiked and Spiked with 0.01, 0.1, 0.5, 1.0, 5.0, and 200 ppm of Metabolites of Direct Black 38

		Recovery of Metabolites ($\bar{x} \pm SE$)[a]						
Metabolites	Analytical Method	Unspiked (ppm)	Spiked with 0.01 ppm (%)	Spiked with 0.1 ppm (%)	Spiked with 0.5 ppm (%)	Spiked with 1.0 ppm (%)	Spiked with 5.0 ppm (%)	Spiked with 200 ppm (%)
Bzd[b]	EC/GC	0.001 ±0.000	c	10.8 ±1.8	53.9 ±10.1	71.9 ±4.8	84.1 ±1.5	d
Bzd	HPLC	0.090 ±0.000	d	d	64.7 ± 0.5	73.8 ±0.6	80.1 ±2.2	d
MoAcBzd[b]	EC/GC	0.032 ±0.002	c	61.9 ±2.4	69.5 ± 6.1	85.8 ±2.2	88.8 ±4.7	72.6 ±2.2
MoAcBzd	HPLC	0.100 ±0.000	d	d	79.8 ± 1.4	89.5 ±2.0	90.1 ±1.2	95 2 ±1.7
DiAcBzd[e]	EC/GC	0.001 ±0.000	50.0 ±0.0	53.0 ±1.0	d	53.6 ±0.3	55.2 ±4.3	d
DiAcBzd	HPLC	0.130 ±0.000	d	d	90.1 ± 1.8	87.8 ±2.4	82.2 ±0.7	d
4-ABP[b]	EC/GC	0.002 ±0.001	37.8 ±1.0	79.7 ±2.6	78.3 ± 3.4	85.0 ±2.1	93.2 ±5.0	d
4-ABP	HPLC	0.430 ±0.000	d	d	59.0 ± 8.0	82.4 ±2.5	90.1 ±3.0	d

[a]Mean and standard error from triplicate assays; corrected for background interferences in the unspiked sample.
[b]Analyzed as the heptafluorobutyryl derivative.
[c]None detected above background.
[d]Not determined.
[e]Analyzed as the heptafluorobutyryl derivative after alkaline hydrolysis to Bzd.

markedly different from human urine in that it normally contains an unusually large amount of suspended solids that may have an adverse effect on the recovery of some compounds spiked at low levels. This phenomenon is evidenced in Table V where Bzd and MoAcBzd were not detected at the 0.01 ppm level, whereas, recoveries of 50.0 and 37.8% were obtained at this level for DiAcBzd and 4-ABP, respectively.

The differences in recovery values between the EC/GC and HPLC methods are slight (Table V), nevertheless, the generally lower recoveries obtained by EC/GC as compared with HPLC are attributed to the larger number of manipulations in the EC/GC procedures. The most critical steps are probably those that involve evaporation to dryness and transfer of residues to other vessels.

No recovery experiments via HPLC were attempted at levels of 0.1 ppm or less since such levels are below the minimum detectable limits of the HPLC procedure. It should be emphasized that the HPLC procedure is an excellent choice for assays of metabolites in dosed animals where ppm amounts of the compounds are present. However, trace assays of metabolites in environmental samples containing sub-ppm levels of residues require the more sensitive EC/GC procedure.

The recovery experiment with hamster urine spiked with 1.0 ppm each for Bzd, MoAcBzd, DiAcBzd, and 4-ABP and extracted with benzene prior to respiking and alkaline hydrolysis resulted in recoveries of 70.0, 82.9, 79.6, and 79.7%, respectively, as determined by HPLC assay. These recoveries indicated efficient removal of the compounds from the urine. After respiking and alkaline hydrolysis, essentially 100% of the Bzd and 82.5% of the 4-ABP were recovered; thus, the hydrolysis procedure did not interfere with recovery of these compounds.

