7. ANALYTICAL METHODS

The purpose of this chapter is to describe the analytical methods that are available for detecting, measuring, and/or monitoring benzidine, its metabolites, and other biomarkers of exposure and effect to benzidine. 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.

7.1 BIOLOGICAL MATERIALS

Several investigators have described means for the determination of benzidine and its metabolites in biological materials, particularly urine, by gas chromatography (GC) and high performance liquid chromatography (HPLC) (IARC 1981b; Jedrejczak and Gaind 1993; Neumeister 1991; Nony and Bowman 1980; Nony et al. 1980). Most studies of the determination of benzidine in biological materials have concentrated on urine as a means of monitoring human exposure to benzidine in the workplace. The metabolites include monacetylated (Hodgson 1987), diacetylated, and conjugated metabolites. Of these, one that is commonly monitored is N,N'-diacetylbenzidine (N,N'-DAB). The analytical methods used to measure benzidine and its metabolites in biological materials are GC (NIOSH 1984b), gas chromatography/mass spectrometry (GC/MS) (Hsu et al. 1996; Hurst et al. 1981 Jedrejczak and Gaind 1993), HPLC (IARC 1981b; Neumeister 1991), thin-layer chromatography (TLC) (NIOSH 1984a), and immunoassay (Johnson et al. 1981). Chemical derivatization is commonly employed in analytical methods based on GC analysis to enhance chromatography and detection (IARC 1981b; Hsu et al. 1996; Jedrejczak and Gaind 1993; NIOSH 1984b).

Benzidine can be extracted from urine with a solvent such as chloroform or benzene, then re-extracted into aqueous HCl as the water-soluble cationic form. One of the reported methods (Jedrejczak and Gaind 1993) converted all acetyl metabolites to benzidine via base hydrolysis prior to analysis to determine total benzidine. Benzidine and its metabolites can also be removed from urine by retention on solid adsorbents, followed by sample clean up and analysis (Hsu et al. 1996; Neumeister 1991).

The technique of ^{32}P post-labeling with HPLC analysis can be employed to detect and quantitate DNA-benzidine adducts formed *in vivo* in mice (Levy and Weber 1988). This method may be used for rapid screening to detect adduct formation and to measure differences in DNA damage caused by variations in metabolic pathways. Talaska et al. (1987) have also studied DNA-benzidine adducts in the livers of mice dosed with benzidine. After an intraperitoneal dose of 38 mg/kg, one adduct in 3.5 million normal nucleotides was detected.

Birner et al. (1990) have investigated adducts of benzidine and benzidine metabolites with hemoglobin. They identified adducts with benzidine, mono-N-acetylbenzidine, and 4-aminobiphenyl. It should be noted that the 4-aminobiphenyl-hemoglobin adduct is also commonly found in tobacco smokers (Hammond et al. 1993) and, thus, would not be specific for benzidine exposure or effect.

Supercritical fluid extraction/chromatography and immunoassay analysis are two areas of intense current activity from which substantial advances in the determination of benzidine and it's metabolites in biological samples can be anticipated. The two techniques are complementary in that supercritical fluid extraction (SFE) is promising for the removal of analytes from sample material (Hawthorne 1988) and immunoassay is very analyte-selective and sensitive (Vanderlaan et al. 1988).

Thermospray techniques used in conjunction with mass spectrometry (MS) with or without high performance liquid chromatographic separation have proven useful for the determination of thermally labile compounds such as toxicant metabolites (Korfmacher et al. 1987) and should be applicable to the determination of benzidine in biological materials (Betowski et al. 1987). More recent liquid chromatography/MS interfaces, such as electrospray and particle beam, increase the potential for selective and sensitive benzidine determination.

Methods for the determination of benzidine in biological samples are summarized in Table 7-1.

