Ph.D. THESIS ABSTRACT

DETERMINATION OF URINARY DI(2-ETHYLHEXYL) PHthalate METabolites AS MARKERS FOR BLOOD TRANSFUSION IN SPORTS DRUG TESTING

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1. PREFACE

Methods of blood doping such as autologous and homologous blood transfusions are some of the most challenging doping practices in competitive sports. Whereas homologous blood transfusion is detectable via minor blood antigens, the detection of autologous blood transfusion is still not feasible. Taking into consideration that for anti-doping testing mainly urinary sampling is conducted and existing methods are based on blood analysis the detection of blood transfusion is limited. Accordingly, it would be a great leap forward to detect both homologous and autologous blood transfusions from urine samples.

The most commonly used plasticiser in flexible polyvinyl chloride (PVC) products, frequently found in medical devices such as blood bags and tubes, is di(2-ethylhexyl) phthalate (DEHP). PVC medical devices contain up to 40% of DEHP by weight. Since DEHP is not chemically bound to the PVC it can easily migrate into the blood and blood products, such as red blood cells, whole blood, platelets and plasma. Therefore, patients receiving medical treatments such as blood transfusion, haemodialysis or nutritional support may be exposed to high amounts of DEHP.

Due to the toxic effects of DEHP, the concentrations of its metabolites in urine have been determined to evaluate the exposure of DEHP to children and the general population. In humans DEHP is rapidly converted into its primary monoester, mono(2-ethylhexyl) phthalate (MEHP), through phase-I biotransformation. Following a multistep oxidative pathway it is further metabolised mainly to mono(2-ethyl-5-hydroxyhexyl) phthalate (5OH-MEHP), mono(2-ethyl-5-oxohexyl) phthalate (5oxo-MEHP), mono(2-ethyl-5-carboxypentyl) phthalate and mono[2-(carboxymethyl)hexyl] phthalate. The metabolites are mainly eliminated as conjugates following phase-II glucuronidation with the highest urinary levels of 5OH-MEHP followed by 5oxo-MEHP and MEHP.

Monitoring the urinary concentrations of DEHP metabolites in order to test for blood transfusion in athletes was proposed by Monfort et al.\(^1\). Analysing urine samples for DEHP metabolites from patients subjected to clinical care or to blood transfusions and from an elite athlete population it was noticed that urine samples collected up to two days after blood

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transfusion contained significantly higher amounts of DEHP metabolites compared to a control group. This indicates that DEHP metabolites are good candidates to be used in sports drug testing as alert markers for the potential misuse of blood transfusion.

2. AIM

The overall aim of this thesis was to investigate the possibility of the detection of blood transfusion by testing athletes’ urine samples for DEHP metabolites. The main objectives were:

- To develop a straightforward and rapid assay for the identification and quantification of three major DEHP metabolites (MEHP, 5oxo-MEHP and 5OH-MEHP) in urine samples.
- To determine the levels of the DEHP metabolites in control samples, in athletes’ samples and in post-transfusion samples.
- To estimate an upper reference limit for the urinary concentrations of DEHP metabolites in subjects without extraordinary DEHP exposure.
- To investigate the possible intra-individual variability of the metabolites over time.
- To integrate the glucuronidated DEHP metabolites into existing screening procedure and apply the method for routine doping control samples.
3. METHODS

3.1. DETERMINATION OF URINARY DI(2-ETHYLHEXYL) PHTHALATE METABOLITES

3.1.1. Analytical procedure

The assay is based on isotope dilution liquid chromatography / electrospray ionisation tandem mass spectrometry (LC-(ESI)-MS/MS) using direct injection of urine specimens after enzymatic hydrolysis of urinary glucuronide conjugates.

A 1 mL aliquot of the urine sample was fortified with 100 ng of the internal standards ($^{13}$C$_4$-MEHP and $^{13}$C$_4$-5oxo-MEHP), then 25 µL of β-glucuronidase (140 U/mL at 37.0°C) was added. The enzymatic hydrolysis was carried out at room temperature within 10 min. The hydrolyzed samples were prepared by appropriate dilution (1:5, v:v) with a mixture of acetonitrile:water (1:1, v:v) and an aliquot of 10 µL was injected into the instrument.

