Toxicology Reports 8 (2021) 1040–1045

Contents lists available at ScienceDirect

Toxicology Reports

journal homepage: www.elsevier.com/locate/toxrep

Phthalates exposure during pregnancy a study in a Mexican cohort

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ARTICLE INFO

Edited by Dr. A.M Tsatsaka

Keywords:
Mono-ethyl phthalate (MEP)
Mono-ethyl-hexyl phthalate (MEHP)
Mono-butyl phthalate (MBP)
Exposure assessment
Pregnancy

ABSTRACT

A prospective cohort study was conducted to measure the concentration levels of three primary phthalate metabolites (MBP, MEHP, MEP) during pregnancy in a group of women from the State of Mexico. The urinary concentration levels of the three phthalate primary metabolites were measured by gas chromatography mass spectrometry during the first, second and third trimesters of pregnancy. The geometric mean and 95 % CI for MBP was 20.38 μg/mL (15.35–27.09); for MEHP 13.43 μg/mL (8.93–20.20), and MEP 52.47 μg/mL (39.88–69.04) adjusted to one g of creatinine. No significant trends were observed among the studied metabolites during the pregnancy period. MBP was higher in less educated women, while women who resided in industrialized zones showed higher levels of MEHP and MEP than women from non-industrialized zones. Consumption of plastic bottled beverages was associated with MBP and MEHP phthalate exposure. Women who used non-registered brands of plastic food containers for storage or for microwave oven use showed the highest levels of MBP and MEP phthalates. The pregnant women in our study were exposed to the three studied primary phthalate metabolites, and this could present a risk to their newborns. To better integrate public health policies, major exploration of potential exposure sources and effects at the regional level is required.

1. Introduction

Phthalates are a group of chemicals distributed ubiquitously in the environment. They are additives commonly used to convert hard polyvinyl chloride (PVC) resins into flexible and workable plastics employed in the fabrication of multiple products such as food containers and medical equipment. High levels of mono-ethyl phthalate (MEP) and mono-ethylhexyl phthalate (MEHP) have been found in foods such as, peanut butter, lard, dairy products and beverages from plastic bottles [1], also in hygiene and beauty products such as, deodorants, shampoos, creams, perfumes, lotions, liquid and bar soaps, conditioners, and makeups [2]. Phthalates are also used in paper manufacture, photographic films, wires and adhesives. The ubiquity and characteristics of these substances allow contamination of food, water, air and soil [3]. It is estimated that two million tons of phthalates are produced annually in the world. 95 % of the production is used for plasticization of PVC. The di-2 ethylhexyl phthalate (DEHP) is the most commonly used [4].

Phthalates are reproductive toxic for male animals. Their adverse biologic effects are caused by their metabolites [5,6]. Animal experiments have shown that phthalates can produce endocrine disruption, not involving androgen receptors [7]. DEHP and di (n-butyl) phthalate (DBP) decrease testosterone levels in male fetus that can be manifested as malformations in the external genitalia, degeneration of the seminiferous tubules, prostate and small seminal vesicles and reduction of the anogenital distance. Intrauterine growth retardation and embryonic malformations were observed in chickens and mice produced by the ingestion of high concentrations of this metabolite [8,9]. DEHP exposure in utero may have adverse effects on both Sertoli and Leydig cell

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https://doi.org/10.1016/j.toxrep.2021.05.006
Received 21 July 2020; Received in revised form 3 May 2021; Accepted 10 May 2021
Available online 12 May 2021

development in males [10]. Human studies suggest that phthalate exposure can be associated with semen parameters, DNA damage and endocrine disruption [11,12]. Other studies have shown that phthalate exposure can shorten pregnancy duration [13], the ano-genital distance of infants [14,15], anemia [16], hypertension during pregnancy [17] and spontaneous abortions [18].

Exposure to pesticides and phthalates in pregnant women have caused lower placental weight, low growth and low fetal weight [19]. Exposure to chemical endocrine disruptors such as phthalates and bisphenol A through complex pathways can alter the development of the fetus, as well as contribute to the development of obesity in children and adults. Exposure to endocrine disruptors can also alter lipid metabolism and adipogenesis through binding to the PPAR-receptor, and thus promoting weight gain, insulin resistance and inhibiting liponectin [20].

