Measurement of Pesticides and Other Toxicants in Amniotic Fluid as a Potential Biomarker of Prenatal Exposure: A Validation Study

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Residential and agricultural pesticide use is widespread in the United States, with approximately one billion pounds of pesticides used annually. Recent studies have demonstrated widespread pesticide exposure to the U.S. population, including pregnant women and children (Berkowitz et al. 2003; Hill et al. 1989, 1995; Kutz et al. 1992; Lu et al. 2001; National Center for Environmental Health 2001; Whyatt et al. 2002). Overall, these studies confirm that children are exposed to pesticides prenatally, when they may be particularly vulnerable to adverse health effects (Eskenazi et al. 1999).

Measurements of pesticides and other chemicals in meconium and amniotic fluid, which are produced by the fetus, are likely to be useful biomarkers of direct fetal exposure. Chemicals in these media may represent cumulative exposures derived from ongoing and/or transient maternal exposures to chemicals that have short half-lives in the body, such as organophosphate (OP) pesticides (Barr et al. 1999; Foster et al. 2000; Hong et al. 2002; Ostrea et al. 1993, 2002; Whyatt and Barr 2001). Although meconium is likely to represent exposures from the second trimester through delivery (Barr et al. 1999; Hong et al. 2002; Ostrea et al. 1993, 2002; Whyatt and Barr 2001), amniotic fluid, collected during amniocentesis, is the only biologic medium available that can be used to characterize early fetal exposures (~15–20 weeks).

Amniotic fluid surounds and protects the developing embryo and fetus. It is present soon after implantation and increases in volume throughout pregnancy (Hytten and Chamberlain 1991). The composition of amniotic fluid, which is primarily fetal urine, varies over the course of gestation, reflecting the sequential maturation of fetal organs and accompanying shifts in sites of fetal metabolism and filtration. Although the fetal lungs and kidneys excrete fluid continuously into the amniotic fluid reservoir, the fetus is also continuously swallowing and “inhaling” amniotic fluid. This cycling suggests that toxic substances excreted into the amniotic fluid may continuously reexpose the fetus. Although the placenta prevents the transfer of some toxicants from the mother to fetus, many chemicals can cross this barrier (McMichael et al. 1986; Moore et al. 1982; O’Leary et al. 1970; Talbot et al. 1988). Additionally, paraplacental transfer, where toxicants transfer directly from maternal blood through the amniotic sac, can also contaminate amniotic fluid (Schmidt 1992).

Studies of organochlorine compounds (including dichlorodiphenyldichloroethylene (DDE) and dichlorodiphenyltrichloroethane (DDT)), therapeutic and illegal drugs, first-hand and environmental tobacco smoke, dust mite allergens, and metals indicate that toxicants are detectable in amniotic fluid and are likely to reflect fetal exposures (Carvalho et al. 2001; Foster et al. 2000; Holloway et al. 2000; Jauniaux et al. 1999; Kim et al. 1998; O’Leary et al. 1970; Polisshuk et al. 1977; Talbot et al. 1988; Winecker et al. 1997). To date, no studies have investigated levels of common nonpersistent pesticides in amniotic fluid. In the present study, we determined whether analytical procedures for measuring nonspecific OP pesticide metabolites (dialkyl phosphates), OP-specific metabolites, pyrethroid pesticide metabolites, and pesticides with chlorinated phenol metabolites in urine were transferable to amniotic fluid, and we report concentration data for several of these analytes.

Materials and Methods

Population. Amniotic fluid samples were collected from 100 women referred to the Children’s Hospital Central California in Madera, California, which serves a cross-section of California residents living in the San Joaquin Valley. The San Joaquin Valley is an agricultural area that also includes several urban centers, including the Madera–Fresno metropolitan area. The 100 samples collected represent a convenience sample. All women were referred for amniocentesis because of an elevated risk for genetic disorders, such as advanced maternal age, family history of genetic disease, and abnormalities on serum screening. Medical records were abstracted from a subset of women (n = 50), selected randomly, for basic demographic and health information. This study was approved by the...
University of California (UC) Berkeley Committee for the Protection of Human Subjects and the Children’s Hospital Central California Institutional Review Board.

