Short communication

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Di(2-ethylhexyl) phthalate metabolites as markers for blood transfusion in doping control: Intra-individual variability of urinary concentrations

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To indicate homologous or autologous blood transfusion in sports drug testing, quantification of increased urinary concentrations of di(2-ethylhexyl) phthalate (DEHP) metabolites presents a promising approach; however, the possible intra-individual variation of the metabolite concentrations over time has not been well characterized.

The aim of this study was to explore the intra-individual variability of urinary DEHP metabolites among seven volunteers without special occupational exposure to DEHP during one week (n = 253) in order to investigate the possibility of increased urinary concentrations of the metabolites caused by, for example, residential, dietary, or environmental exposure.

Quantification of three DEHP metabolites – mono(2-ethylhexyl) phthalate, mono(2-ethyl-5-oxohexyl) phthalate, and mono (2-ethyl-5-hydroxyhexyl) phthalate – was accomplished after enzymatic hydrolysis of urinary glucuronide conjugates and direct injection using isotope-dilution liquid chromatography-tandem mass spectrometry.

Although urinary concentrations of DEHP metabolites showed considerable intra-individual variation, no increased values were observed comparable to the concentrations measured in urine specimens collected after blood transfusion.

Supporting information may be found in the online version of this article.

Keywords: DEHP metabolites; sports drug testing; liquid chromatography mass spectrometry; blood transfusion

Introduction

Di(2-ethylhexyl) phthalate (DEHP) is predominantly used as a plasticizer in the production of polyvinyl chloride (PVC) products to improve their flexibility. It is present in a variety of consumer products, building materials, and medical devices, such as blood bags and tubes. Since it is not chemically bound to PVC, it can easily leach into blood and blood products.[1]

In humans, DEHP is rapidly converted to its monoester, mono(2-ethylhexyl) phthalate (MEHP), which is further metabolized to several oxidative metabolites (phase-I biotransformation). The main secondary metabolites are mono(2-ethyl-5-hydroxyhexyl) phthalate (5OH-MEHP), mono(2-ethyl-5-oxohexyl) phthalate (5oxo-MEHP), mono-(2-ethyl-5-carboxypentyl) phthalate (5cx-MEPP), and mono-[2-(carboxymethyl) hexyl] phthalate (2cx-MMHP).[2] The metabolites are eliminated in urine after glucuronidation (phase-II biotransformation).[3,4] After exposure, the urinary concentration of MEHP peaked at 2 h, and those of the secondary metabolites at 4 h. The short half-life times of elimination and fast excretion of the metabolites can result in a high intra-individual variability of their urinary concentrations over time.

Determination of urinary concentrations of DEHP metabolites has a high potential to detect blood transfusion in sports drug testing and it can provide supporting evidence to prove blood doping.[5,6] However, only poor data on the possible variance of concentration levels within one subject is available. To investigate the possibility of increased DEHP exposure via non-medical routes, intra-individual variability of urinary DEHP metabolites was tested among seven volunteers during one week.

Experimental

Chemicals

MEHP, 5oxo-MEHP, 5OH-MEHP, $^{13}C_4$-MEHP and $^{13}C_4$-5oxo-MEHP were obtained from Cambridge Isotope Laboratories Inc. (Andover, MA, USA) and they were of analytical purity. β-Glucuronidase (from *E. coli* K12, 140 U/mL at 37.0 °C) was acquired from Roche Diagnostics GmbH (Mannheim, Germany). Acetic acid and ammonium acetate were purchased from Sigma (Steinheim, Germany). All reagents were of analytical grade. Acetonitrile (LC-MS grade) was supplied by VWR International GmbH (Darmstadt, Germany). Standard solutions and other aqueous solutions were prepared using deionised water (Sartorius Stedim Biotech S. A., Aubagne, France).