The first report of phthalate exposure in general population was published in 2000 by Blount 2000a [21], followed by the second report of the National Health and Nutrition Examination Survey (NHANES) [22]. Since then several biomonitoring studies have been performed in pregnant women, newborns, children and adults using different biological matrices such as: urine, amniotic liquid, milk, blood, hair and meconium. For the above it is well documented that phthalates exposure is a public health problem. Fortunately; in some countries, strict regulation applied for phthalates use and the emergence of new alternatives plasticizers may decrease the human exposure in the future [23,24].

Phthalates are chemicals worldwide used found in daily used products and industry. Their metabolites produce a wide spectrum of public health problems. In Mexico, there is not research regarding to this topic. The purpose of this study was to measure phthalate exposure during pregnancy in a group of Mexican women who gave birth male children to evaluate in this population the phthalates effect on malformations in the external genitalia and reduction of the anogenital distance.

2. Materials and methods

2.1. Population and study design

Pregnant women were recruited during their first pre-natal visit, between the seventh and thirteenth week of pregnancy, in the community and hospitals in the metropolitan area of Toluca, a city located at west of Mexico City. Women were visited at their homes every three months until they gave birth. At each visit, a urine sample was collected to measure phthalate exposure.

To be included in the study, pregnant women must reside in the Toluca area at least two years. An informed consent was obtained, a basic questionnaire was applied to obtain sociodemographic data, reproductive and occupational history, eating habits especially of milk products and habitual patterns of plastic products used. At the second and third trimester, a questionnaire was obtained to update any changes such as, address, activities, state of pregnancy, or refusal to continue participating in the study.

Original cohort of 220 women was studied. From this cohort, only women who gave birth to male children were selected since the main study contemplated the evaluation of the effects on male infants. All subjects gave their informed consent for inclusion before they participated in the study. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of the Instituto Nacional de Salud Pública (INSP) under the number CI-056.

2.2. Exposure assessment

First urine sample (approximately 250 mL) was recollected in dark glass flasks, with metallic covers and Teflon coupling, pre-washed with acetone and dichloromethane and analyzed to discard contamination. Samples were preserved in an ultra-freezer at -20 °C until processing.

2.3. Analytical method for the analysis of the urine samples

Phthalates quantification was performed by Gas Chromatography-Mass Spectrometry (CG/MS) using an equipment brand VARIAN model 3600, model Saturn II, with a Restek capillary column RTX-5MS phase of 30 m x 0.25 DI and 0.1 film thickness with the program: initial temperature 60 °C for 3 min up to 300 °C with a ramp of 10 °C/minute and at 300 °C for 10 min. Splitless injector at 280 °C, transfer line at 280 °C, Helium UAP gas flow of 1 mL/minute and mass detector reading in the electronic impact mode with intervals from 50 to 400 uma. Sample was an injection volume of 2 μL.

To test the accuracy the following parameters were determined: linearity, detection limit, quantification limit, reproducibility and recovery percentage. Standard curves were made using a mixture of phthalate metabolites such as: mono ethyl phthalate (MEP), mono butyl phthalate (MBP) and mono-ethylhexyl phthalate (MEHP) at final concentrations of 3, 5, 10, 20, 30 and 50 μg/mL. Reproducibility was determined analyzing by triplicate, by two analysts at two different days each other the standard solution of 20 μg/mL. Recovery percentage was calculated adding the same concentration to a solution of deionized water, phosphate buffer pH 6.8 (optimum pH for glucoronidase *E coli*) and sodium chloride 0.1 %, then its extraction and analysis was performed by GC/MS.

