6. ANALYTICAL METHODS

The purpose of this chapter is to describe the analytical methods that are available for detecting, and/or measuring, and/or monitoring 3,3'-dichlorobenzidine, its metabolites, and other biomarkers of exposure and effect to 3,3'-dichlorobenzidine. The intent is not to provide an exhaustive list of analytical methods. Rather, the intention is to identify well-established methods that are used as the standard methods of analysis. Many of the analytical methods used for environmental samples are the methods approved by federal agencies and organizations such as EPA and the National Institute for Occupational Safety and Health (NIOSH). Other methods presented in this chapter are those that are approved by groups such as the Association of Official Analytical Chemists (AOAC) and the American Public Health Association (APHA). Additionally, analytical methods are included that modify previously used methods to obtain lower detection limits, and/or to improve accuracy and precision.

6.1 BIOLOGICAL SAMPLES

Methods for the determination of 3,3'-dichlorobenzidine and its metabolites in biological materials are summarized in Table 6- 1.

The compound 3,3'-dichlorobenzidine has been measured most often in urine and serum using gas chromatography (GC) (Bowman and Nony 1981; Bowman and Rushing 1981; Hoffman and Schmidt 1993; Joppich-Kuhn et al. 1997; Nony and Bowman 1980; Nony et al. 1980) and high performance liquid chromatography (HPLC) (Birner et al. 1990; Bowman and Nony 1981; CPMA 1998; Nony and Bowman 1980; Nony et al. 1980; Zwirner-Baier and Neumann 1994). A method for 3,3'-dichlorobenzidine in fish using GC (Diachenko 1979) has been reported. GC methods usually relied upon selective detection of the fluorinated derivatives while HPLC methods relied on absorbence or electrochemical detection. In addition, one method of analysis in urine used a spectrophotometric approach (Roberts and Rossano 1982). Several of the reported methods can also be used to determine the mono- and di-acetylated metabolites. The studies of Birner et al. (1990), Joppich-Kuhn et al. 1997, and Zwirner-Baier and Neumann (1994) reported the determination of 3,3'-dichlorobenzidine and monoacetyl-3,3'-dichlorobenzidine following hydrolysis of the analyte-hemoglobin adducts (the adduct is a marker of exposure). Although most of these methods have been developed using animal samples, they should also be applicable to the determination of 3,3'-dichlorobenzidine and its metabolites in samples of human origin. Limits of detection in the low to mid ppb range

Table 6-1. Analytical Methods for Determining 3,3'-Dichlorobenzidine and Metabolites in Biological Samples

Sample type	Extraction/cleanup	Detection	Limit of detection	Percent recovery	Reference
Human hemoglobin adducts (dichloro-benzidine, monoacetyl-dichlorobenzidine)	Isolation of hemoglobin, removal of water, followed by alkaline hydrolysis of adducts; addition of 2,2'-dichloro-benzidine as internal standard; extraction suing toluene containing 5% 2-propanol; derivatization using HFBA.	GC/NCI-MS	<0.1 ng/g (ppb)	65-85% over range 0-150 ng/g. (7% RSD for dichloro-benzidine, 16% RSD for monoacetyl-dichloro-benzidine)	Joppich-Kuhn et al. 1997
Rat hemoglobin adducts (dichloro-benzidine, monoacetyl-dichlorobenzidine)	Isolation of hemoglobin followed by alkaline hydrolysis of adducts, cleanup using C_{18} SPE, addition of internal standard.	HPLC/EC	No data	>90	Birner et al. 1990
Rat hemoglobin adducts (dichloro-benzidine, monoacetyl-dichlorobenzidine)	Isolation of hemglobin followed by alkaline hydrolysis of adducts, addition of recovery standard, cleanup using C_{18} SPE, addition of internal standard.	HPLC/EC	6 ng/g (1 ppb, wt:wt)	92–98	Zwirner-Baier and Neumann 1994
Fish tissue	Digestion with NaOH, extraction with benzene, extraction with dilute H_2SO_4, water removal and volume reduction; GPC cleanup .	GC/HCD (N mode)	<20 ppb	65 (20% RSD)	Diachenko 1979
Rat urine and serum	Addition of internal standard and sodium bicarbonate followed by extraction with diethyl ether; evaporation to dryness and redissolution in toluene.	GC/NPD	5 ng/mL (ppb)	No data	Hoffman and Schmidt 1993

