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Phthalate Esters in Blood, Urine and Breast-milk Samples of Transfused Mothers in Some Hospitals in Ibadan Metropolis Southwestern Nigeria

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Abstract

Phthalate esters which are plasticizers in plastics, other polymers, blood bags and infusion tubes are known to leach extensively into the content. Phthalate esters have been implicated as endocrine disruptors in humans. Therefore, clinically; phthalate esters pose serious health risk, as transfused patients are vulnerable to phthalate esters contamination. In this study the concentrations of some phthalate esters and phthalate esters metabolites in blood serum, urine and breast-milk of some transfused nursing mothers. The study was carried out in some major hospitals in Ibadan metropolis after the receipt of ethical approval. Blood, urine and breast milk samples were obtained from twenty consented transfused nursing mothers and the samples were obtained in replicates.

Hexane: dichloromethane 87.5:12.5 v/v was used to extract blood serum. Hexane: dichloromethane 87.5:12.5 v/v was used to extract urine. Hexane: diethyl ether, 50:50 v/v was used to extract breast-milk sample. All extractions were done using ultrasonicator. The extracts were cleanup respectively in a column of silica gel and eluted with hexane: acetonitrile 99.3:0.7 v/v, the cleanup extract was analyzed using HPLC-UV. The concentration of diethyl phthalate was in the range from not detected (nd)-3.03, nd-0.30 and nd-0.94 µg/ml; in breast-milk, urine and blood serum respectively. Dipropyl phthalate ranged from 0.09-1.55, nd-1.2 and nd-7.68 µg/ml; in breast milk, urine and blood serum respectively. Dibutyl phthalate ranged from 0.07-2.22, nd-1.2 and nd-10.74 µg/ml; in breast-milk, urine and blood serum respectively. Monobutyl phthalate ranged from 0.62-15.04, nd-3.2 and nd-7.45 µg/ml; in breast-milk, urine and blood serum respectively. Mono-2-ethyl-5-oxo-hexyl phthalate ranged from 0.84-4.29, nd-1.5 and nd-14.68 µg/ml; in breast milk, urine and blood serum respectively. Level of phthalate esters and metabolites in the samples were high; which is suggestive that the infusion tubes and blood bags may be one of the major clinical sources of phthalate esters contamination in transfused patients.

Keywords: Phthalate esters; Blood transfusion; Blood bags; Infusion tubes

Introduction

Phthalate esters are esters of benzene 1, 2 dicarboxylic acid; which are used as plasticizers in plastics and other polymer products. They are not chemically bound to the matrices of these materials. They therefore leach into the content of the materials or to the immediate environment. Thus, are found to be ubiquitous in the environment and in biological systems [1,2]. Rael reported between 13-20-fold increase in phthalate esters concentration, in blood stored in blood bags for day 1 and day 42 [3]. Studies have also reported that blood stored at 4°C in polyvinyl chloride bags increase in phthalate esters concentration, from 100 to 275 mg/L in one week of storage, and from 200 to 300 mg/L in three days when stored at ambient temperature [4]. The phthalate esters content of finished plastic or polymer material is in the range of 10-60% by weight [5]. These polymer materials therefore constitute a major route to phthalate esters exposure.

The main route of exposure to phthalate esters includes inhalation, ingestion, skin contact and intravenous injection [6,7]. All other modes must pass through absorption to be incorporated into the blood stream. Nevertheless, the intravenous injection seems to be the most lethal. As it bypasses absorption process and it is delivered directly into the circulatory system of the recipient. Though, there were claims that phthalate esters do not seem to bio-accumulate but their effects are trans-generational. Studies have shown that they are carcinogenic, toxic to testes, ovary, hormones and kidneys in animals [8-10]. Prenatal exposure to phthalate esters in human infants has been linked to sterility [11]. Researches have also shown that obesity and thyroid malfunction in humans have been linked to phthalate esters [12,13].

Diethyl phthalate has been shown to reduce sperm motility in humans [14]. Also, metabolite of diethyl hexyl phthalate (DEHP); mono-2-ethyl-5-oxohexyl phthalate (MEOHP) alongside metabolite of diethyl phthalate; monoethyl phthalate (MEP) and monobutyl phthalate (MBP) have been implicated in obesity [12]. High mono-2-ethyl-5-oxohexyl phthalate in urine has been associated with attention deficit hyperactivity disorder in children [15]. Monoethyl phthalate and mono-2-ethyl-5-hydroxyl hexyl phthalate in human urine have been related with decrease in sperm motility, decreased testosterone level, estradiol, increased sperm damage and high blood pressure [16-18]. High concentration of Dibutyl phthalate and its metabolite monobutyl phthalate in mothers have been linked to reduce ano-genital distance in babies [19].