2.4. Metabolites extraction from urinary samples

Phthalate metabolites are excreted as glucorinated or sulfates conjugates [25], thus, they must be released adding 50 μL of a solution of β-glucoronidase from de E coli (12 U/mL) to urine samples previously adjusted to pH 6.8 with 0.2 M (±10 mL) phosphate buffer, for optimal enzymatic activity in a volumetric flask, this enzyme selectively hydrolyzes glucorinated metabolites to be analyzed in their free form [21]. Samples were incubated at 40 °C for twelve hours, at the end of the time pH was adjusted to 2 using concentrated HCl, subsequently 0.1 g of NaCl and 1 mL of HPLC grade methanol were added. Then metabolites extraction was carried out with three portions of dichloromethane. Organic phase was passed through an anhydrous sodium sulfate bed and evaporated at room temperature to final volume of 0.5 mL. The extracted metabolites were derivate using clorometil silane and the volume was adjusted to 0.5 mL with methanol. The extracts were injected into the mass gas chromatograph and quantified with the respective calibration curves. The results were reported as μg/mL adjusted to one g of creatinine [15].

2.5. Derivation of metabolites

The derivatization of acid metabolites (mono alkyl phthalates) to methyl alkyl phthalate (their more volatile equivalents) was achieved adding 2.0 mL of chlorotrimethylsilane 10 % in methanol to the extract urinary metabolites [26–28], then the samples were concentrate to 0.5 mL. Finally, extracts were evaporated to dryness at room temperature in an extraction hood and resuspended in 100 mL of methanol and transferred to an amber vial for storage at -20 °C until their CG/MS analysis [15].

2.6. Accuracy determination

First, creatinine was determined in urine samples using the RANDOX brand kit as recommended by Adibi 2008 [29]. Then, method linearity was estimated using a standard curve for each metabolite. Linearity was considered as optimum if variation coefficient (r^2) was greater than 0.98 which meets the recommended acceptance criteria by García 2002 [30]. Detection (LD) and quantification (LC) limits were determined using the formulas by García 2002.

$$LD = \frac{3.3xS_b}{b_1} \quad LC = \frac{10xS_b}{b_1}$$

Where:

S_b = standard deviation of the blanks

b_1 = slope of the standard curve of each metabolite.

Reproducibility was calculated per analyst and per day with. It was considered acceptable if variation coefficient was less than 2% for each metabolite. Recovery percentage (%R) for each metabolite was determined using the following formula:

$$\%R = \frac{Recovered\ concentration}{initial\ concentration} x\ 100$$

2.7. Statistical analyses

Statistical analyses included the phthalate metabolite concentrations by trimester of pregnancy, geometric means and 95 % CI since they did not follow a normal distribution (skewed to the right).

To evaluate potential exposure sources of phthalates, we compared the medians of the phthalates urine levels by age, education, occupation, place of residence, variables related to the use of plastic products in the conservation and preparation of food, use of special recipients to store or heat food, registered brand products where the producer specifies their micro-wave usage, use of micro-wave ovens, tobacco use, use of medication during pregnancy, common use of make-up, and consumption of milk products by week.

To estimate the lack of independence of phthalates levels, regression models (GEE) for longitudinal data were used [31]. The models included variables that in the bi-variate analysis were statistically significant or that could be important according to theoretical framework. The dependent variable (concentrations of each metabolite) was log transformed. All analyses were performed with the statistical package Stata 8 [32].

3. Results

Initial cohort was of 220 pregnant women, 8.2 % had spontaneous abortions, and 7.7 % were lost to follow-up due to change of address, and refusal to participate in the study. A total of 177 infants were born, 100 females and 77 males. Seventy-seven pregnant women were included in the analyses. The average age of the women was 24 years old, with a SD of 4.7 years. Recovery percentage (%R) for all metabolites was in a range of 97.98–98.80% with a CV $\leq$ 2%.

Standard curves for each phthalate metabolites tested, detection (LD) and quantification (LC) limits were as follow: mono-ethyl phthalate (MEP) $y = 1116.4x + 315.83$; $r^2 = 0.9944$; LD = 0.1 μg/mL; LC = 0.4 μg/mL. Mono-butyl phthalate (MBP) $y = 8875.9x - 17367$; $r^2 = 0.9902$; LD = 0.01 μg/mL; LC = 0.03 μg/mL. Mono-ethyl-hexyl phthalate (MEHP) $y = 544.0x - 21109$; $r^2 = 0.9893$; LD = 0.07 μg/mL; LC = 0.2 μg/mL. Where y = area under the curve obtained from of chromatogram; x = metabolite concentration in μg/mL and r2 = coefficient of determination.

