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Phthalate exposure: new clue to suspect illicit blood transfusion

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Abstract

Humans are exposed to di-(2-ethylhexyl)phthalate (DEHP), a plasticizer used in polyvinyl chloride (PVC) products. DEHP is also present in bags used in blood transfusions, subsequently patients submitted to blood transfusions for medical reasons or athletes using blood doping are exposed to this chemical, for which some toxic effects are known.

DEHP is metabolized and its metabolites are excreted in urine, and the metabolites have been used as markers of DEHP exposure. In the first step of this study, an analytical methodology to measure three DEHP metabolites in urine has been developed.

In a second step, the concentration of DEHP metabolites were evaluated in different population groups: healthy volunteers, patients receiving blood transfusions, hospitalized patients receiving medical care involving plastic materials, and, athletes.

The results indicated significantly higher concentrations of DEHP metabolites in subjects submitted to blood transfusion in comparison to the other population groups. These differences suggest that DEHP metabolites could be selective markers of the blood transfusion misuse. This selective and rapid methodology could be applied as screening test in urine in the antidoping control laboratories to suspect those prohibited practices.

Introduction

The use of blood transfusion in sports is widely used and it is forbidden by the World Anti-Doping Agency. Nevertheless, the detection of these practices is limited by the need of blood sampling and the limitations of present methodologies. Thus, no method is available at present for the laboratory detection of the autologous blood transfusion [1,2].

Phthalates are used as plasticizers in polyvinyl chloride (PVC) plastic materials and humans can be exposed to DEHP through different products [3,4]. The blood bags used to store blood for transfusion contain this plasticizer; therefore transfused subjects are additionally exposed to it [5].

DEHP is metabolized to mono-(2-ethylhexyl)phthalate (MEHP), mono-(2-ethyl-5-hydroxy-hexyl)phthalate (OH-MEHP), and mono-(2-ethyl-5-oxo-hexyl)phthalate (OXO-MEHP), and those metabolites are excreted in urine as glucuronide conjugates [6,7].

In this paper, the use of DEHP metabolites as markers of blood transfusion misuse has been evaluated.

Experimental

Subjects

Urinary concentrations of DEHP metabolites were evaluated in different populations groups: healthy subjects (Group 1, n=30, reflecting common environmental DEHP exposure); hospitalized patients exposed to different medical treatments involving plastic materials but not subjected to blood transfusions (Group 2, n=39); and hospitalized transfused subjects (Group 3, n=25). In groups 2 and 3, samples were collected for the periods from 0 to 24h and from 24 to 48h after the last exposition. Stored urine samples from actual doping control programs were also tested (Group 4, n=127).

Measurement of DEHP metabolites

Quantitative determination of MEHP, OH-MEHP, and OXO-MEHP in urine was performed by liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS).

To 1 mL of urine samples, the internal standards (MEHP$^{13}C_4$ and OXO-MEHP$^{13}C_4$, Cambridge Isotope Laboratories, Inc. Andover, MA, USA) were spiked, and the pH was adjusted to 6.5 with ammonium acetate buffer (250µL). After enzymatic hydrolysis with beta-glucuronidase, samples were acidified with 0.67 M phosphate buffer pH 2 (2 mL) and extracted with 8 mL of ethyl acetate for 20 min. The organic layers were evaporated to dryness under nitrogen stream and finally, the extracts were reconstituted with 100 µL of a mixture of deionized water:acetonitrile (80:20,v/v) and aliquots of 5 µL were analyzed by UPLC-MS/MS.