Table 6-1. Analytical Methods for Determining 3,3'-Dichlorobenzidine and Metabolites in Biological Samples (continued)

Sample type	Extraction/cleanup	Detection	Limit of detection	Percent recovery	Reference
Hamster urine (dichlorobenzidine, mono- and di-acetyldichlorobenzidine, conjugates)	Adjustment of pH, extraction with benzene, volume reduction, formation of heptafluorbutyryl derivatives; for conjugates: alkaline hydrolysis of aqueous phase followed by derivatization as above.	GC/ECD	7–48 µg/L	No data	Bowman and Nony 1981; Nony and Bowman 1980; Nony et al. 1980
Urine (dichlorobenzidine, mono- and di-acetyldichlorobenzidine)	Adjustment of pH, extraction with benzene, volume reduction.	HPLC/UV	525 to 660 µg/L	No data	Bowman and Nony 1981; Nony and Bowman 1980; Nony et al. 1980
Urine	Adjustment of pH to 8, adsorption onto C_{18} SPE cartridge and elution with methanol.	HPLC/EC	5 µg/L (ppb)	No data	CPMA 1998
Urine	Adsorption onto XAD-2 resin, elution with acetone followed by clean up using acid-base partitioning and silica gel, formation of penta fluoropropyl derivative.	GC/ECD	≈1 µg/kg (ppb)	41±8	Bowman and Rushing 1981
Urine	Addition of sodium chloride, pH adjustment to 6, extraction with chloroform, extraction of chloroform extract with 3 N HCL; addition of chloramine-T and extraction of colored product into chloroform.	Absorbance at 457 nm	1–2 ppb (µg/L)	68 (4.6% RSD)	Roberts and Rossano 1982

GC = gas chromatography; EC = electrochemical detector; ECD = electron capture detector; HCD = Hall conductivity detector; HFBA = heptafluorobutyric anhydride; HPLC = high performance liquid chromatography; NCI-MS = mass spectrometry in the negative chemical ionization mode; NPD = nitrogen-phosphorus detector; ppb = parts per billion; UV = ultraviolet absorption; SPE = solid phase extraction; wt:wt = weight:weight

have been reported, although the hemoglobin adduct method of Joppich-Kuhn et al. (1997) reported a limit of detection of less than 0.1 ng/g (ppb). These sensitive methods are potentially useful for the assessment of human exposure to 3,3'-dichlorobenzidine.

6.2 ENVIRONMENTAL SAMPLES

Methods for the determination of 3,3'-dichlorobenzidine in environmental samples are summarized in Table 6-2.

The determination of 3,3'-dichlorobenzidine in environmental samples is most commonly achieved by GC/mass spectrometry (GC/MS) (Diachenko 1979; EPA 1982b, 1986a, 1984a; Greenberg et al. 1992) and HPLC (Armentrout and Cutie 1980; EPA 1982a; Morales et al. 1981; NIOSH 1994; Riggin and Howard 1979). Sample preparation typically employs liquid-liquid or liquid-solid extractions for water, waste water, soils, sediments, and solid waste. Supercritical fluid extraction has also been shown to provide good recovery of 3,3'-dichlorobenzidine from a spiked, dried soil (Oostdyke et al. 1995). Lopez-Avila et al. (1996) demonstrated that microwave-assisted extraction using a hexane-acetone solvent system gave recoveries from spiked (5 mg/kg), standard soil of 96%. The same solvent system in Soxhlet extraction resulted in only 47% recovery.

Solid phase extraction followed by capillary zone electrophoresis with UV absorbence detection has been shown to be applicable to the isolation and determination of 3,3'-dichlorobenzidine in water at ppm levels (Cavallaro et al. 1995).