Table 1 shows the ions used to quantify and the identify metabolites and phthalates in the urine samples.

Table 1
Ions used to quantify and the identify metabolites and phthalates in the urine samples.

Metabolite	Ion quantification m/z	Ion of identification m/z
MEP	149	163, 176
MBP	149	163, 184, 77
MEHP	149	163, 181
MBzP	149	163, 91, 77
MOP	149	163, 181, 77
DEHP	149	181

Table 2 presents the concentrations of primary metabolites during pregnancy. In general, the levels found were higher for MEP, followed by MBP and MEHP phthalate. Concentrations remained similar during the entire pregnancy period, without any statistical differences in the medians.

Table 3 presents sociodemographic, occupational and source of exposure characteristics of the pregnant woman by their metabolite median exposure concentration levels. Women $\geq$ 25 years old and older presented the highest concentration levels of MEP. Less educated women (elementary school or less) showed the highest concentration levels of all the studied metabolites. Women who lived in industrialized zones presented highest level of MEHP compared to those living in non-industrialized zones. Higher concentration levels of MBP and MEHP were observed in women who consumed plastic bottled beverages. Women who used non-registered brand plastic recipients to store or heat food in the micro-wave oven presented higher levels of MEP and of MBP. No statistically significant difference was observed in the metabolite levels associated with medication during pregnancy, habitual use of cosmetics, tobacco use or consumption of milk products during the week before the interview (data not reported in Table 3); p values which show significant differences are denoted by the symbol †.

Table 4 shows longitudinal analysis of association between studied variables and concentration of phthalate metabolites that are expressed in logarithm scale. MBP was positively associated with consumption of bottled beverages and negatively with the use of registered brand special plastic recipients for food storage. MEHP reduction was associated to residential status (urban versus industrialized) and to consumption of bottled beverages; however, the coefficients were not statistically significant. MEP was positively associated with the occupation and use of microwave oven, but negatively associated with residential status.

4. Discussion

The findings in our study are relevant to show phthalates exposure during perinatal period and potential exposure sources as shown in previous studies. There is a paucity of research on human exposure to phthalates in pregnant women. Our findings in urinary phthalate levels were similar in all pregnancy period, they could be important in future studies. Although repeated measurements during pregnancy can reduce the variance of estimations, measuring urine samples at the beginning or at the end of the pregnancy could be enough to assess exposure during the pregnancy period [14,33,34].

Urine phthalate levels in our study were lower than those reported in adults (Blount 2000a; 2000b), and pregnant women in New York and Poland [35] (Table 5), and lower than those found in infants born in Toluca City hospitals [36]. However, comparison of these populations is difficult due to potential differences in the metabolism between children and adults. The sources of hospital exposures require a separate analysis.

The levels of urinary metabolites in this study were variable compared to other studies (Table 5). It may be due to the great variability in terms of the number of people involved in the studies and different effects that could modify excretion levels, such as age, socioeconomic status, occupational exposure, smoking and others [37].

Our study agrees with the results obtained by Serrano 2014 [1] where MEP was the most excreted; the samples were taken every trimester, with non-significant differences. It may be because pregnant women tend not to change their lifestyle during the pregnancy period, thus, phthalates exposure could remain constant.

Daily usage of hygiene and beauty products with the highest percentage of phthalates used by the pregnant women in our study were deodorants 91 %, shampoos and creams 80 %, perfumes or lotions 70 %, liquid soaps 68 %, bar soaps 65 %, cream or conditioners 65 %, makeup 54 %. MEP was the most frequent phthalate metabolite found in pregnant women in our study. Other studies found DEHP as the most frequent phthalate associated with the use of makeup products [2]. This contrast could be due to differences in phthalates concentrations in the

Table 2
Primary phthalate metabolite percentile levels by trimester of pregnancy in a cohort of pregnant women from the state of Mexico.