**Sample collection method.** Medical staff collected 15–20 mL of amniotic fluid according to standard clinical procedures for amniocentesis, which were then centrifuged. Cells were removed for cyrogenetic analysis, and 2 mL were aliquoted for laboratory tests. According to California State requirements, 4 mL of fluid were stored at –80°C for at least 30 days in case the clinical or laboratory tests were positive or inconclusive and required follow-up testing. After 30 days, these 4-mL samples are normally discarded. We selected 100 of these samples that were slated for disposal. Samples were bar coded and shipped on dry ice to the Centers for Disease Control and Prevention (CDC; Atlanta, GA), where they were stored at –80°C until analysis. Before collection and analysis, all clinical and laboratory equipment, including syringe components, centrifuge containers, and storage vials, were evaluated by the CDC for potential interferences that could adversely affect laboratory analyses.

**Chemical analysis method.** Of the 100 4-mL samples, 20 were pooled for method validation and laboratory quality assurance and control. The remaining 80 samples were divided into four sets of 20 and analyzed by four separate mass spectrometry–based methods to measure a) synthetic pyrethroids, b) chemical-specific metabolites of OP pesticides, c) class-specific metabolites of OP pesticides, and d) pesticides, including carbamates, herbicides, and pesticides with chlorinated phenol metabolites, such as disinfectants. These target analytes and their respective detection limits are listed in Tables 1 and 2. Laboratory methods involved a solid-phase or liquid partitioning extraction with analysis using tandem mass spectrometry and isotope dilution gas chromatography–mass spectrometry methods previously developed by the CDC for urine. Detailed descriptions of these analytical procedures are presented elsewhere (Baker et al. 2000; Barr et al. 1999; Beeson et al. 1999; Bravo et al. 2002; Shealy et al. 1996; Whyatt and Barr 2001). Because amniotic fluid is similar to urine, no major modifications in methodology were required, although minor changes were necessary (described in “Results”). Laboratory quality assurance included repeat analysis of spiked matrix pools (10 µg/L of each analyte) and inclusion of these spiked pools in each run. Results from these samples provided data on recovery and precision. An analytical run was considered “out of control” if a) either of the two quality control (QC) values was outside the 99th percentile confidence limits (mean + 3 SD); b) both QC values were outside of the 95th percentile confidence limits (mean + 2 SD) even if on opposite sides of the mean; c) one QC value was outside the 95th percentile confidence intervals and the preceding nine measurements were on the same side of the mean (www.westgard.com). Data were not reported from runs considered “out of control.” To determine the stability of analytes in amniotic fluid, aliquots of amniotic fluid were kept either at room temperature, under refrigeration, or frozen for 0–12 hr and analyzed every hour.

### Table 1. Concentrations of chemicals detected in amniotic fluid (µg/L) and laboratory parameters for amniotic fluid compared with historical results for urine.a

| Analyte | Detects | Mean recovery | Historical urine results |
|---------|---------|---------------|-------------------------|
|         | [n (%)] | ± CV (%)      | LOD ± CV (%)            |
| 1- or 2-Naphthol | 14 (70) | 0.72 | 0.61 | 4.19 | 0.10 | 98 ± 4 | 0.20, 0.40 | 96 ± 2 |
| 2,5-Dichlorophenol | 11 (55) | 0.39 | 0.37 | 0.43 | 0.10 | 91 ± 7 | 0.10 | 93 ± 3 |
| Carbofuran | 1 (5) | 0.12 | 0.12 | 0.12 | 0.10 | 91 ± 4 | 0.20 | 92 ± 5 |
| α-Phenylphenol | 6 (30) | 0.13 | 0.10 | 0.17 | 0.10 | 91 ± 4 | 0.20 | 93 ± 2 |
| Pentachlorophenol | 3 (15) | 0.23 | 0.15 | 0.54 | 0.10 | 91 ± 4 | 0.50 | 94 ± 3 |
| DFP | 2 (10) | 0.31 | 0.26 | 0.38 | 0.20 | 81 ± 9 | 0.20 | 95 ± 8 |
| DMP | 2 (10) | 0.32 | 0.30 | 0.34 | 0.20 | 85 ± 22 | 0.50 | 90 ± 20 |
| DMTP | 1 (5) | 0.43 | 0.43 | 0.43 | 0.20 | 92 ± 8 | 0.50 | 99 ± 7 |

Abbreviations: CV, coefficient of variability; GM, geometric mean; max, maximum; min, minimum. *Historical results for CDC method development projects (Barr D. Personal communication). g = 5 spike samples; extraction recovery is based on spike at 10 µg/L for amniotic fluid. n = 25 samples; urine spiking level varied from 2.5 to 20 µg/L. Levels of detection provided for 1-naphthol and 2-naphthol, respectively, in urine.