Mothers at the point of child delivery sometimes require blood transfusion to maintain a safe blood level, due to blood loss during child delivery [20]. The blood transfused is usually stored in blood bags, which are made of polymer materials [3]. The infusion tubes too are made of polymer materials. Phthalate esters therefore leach extensively from the blood bags into the blood transfused to these mothers.

There is dearth of information and paucity of data on phthalate esters and metabolites in blood, urine and breast-milk of nursing mothers after transfusion, in most hospitals within Ibadan metropolis. Determination of phthalate esters and metabolites concentrations in breast-milk through leaching from blood bags and infusion tubes will serve to determine the extent of exposure to neonates who depend solely on their mothers' breast-milk; the mothers who have been exposed to these hazardous substances through blood transfusion.

Materials and Methods

The analytical reference standards; monobutyl phthalate, dibutyl phthalate, diethyl phthalate, dipropyl phthalate were from Sigma Aldrich (Munich, Germany), while mono2ethyl oxohexyl phthalate was from Cambridge Isotope Laboratory (Andover Massachusetts, U.S.A.). Dichoromethane, hexane, methanol and ethyl acetate were from Sigma Aldrich (Munich, Germany). The 60-300 mesh size silica gel was from Sigma Aldrich (Munich, Germany).

Sample sites

Ibadan is in the southwestern part of Nigeria and it is about 3 million in population [21]. The three major government hospitals in Ibadan vis; University College Hospital (U.C.H.), Adeoyo Maternity Teaching Hospital Yemetu and Adeoyo State Hospital Ring Road Ibadan were chosen as study. The study sites were chosen for their high patronage by the public and their strategic location. The hospitals also have the highest cumulative number of child delivery per annum within the metropolis.

Volunteers statistics

Twenty transfused nursing mothers were recruited for this research. Eighty five percent (85%) of those recruited for this study were of low income class; while 15% were of high income class. Thirty percent (30%) of the recruited participants had post-secondary school education, 60% had secondary education and 10% of all participant recruited had no conventional education at all. None of the recruited transfused nursing mothers recruited for this study was a beautician or works in polymer manufacturing industry or skin care product manufacturing industry or paint industry. These eliminate phthalate esters exposure in work environment for recruited participants in the study. The range of age of the transfused nursing mothers recruited was 20-39 years and the mean age was 28 years. Ninety five percent of the participants recruited lived in the urban communities in Ibadan metropolis. The range of weight of the babies of the recruited transfused nursing mothers was between 2.0-3.1 kg with the mean weight being 2.7 kg.

Sample collection

The ethical approval was obtained from the Ethical Review Committee of the University of Ibadan/University College Hospital (U.I./U.C.H.), to obtain biological samples from University College Hospital (U.C.H.) and the Oyo State Ethical Review Committee to obtain biological samples from Adeoyo Maternity and Teaching Hospital, Yemetu, Ibadan, and Adeoyo State Hospital, Ring road, Ibadan. The samples were collected from volunteer transfused nursing mothers by trained physicians, after the administration of questionnaires and signing of the informed consent forms by the recruited transfused nursing mothers. The blood samples were collected into bijou bottles with stainless steel needle by trained physicians and were centrifuged at four thousand revolutions per minute (4000 r.p.m.) for ten minutes, within four hours of collection to obtain the blood serums, the serums obtained were stored in the freezer at -20°C, after the addition of ortho-phosphoric acid to the serums to prevent enzymatic hydrolysis, and then preserved in the freezer until the time of the analysis. The breast milk samples were obtained from the transfused nursing mothers into bijou bottles by hand expression after the mothers have abstained from skin care products and proper hand washing. The expressed milk samples were preserved at -20°C after the addition of ortho-phosphoric acid until the time of analysis. The urine samples were collected into bijou bottles and ortho-phosphoric acid was added to them and thereafter stored in the freezer at -20°C until the time of the analysis.