Phthalate Metabolites*	Trimester	Percentiles			P**	General Geometric Mean	95 % CI
		5th	50th	95th			
MBP*	1	2.80	10.86	3,096.50		20.38	15.35, 27.09
	2	2.32	10.00	4,018.00			
	3	2.00	15.15	9,145.20	0.14		
MEHP*	1	0.42	3.49	2020.26		13.43	8.93, 20.20
	2	0.30	3.48	1563.18			
	3	0.30	3.49	5,275.51	0.6		
MEP*	1	4.20	30.61	5.515.96		52.47	39.88, 69.04
	2	3.48	39.47	2,103.10			
	3	3.48	34.88	6,122.59	0.9		

* MBP = Mono-Butyl phthalate, MEHP = Mono-Ethyl-Hexyl phthalate, MEP = Mono-Ethyl phthalate, μg/g of creatinine.
** Kruskal Wallis test for median differences.

Table 3
Median phthalate exposure levels in a cohort of pregnant women from the State of Mexico by their sociodemographic, occupational and sources of exposure.

Characteristics	(N)	MBP*	P**	MEHP*	P**	MEP*	P**
Age (years)							
≤20	(17)	10.0		2.94		34.88	
21−24	(31)	10.0		3.93		55.56	
≥25	(29)	13.30	0.087	3.48	0.54	25.0	0.05†
Education							
<6 years	(23)	2.82		4.77		47.0	
≥6 years	(54)	2.30	0.03†	3.0	0.14	30.0	0.23
Occupation							
Textile	(1)	2892.28		586.01		657.31	
Sales	(7)	25.00		3.94		38.46	
Housewives	(62)	11.23		3.61		34.88	
Other***	(7)	6.25	0.002†	0.69	0.02†	12.29	0.04†
Residential zone							
Urban	(56)	10.0		3.0		39.47	
Industrial	(21)	15.15	0.69	5.0	0.02†	34.88	0.20
Beverage consumption (plastic containers)							
No	(73)	10.0		3.48		34.88	
Yes	(4)	33.67	0.04†	368.0	0.06†	52.72	0.42
Microwave oven use (plastic containers)							
No	(61)	11.23	0.74	3.48	0.90	30.61	
Yes	(16)	12.47		3.01		65.95	0.02†
Use of plastic Containers							
Non-brand	(19)	26.31	0.001†	5.0	0.31	39.47	0.98
Brand	(58)	10.0		3.0		34.88	0.98

* MBP = Mono-Butyl phthalate, MEHP = Mono-Ethyl-Hexyl phthalate, MEP = Mono-Ethyl phthalate, μg/g of creatinine.
** Kruskal Wallis test for medians differences.
*** Includes secretaries, clerks and teachers.
† p values which show significant differences.

Table 4
Regression coefficients for the association between phthalate metabolite* concentration levels and sociodemographic characteristics and sources of exposure in a cohort of pregnant women from the State of Mexico adjusted by creatinine and the variables included in the model.

Characteristics	MBP log			MEHP log			MEP log		
	β	95 % CI	P-value	β	95 % CI	P-value	β	95 % CI	P-value
Residential area Urban vs Industrial				0.82	−0.07, 0.72	0.07	−0.065	−1.32, 0.03	0.06
Occupation Salesperson vs. Other							1.27	0.19-, 2.33	0.02
Beverage consumption (Plastic bottles)	1.75	0.39, 3.11	0.001	1.58	−0.022, 3.37	0.08			
Use of plastic containers Brand vs. Other	−0.90	−1.60, -0.20							

* MBP = Mono-Butyl Phthalate, MEHP = Mono-Ethyl-Hexyl Phthalate, MEP = Mono-Ethyl Phthalate.

air according to geographical zones of residence or occupation, diet, use of plastic recipients and micro-wave ovens. Respiratory exposure by air was not directly evaluated by environmental monitoring in our study, but it was indirectly evaluated by the residential zones, urban vs. industrial in Toluca city, Mexico State.

Food contamination has been reported as the main source of exposure for general population [38]. Although we did not evaluate food contamination directly, we observed increased phthalates levels in women who used non-brand plastic containers to heat food in microwave ovens, and consumption of beverages in plastic bottles.

