Identification of Phthalate Esters in the Serum of Young Puerto Rican Girls with Premature Breast Development

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Premature breast development (thelarche) is the growth of mammary tissue in girls younger than 8 years of age without other manifestations of puberty. Puerto Rico has the highest known incidence of premature thelarche ever reported. In the last two decades since this serious public health anomaly has been observed, no explanation for this phenomenon has been found. Some organic pollutants, including pesticides and some plasticizers, can disrupt normal sexual development in wildlife, and many of these have been widely used in Puerto Rico. This investigation was designed to identify pollutants in the serum of Puerto Rican girls with premature thelarche. A method for blood serum analysis was optimized and validated using pesticides and phthalate esters as model compounds of endocrine-disrupting chemicals. Recovery was > 80% for all compounds. We performed final detection by gas chromatography/mass spectrometry. We analyzed 41 serum samples from thelarche patients and 35 control samples. No pesticides or their metabolite residues were detected in the serum of the study or control subjects. Significantly high levels of phthalates [dimethyl, diethyl, dibutyl, and di-(2-ethylhexyl)] and its major metabolite mono-(2-ethylhexyl) phthalate were identified in 28 (68%) samples from thelarche patients. Of the control samples analyzed, only one showed significant levels of di-isooctyl phthalate. The phthalates that we identified have been classified as endocrine disruptors. This study suggests a possible association between plasticizers with known estrogenic and antiandrogenic activity and the cause of premature breast development in a human female population. *Key words*: endocrine-disrupting chemicals, phthalate esters, premature thelarche. *Environ Health Perspect* 108:895–900 (2000). [Online 8 August 2000]
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In humans, the onset of puberty is primarily assessed by the physical changes that occur in the genitalia of both sexes. In females, the physical signs associated with the onset of puberty are enlargement of the breasts, ovaries, and uterus, as well as the growth of pubic and axillary hair with apocrine secretion. The process by which puberty occurs is primarily regulated by the endocrine system through its chemical messengers, specifically the sexual hormones (*1*). The onset of pubertal changes at an earlier age than expected may occur secondary to a varied group of disorders. When the cause of premature sexual development is unknown, the condition is considered idiopathic. Premature sexual development in the human female is presently defined as the appearance of any physical change characteristic of puberty with onset before 8 years of age (*2*).

Hyperplasia of breast tissue in neonates is a normal physical finding that may be present during the first 6 months of life as a result of placental passage of maternal estrogenic hormones. When this condition persists beyond this period or occurs before 8 years of age in females, it is known as premature or precocious thelarche (Figure 1). The majority of cases occur before 2 years of age.

Girls diagnosed with premature thelarche have one or both breasts enlarged without any other physical signs of the onset of puberty (*3*).

Since 1979, pediatric endocrinologists in Puerto Rico have detected an alarming increase in the number of patients with premature thelarche (*4,5*). Among the hypotheses proposed to explain the observed premature sexual development in this U.S. Caribbean Island territory, the most controversial theory associated thelarche with the subject's diet. Sáenz et al. (*6*) suggested that dairy and meat products were contaminated with anabolic estrogenic chemicals, which are used for increasing muscle mass in cattle and poultry. In 1985, studies conducted by the U.S. Department of Agriculture in conjunction with a scientific commission from the Puerto Rico Department of Health led to the conclusion that no abnormal levels of the suspected chemicals were present in the approximately 800 samples of meat and dairy products that were analyzed (*7*). Other theories are still under consideration, such as the association with ovarian cysts, premature endogenous production of sexual hormones, and environmental contamination by pharmaceutical waste products. These theories do not establish a strong association with the majority of the cases reported (*8*). Also, a genetic predisposition of Puerto Rican girls for developing premature thelarche is unlikely. Investigation among this ethnic group in the Philadelphia, Pennsylvania, area did not reveal a similar pattern of early sexual development (*8*). Moreover, other ethnic groups living in Puerto Rico are also affected by the condition (*8*).