### Table 2. Levels of detection (µg/L) and laboratory recovery for analytes not detected in amniotic fluid compared with historical laboratory results for urine.a

| Analyte | Mean recovery ± CV (%) | LOD ± CV (%) |
|---------|------------------------|--------------|
| 2-Isopropoxophenol | 0.10 | 86 ± 12 | 1.04 ± 9 |
| 2,4-Dichlorophenol | 0.10 | 86 ± 4 | 0.30 | 94 ± 3 |
| 2,4,5-Trichlorophenol | 0.10 | 82 ± 6 | 0.90 | 80 ± 2 |
| 2,4,6-Trichlorophenol | 0.10 | 83 ± 6 | 1.30 | 91 ± 6 |
| 3,5,6-Trichloro-2-pyridinol | 0.10 | 98 ± 4 | 0.40 | 101 ± 4 |
| 4-Nitrophenol | 0.10 | 91 ± 2 | 0.10 | 94 ± 2 |
| Acetophenone | 1 | 35 ± 6 | 0.80 | 34 ± 8 |
| 3-Chloro-4-methyl-7-hydroxycoumarin | 1 | 90 ± 2 | 0.20 | 91 ± 3 |
| Diethylidithiophosphate | 0.20 | 91 ± 6 | 0.10 | 89 ± 7 |
| Diethylthiophosphate | 0.20 | 91 ± 6 | 0.20 | 89 ± 5 |
| Dimethylidithiophosphate | 0.20 | 96 ± 2 | 0.10 | 98 ± 4 |
| Methyl-1,2,3-benzotrizain-4-one | 1 | 86 ± 4 | 6 | 90 ± 7 |
| 5-Chloro-1,2-dihydro-1-isopropyl-[1H]-1,2,4-triazol-3-one | 1 | 86 ± 3 | 1 | 88 ± 6 |
| Methanidaphor | 1 | 21 ± 10 | 0.2 | 21 ± 13 |
| 2-Diethylamino-6-methyl pyrimidin-4-ol | 1 | 92 ± 3 | 0.2 | 98 ± 3 |
| 3-Phenoxybenzoic acid | 0.80 | 78 ± 6 | 0.3 | 77 ± 10 |
| 4-Fluoro-3-phenoxybenzoic acid | 0.50 | 78 ± 6 | 0.3 | 79 ± 6 |
| cis-DBCA | 0.30 | 90 ± 3 | 0.2 | 88 ± 10 |
| cis-DCCA | 0.20 | 90 ± 6 | 0.5 | 91 ± 12 |
| trans-DCCA | 0.40 | 91 ± 6 | 0.5 | 93 ± 7 |
| 2-Isopropyl-4-methyl-6-hydroxypyrimidine | 0.04 | 93 ± 4 | 0.2 | 93 ± 3 |
| 2,4-Dichlorophenoxyacetic acid | 0.50 | 95 ± 8 | 0.2 | 96 ± 9 |
| 4,5-Phenoxyphenolacetic acid | 0.50 | 91 ± 10 | 0.1 | 93 ± 7 |
| 3-Phenoxybenzoic acid | 1 | 78 ± 5 | 0.1 | 72 ± 6 |
| Acetochlor mercapturate | 0.5 | 93 ± 5 | 0.1 | 97 ± 4 |
| Alachlor mercapturate | 10 Not determined | 3 | 95 ± 6 |
| Atrazine mercapturate | 0.60 | 97 ± 6 | 0.1 | 99 ± 5 |
| Diethyltoluamide | 0.40 | 100 ± 3 | 0.1 | 98 ± 4 |
| Malathion diacid | 0.60 | 75 ± 6 | 0.3 | 78 ± 5 |
| Metolachlor mercapturate | 0.90 | 90 ± 7 | 0.2 | 91 ± 9 |

Abreviations: cis-DBCA, cis-3-(2,2-dibromovinyl)-2,2-dimethylclopropano-1-carboxylic acid; cis-DCCA, cis-3-(2,2-dichlorovinyl)-2,2-dimethylclopropano-1-carboxylic acid; CV, coefficient of variability; trans-DCCA, trans-3-(2,2-dichlorovinyl)-2,2-dimethylclopropano-1-carboxylic acid. *Historical results for CDC method development projects (Barr D. Personal communication). g = 5 spike samples; extraction recovery is based on spike at 10 µg/L for amniotic fluid. n = 25 samples; urine spiking level varied from 2.5 to 20 µg/L.