Study subjects

In order to determine the intra-individual variability of urinary concentrations of DEHP metabolites, urine samples were collected

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from seven healthy volunteers (3 female, 4 male; aged 27–37 years) during one week (29–45 samples per volunteer, n = 253). The samples were collected from different starting dates and during a different period of time (153 h–193 h) from each volunteer. The study was not a specially designed experiment. The volunteers did not follow any restrictions and did not answer questionnaires. All samples were stored in polyethylene bottles until analysis. An informed consent was obtained from each volunteer.

Analytical method

The analytical method used to quantify urinary DEHP metabolites has been described previously.[6] After enzymatic hydrolysis of the glucuronides, the samples were diluted and directly injected to the liquid chromatography-tandem mass spectrometry (LC-MS/MS) system. Analytes were recorded in multiple reaction monitoring (MRM) mode after negative electrospray ionization and quantified by isotope dilution utilizing an external calibration. Table 1 shows the chromatographic and mass spectrometric parameters for the analytes. Metabolites determined were MEHP, 5OH-MEHP, and 5oxo-MEHP. Quantification was performed for each sample using the peak area ratios of the quantifier ion transitions of the analytes and the respective internal standards (ISTDs; $^{13}C_4$-MEHP for MEHP, $^{13}C_4$-5oxo-MEHP for 5oxo-MEHP, and 5OHMEHP). Concentrations of DEHP metabolites were determined by linear regression derived from analyzing spiked blank urine samples at concentration levels of 1, 10, 50, 100, 150, 200, and 250 ng/ml. Due to the lack of phthalate-free urine matrices, the quantitative results were corrected to the amount of the respective analytes in the used blank matrix. All quantitative results were corrected for specific gravity of 1.020 g/ml according to World Anti-Doping Agency (WADA) guidelines,[7] including those samples with specific gravity lower than 1.020 g/ml. Limits of quantification (LOQ) were 1 ng/ml for all analytes.

Statistical evaluation

Statistical analyses were performed using STATISTICA software version 8.0 (StatSoft Inc., Tulsa, OK, USA). Phthalate metabolite concentrations below the LOQ were substituted with the value of the LOQ divided by two (LOQ/2). Within-subject variability was assessed using intra-class correlation. Intra-class correlation coefficients (ICCs) were calculated for each metabolite using random effects models, which were applied for the log-transformed data. As markers for reliability, ICCs range from 0 to 1, with values near 1 indicating high reliability.

Results and discussion

Within this study, the primary metabolite MEHP was quantified in 57% of the samples, the secondary metabolites 5oxo-MEHP and 5OH-MEHP could be quantified in 87% and 96% of the specimens, with maximum concentrations for MEHP, 5oxo-MEHP and 5OH-MEHP of 56 ng/ml, 78 ng/ml, and 154 ng/ml, respectively (Figure 1). The calculated median values ranged from 2.5 to 10.1 ng/ml for 5oxo-MEHP, from 10.8 to 22.9 ng/ml for 5OH-MEHP and from 1.0 to 3.8 ng/ml for MEHP, respectively.

In Figure 1, the urinary concentrations of MEHP, 5oxo-MEHP, and 5OH-MEHP corrected to specific gravity are plotted for each subject.

In accordance with earlier studies, moderate ICCs for the DEHP metabolites were observed, with calculated values of 0.43, 0.19, and 0.22 for MEHP, 5oxo-MEHP, and 5OH-MEHP for the concentrations corrected to specific gravity. The values indicate high within-subject variability and low reliability of the measurement over time. As illustrated in Figure 1, some of the studied subjects demonstrated only weak variability (volunteers 1–5) while others showed considerable variance (volunteers 6 and 7). No correlation between the elevated values of volunteers 6 and 7 could be identified.

