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Phtalates in urine as markers of blood transfusion in sports: Population concentrations of five DEHP metabolites and reference limits

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Abstract

Di-(2-ethylhexyl)-phthalate (DEHP) is the most widely used plasticizer authorized for blood storage bags addressed to blood transfusions. The urinary metabolites of DEHP have been proposed as markers to suspect the misuse of blood transfusions in sports. However, DEHP is also present in different polyvinyl chloride products and the general population is continuously exposed to it. In this regard, the knowledge of the concentrations in normally exposed subjects and the establishment of reference limits (RL) of this common exposure are needed to identify a high exposure resulting from a blood transfusion.

In the present study, urinary concentrations of the five main DEHP metabolites were determined in different healthy population groups by ultraperformance liquid chromatography coupled to tandem mass spectrometry. The groups studied were control group (n=30), athletes of different sports (n=464), and young Spanish females (n=535). The concentrations obtained in all groups were in the low range for all the metabolites in accordance with published data. The concentrations obtained in the athletes group were used to establish RL for a risk of 1:1000 of false positive results. The RL were established at 338.8, 158.5, 331.1, 229.1 ng/mL for the metabolites mono-(2-ethyl-5-hydroxyhexyl)phthalate, mono-(2-ethyl-5-oxohexyl)phthalate, mono-(2-ethyl-5-carboxypentyl)phthalate, and mono-(2-carboxymethyl-hexyl)phthalate, respectively. The proposed RL could be applied to the urines collected in doping control programs to distinguish a common exposure to DEHP from a high exposure in order to suspect of the use of a blood transfusion.

Introduction

Di-(2-ethylhexyl)phthalate (DEHP) is the plasticizer authorized for blood storage in blood transfusions. The urinary metabolites of DEHP (Figure 1) have been proposed as markers to suspect of blood transfusions in sports because of the high exposure to this compound during a blood transfusion process [1-3].
DEHP is also present in several polyvinyl chloride products, so the general population is continuously exposed to it. For this reason, the common concentrations of DEHP metabolites in healthy subjects need to be known.
The aim of this study was to determine concentrations of the main five DEHP metabolites in different population groups. Additionally, reference limits (RL) have been established in order to discriminate a normal exposure from a high exposure that could indicate the use of a blood transfusion.

Experimental

Samples
Samples from three different groups were studied: control group (n=30; 23.7±2.3 years, 24h urine samples), athletes (n=464, spot urine samples), and young pregnant women living in Sabadell, Spain (n=325; 30.6±4.0 years; spot urine samples collected during the first, n=320, and third, n=215, trimester of pregnancy).

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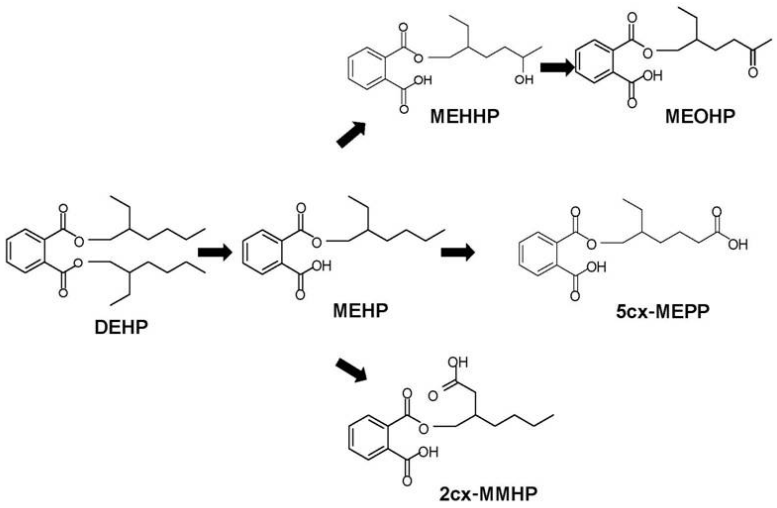

Figure 1. Main DEHP metabolites.

Analysis of DEHP metabolites in urine

Samples of the control and the athletes group were analysed according to a previously described protocol [4].