Extraction

The extraction of urine was a modification of the method of Kondo as well as Fatoki and Ogunfowokan; five milliliters (5 ml) of urine sample was taken into a test tube and 1 g of sodium chloride was added to it, 3 × 5 ml of dichloromethane: hexane 12.5:87.5 v/v was used to extract the sample in an ultrasonic bath and the extracts were pooled; Further extraction was performed with (3 × 5 ml) of 0.1 M of sodium carbonate [22,23]. The extract was then concentrated to 2 ml, and afterward was subjected to cleanup. Serum extraction followed modification of Colon method in tandem with Fatoki and Ogunfowokan method [23,24]; three milliliters (3 ml) of serum was taken into a 25 ml test tube, 1 ml acetonitrile was added to it to precipitate plasma and serum protein and was extracted with 3 × 5 ml of dichloromethane: hexane 12.5:87.5 v/v in an ultrasonic bath. The extract was pooled and further extracted with 3 × 5 ml of 0.1M sodium carbonate and the extract was then concentrated to 2 ml for cleanup. The extraction of breast milk was by modified Sorensen method in tandem with Fatoki and Ogunfowokan methods [23,25]; five milliliters (5 ml) of milk sample was taken into a test tube and was extracted with 3 × 5 ml of hexane-diethyl ether 50:50 v/v in an ultrasonic bath, the extract was pooled and further extracted with 3 × 5 ml of 0.1 M sodium carbonate. The extract was then concentrated to 2 ml and thereafter, cleanup was carried out in a similar procedure described below.

Clean up

The cleanup of the extracts was by Sorensen method in tandem with Fatoki and Ogunfowokan method were modified to obtain the procedure [23,25]. Two milliliters (2ml) of the serum extract was mixed with 1.5 g of deactivated silica gel and made into slurry after which it was dried; two grams of anhydrous sodium sulphate was loaded in column as drying agent, after which the already dried slurry was loaded to the same column. Phthalates was then eluted with 0.7% ethyl acetate in hexane, the eluate was then concentrated to 1ml, and was reconstituted to 1 ml acetonitrile and kept at -20°C until analysis. The same cleanup procedure was followed for breast milk and urine extracts.

HPLC analysis

The samples were run in Agilent Technologies 1200 series HPLC, the column was Zorbax XDB RP C8 150 × 4.6 mm, 5 µm, with a UV detector, the mobile phase used was Acetonitrile: Water (80:20 v/v). The temperature of operation was ambient while the injection volume was 10 µL; the flow rate was 1 ml/minute, wavelength of detection of phthalate esters and their metabolites was 226 nm, while, the mode of quantification was peak area. All the results were processed on Microsoft excel 2007 to obtain the calibration curve and to obtain the mean concentration of phthalate esters.

Results

The overall minimum and maximum; mean and median of age in years of the twenty (20), transfused nursing mothers as well as weight in kilograms of the transfused nursing mothers before pregnancy were as expressed in Table 1, the weight of babies in gram as well as age in days of the live babies as at the day of sampling were highlighted.

	Mean	Median	Min	Max
Nursing Mothers' Age (years)	28	29	20	39
Weight before pregnancy (Kg)	66	65	59	75
Birth weight of the child (g)	2680	2700	2000	3100
Children's age at sampling (days)	3	4	2	6

Table 1: Personal data of consented transfused mothers.

From Table 1, it was observed that all the nursing mothers were within the productive years with healthy weight and they had live babies as well. The weightier babies seem to be more than the lighter babies.

Three phthalates viz; diethyl phthalate, dipropyl phthalate, dibutyl phthalate and two phthalate metabolites viz; monobutyl phthalate and mono-2-ethyl-5-oxohexyl phthalate were analyzed in this study. The detection rate of each of the phthalates and metabolites in blood serum, urine and breast milk samples of the transfused nursing mothers were expressed in Table 2. The detection rate was the expression of the percentage of the samples with detectable phthalates and metabolites concentration out of the total samples analyzed.

Phthalate esters	Detection rate		
	Blood	Urine	Breast milk
Total number of subject (20)			
Dibutyl phthalate	85%	70%	100%
Dipropyl phthalate	65%	40%	100%
Diethyl phthalate	75%	40%	65%
Mono2ethyl5oxohexyl phthalate	95%	70%	100%
Monobutyl phthalate	90%	85%	100%

Table 2: Detection rate of each phthalate esters as a percentage of the total sample.

Detection rate=Number of samples with detectable phthalate concentration/Total number of samples.

From Table 2, it was observed that the order of frequency of occurrence of phthalate esters and metabolites in the biological samples followed the trend; breast-milk>blood>urine. The low frequency of occurrence of phthalate and metabolites in urine may be attributed to time gap between phthalate intake and metabolism of phthalate in the system excretion in urine samples. Phthalates are lipophilic and breast milk contains about 2-4% fat, therefore phthalate and metabolites were higher in the breast milk of mothers examined than urine and even blood [26]. The high concentration of phthalate and metabolites in nursing mothers breast milk may also be an indication that the neonates are exposed to the phthalate and metabolites from their mothers' breast-milk; even though it was said that phthalates do not bio-accumulate, yet their effect could be seen in the offspring of the exposed mothers. Also, it was observed that all the transfused mothers had high level of phthalates and metabolites in their biological samples, which could be an indication that transfusion indeed exposed the transfused nursing mothers to phthalates and metabolites and in turn the circulatory system of the nursing mothers. The phthalate and the metabolites exposed to by the nursing mothers were also excreted in the urine of the transfused nursing mothers. The fact that not all the phthalates and metabolites were found in each of the biological samples may be an indication that contamination was not a systemic problem in this research [27].