7.2 ENVIRONMENTAL SAMPLES

Benzidine in environmental samples is most commonly determined by GC/MS (EPA 1982b) and HPLC (EPA 1982a). Methods using GC with Fourier transform infrared (GC/FT-IR) spectrophotometry have also been reported (EPA 1994). Although methods for the determination of benzidine in soils are reported, problems are so severe that Superfund has dropped benzidine from the analyte list in the current laboratory programs (Brumley and Brownrigg 1994).

Table 7-1. Analytical Methods for Determining Benzidine in Biological Samples

Sample matrix	Preparation method	Analytical method	Sample detection limit	Percent recovery	Reference
Urine	Acidification, addition of sodium nitrite and extraction with ether or acetone; destruction of excess nitrite and addition of 8-hydroxyquinoline; basification then extraction with 3-methyl-1-butanol	UV-Vis spectrophoto-metry	<30 µg/L (0.03ppm)	95%	Upadhyay and Gupta 1985
Urine (for benzidine and metabolites)	Extraction with benzene, heptafluorobutyryl derivatization of non-conjugated compounds; base hydrolysis aqueous phase repeat extraction and derivatization for determination of conjugated metabolites	GC/ECD	1 µg/L (0.001ppm)	53 (7.9% RSD) at 0.01 ppm; 84 (6.0% RSD) at 0.1 ppm	IARC 1981b; Nony and Bowman 1980; Nony et al. 1980
Urine (for benzidine and metabolites)	Extraction with benzene and solvent exchange to methanol; hydrolysis of aqueous phase; extraction and solvent exchange	HPLC/UV	180 µg/L (0.18ppm)	64.7 at 0.5 ppm to 80.1 at 5 ppm (3% RSD)	IARC 1981b; Nony and Bowman 1980; Nony et al. 1980
Urine (total benzidine)	Base hydrolysis, addition of deuterated benzidine as internal standard, extraction with ethyl acetate/pyridine and derivatization with pentafluoropropionic anhydride	GC/MS (NICI)	0.5 µg/L (0.5 ppb)	75 at 10 µg/L; 87 at 100 µg/L (precision = 8.7% RSD at 10 µg/L)	Jedrejczak and Gaind 1993

Table 7-1. Analytical Methods for Determining Benzidine in Biological Samples *(continued)*

Sample matrix	Preparation method	Analytical method	Sample detection limit	Percent recovery	Reference
Urine	Conjugation hydrolysis at pH=12 and isolation of free amine using C_{18} SPE, elution with benzene	HPLC/UV, HPLC/EC	1.5 µg/L or 0.0015 ppm (UV); 0.24 µg/L or 0.24 ppb (EC)	>89	Neumeister 1991
Urine	Chloroform extraction, HCl extraction, derivatization with trinitrobenzene sulfonic acid	TLC/Spec (method 8304; screening test)	1 µg/L	70 at 5 µg/L	NIOSH 1984a
Urine (for benzidine and mono- and di-acetylbenzidine conjugates	Base hydrolysis, extraction with benzene, derivatization with heptafluorobutyric anhydride, Florisil cleanup	GC/ECD (method 8306; quantitative method)	5 µg/L	92 (11% RSD at 10 µg/L)	NIOSH 1984b
Urine	No data	GC/MS	No data	No data	Hurst et al. 1981
Urine	Centrifugation of urine, pH adjustment to 6.8–7.2	RIA[a]	10 pg (low ppm given volume used)	92.7±0.8%	Johnson et al. 1981
Urine	Solid-phase extraction followed by successive washing and derivatization with pentafluoropropionic anhydride	GC/MS	0.5 µg/L (ppb)	87–89%	Hsu et al. 1996

[a]RIA is for diacetyl metabolite of benzidine.