Generally, the median values within this study were in agreement with earlier results, [5,6,8–12] although slightly higher median concentrations were observed in some cases.[13,14] Regarding the maximum concentrations, the values were consistent with our earlier findings.[6] However, some studies reported significantly higher concentrations. In 2007, Fromme *et al*. demonstrated a substantial within-subject variability of urinary DEHP metabolites by investigating 50 healthy volunteers (Munich, Germany), with maximum concentrations for 5oxo- and 5OH-MEHP ranged from 439.9 to 674.3 ng/ml for women and from 215.4 to 309.3 ng/ml for men.[13] Inter-individual variability of urinary DEHP metabolites was also studied by Preau *et al*. analyzing samples collected from eight volunteers (Atlanta, GA, USA) over one week. While some of the subjects demonstrated moderate intra-individual variability others showed substantial variance with maximum concentration for 5OH-MEHP of 706.3 μg/g creatinine.[15] Analyzing samples from 25 men working in dental laboratories (Seoul, Korea) with possible occupational exposure to DEHP a significant difference was found in the concentrations of urinary DEHP metabolites before and after work with maximum post-shift concentrations for 5oxo- and 5OH-MEHP of 97.9 and 276.0 ng/ml.[11] Contrarily, when analyzing first morning urine samples the level of DEHP metabolites was observed to be more reproducible over time.[10,16]

As described elsewhere, samples of transfused patients and reference populations were investigated, and the determined 99.9% upper reference values of an athletes' population ($n = 468$) provided values at 157.3 ng/ml for 5oxo-MEHP and 193.0 ng/ml for 5OH-MEHP.[6] In this work, the determined concentrations of each subject did not exceed this upper reference values.

Table 1. Summary of chromatographic and mass spectrometric parameters

Abbreviation	RT	Precursor Ion (m/z)	Product Ion I (m/z)	Collision Energy I (eV)	Product Ion II (m/z)	Collision Energy II (eV)	Product Ion III (m/z)	Collision Energy III (eV)	Declustering Potential (V)
MEHP	10.96	277	134	−22	127	−24	77	−36	−70
5oxo-MEHP	9.41	291	143	−20	121	−26	77	−46	−70
5OH-MEHP	9.25	293	121	−28	77	−46	145	−20	−70
$^{13}C_4$-MEHP	10.95	281	137	−22	79	−38	127	−24	−70
$^{13}C_4$-5oxo-MEHP	9.40	295	143	−20	124	−26	79	−46	−70