For the HPLC determination of 3,3'-dichlorobenzidine in water, a relatively complicated procedure may be used (EPA 1982a) in which the analyte is extracted into chloroform, back-extracted with acid, neutralized, and extracted with chloroform. The chloroform is exchanged to methanol and concentrated using a rotary evaporator and nitrogen blowdown, then brought to a 5 mL volume with an acetate buffer. HPLC with electrochemical detection is used, providing for a method detection limit of 0.13 μg/L; single operator accuracy and precision for 30 analytes of 5 different types of water samples over a spike range of l-5 μg/L gave an average recovery of 65% and a standard deviation of 9.6% (EPA 1982a). The more complicated the matrix, the more extensive the sample preparation methods generally need to be. In certain circumstances (i.e., relatively clean water samples), water matrices can be introduced directly into the

Table 6-2. Analytical Methods for Determining 3,3'-Dichlorobenzidine in Environmental Samples

Sample type	Extraction/cleanup	Detection	Limit of detection	Percent Recovery	References
Air	Pumping of an aliquot of air through a glass fiber filter, elution with triethylamine in methanol.	HPLC/UV (Method 5509)	0.5 µg/m^3	96	NIOSH 1994
Air (dichlorobenzidine and its salts)	Pumping of an aliquot of air through a glass fiber filter and silica gel, extraction with triethylamine-methanol.	HPLC/UV	3 µg/m^3 for 50 L sample	No data	Morales et al. 1981
Water, wastewater	Extraction with methylene chloride at pH>11 and again at pH<2, removal of water followed by volume reduction.	GC/MS (Standard Method 6410)	16.5 µg/L	110 at 100 µg/L (100 ppb)	Greenberg et al. 1992
Waste water	Extraction with chloroform, solvent exchange to methanol, volume reduction.	HPLC/EC (EPA Method 605)	0.13 µg/L	64 (96% RSD)	EPA 1982a
Water	Adjustment of pH to 6.5–8 followed by filtration and isolation of analyte using SPE with elution using 150 mM phosphoric acid in water-acetone (80:20).	CZE/UV	1.5 mg/L (ppm)	82 (2.4% RSD) at 20 mg/L.	Cavallaro et al. 1995
Water	Adjustment of pH to 11, extraction with solvent such as dichloromethane, removal of water, volume reduction.	GC/MS (EPA Method 625)	16.5 µg/L	143 (145% RSD)	EPA 1982b
Waste water	Addition of isotopically-labeled standard, extraction with methylene chloride at pH 12–13, then at pH <2, removal of water, volume reduction, addition of internal standard.	GC/IDMS (EPA Method 1625)	50 µg/L	106 (25% RSD) at 100 µg/L	EPA 1984a
Waste water	Direct injection into HPLC.	HPLC/UV HPLC/EC	3 ppb (µg/L) with 500 µL injection, EC	87 over range 3 to 12 ppb	Armentrout and Cutie 1980

Table 6-2. Analytical Methods for Determining 3,3'-Dichlorobenzidine in Environmental Samples (continued)

Sample type	Extraction/cleanup	Detection	Limit of detection	Percent Recovery	References
Waste water	Extraction, conversion of 3,3'-dichloro-benzidine to pentafluoropropionamides.	HPLC/EC	0.2 pg		Kawahara et al. 1982
Waste water	Isolation via extraction with chloroform or SPE, addition of or elution with methanol, volume reduction.	HPLC/EC	50–100 ng/L	94 (4% RSD)	Riggin and Howard 1979
Dried soil	Addition of internal standard followed by extraction of soil by SFE with nitrous oxide/methanol/1,6-hexanediamine, expansion of fluid into methylene chloride, volume reduction.	GC/MS	No data	98	Oostdyke et al. 1995
Fish tissue	Digestion with NaOH, extraction with benzene, extraction with dilute H_2SO_4, water removal and volume reduction; GPC cleanup.	GC/HCD (N mode)	<20 ppb	65 (20% RSD)	Diachenko 1979
Waste water, soil, sediment, solid waste	Extraction (liquid-liquid, Soxhlet, sonication) with organic solvent such as dichloromethane, removal of water, volume reduction.	GC/MS (EPA method 8270)	20 µg/L (ppb) for wastewater; 1,300 µg/kg (ppb) for low soil, sediment	110 at 100 µg/L (100 ppb)	EPA 1986a

CZE = capillary zone electrophoresis; EC = electrochemical detector; GC = gas chromatography; HCD = Hall conductivity detector; HPLC = high performance liquid chromatography; IDMS = isotope dilution mass spectrometry; MS = mass spectrometry; RSD = relative standard deviation; SFE = supercritical fluid extraction; SPE = solid phase extraction; UV = ultraviolet absorbance detection

analysis step without prior treatment (Armentrout and Cutie 1980). GC separation methods can be applied also to the extracts obtained for HPLC analyses. Detection of the free amine, in addition to fluorinated derivatives, has been demonstrated by GC methods.