Analyses of the DEHP metabolites in samples of pregnant women were performed using a method which allows the quantification of DEHP metabolites together with metabolites of other common phthalates. Briefly, an enzymatic hydrolysis with b-glucuronidase from E.coli was applied to urines (2 mL) followed by SPE using Oasis MAX columns. The columns were conditioned with methanol and water and, then, the sample was loaded. After two washing steps (2 mL 5% ammonia solution and 2 mL methanol), the analytes were eluted with acetonitrile with formic acid (2%) (2 mL). The organic phase was evaporated to dryness and the residue was reconstituted with a mixture of water, acetonitrile and formic acid, and analyzed by ultraperformance liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS). All the DEHP metabolite standards and the labelled analogues were obtained from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA).

A triple quadrupole mass spectrometer provided with an electrospray interface (Xevo TQ MS) coupled to an Acquity UPLC system was used (Waters Corporation, Milford, MA, USA). The separation was achieved in 26 min using an Acquity BEH C18 column (100 x 2.1 mm i.d., 1.7mm) (Waters Corporation) and a gradient program using acetonitrile and water with formic acid (0.01%) as mobile phases. Analyses were performed in SRM mode (Table 1).

Metabolite	MM	RT (min)	ESI	CV (eV)	CE (eV)	Ion transitions
MEHP	278.15	19.11	neg	25	15	277>134
MEOHP	292.13	12.72	neg	20	15	291>143
MEHHP	294.15	12.74	neg	25	15	293>145
5cx-MEPP	308.13	12.42	neg	15	15	307>159
2cx-MMHP	308.13	13.64	neg	15	15	307>159
MEHP $^{13}C_4$	282.15	19.11	neg	25	15	281>137
MEOHP $13C_4$	296.13	12.72	neg	20	15	295>143
5cx-MEPP $^{13}C_4$	312.13	12.42	neg	15	10	311>159

Table 1. Monitoring conditions in the LC-MS/MS system for DEHP metabolites. MM, monoisotopic mass; RT, retention time; ESI, electrospray ionization: pos, positive; neg, negative; CV, cone voltage; CE, collision energy.

Statistical analysis

The statistical analysis was performed with the computer software SPSS 12.0 (SPSS, Inc., Chicago, IL) and the program RefVal (RefVal 4.11, Oslo, Norway) was used for the determination of the RL.

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Results and Discussion

Concentrations of DEHP metabolites in the different populations studied are summarized in Table 2. The concentrations of the DEHP metabolites found in the control and in athletes group were low for all metabolites, although the samples belonging to the control group presented slightly higher concentrations (they were 24h urine samples as compared with spot samples in the other groups). In the case of the pregnant women group, the concentrations obtained were also low and, additionally, differences were not observed between samples collected in the first and the third trimester of pregnancy.

		Percentile (ng/mL)				
Group	Metabolite	10th	25th	50th	75th	90th
Control (n=30)	MEHP	5.1	8.1	16.0	22.7	26.4
	MEHHP	11.8	31.1	51.4	80.2	112.7
	MEOHP	9.1	20.2	38.2	65.1	111.7
	5cx-MEPP	17.4	33.2	55.0	109.2	143.1
	2cx-MMHP	10.7	22.5	34.0	60.5	169.0
Athletes (n=464)	MEHP	2.5	3.5	5.5	9.4	15.3
	MEHHP	10.9	15.8	27.3	44.3	76.0
	MEOHP	5.1	8.5	13.6	22.0	39.8
	5cx-MEPP	12.3	18.8	28.4	46.7	81.8
	2cx-MMHP	8.4	12.8	21.1	33.8	66.2
Women (n=320) 1st trimester	MEHP	3.1	4.8	7.8	13.3	22.7
	MEHHP	5.5	10.4	21.5	41.2	66.5
	MEOHP	4.0	7.8	15.7	27.2	45.5
	5cx-MEPP	10.6	17.2	28.3	51.8	80.0
	2cx-MMHP	11.8	18.9	32.3	53.0	88.4
Women (n=215) 3rd trimester	MEHP	2.8	4.3	7.7	13.8	23.5
	MEHHP	5.7	10.2	19.3	30.8	62.5
	MEOHP	4.5	9.2	16.5	26.8	56.9
	5cx-MEPP	9.1	18.0	29.2	51.9	90.7
	2cx-MMHP	10.4	18.9	32.9	55.4	94.0

*For control group and athletes group, concentrations were adjusted by specific gravity. The results of pregnant women group correspond to absolute concentrations.