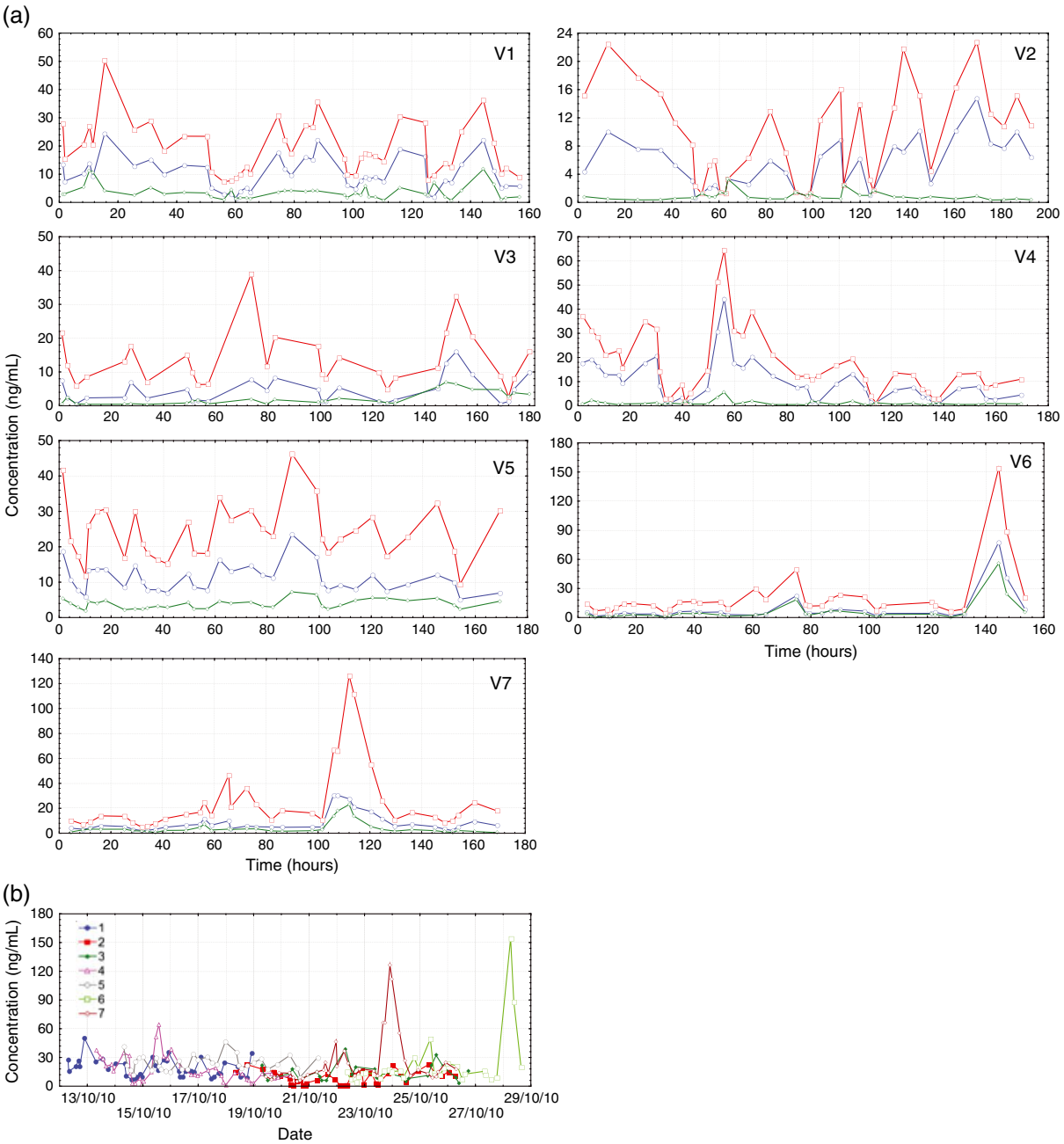

Figure 1. (a) Concentrations of 5oxo-MEHP (blue line), 5OH-MEHP (red line) and MEHP (green line) corrected to specific gravity in urine samples collected from 7 volunteers (V1-V7). (b) Gravity–adjusted concentrations of 5OH-MEHP represented in uniform time-scale for all volunteers.

Conclusion

Although urinary concentrations of DEHP metabolites showed considerable intra-individual variation, no values have been observed comparable to the concentrations measured in samples after blood transfusion (5OH-MEHP: from 876 to 8605 ng/ml, 5oxo-MEHP: from 741 to 4995 ng/ml[6]). The results confirm our previous findings that determination of the urinary concentration of DEHP metabolites has a high potential to indicate for homologous or autologous blood transfusion, and may provide supporting evidence to prove blood doping. Additionally, longitudinal studies would present valuable data that can be utilized for interpretation of abnormally high DEHP metabolite concentrations of athletes. It is noted, that in different countries there is a trend in substituting DEHP according to its toxicity. This may allow to suggest a lower general exposure in humans, and thus, decreasing urinary concentrations over time.

Supporting Information

Supporting information may be found in the online version of this article.