Dichlorobenzidine and its salts are collected from air matrices using adsorption/filtration approaches (Morales et al. 1981; NIOSH 1994) and recovered from the adsorbent using methanol containing a small amount of triethylamine (TEA). The addition of TEA converts any salt to the corresponding amine, thus rendering it soluble in the organic solvent. Limits of detection in the low $\mu g/m^3$ (low to sub-ppb) range have been reported. The compound 4,4'-methylenebis(2-chloroaniline) was reported to interfere with 3,3'-dichlorobenzidine (Morales et al. 1981; NIOSH 1994).

6.3 ADEQUACY OF THE DATABASE

Section 104(i)(5) of CERCLA, as amended, directs the Administrator of ATSDR (in consultation with theAdministrator of EPA and agencies and programs of the Public Health Service) to assess whether adequate information on the health effects of 3,3'-dichlorobenzidine is available. Where adequate information is not available, ATSDR, in conjunction with the NTP, is required to assure the initiation of a program of research designed to determine the health effects (and techniques for developing methods to determine such health effects) of 3,3'-dichlorobenzidine.

The following categories of possible data needs have been identified by a joint team of scientists from ATSDR, NTP, and EPA. They are defined as substance-specific informational needs that if met would reduce the uncertainties of human health assessment. This definition should not be interpreted to mean that all data needs discussed in this section must be filled. In the future, the identified data needs will be evaluated and prioritized, and a substance-specific research agenda will be proposed.

6.3.1 Identification of Data Needs

Methods for Determining Biomarkers of Exposure and Effect. Methods for the determination of 3,3'-dichlorobenzidine in urine and serum have been reported (Birner et al. 1990; Bowman and Nony 1981; Bowman and Rushing 1981; Hoffman and Schmidt 1993; CPMA 1998; Nony and Bowman 1980; Nony et al. 1980; Zwirner-Baier and Neumann 1994). Some of the methods have been shown to be

suitable for the determination of the acetylated metabolites (Bowman and Nony 198 1; Nony and Bowman 1980; Nony et al. 1980). The methods of Birner et al. (1990), Joppich-Kuhn et al. (1997), and Zwirner-Baier and Neumann (1994) permit the analysis of hemoglobin adducts of 3,3'-dichlorobenzidine and its monoacetyl metabolite. Limits of detection for 3,3'-dichlorobenzidine in urine and serum were reported to be as low as 1 to 5 ppb (Bowman and Rushing 1981; Hoffman and Schmidt 1993; Roberts and Rossano 1982), with detectable concentrations of the acetylated metabolites somewhat higher. Most of these studies were performed with samples from rats; the methods should be tested to determine if they are applicable to samples of human origin. In addition, the levels of these biomarkers associated with exposures to 3,3'-dichlorobenzidine of toxicological concern should be defined in order to increase their utility.

Methods for Determining Parent Compounds and Degradation Products in Environmental Media. Methods have been described for the determination of 3,3'-dichlorobenzidine in air, with reported limits of detection of 0.5 μg/m^3 (NIOSH 1994) and 3 μg/m^3 (Morales et al. 1981). Methods for the analysis of 3,3'-dichlorobenzidine in water and waste water have also been described, with reported detection limits of 16.5 μg/L (ppb) (EPA 1982b; Greenberg et al. 1992), 50 μg/L (ppb) (EPA 1984a), 3 ppb (Armentrout and Cutie 1980), 0.13 μg/L (ppb) (EPA 1982a), and 50 to 100 ng/L (ppt) (Riggin and Howard 1979). The only method found for 3,3'-dichlorobenzidine in food (fish) reported a limit of detection of less than 20 ppb (Diachenko 1979). It does not appear that additional methods for the determination of 3,3'-dichlorobenzidine in air or water are needed. Additional methods for 3,3'-dichlorobenzidine in foods are needed. If MRLs were established, the needs could be defined more precisely.

6.3.2 Ongoing Studies

No ongoing studies in which new methods for the determination of 3,3'-dichlorobenzidine are being developed were found in a search of the Federal Research in Progress database (FEDRIP 1998).