The overall mean concentration, standard deviation, median concentration, limit of detection of each phthalate and metabolite as well percentage of samples with concentration of phthalates and metabolites below the limit of detection for each of the phthalates and metabolites in blood serum samples of the transfused nursing mothers were expressed in Table 3.

Phthalate	Limit of detection (LOD) (µg/ml)	%< LOD	Median (µg/ml)	Mean (µg/ml)	SD
DBP	0.002	15%	0.12	1.3	2.75
DPP	0.004	35%	0.54	1.8	2.7
DEP	0.004	25%	0.11	0.23	0.28
mEOHP	0.007	5%	0.6	2.8	3.8
mBP	0.01	10%	0.7	1.5	2.1

Table 3: Concentration of phthalates and metabolites in blood serum samples.

From Table 3; the limits of detection for each of the phthalate using high performance liquid chromatography methods were low. The low limit of detection of HPLC resulted in better quantification of phthalate concentration in the samples. It was also observed that the concentration of the phthalate ester in blood serum samples were high which is an indication that transfusion is a viable means of exposure to phthalate esters.

The overall mean concentration, standard deviation (Sd), median, limit of quantification (LOQ) for each phthalate and metabolites as well as percentage of urine samples with concentration less than limit of detection of phthalates and metabolites on HPLC (i.e., Frequency of occurrence) were expressed in Table 4.

Phthalate	Quantification limit (LOQ) (µg/ml)	% < LOD	Median (µg/ml)	Mean (µg/ml)	SD
DBP	0.006	30%	0.04	0.15	0.4
DPP	0.015	60%	0.09	0.22	0.35
DEP	0.014	60%	0.05	0.1	0.09
mEOHP	0.025	30%	0.15	0.44	0.44
mBP	0.03	15%	0.28	0.7	0.87

Table 4: Concentration of phthalates and metabolites in urine samples.

From Table 4; it was observed that frequency of occurrence of phthalates (% < limit of detection), in urine samples were lower than frequency of occurrence of the metabolites in urine samples, the higher frequency of occurrence of phthalate metabolites may seems to suggest that phthalates had just been exposed to recently. However, it could be observed that phthalate metabolites though higher in frequency of occurrence, also had higher concentration than the parent phthalate; which suggests that after exposure the phthalate esters not accumulate but are easily excreted out of the circulatory system of the exposed patient.

The overall mean concentration, standard deviation (Sd), median and percentage of breast milk samples with concentration of phthalates and metabolites below the limit of detection on HPLC were expressed in Table 5.

Phthalate	% < LOD	Median (µg/ml)	Mean (µg/ml)	Sd
DBP	0%	0.11	0.8	1
DPP	0%	0.56	0.76	0.61
DEP	35%	1.79	1.79	1.24
mEOHP	0%	1.85	2.33	1.45
mBP	0%	1.5	5.72	6.6

Table 5: Concentration of phthalates and metabolites in breast milk samples.

From Table 5; phthalates and metabolites were observed in all the milk samples except diethyl phthalate which was not detected in some, we also observed that the metabolites had higher concentration than their parent phthalates which tends to show that phthalates do not accumulate in the body system; it could be said that the lower level of phthalates than the metabolites, was an indication that the sampling and sample pretreatment methods might not have introduced contaminant into the samples.

Discussion

The concentration of monobutyl phthalate (mBP) in blood serum was higher than that found in Korea with patients with congenital hypothyroidism [28]. This observation is suggestive that the exposure of nursing mothers to blood transfusion may result in hormonal dysfunction. The mean concentration of mBP in urine of the transfused nursing mothers was higher than that found in adult women in the U.S. which was 27.6 µg/g [12]. The mean concentration of mBP obtained in milk samples in this study is higher than that obtained in breast milk of women in Germany which had a mean 2.6 µg/L [27]. Monobutyl phthalate due to its lipophilic nature was found to be higher in breast milk samples than the urine samples in this study.