Chromatographic separation was carried out on a Waters Acquity UPLC system using an Acquity BEH C_{18} column (100 mm x 2.1 mm i.d., 1.7 µm particle size) (Waters Corporation, Milford, MA) with a mobile phase consisting of a mixture of water and acetonitrile with 0.01% formic acid and a gradient elution. The UPLC system was coupled to a Quattro Premier XE triple quadrupole mass spectrometer (Micromass, Waters Corp.) with an electrospray ionization source working in positive ionization mode and using argon and nitrogen gas as collision and desolvation gas, respectively. The following selective precursor and product ion transitions were monitored: 279>149, 293>127, 295>167, 283>153, and

297>127 for MEHP, OXO-MEHP, OH-MEHP, MEHP$^{13}C_4$, and MEOHP$^{13}C_4$, respectively. The mass spectrometric working parameters (cone voltage and collision energy) were optimized for each compound to maximize the signal detected.

Results

The method developed was validated. The extraction recoveries were all higher than 80% and the limits of quantitation were less than 5ng/mL for all the metabolites. A good linearity was obtained for the method. The intra-day and intermediate precision were estimated by using two quality control samples at two concentration levels (a low and a high control). The intra-day precision (expressed as the relative standard deviation,RSD, of three replicates) was less than 10 and 25% for the high and low control, respectively. The RSD obtained for the intermediate precision was less than 25% for all the metabolites.

To evaluate the exposition of the general population, samples of Group 1 were analyzed. The samples of Group 2 and 3 were tested to determine the DEHP metabolites concentrations after different medical treatments. Concentration of DEHP metabolites in samples of Groups 1, 2 and 3 are shown in the **Figure 1**. Samples corresponding to Group 1 had concentration below 50 ng/mL but an important increase of these levels was observed in urine samples of transfused subjects, Group 3, with concentrations around 200 ng/mL. In the case of samples of Group 2, the concentrations were statistically different from those of transfused patients ($p<0.001$) but not from the samples of healthy subjects ($p>0.001$). The concentration of MEHP in urine samples of Group 3 collected up to 2 days after blood transfusion showed remarkable differences as compared to samples of Group 1 and 2. In the case of OH-MEHP and OXO-MEHP significant higher concentrations have been detected in transfused subjects in samples collected from 0 to 24 h after the transfusion. Consequently, the accumulation of DEHP metabolites in the urine of transfused subjects was significantly higher than in the other groups. In **Figure 2** typical chromatograms obtained are presented and the different concentration of DEHP metabolites in samples of non-transfused and transfused subjects can be observed.

The results of the analysis of the samples of athletes indicate similar DEHP metabolites concentrations to those of the Group 1 and 2 samples, except for four samples (A,B,C,D in **Figure 3**) which appear as far-outliers in the boxplot figure.

Discussion

The work shows the potential use of DEHP metabolites as markers of DEHP exposure as a consequence of a blood transfusion process because high levels of DEHP metabolites appear in urine after this kind of invasive treatment. The athletes samples analyzed corroborate low levels of DEHP metabolites in urine of that characteristic population. When high concentrations are observed in an athlete's samples after the analysis here proposed (for example, subjects A,B,C,D in Figure 3) they could be regarded as a consequence of a potential blood transfusion process and a follow up of the case would be needed by more specific methods.

In conclusion, this approach opens the door to a suitable easy tool to suspect blood transfusion either autologous or homologous in athletes, using a cheap and simple sample preparation and analysis. This screening methodology may be applied to all urine samples submitted to doping control in any accredited laboratory.

Given that DEHP is widely used worldwide as plasticizer and different sources of DEHP exposure could increase the DEHP metabolites in urine [8,9], it could be useful to incorporate the basal concentrations of urinary DEHP metabolites into the individual athlete biological passport in order to detect sudden and unexplained increases of DEHP metabolites.