In 1987, the Puerto Rico Department of Health created by law the Premature Thelarche and Early Sexual Development (PTESD) Registry in response to the observed increase in cases (*9*). This is the only world registry created for the study of premature sexual development in a human population. The objectives of this epidemiologic surveillance system are to define the epidemiologic, clinical, and etiologic aspects of the different manifestations of premature sexual development on the island. Although the registry was established in 1988, retrospective data to 1969 and prospective data to 1998 have been collected. In this time period, 6,580 cases of premature sexual development have been registered, of which 4,674 (71%) are premature thelarche cases. Based on the data accumulated by the registry, the estimated annual average incidence rate of premature thelarche in Puerto Rican girls 6–24 months of age is 8 cases per 1,000 live female births from 1984 to 1993 (*10*). This incidence is, to our knowledge, the highest ever reported. Compared to a study conducted in Minnesota (*11*), the incidence of premature thelarche in the Puerto Rican female population is 18.5 times higher.

In the last decade, there has been a growing interest and concern for the study of the impact of man-made chemicals on wildlife and humans. These studies have suggested that synthetic and naturally occurring substances in the environment may affect the normal function of the endocrine system. These substances are also referred to as endocrine-disrupting chemicals (EDCs). In wildlife, alterations in sexual reproductive behavior have been observed in areas of contamination with EDCs. For example, malformations in the sexual organs of alligators have been reported in Lake Apopka, Florida, where high concentrations of DDT and its degradation products have been detected (12,13), and feminization of trout in the Great Lakes has been associated with the high levels of polychlorinated biphenyls (PCBs) in water samples (14). Other studies have indicated that many chemicals, including phthalate esters, may affect development and reproduction, including germ cells, sperm motility, chryptorchidism, and hypospadias, in laboratory animals (15–19). The specific mechanisms by which these chemicals may affect human health are unknown. Extrapolations to humans of the effect of these substances on wildlife are difficult. A limited number of reports (20–22) in the scientific literature describe the accidental exposure of humans to chemicals such as lindane and other organochlorinated pesticides, dioxins, and PCBs with known endocrine-disrupting properties. The study of these exposures has led to the conclusion that these compounds can alter the female-to-male ratio in offspring (20) and cause learning disabilities, behavioral problems, suppression of the immune system, and gynecomastia in the exposed subjects (21–22).

At present, the cause of the observed high incidence of premature sexual development in Puerto Rico is unknown, and the long-term consequences of the aberrant premature sexual development in this population are also unknown. Many of the chemicals that are classified as EDCs have been imported or

Figure 1. Twenty-three-month-old Puerto Rican girl with premature breast development (thelarche).

produced in high quantities in Puerto Rico. Until 1988, a total of 450 million pounds of chlorinated pesticides were imported to the island (23). Although many of these pesticides are currently banned for use in U.S. territories, these pesticides are known to bioaccumulate and to have a long persistence in the environment. Also, many of these substances such as phthalate esters, alkyl phenols, and surfactants are present in commercial products commonly used for packaging, storing, and preserving food (24). The environmental load of plasticizers in Puerto Rico is unknown, but it is assumed to be significant because of the high level of consumption of dietary products in plastic containers imported to the island. Based on the high exposure to these substances in the general population in Puerto Rico and the fact that exposure of human fetuses, newborns, and young girls to exogenous estrogenic chemicals may lead to adverse effects in their sexual development, we designed this study to search for known EDCs in the serum of Puerto Rican girls with premature thelarche. We focused specifically on girls with premature thelarche because this condition represents the majority (71%) of the total cases of premature sexual development reported on the island.

Methods

This research protocol was approved by the San Juan City Hospital's Institutional Review Board before the initiation of the study.

Case and control subjects. Study subjects were females from 6 months to 8 years of age (mean age 31 months; median age 20 months), all diagnosed with premature thelarche. We analyzed all samples from thelarche patients taken from January 1994 to April 1998. Control subjects were females from 6 months to 10 years of age (mean age 70 months; median age 46 months). We obtained control serum samples from the San Juan City Hospital general clinical laboratory. Control individuals did not have evidence of premature sexual development or any other endocrine disease (they were seen in the institution for general pediatric care).