EC = electrochemical detection; ECD = electron capture detector; GC = gas chromatography; HPLC = high performance liquid chromatography; MS = mass spectrometry; NICI = negative ion chemical ionization mass spectrometry; NIOSH = National Institute for Occupational Safety and Health; RIA = radioimmunoassay; SPE = solid phase extraction; TLC = thin-layer chromatography; UV = ultraviolet absorption spectrophotometry

The GC/MS determination of benzidine in liquid and solid samples involves extraction into methylene chloride or chloroform followed by separation without derivatization on a gas chromatographic fused-silica capillary column coated with a slightly polar silicone material and detection with a mass spectrometer. Unfortunately, benzidine can be subject to oxidative losses during solvent extraction and concentration and its gas chromatographic separation properties are poor. Chromatographic problems can be overcome through the use of chemical derivatization; such is the case for benzidine in air where the pentafluoropropyl derivative is formed after extraction from the sorbent (Roussel et al. 1991). Benzidine and similar compounds can photodegrade (Brumley and Brownrigg 1994). Benzidine has been shown to be thermally-labile, and this could decrease the accuracy of GC-based methods (Riggin and Howard 1979). Sodium sulfate is commonly used to dry extracts prior to GC analysis and has been shown to adsorb benzidine (Riggin and Howard 1979); this can also impact the accuracy of the methods.

For the HPLC determination of benzidine in water, a relatively complicated procedure may be used (EPA 1982b) in which the analyte is extracted into chloroform, back-extracted with acid, neutralized, and extracted again 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. Conditions are used that permit the separation of benzidine compounds by HPLC with electrochemical detection, which is now currently favored over spectrophotometric measurement (Trippel-Schulte et al. 1986). The method detection limit with HPLC separation and electrochemical detection is reported to be 0.08 µg/L, and single-operator accuracy and precision for 30 analyses of 5 different types of water samples over a spike range of 1.0–50 µg/L gave an average percent recovery of 65% and a standard deviation of 11.4% (EPA 1984). While this is an accepted method for benzidine analyses in water, some laboratories do not permit the use of chloroform and the electrochemical detector requires an operator with specialized skills (Hites and Budde 1991).

HPLC separation with ultraviolet (UV) absorption detection is used for the determination of benzidine in air (NIOSH 1985b). The analyte at levels in a range of 0.2–7 µg per sample can be collected in a silica gel collection tube from up to 100 L of air. The estimated method limit of detection is 0.05 µg/sample.

Methods for the determination of benzidine in environmental media are summarized in Table 7-2. While various methods have been employed to monitor exposures to benzidine in environmental materials, possible interferences may occur, reducing their sensitivity. Metal ions forming hydroxides in alkaline media may be expected to interfere in spectrophotometric analyses (Upadhyay and Gupta 1985). Also,

Table 7-2. Analytical Methods for Determining Benzidine in Environmental Samples

Sample matrix	Preparation method	Analytical method	Sample detection limit	Percent recovery	Reference
Air	Glass fiber filter and silica gel; extraction with methanol containing triethylamine	HPLC	3 µg/m^3 (0.02 ppm)	70–97	IARC 1981a
Air	Silica gel sorption, desorption using methanol containing triethylamine	HPLC/UV (method 5509)	0.05 µg/sample 0.5–2.5 µg/m^3 or 0.004–0.0019 ppm, (depending on volume sampled)	No data	NIOSH 1985b
Air	Glass fiber filter impregnated with 5% sulfuric acid; release by sonication in acidic water; basification, extraction with toluene, and derivatization with pentafluoropropionic acid	GC/MS	1ng/m^3	76	Roussel et al. 1991
Waste water	Addition of stable, isotope-labeled analogue of benzidine; basification, extraction with methylene chloride, solvent removal	GC/IDMS (method 1625)	50 µg/L (50 ppb)	Variable	EPA 1990b
Waste water	Extraction with chloroform, back-extraction with acid, neutralization and re-extraction with chloroform, exchange to methanol, solvent removal	HPLC/ELCD (method 605)	0.08 µg/L, depending on nature of interferences	0.70C + 0.06 where C = conc. in µg/L	EPA 1984
Waste water[a]	Extraction with methylene chloride at pH>11, solvent removal	GC/MS (method 625)	44 µg/L (44 ppb)	63–87	EPA 1982b

Table 7-2. Analytical Methods for Determining Benzidine in Environmental Samples *(continued)*