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Figure 1. Concentration of DEHP metabolites in urine samples: 1 *(healthy subjects),* ***2A*** *(hospitalized but not transfused subjects, 0 to 24 hours),* ***2B*** *(hospitalized but not transfused subjects, 24 to 48 hours),* ***3A*** *(transfused subjects, 0 to 24 hours),* ***3B*** *(transfused subjects, 24 to 48 hours). High concentrations in one subject corresponding to 3535 ng/mL, 5174 ng/mL and 2362 ng/mL (MEHP, OH-MEHP and OXO-MEHP, respectively) and one value of another subject (1972 ng/mL for OH-MEHP) are not shown in 3A and 3B respectively. . The median value is shown as a line across the box. The upper edge of the box indicates the 75th percentile of the data, and the lower edge indicates the 25th percentile. The whiskers indicate the minimum and maximum data values excluding the outliers. Small circles illustrate the outside values (values that are at the most 3 box lengths above the upper quartile). Asterisks indicate the far outside values (values that are at least 3 box lengths above the upper quartile).*

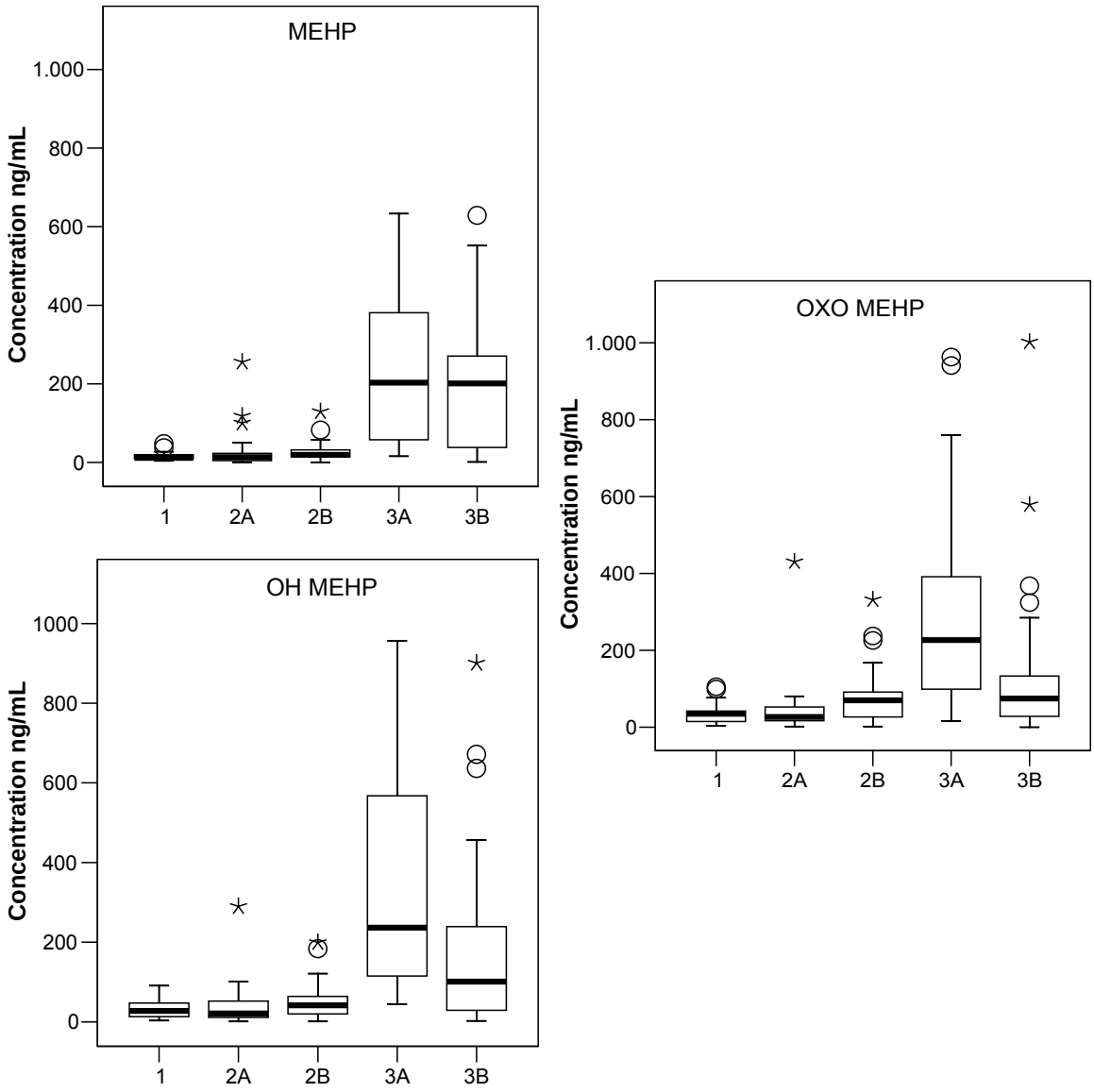

Figure 2. *Chromatograms of a blank sample corresponding to a healthy subject (left) and chromatograms of a typical urine sample of a transfused subject (right).*

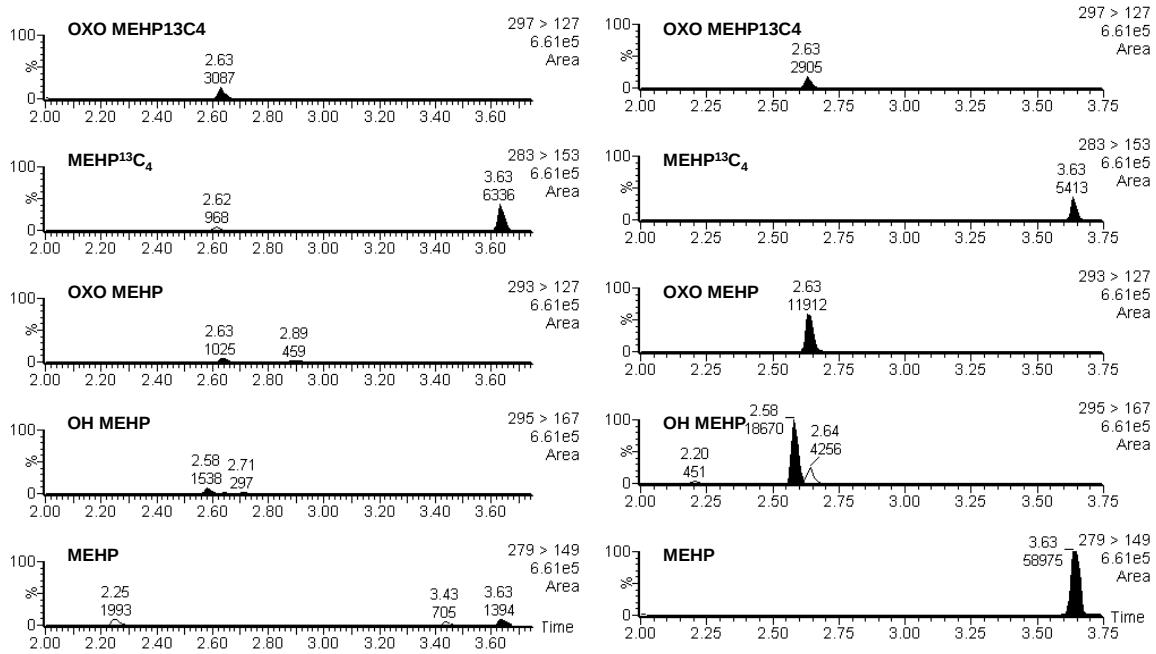

Figure 3. Concentrations of DEHP metabolites in urine samples of athletes compared with healthy subjects (Group 1). *Four subjects (A,B, C and D) in the athletes group presented outlier results in rewspect the full sportive population studied. The median value is shown as a line across the box. The upper edge of the box indicates the 75th percentile of the data, and the lower edge indicates the 25th percentile. The whiskers indicate the minimum and maximum data values excluding the outliers. Small circles illustrate the outside values (values that are at the most 3 box lengths above the upper quartile), Asterisks indicate the far outside values (values that are at least 3 box lenghts above the upper quartile). A, B, C, and D indicate four outlier subjects.*