The concentration of dibutyl phthalate (DBP) in blood serum in this study was found to be many folds higher than that obtained in Korean study on women with congenital hypothyroidism which was 51.11 ± 27.5 ng/ml [28]. It was higher than obtained in India with women with endometriosis, whose mean was 0.98 ± 0.96 µg/ml [29]. The concentration of DBP in urine in this study was higher than that obtained in urine in Chinese women undergoing parturition which was 24.93 ± 18.67 µg/L [30]. The concentration of DBP in breast milk sample in this study was several folds higher than that obtained in lactating mothers in Italy which was 1.5 µg/L [26]. DBP in this study was also higher than that obtained by Fromme, in mothers in Germany whose mean was 1.2 ng/g [27]. The high concentration of DBP in transfused mothers' milk shows that babies were exposed to DBP from their mother's milk. In general, it was observed that phthalates concentrations were higher in breast milk samples than urine samples due to their lipophilic nature. However, phthalate esters concentrations in blood serum were comparable to that obtained in breast milk, probably, because blood had direct contact with the plasticizers in blood bags and infusion tubes.

Mean diethyl phthalate (DEP) in blood serum in this study was found to be lower than that observed in China by Xu, in women with polycystic ovary syndrome, which was 0.45 µg/ml [31]. The mean DEP in urine in this study is lower than that obtained by Ogunfowokan in sewage treatment lagoon and receiving stream which had overall mean of 25.08 ± 24.85 mg/L [32]. The mean DEP in breast-milk samples in this study was higher than that obtained by Zhu, in a six-month postpartum study of phthalate concentration in breast-milk of women in Canada; the mean concentration obtained in the study was 0.31 ng/g [33]. The high DEP concentration obtained in this study represents the concentration ingested directly by the neonates from mothers' breast-milk, which may significantly affect future reproductive health of these neonates.

Mean mono2ethyl 5oxohexyl phthalate in blood serum samples analysed in this study was very much higher than that obtained by De Cock, in blood plasma which was 0.29 ng/ml [34]. Such high concentration of phthalate could lead to reduced chances of sustaining healthy pregnancies in the mothers [9,35]. The mean mono-2-ethyl-5-oxohexyl phthalate in urine samples analysed in this study was higher than that obtained by Zhu, in urine samples of women of the same age whose mean was 7.25 ng/ml [36]. Mono-2-ethyl-5-oxohexyl phthalate has been linked with obesity, insulin resistance, and some other clinical disorder in humans [12,15]. It was said that it reduces the chances of

women getting pregnant [35]. Mono-2-ethyl-5-oxohexyl phthalate in breast-milk samples was higher than that obtained by Hines, in North Carolina lactating mothers who had mono-2-ethyl-5-oxohexyl phthalate in milk with a mean 0.3 µg/L [6]. This high concentration in others may be harmful to the reproductive health of the babies taking the seemingly innocuous breast milk of their mothers.

Mean dipropyl phthalate in blood serum analysed in this study was higher than that observed by Lu, in physiological saline solution with mean nd [37]. There seems to be paucity of data on dipropyl phthalate on human biological samples, however, higher dipropyl phthalate in blood is an indication that blood transfusion seems to increase phthalate esters in the human circulatory system than taking saline solution. The mean dipropyl phthalate in urine in this study is higher than that obtained by Zhang M et al., in domestic water out of water pipe, whose mean concentration was 0.12 ng/ml [38]. This may suggest that blood transfusion seems to introduce more phthalate esters into the human system than that which could be encountered in phthalate esters contaminated domestic water. High level of dipropyl phthalate in urine has been said to be toxic to human reproductive system with possible teratogenic effect [39]. The mean dipropyl phthalate in human breast milk analysed in this study is higher than the mean obtained by Zhang M et al., in raw milk with a mean nd [40]. This may seems to suggest that blood transfusion leads to more exposure to phthalate in the human breast milk of transfused mothers than taking raw milk analyzed by Zhang M et al. [40].

Conclusion

The result obtained from this study showed high level of phthalate esters in all the blood samples of the transfused nursing mothers analyzed. It was higher than those found in literatures. This is suggestive that blood transfusion is a viable and major source of exposure to phthalate esters. Phthalate esters and metabolites were also found in urine samples of the transfused nursing mothers; indicative that phthalate esters were excreted out in the body fluids and those phthalate esters does not accumulate. High phthalate esters in milk samples may infer that; neonates were exposed through their mothers' milk and whose effect is trans-generational. As the effect of the phthalate has been found to include sexual development of neonates, this may damage the reproductive health of those neonates in the future. Breast feeding should not be discouraged as it provides necessary nutrition and immunity to neonates against diseases. However, measures to stop the use of phthalate esters as plasticizers in polymers should be taken so as to reduce exposure through blood transfusion.

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Conflict of Interest

The Authors declare no conflict of interest.

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