Serum samples were provided by the Pediatric Endocrinology Section of the San Juan City Hospital. This hospital is the only municipal tertiary care public health institution for the city of San Juan and primarily serves an indigent population, including the subjects and controls for this study (25).

We used a number coding system for the handling of samples during the analysis. To assure confidentiality, subject's names and clinical data were not available to the University of Puerto Rico's Environmental Analytical Chemistry Laboratory personnel during the study.

Moni-Trol samples. Moni-Trol (Dade, EF Baragaño, Puerto Rico), a lyophilized product derived from human serum, is available commercially for optimization and calibration of instrumentation in clinical laboratories. We reconstituted the lyophilized samples as needed with 5.00 mL of a carbonate diluent supplied by the manufacturer. Samples were stored at 4°C and protected from light.

Serum samples. Whole blood samples were collected by venipuncture into glass tubes with no chemical additives. After collection, the samples were allowed to coagulate and were then centrifuged. Serum samples were stored at –23°C in glass tubes and protected from light until analysis.

Optimization of the extraction procedure. Moni-Trol samples were prepared, and 1 mL samples were spiked with standard solutions of diethyl phthalate (DEP) and dibutyl phthalate (DBP) as model compounds of the phthalate ester family. The spiked concentrations were between 50 ppb and 25 ppm. The samples were stored at 4°C protected from light for 18 hr. After reaching room temperature, the samples were extracted with hexane/dichloromethane (8:1). The extracts were concentrated to 1.0 mL with a flow of nitrogen and 1 μL was injected into the gas chromatography/mass spectrometry (GC/MS) system (HP 5890/5971; Hewlett Packard, Wilmington, DE). The samples were analyzed by selected ion monitoring (SIM) using a SPB-1 column (100% polydimethyl siloxane; Supelco, Bellefonte, PA). Samples were heated to an initial temperature of 70°C for 4 min and then heated to 250°C at 10°C/min. We compared the relative area of the peaks for each standard to the area of standards in a calibration curve generated under the same conditions and calculated the percent of recovery for each concentration. All methodology was tested using serum samples as well. Serum samples were spiked with the model compounds in concentrations ranging from 20 to 80 ppb.

Extraction of organic compounds from serum samples. Serum samples were transported to the analytical laboratory in glass tubes stored at 0°C. Precipitation of serum and plasma proteins was accomplished by adding 1 mL acetonitrile to 1.0 mL sample. Five milliliters of the extraction mixture, which consisted of an 8:1 solution of hexane and dichloromethane, was added to each sample. Samples were then submitted to ultrasound extraction for 5 min in a sonicator bath (Fisher Scientific, Cayey, Puerto Rico). The phases were allowed to separate, and the extract was transferred to a centrifuge tube. The extraction procedure was performed twice and the extracts were combined.

Concentration and analysis. The combined extracts were concentrated to 0.5 mL with a

flow of nitrogen. The concentrated extract (1 μL) was injected into the GC/MS system. The samples were heated to an initial temperature of 70°C for 4 min and then raised to 130°C at 5°C/min and then to 250°C at a rate of 10°C/min.

Special precautions. We applied essential quality control standards to avoid incorrect interpretation of results. This is particularly important when the expected concentrations of the analytes occur at trace levels. Phthalate esters have been detected as interferences in many chromatographic analyses (26,27). We analyzed adequate sets of analytical blanks before sample analysis; these included system and solvent blanks as well as blanks for the sampling device, pipettes, and storage tubes.

Results

Optimization of the extraction and analysis procedures. For the optimization of the extraction procedure, Moni-Trol samples spiked with DEP and DBP (concentrations ranging from 50 ppb to 25 ppm) were extracted and analyzed by GC/MS. We compared the relative area for each compound in the extract to the relative area for standards in a calibration curve. The calibration curves had R^2 values > 0.99 for the model phthalates. We calculated the amount extracted (in parts per million) for each concentration and generated recovery curves for each compound by plotting the amount extracted as a function of the

amount of standard added to the sample. The slope of the regression line gives the average percent of recovery for each compound for the concentration range. In Moni-Trol samples, the average percent recovery (± SD) for DEP was 79.9 ± 0.4%, whereas the average percent recovery for DBP was 88.2 ± 0.2%. The method for extraction and analysis showed excellent linear correlation (R^2 values > 0.99) for the concentration range tested.