Sample matrix	Preparation method	Analytical method	Sample detection limit	Percent recovery	Reference
Drinking water, surface water, and waste water[a]	Extraction with methylene chloride followed by separation with reverse-phase HPLC	HPLC/PB/MS method 8325	No data	96–97	EPA 1996
Drinking water, surface water, and waste water[a]	Extraction with methylene chloride at pH>11, solvent removal	GC/MS (method 6410)	44 µg/L (44 ppb)	No data	APHA 1992
Water, soil, sediment	Water: pH adjustment to 8.5, extraction with methylene chloride, solvent removal and redissolution in mobile phase	CZE	Water: 0.1 mg/L (0.1 ppm)	Water: 82% at 1 g/L, 59% at 0.1 g/L	Brumley and Brownrigg 1994
	Soil, sediment: Soxhlet extraction, solvent removal and redissolution in mobile phase		Soil, sediment: approximately 1 mg/kg (1 ppm)	Soil: 56% at 0.9 g/kg	
Soil, sediment, solid waste[a]	Extraction with methylene chloride, solvent removal	GC/MS (method 8270D)	No data	No data	EPA 1998i
Food dyes	Reduction of bound amines with sodium dithionite, followed by chloroform extraction and derivatization with 2-naphthol-3,6-disulfonic acid, disodium salt	HPLC	3–4 ng/g	46%	Lancaster and Lawrence 1999

[a]Benzidine is subject to oxidative losses during extraction and evaporation.

APHA = American Public Health Association; CZE = capillary zone electrophoresis; ELCD = electrochemical detector; EPA = Environmental Protection Agency; FT-IR = Fourier transformed infrared spectrometry; GC = gas chromatography; HPLC = high performance liquid chromatography; IDMS = isotope dilution mass spectrometry; MS = mass spectroscopy; NIOSH = National Institute for Occupational Safety and Health UV = ultraviolet absorption spectrophotometry

certain amines can also interfere with this method at high concentrations. p-Nitroaniline and toluidine caused positive interferences (Upadhyay and Gupta 1985).

Studies designed to improve the analysis of semivolatile compounds will continue to yield refinements and improvements in the determination of benzidine in the environment. The current high level of activity in supercritical fluid extraction of solid and semi-solid samples should yield improved recoveries and sensitivities for the determination of benzidine in solid wastes, avoiding troublesome problems of analyte oxidation. The combination of SFE and supercritical fluid chromatography (SFC) has been described for the determination of sulfonylurea herbicides and their metabolites in complex matrices, including soil, plant materials, and a cell culture medium (McNally and Wheeler 1988). The approach described in this work should be applicable to many other toxicologically and environmentally significant analytes, including benzidine, and might well overcome the oxidation problems (EPA 1996) associated with the extraction and concentration of benzidine. Indeed, the ability to recover benzidine from spiked sand (Oostdyk et al. 1993a) and soil (Oostdyk et al. 1993b) is currently being investigated. In SFE, the addition of 1,6-hexanediamine in methanol to the extraction fluid was found to be needed to recover benzidine from spiked soil at the 79–82% range (Oostdyk et al. 1993b). The addition of the amine helps to displace benzidine from acidic surface sites on the soil. SFE has not been applied to soils containing many potentially interfering compounds. Benzidine should be amenable to SFC analysis, and this would reduce the need for chemical derivatization to improve chromatographic efficiency. Capillary zone electrophoresis (CZE) holds promise as a rapid determinative method that can provide for greater resolution and sensitivity than HPLC (Brumley and Brownrigg 1994). Sample preparation is still problematic. Immunoassay analysis (Vanderlaan et al. 1988) is an area of intense current activity from which substantial advances in the determination of benzidine in environmental samples can be anticipated.