In a cohort study conducted by Serrano 2014 [1] in pregnant women during the first pregnancy trimester, they found in urine lower levels of phthalate metabolites in women who purchased organic products and unprocessed foods. MEP was the most frequent metabolite detected, followed by MBP in women with low educational level. MEP also was found in the consumption of dairy processed products. Although, in

Table 5
Comparison of urinary phthalates metabolite median levels in the NHANES and among pregnant women from New York City (NYC), Denmark, Canada, several United States (US) cities*, Boston, Mexico and Greece.

Phthalate Metabolites **	NHANES 1988−94	NHANES 1999−2000	NYC Adibi et al. (2003) [35]	Denmark Toft et al. (2012) [18]	NY Kobrosly et al. (2014) [39]	Canada Arbuckle et al. (2014) [40]	Several US cities ** Serrano et al. (2014) [1]	Boston Braun et al. (2014) [41]	Canada Fisher et al. (2015) [42]	Mexico Bustamante-Montes et al. (2013) [15]	Crete, Greece Katsikantami et al. (2020) [24]
MBP***	41.0	26.0	42.6	225.1	13.61	–	–	77.8	23	11.23	28.1
MEHP***	2.7	3.2	4.60	16.2	3.65	2.24	2.54	–	2.26	3.48	6.1
MEP***	305.0	164	236.0	405.8	81.01	32.02	35.63	309.5	42.5	34.88	–
MBzP***	21.2	17.0	12.1	20.3	6.59	5.20	4.28	–	10.7	–	46.7

* Minneapolis, Rochester, Seattle and San Francisco.
** μg/L, μg/g of creatinine.
*** MBP = Mono-Butyl Phthalate, MEHP = Mono-Ethyl-Hexyl Phthalate, MEP = Mono-Ethyl Phthalate, MBzP = Mono-Benzyl Phthalate.

smaller quantity, similar findings were observed in our study. MEP had the highest concentration levels in pregnant women, followed by MBP and MEHP. Older women showed lower MEP levels that women who had low educational status. These results suggest that further studies are needed to analyze the role that personal practices play particularly during pregnancy.

As in all observational studies, the interpretation of our results should consider the potential limitations of selection bias. Selection bias was avoided by reducing loss to follow- up with the conduct of a home visit when the mothers could not attend the appointments, and in those cases, they were driven to the clinic or the samples and application of the questionnaire were taken at her home by the study personnel in charge of the project. Selection bias was additionally evaluated comparing sociodemographic information from the participants and those who decided not to participate or refuse to continue in the study, and no differences were observed between the women who continued in the study vs. those who were lost to follow-up.

Exposure assessment to phthalates is one of the first steps to evaluate their effects in human health. Our study adds information to literature, but there is still a long way to go if we want to characterize exposure for the most susceptible populations. To get better politics in public health, it should explore the potential exposure sources and effects at regional level.

Previous discussion was done comparing phthalates concentration only in urine samples as biological matrices. A recent study that used amniotic liquid and urine found that in urine are detected higher phthalates levels and more molecules than in amniotic liquid; as well as, the mono ethylhexyl phthalate (mEHP) was the most abundant phthalate in both biological matrices and was strongly associated with the usage of plastic food containers [23]. Urine is the preferred sample for biomonitoring phthalates exposure in human even in not pregnant women due to in this sample could be detected more molecules in type and quantity. In this sense; many studies in open population have been done in Europe (Germany, Netherlands, Denmark, Norway, Sweden, Greece, the Czech Republic, Hungary, Slovakia, and Spain) Asia (Japan, China, South Korea, India, Taiwan, Vietnam, Saudi Arabia, Malaysia, and Kuwait) and United States. Phthalate concentrations have a large variation among countries [43].

Author contributions

LPBM Funding Acquisition; VHBA, MHV, MMGF and PBB Methodology; RGA Data Curation; GAAG Writing – Review.

Declaration of Competing Interest

The authors declare no conflict of interest.

Acknowledgment

This research had the support of the “National Science and Technology Council”, through the Health Sector Fund (C01-017) and the Universidad Autónoma de Guadalajara. We thank the study participants for their cooperation and participation in the study; the Fulbright Scholarship Program through the scholarship provided to Dr. Hernández-Valero, and the National Institutes of Health (NCMHD P60-MD000503) for the funds provided for Dr. Hernández-Valero research.

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