When we tested the optimized method using real blood serum instead of Moni-Trol for the sample preparation and recovery curves, we used concentrations ranging from 20 to 80 ppb; the recovery curves are presented in Figure 2. Samples were analyzed by GC/MS in the SIM mode. As calculated from the recovery curves, the average percent of recovery (± SD) was 118 ± 13% for DBP and 88 ± 3% for DEP.

Serum samples from thelarche patients and control samples. We analyzed 41 samples from patients diagnosed with premature thelarche at the San Juan City Hospital's Pediatric Endocrinology Division and included in the Premature Thelarche and Early Sexual Development Registry of the Puerto Rico Department of Health. We used a GC/MS instrument operating in the scan mode to analyze the samples. Figure 3 shows a representative total ion chromatogram for serum samples from study subjects. Figure 4 shows the extracted ion chromatogram for

m/z = 149 for the same chromatogram. The m/z = 149 is one of the characteristic ions for phthalate ester detection. As confirmed by mass spectral data, four of the peaks in the extracted ion chromatogram correspond to compounds of the phthalate ester family. Phthalate esters were consistently detected at significant concentration levels (ranging from tens of parts per billion to units of parts per million) in 28 of 41 (68%) serum samples obtained from the thelarche patients. The concentration of phthalate esters was

Table 1. Average concentrations (μg/L) of phthalate esters in thelarche patient samples analyzed by GC/MS in the SCAN mode.

Phthalate ester	Sample ID no.	Age (months)	Conc (μg/L)
DBP	1	19	115
	2	19	134
	3	17	182
	4	47	276
	5	20	91
	6	37	43
	7	24	79
	8	20	57
	9	36	252
	10	20	120
	11	26	125
	35	32	38
	39	21	15
DEP	2	19	8.0
	3	17	37
	4	47	19
	5	20	12
	6	37	22
Benzyl butyl phthalate (BBP)	5	20	117
	6	37	54
di-n-Octyl phthalate (DOP)	15	36	438
di(2-Ethylhexyl) phthalate (DEHP)	3	17	1,809
	4	47	2,098
	5	20	565
	6	37	578
	7	24	1,602
	8	20	1,447
	9	36	935
	10	20	907
	11	26	607
	12	10	681
	13	83	855
	25	78	633
	26	12	721
	27	29	392
	28	72	326
	29	15	444
	30	21	470
	31	18	532
	32	16	417
	33	36	468
	34	32	353
	35	19	246
	37	20	454
	38	16	187
	39	16	349
Mono-(2-ethylhexyl) phthalate (MEHP)	2	19	18
	3	17	38
	4	47	33
	5	20	11
	6	37	6.3

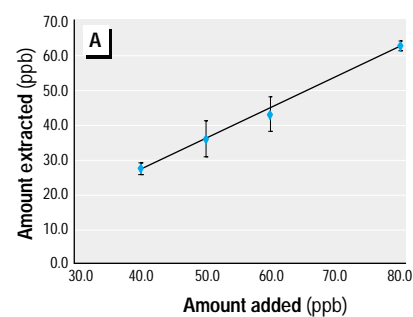Abbreviations: conc, concentration; ID, identification.