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References

[1] ATSDR. Toxicological Profile for Di (2-ethylhexyl) phthalate. US Department of Health and Human Services, Public Health Service, Agency for Toxic Substances and Disease Registry, Atlanta, GA, **2002**.
[2] P. W. Albro, R. Thomas, L. Fishbein. Metabolism of diethylhexyl phthalate by rats, isolation and characterisation of the urinary metabolite. *J. Chromatogr.* **1973**, *76*, 321.
[3] H. M. Koch, H. M. Bolt, J. Angerer. Di(2-ethylhexyl)phthalate (DEHP) metabolites in human urine and serum after a single oral dose of deuterium-labelled DEHP. *Arch. Toxicol.* **2004**, *78*, 123.
[4] M. J. Silva, D. B. Barr, J. A. Reidy, M. Kato, N. A. Malek, C. C. Hodge, D. Hurtz, A. M. Calafat, L. L. Needham, J. W. Brock. Glucuronidation patterns of common urinary and serum monoester phthalate metabolites. *Arch. Toxicol.* **2003**, *77*, 561.
[5] N. Monfort, R. Ventura, A. Latorre, V. Belalcazar, M. López, J. Segura. Urinary di-(2-ethylhexyl)phthalate metabolites in athletes as screening measure for illicit blood doping: a comparison study with patients receiving blood transfusion. *Transfusion* **2010**, *50*, 145.
[6] E. Solymos, S. Guddat, H. Geyer, U. Flenker, A. Thomas, J. Segura, R. Ventura, P. Platen, M. Schulte-Mattler, M. Thevis, W. Schänzer. Rapid determination of urinary di(2-ethylhexyl) phthalate metabolites based on liquid chromatography/tandem mass spectrometry as a marker for blood transfusion in sports drug testing. *Anal. Bioanal. Chem.* **2011**, *401*, 517.
[7] WADA website. Available at: http://wwwwada-amaorg/Documents/World_Anti-Doping_Program/WADP-IS-Laboratories/WADA_TD2009NA_ENpdf [November 2009].
[8] CDC. Fourth National Report on Human Exposure to Environmental Chemicals. U.S. Centers for Disease Control and Prevention, National Center for Environmental Health, Division of Laboratory Sciences, Atlanta, GA, **2009**.
[9] CDC. Fourth National Report on Human Exposure to Environmental Chemicals - Updated Tables. U.S. Centers for Disease Control and Prevention, National Center for Environmental Health, Division of Laboratory Sciences, Atlanta, GA, **2010**.
[10] J. D. Peck, A. M. Sweeney, E. Symanski, J. Gardiner, M. J. Silva, A. M. Calafat, S. L. Schantz. Intra- and inter-individual variability of urinary phthalate metabolite concentrations in hmong women of reproductive age. *J. Expo. Sci. Environ. Epidemiol.* **2010**, *20*, 90.
[11] M. S. Park, Y. J. Yang, Y. P. Hong, S. Y. Kim, Y. P. Lee. Assessment of Di (2-ethylhexyl) phthalate exposure by urinary metabolites as a function of sampling time. *J. Prev. Med. Public Health* **2010**, *1043*, 301.
[12] J. J. Adibi, R. M. Whyatt, P. L. Williams, A. M. Calafat, D. Camann, R. Herrick, H. Nelson, H. K. Bhat, F. P. Perera, M. J. Silva, R. Hause. Characterization of phthalate exposure among pregnant women assessed by repeat air and urine samples. *Environ. Health Perspect.* **2008**, *116*, 467.
[13] H. Fromme, G. Bolte, H. M. Koch, J. Angerer, S. Boehmer, H. Drexler, R. Mayer, B. Liebl. Occurrence and daily variation of phthalate metabolites in the urine of an adult population. *Int. J. Hyg. Envir. Heal.* **2007**, *210*, 1.
[14] H. M. Koch, B. Rossbach, H. Drexler, J. Angerer. Internal exposure of the general population to DEHP and other phthalates - determination of secondary and primary phthalate monoester metabolites in urine. *Environ. Res.* **2003**, *93*, 177.
[15] J. L. Preau Jr, L.-Y. Wong, M. J. Silva, L. L. Needham, A. M. Calafat. Variability over one week in the urinary concentrations of metabolites of diethyl phthalate and Di(2-Ethylhexyl) phthalate among 8 adults: an observational study. *Environ. Health Perspect.* **2010**, *118*, 1748.
[16] J. A. Hoppin, J. W. Brock, B. J. Davis, D. D. Baird. Reproducibility of urinary phthalate metabolites in first morning urine samples. *Environ. Health Perspect.* **2002**, *110*, 515.