### Results

**Population characteristics.** For the subset of 50 women with demographic and medical information, the mean age was 34.4 ± 6.1 years, with a range of 18–43 years. The mean gestational age was 18 ± 2.6 weeks. White women comprised 44% of the sample; the remaining 56% were of unspecified ethnicity. Reasons for referral for amniocentesis included advanced maternal age (72%), positive screen for Down syndrome (14%), a positive serum-extended α-fetoprotein test (6%), abnormal ultrasound (6%), and...
chromosomal or other abnormalities (22%) (total sums to > 100% because of multiple indicators for some women).

**Laboratory analysis.** Tables 1 and 2 present the limits of detection (LODs) and percent recovery for repeat analysis of spiked amniotic fluid samples and historical results for urine from CDC method development projects. Mean recovery of spiked analytes in amniotic fluid ranged from 21% to 100% and were almost identical to historical results for urine. Mean recoveries were below 75% for only three analytes (pentachlorophenol, acephate, and methamidophos; Tables 1 and 2). Precision of repeat measurements were consistent with historical results for urine, with the average coefficient of variability (CV) equal to 8%, and only eight samples with CVs greater than 8%. LODs for the amniotic fluid measurements were also similar to historical results for urine (Tables 1 and 2). Interferences not present in ordinary urine initially reduced sensitivity of detection methods for OPs; however, adjustment of ion monitoring parameters mitigated these problems. Most analytes were stable in spiked amniotic fluid at ambient temperature and refrigeration for 12 hr, and all analytes were stable at −20°C for more than 2 weeks. Calculated concentrations were similar to spiked concentrations (Figure 1). Slopes of the amniotic fluid–spiked calibration plots were linear (Figure 2) and were similar to those of neat standards.

Table 1 presents results for the chemicals detected in amniotic fluid. Five compounds were detected in the chlorinated phenols assay. Eighteen (90%) samples had detectable levels of at least one chlorinated pesticide, with concentrations ranging from 0.10 to 4.19 µg/L. Five of 20 samples (25%) in the nonspecific OP pesticide metabolite assay had detectable levels of diethylphosphate (DEP), dimethylphosphate (DMP), or dimethylthio- phosphate (DMTP), with concentrations ranging from 0.26 to 0.43 µg/L.

No chemicals analyzed in the pyrethroid or chemical-specific OP assays were detected (see Table 2).

**Discussion**

Laboratory methods for measuring pesticides and other toxicants in urine were transferable to amniotic fluid with minor modifications. Detection limits, precision, and analytical recovery were similar to urine methods (Baker et al. 2000; Barr et al. 1999; Beeson et al. 1999; Bravo et al. 2002; Shealy et al. 1996). Metabolites derived from naphthalene, carbaryl, para-dichlorobenzene, ortho-phenylphenol, carbofuran, pentachlorophenol, and OP pesticides were detected in this small sample of amniotic fluid samples, indicating that fetal exposures to these compounds occur as early as 15–18 weeks gestation, possibly during sensitive developmental periods.