Figure 2. Recovery curve for real serum samples spiked with (A) DEP ($y = 0.8334x − 8.2971$; $R^2 = 0.9939$) and (B) dibutyl phthalate ($y = 1.18x − 17$; $R^2 = 0.9504$).
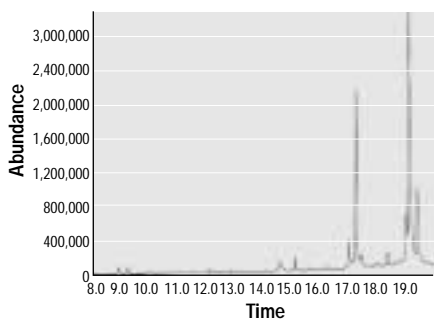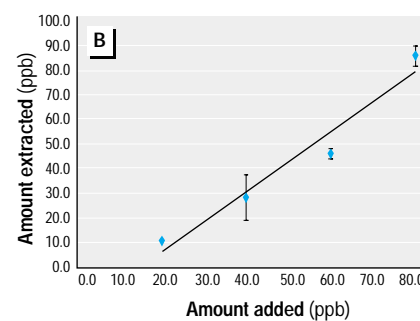

Figure 3. Total ion chromatogram representative of those obtained for samples from thelarche patients.
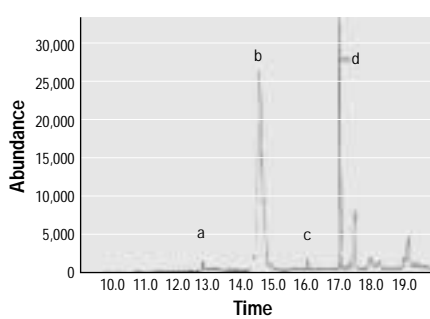

Figure 4. Extracted ion chromatogram for the total ion chromatogram in Figure 2. The peaks identified as a, b, c, and d correspond to compounds of the phthalate ester family.

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	26	12	721
	27	29	392
	28	72	326
	29	15	444
	30	21	470
	31	18	532
	32	16	417
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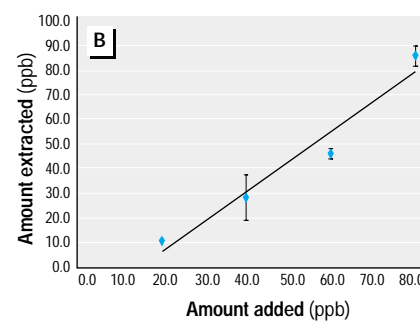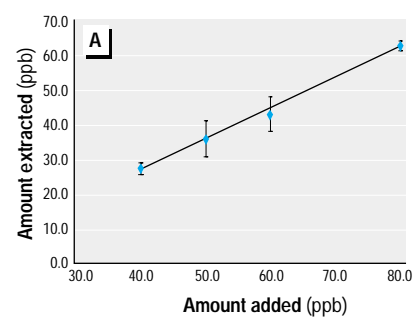Abbreviations: conc, concentration; ID, identification.

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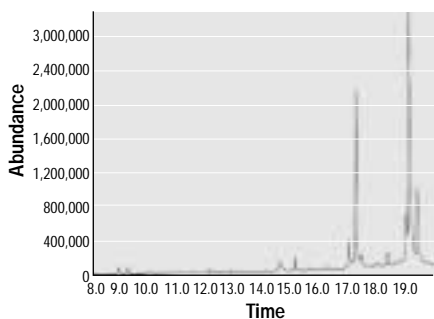

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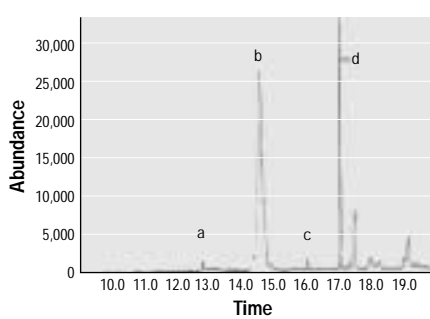

Figure 4. Extracted ion chromatogram for the total ion chromatogram in Figure 2. The peaks identified as a, b, c, and d correspond to compounds of the phthalate ester family.

Table 4. Average concentrations (μg/L) of phthalate esters detected in blanks.