Table 2. Distribution of concentrations of DEHP metabolites in different population groups*.

Concentrations obtained in the athletes group were used to establish the RL for a risk of 1:1000 for four DEHP metabolites. The RL obtained were 338.8, 158.5, 331.1, 229.1 ng/mL for the metabolites MEHHP, MEOHP, 5cx-MEPP and 2cx-MMHP, respectively. The RL for MEOHP was in accordance with the value previously proposed by other authors [2], however the RL of MEHHP obtained in our study was higher.

The calculated RL were applied to the pregnant women group (Figure 2). The concentrations of the different DEHP metabolites were clearly below of the RL and all the distribution of concentrations indicated a common exposure to DEHP during pregnancy. Only one female volunteer had concentrations higher than the reference limits proposed for all metabolites (A in Figure 2).

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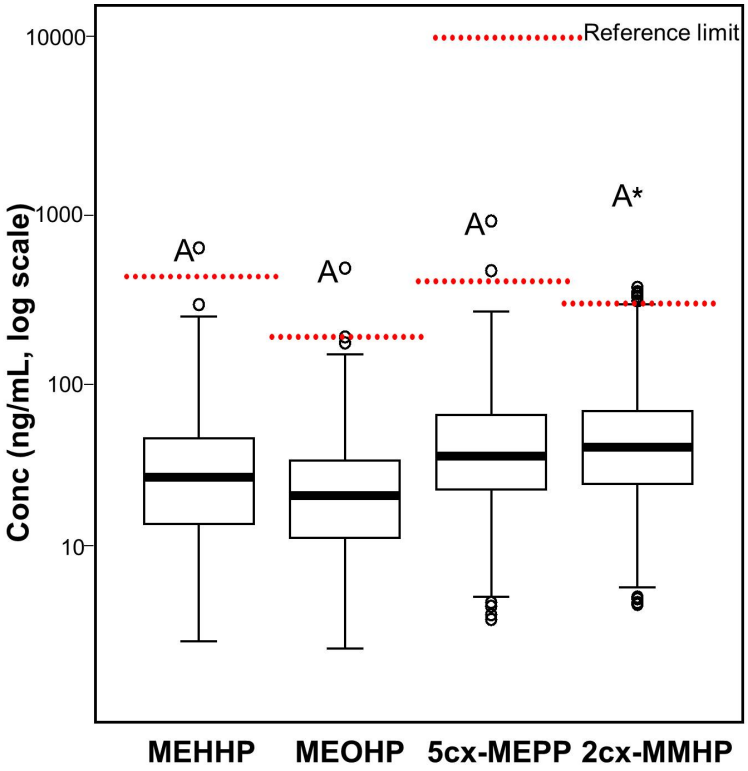

Figure 2. Application of the calculated reference limits to pregnant women (n=535, the boxplots include samples of the 1st and 3rd trimester of pregnancy; the results exceeding the RL corresponding to the same woman are indicated with A; mild outliers and extreme outliers are marked with (°) and (*), respectively; values exceeding the third quartile plus 1.5 times interquartile range are considered mild outliers, and values exceeding the third quartile plus 3 times interquartile range are considered extreme outliers).

The suitability of the RL proposed was evaluated using the results obtained in a previous study of autologous blood transfusion to 25 subjects [3]. Taking into account these results, MEHHP, MEOHP and 5cx-MEPP could be used as markers to detect blood transfusion during the first hours after the transfusion. Regarding 2cx-MMHP, it could be used to suspect beyond 24 hours after the transfusion because of its longer half-life.

Conclusions

Concentrations of five DEHP metabolites have been measured in different population groups. The results obtained in all cases were low for all the metabolites. Moreover, RL were calculated for a risk of 1:1000 for the metabolites MEHHP, MEOHP, 5cx-MEPP and 2cx-MMHP. These RL could be used as threshold concentrations in order to distinguish common exposure from a higher one that could lead to suspicion of the use of a blood transfusion.

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