In the experiment with hamster urine spiked with 1.0 ppm of each of the possible metabolites of Pigment Yellow 12, recoveries obtained via HPLC for DiClBzd, MoAcDiClBzd, and DiAcDiClBzd were 78.9, 83.7, and 80%, respectively, and the backgrounds were 0.240, 0.260, and 0.200 ppm, respectively. Assays by EC/GC of urine spiked at the 1.0 ppm level yielded recoveries for DiClBzd, MoAcDiClBzd, and DiAcDiClBzd of 89.0, 85.4, and 58.0%, respectively. At the 0.1 ppm level, recoveries of these compounds diminished to 53.6, 61.7, and 45.0%; the backgrounds from unspiked samples were 0.004, 0.024, and 0.004 ppm, respectively. None of these compounds could be detected in samples spiked at the 0.01 ppm level.

Results from triplicate EC/GC assays of human urine unspiked and spiked with 0.001, 0.01, and 0.1 ppm of the metabolites of Direct Black 38 and also spiked only with MoAcBzd at the 1.0 ppm level are presented in Table VI. All recoveries were excellent at the 0.1 ppm level; however, recoveries of all compounds decreased appreciably at the 0.01 ppm level. Only DiAcBzd (20.3%) was recovered at the 0.001 ppm level.

The recovery experiment with human urine spiked with 1.0 ppm each of Bzd, MoAcBzd, DiAcBzd, and DiAmAzBz and extracted with benzene prior to being respiked and subjected to alkaline hydrolysis resulted in recoveries of 76.8, 87.5, 85.7, and essentially 100%, respectively, as determine by HPLC assay. After the urine was respiked and subjected to alkaline hydrolysis, essentially 100% of all four compounds were recovered since the theoretical amounts of Bzd and DiAmAzBz were obtained.

Previously, assays for free aromatic amines in the urine has been the basis for monitoring humans who are potentially exposed (10). However, the present procedure which determines free amines, metabolites, and alkaline hydrolyzable conjugates provides vastly improved sensitivity. In fact, the authors propose that in assays to monitor human urine, the samples be hydrolyzed at the outset to provide results that include both free and conjugated residues. Methods previously developed for monitoring urine that do not include a hydrolysis step will obviously fail to detect the conjugated amines.

For efficient monitoring of human urine it is suggested that equal portions of 5 samples be composited and analyzed by the hydrolysis procedure to determine if the composite is positive or negative for Bzd. If a given composite is negative, then each sample in the composite may be considered

Table VI Analysis of Human Urine Unspiked and Spiked with 0.001, 0.01, 0.1, and 1.0 ppm of Metabolites of Direct Black 38[a]

	Recovery of Metabolites ($\bar{x} \pm SE$)[b]				
Metabolite	Unspiked (ppm)	Spiked with 0.001 ppm (%)	Spiked with 0.01 ppm (%)	Spiked with 0.1 ppm (%)	Spiked with 1.0 ppm (%)
Bzd	0.0002 ±0.0001	*c*	53.4 ±4.2	87.0 ±5.2	*d*
MoAcBzd	0.0014 ±0.0000	*c*	5.29 ±0.36	61.1 ±1.5	76.9 ±2.5
DiAcBzd	0.0003 ±0.0000	20.3 ±4.0	65.5 ±3.8	94.4 ±2.3	*d*
DiAmAzBz	0.0008 ±0.0003	*c*	37.6 ±0.6	69.8 ±1.7	*d*
4-ABP	0.0010 ±0.0000	*c*	41.3 ±1.9	76.5 ±3.0	*d*

[a]Analyzed by EC/GC as the HFB-derivatives. DiAcBzd analyzed after alkaline hydrolysis to Bzd.
[b]Mean and standard error from triplicate assays; corrected for background interferences in the unspiked sample.
[c]None detected above background.
[d]Not determined

negative. If a composite is positive, then individual assays of the reserved samples (stored at -20°C) are required to identify the positive sample(s). The benefits of this approach towards monitoring the urine of workers are evident since the method will also detect any other derivatizable aromatic amines and thus allow some judgement by the analyst concerning the identity or nature of the chemical(s), dye(s), or pigment(s) that could have caused a given occupational exposure.

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