The FDA has developed a method to quantify total benzidine (free and combined) in azo dyes that are currently approved for use in foods. The method involves reduction of the dye matrix with dithionite to free the combined benzidine, then extraction, diazotization, coupling with pyrazolone T, and analysis by HPLC with a photodiode array detector (Prival et al. 1993). The FDA has set a limit for free benzidine in these dyes at 1 ppb (see Chapter 8), but the detection limit for this method is reported as 5 ppb. This technique has been modified slightly, and a detection limit of 3–4 ng/g has been achieved (Lancaster and Lawrence 1999).

7.3 ADEQUACY OF THE DATABASE

Section 104(i)(5) of CERCLA, as amended, directs the Administrator of ATSDR (in consultation with the Administrator of EPA and agencies and programs of the Public Health Service) to assess whether adequate information on the health effects of benzidine is available. Where adequate information is not available, ATSDR, in conjunction with the National Toxicology Program (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 benzidine.

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.

7.3.1 Identification of Data Needs

Methods for Determining Biomarkers of Exposure and Effect. Methods are available for the determination of benzidine and/or its metabolites in urine (IARC 1981b; Hsu et al. 1996; Jedrejczak and Gaind 1993; Neumiester 1991; NIOSH 1984a; Nony and Bowman 1980; Nony et al. 1980; Upadhyay and Gupta 1985). These permit the confirmation of human exposure to benzidine, its metabolites, and associated compounds such as the benzidine-based dyes. If an intake of 0.04 mg/kg/day is assumed, then a 70 kg person would ingest 2.59 mg/day. At this exposure, current analytical methods are sufficient to monitor resulting urinary concentrations at levels which may pose a human health threat. Reported methods for urine allow detection of benzidine and metabolites to below the µg/L concentration. Benzidine and it metabolites have a half-life in the body of only 5–6 hours (Neumeister 1991), so the methods are useful for detecting exposure for a short period after the exposure has occurred. More sensitive methods would be required if there is a need to increase the time from exposure termination to the collection of a sample containing measurable benzidine.

Adducts of benzidine and benzidine metabolites have been found in DNA (Levy and Weber 1988; Talaska et al. 1987) and hemoglobin (Birner et al. 1990; Hammond et al. 1993). In addition to hemoglobin adducts of the N-acetylated benzidines, an adduct with 4-aminobiphenyl has been measured (Birner et al. 1990). As noted above, 4-aminobiphenyl-hemoglobin adduct is also commonly found in

tobacco smokers (Hammond et al. 1993) so the presence of this adduct would not be specific for benzidine exposure or effect. Additional information is needed regarding the quantity of adduct formed after a given exposure and how the quantities of adduct relate to effect. It is likely that more sensitive and specific methods for adducts are needed to properly identify and quantify the exposures and potential effects.

Methods for Determining Parent Compounds and Degradation Products in Environmental Media. Standard methods are available for the determination of benzidine in environmental samples including air, water, soil, sediment, and solid waste (APHA 1992; EPA 1982b, 1984, 1990b, 1996, 1998i; NIOSH 1985b). Assuming an oral exposure of 0.04 mg/kg/day, analytical methods for benzidine in drinking water are sufficient (2 L consumed per day by a 70 kg person). Problems need to be solved regarding the oxidation of benzidine during extraction from environmental samples and subsequent concentration. This is especially true if the quantitation of benzidine in soils is required. More sensitive methods are needed in order to accurately measure benzidine levels in soils and sediment. This is particularly true where benzidine may be present in significant quantities, such as at hazardous waste sites. It should be noted that background levels of benzidine are expected to be very low in most environmental media, and may be below the detection limits regardless of the sensitivity of the technique employed. No MRL has been established for inhalation exposures but many of the analytical methods for benzidine in air are very sensitive (e.g., Roussel et al. 1991; 1 ng/m^3; and NIOSH 1985b; 0.5 µg/m^3), so it is not likely that new methods would be required. If MRLs are established for inhalation exposure, the values will dictate whether additional methods are needed.

7.3.2 Ongoing Studies

No information could be identified on ongoing studies related directly to the development of analytical techniques used to quantify benzidine in biological or environmental media.