Phthalate ester	Sampling butterfly (μg/L)	Distilled water (μg/L)	Extraction solvent (μg/L)	Glass tube (μg/L)	Plastic tube (μg/L)
DBP	ND	263	62	457	870
Benzyl butyl phthalate (BBP)	ND	227	ND	149	ND
DEP	ND	ND	67	96	183
bis 1-Methylethyl phthalate	ND	ND	ND	ND	38

ND, not detected (concentration below 10 μg/L).

metabolite residues in samples. This optimized method permits the use of the SIM modality when lower detection limits are required. This methodology may allow the development of other analytical research protocols that could result in enhanced detection limits, thus offering investigators the possibility of assessing levels of phthalate esters and other known pollutants in blood samples from the general population.

We detected high levels of phthalate esters in 68% of the samples from thelarche patients. DEP, DBP, and DEHP were detected in levels ranging from tens of parts per billion (nanograms per milliter) to units of parts per million (milligrams per milliliter) in case samples. MEHP, one of the major metabolites of DEHP, was detected in five case samples (Table 1). The presence of this phthalate ester was not caused by sample contamination, because contamination by a metabolite rarely occurs. We detected DEHP in 14% of the control samples. The concentration of this phthalate in control samples was significantly lower than the levels in study samples (Table 2).

Many of the phthalate esters detected in study samples have been shown to be estrogenic when assayed by the recombinant yeast screen test (*32*). Although phthalates have low estrogenic activity compared to estradiol and other potent estrogenic substances (*33*), these chemicals may cause endocrine disruption through several mechanisms. Chemicals with weak estrogenic activity may cause disruption in biologic systems if they act at critical periods of development (*34*). The intrauterine period of human embrionic differentiation and development is particularly sensitive to weak estrogens. Besides their estrogenic activity, phthalates such as DBP have blocking antiandrogen action (*35*). If these properties of phthalate esters play a role in the cause of premature breast development in Puerto Rican females, further research should address this possibility.

The detection of high levels of phthalate esters in this group of Puerto Rican girls could reflect an elevation in the exposure of the general Puerto Rican population to these substances. Further research will address this issue. The total daily consumption of DEHP from all sources of exposure has been estimated at 5.8 mg in the United States and 2.1 mg in Japan (*36*). The most important sources of exposure for children are ingestion of contaminated formulas, food, and water from contact with plastic wrappings and containers and mouthing of plastic toys and pacifiers (*36–38*). There is mounting concern for exposure to phthalate esters (specifically DEHP), especially through polyvinyl chloride plastic medical equipment.

The present study provides the first analytical evidence of the presence of plastic additives with known estrogenic activity in girls with premature thelarche. In a study conducted in 1997 that included 17,077 girls, Herman-Giddens et al. (*39*) reported that girls in the United States are developing pubertal characteristics at younger ages than previously reported. These authors concluded that

the possibility that the increasing use of certain plastics and insecticides that degrade into substances that have estrogen-related physiological effects on living things should be investigated in relation to the earliest onset of puberty.

The findings of this study cannot be interpreted as the cause of premature thelarche in Puerto Rican girls at present. It may well be that the etiology of the various manifestations of premature sexual development (including thelarche) on this island is multifactorial. Further research should be performed to clarify if phthalate esters by themselves, or in association with other endogenous or exogenous estrogenic compounds, are capable of inducing precocious sexual development in animals and humans. Other possible environmental cofactors related to exposure should also be considered, especially those unique to the Puerto Rican environment. The following have already been associated with premature sexual development in Puerto Rico: the presence of anabolic steroids in poultry (*6*) and consumption of soy-based formula with a high phytoestrogen content by Puerto Rican infants (*8*). The higher exposure to phthalate esters in the Puerto Rican infant population is supported by the high importation of plastic packaged foods and because Puerto Rico is a tropical island with year-round high temperature and humidity, which promotes closed environments with the frequent use of air conditioners in homes and public buildings (*36*).

The issue of endocrine disruptors causing adverse health effects to humans and wildlife is not free of controversy (*40,41*).

Other studies should address this issue, particularly that of critical stage susceptibility to endocrine-disrupting chemicals and alterations in sexual development of humans and other animal species. If the hypothesis holds true, premature sexual development in Puerto Rico may prove to be an unfortunate example of the impact of endocrine-disrupting environmental chemicals at a critical stage of human development.

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