The presence of these metabolites may reflect exposures to common ingredients of consumer products. For example, naphthalene and para-dichlorobenzene are used in various moth repellents as well as in toilet and diaper pail deodorizers (Sciences International Inc. 2002). ortho-Phenylphenol is a common industrial disinfectant and is used as a postharvest food preservative on citrus fruits and vegetables (OEHHAS 2000). Carbofuran, a carbamate pesticide, and the OP metabolites derive from cholinesterase-inhibiting pesticides used in agriculture. Potential exposure sources include residues in the diet (U.S. Department of Agriculture 2000) and home or agricultural pesticide use (Bradman et al. 1997; Curl et al. 2002; Fenske et al. 2002; Lu et al. 2000; Simcox et al. 1995). Pentachlorophenol, a wood preservative that is now banned, is still widely dispersed in the environment. Both carbaryl and naphthalene are sources of the 1-naphthol metabolite. Naphthalene is also metabolized to 2-naphthol. Levels of 1- and 2-naphthol are usually correlated when they are both derived from naphthalene (Hill et al. 1995; Shealy et al. 1997). In our study, however, only one sample had detectable levels of 2-naphthol, whereas 1-naphthol was detected in 13 other samples. Because 1- and 2-naphthol levels are not correlated in this study, it is possible that carbaryl may have been an additional source of 1-naphthol (Shealy et al. 1997).

The levels of the chemicals detected in our study were lower compared with previously reported levels in meconium, urine, and blood. For example, Whyatt and Barr (2001) detected DEP and diethylthiophosphate in 95 and 100% of 20 meconium samples, respectively, with ranges of 800–5,600 µg/L, whereas diethylthiophosphate and DMP were detected in only 1 of 20 meconium samples at 1,800 and 16,000 µg/L, respectively. Hill et al. (1989) reported median levels (and detection frequencies) of pentachlorophenol and 2,5-dichlorophenol of 14 µg/L (100%) and 9 µg/L (96%), respectively, in urine samples from children 2–6 years of age living near a herbicide manufacturing plant. In a study of U.S. residents without known sources of pentachlorophenol exposure, Cline et al. (1989) reported a median level in urine of 3 µg/L and 40 µg/L in blood. Finally, for U.S. children 6–11 years of age, median levels of DEP, DMP, and DMTP were 1.4, 1.0, and 4.1 µg/L, and 1-naphthol, 2.5-dichlorophenol, and ortho-phenylphenol levels were 1.1, 9.0, and 0.49 µg/L (National Center for Environmental Health 2003).

Because of the small sample size (n = 20 for each analysis), it is not possible to generalize our findings to other populations. Pyrethroid pesticide metabolites were not detected in any samples, although they are used widely in homes (Landrigan et al. 1999). Similarly, none of the herbicides, including atrazine, alachlor, and acetachlor, were detected in the amniotic fluid samples. These chemicals may not have been detected for several reasons: a) The LODs may have been too high, b) the toxicant may not cross the maternal–fetal barrier, or c) no exposure to the mother may have occurred. It is possible that these compounds would be detected in larger volumes of amniotic fluid, in larger surveys, in women with pesticide exposure risk factors or in different geographic areas. Additionally, future studies are likely to benefit from technical improvements in laboratory analysis methods, resulting in lower detection limits.

Because of risks to the fetus, amniocentesis is conducted only when there is an elevated...
risk of congenital defects. Therefore, the population sampled will always be biased and cannot represent the total population of pregnant women. For women already undergoing the procedure, collection of the amniotic fluid for research purposes is noninvasive. Given that many women receive amniocenteses annually (>40,000 in California alone; Goldman S. Personal communication), a large source of material is safely available for testing to establish background levels of pesticides and other toxicants in amniotic fluid and to identify sources of fetal exposures. Amniotic fluid can also be collected at delivery. Thus, it may also be possible to conduct exposure and health studies that are representative of the general population. Studies using amniotic fluid collected at delivery, however, would not allow distinction between exposures early and late in gestation.

This study is a first step toward validating measurements of nonpersistent pesticides in amniotic fluid as an exposure biomarker. Next steps should include larger surveys that include women from diverse geographic areas and who have potential pesticide exposure risk factors to define which chemicals are present in this medium. Additional studies that measure nonpersistent pesticides in amniotic fluid and concurrently sampled maternal blood and urine samples would also provide data on the interrelationships of maternal and fetal exposures and aid in the development of physiologically based pharmacokinetic (PBPK) models for pregnant women and fetuses. Several researchers are currently developing PBPK models describing toxicant dynamics during pregnancy (Luecke et al. 1994, 1997a, 1997b; Young 1998; Young et al. 1997), which may become valuable tools for estimating fetal exposures for risk assessment or epidemiologic studies investigating prenatal exposures where only maternal biologic samples are available. Given the initial promising findings of this study, additional research is needed to better characterize contaminants in amniotic fluid and the potential health risks to the fetus and developing child.

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