Reversed phase liquid chromatography was performed on a Phenomenex Gemini C$_6$-phenyl column (100 x 2 mm; 3 µm) connected to a Phenomenex Gemini C$_6$-phenyl pre-column (2 x 4 mm). The mobile phase consisted of 5 mM ammonium acetate buffer containing 0.1% acetic acid (pH = 3.5, mobile phase A) and acetonitrile (mobile phase B). The flow rate was set to 0.25 mL/min. The gradient program started at 0% B, then increased to 100% B in 8 min and then was held at 100% B for 2 min. The analysis run time was 10 min with a 4.5 min post-run equilibration time resulting in a 14.5 min injection-to-injection duration.

Mass spectrometric detection was carried out using a hybrid triple quadrupole/linear ion trap mass spectrometer (AB SCIEX 5500 QTrap; Darmstadt, Germany) using negative electrospray ionisation in multiple reaction monitoring (MRM) mode. The ion source was operated at 400°C and the applied ionspray voltage was set to -4500 V. Collision energy and declustering potential were optimised for each analyte via direct injection of pure reference compounds using a 1 mL syringe at a flow rate of 10 µL/min. Enhanced product ion spectra were acquired using linear ion trap (LIT) mode.
For quantification purposes the most suitable ion transitions were \( m/z \) 277/134 for MEHP, \( m/z \) 291/143 for 5oxo-MEHP and \( m/z \) 293/121 for 5OH-MEHP. The chosen qualifier transitions were \( m/z \) 277/127 and \( m/z \) 277/77 for MEHP, \( m/z \) 291/121 and \( m/z \) 291/77 for 5oxo-MEHP, and \( m/z \) 293/77 and \( m/z \) 293/145 for 5OH-MEHP. Compound identification was performed based on the relative retention time and the relative ratios of three ion transitions for each analyte.

To enable quantification of the target compounds the peak area ratios of the quantifier ion transitions of the analytes and the respective internal standards (MEHP to \( ^{13}\)C₄-MEHP, 5oxo-MEHP and 5OH-MEHP to \( ^{13}\)C₄-5oxo-MEHP) were used. Calibration graphs were obtained by analysing spiked blank urine samples in the concentration range of 1-250 ng/mL. Samples with concentrations above the highest calibration point were diluted with water to fit the calibration range.

Due to the lack of phthalate-free urine matrices the quantitative results were corrected to the physiological amount of the respective analytes in the used blank matrix and adjusted to a standard urine density.

3.1.2. Study subjects

Urinary DEHP metabolites were measured in control groups without special exposure to DEHP (\( n = 100 \) from Cologne and \( n = 356 \) from Bochum), in athletes being subject to routine doping control (\( n = 468 \)) and in hospitalised patients receiving blood transfusions (\( n = 10 \) from Neuss and \( n = 25 \) from Bochum).

The log-transformed data of MEHP demonstrated a bimodal distribution compared to secondary metabolites that yielded approximate gaussianity. Additionally, the maximum concentration for MEHP in the investigated control group was higher than the minimum concentration determined in samples after blood transfusion. Thus, 5oxo-MEHP and 5OH-MEHP represented more appropriate markers to indicate blood transfusion.

This investigation demonstrates that significantly increased levels of secondary DEHP metabolites were found in urine samples collected within 24 hours after blood transfusion. The maximum concentrations of 5oxo-MEHP and 5OH-MEHP in the control samples were 18- and 13-times lower than the minimum concentrations determined after transfusion. The 99.9% upper reference limits of the athletes’ group were determined as 157.3 ng/mL for 5oxo-MEHP and 193.0 ng/mL for 5OH-MEHP which are 4- and 5-times lower than the lowest concentration received after blood transfusion. It is emphasised that this assay presents
additional data in the interpretation of the Athlete Biological Passport and it is not intended to be used separately as a proof of blood doping.

To investigate the possibility of increased urinary concentrations of the metabolites caused by e.g. residential, dietary or environmental exposure the intra-individual variability of urinary DEHP metabolites among seven volunteers without special occupational exposure to DEHP during one week ($n = 253$) was accomplished. ICCs calculated from the concentrations of DEHP metabolites corrected to specific gravity were 0.43, 0.19 and 0.22 for MEHP, 5oxo-MEHP and 5OH-MEHP respectively. Although the values indicate considerable intra-individual variation, no increased values have been observed comparable to the concentrations measured in urine specimens collected after blood transfusion. Additionally, longitudinal studies would present valuable data that can be utilised for interpretation of abnormally high DEHP metabolite concentrations in athletes.

### 3.2. INTEGRATION OF DI(2-ETHYLHEXYL) PHTHALATE METABOLITES INTO AN EXISTING SCREENING PROCEDURE FOR SPORTS DRUG TESTING

#### 3.2.1. Analytical procedure

The phthalate metabolites are excreted into urine mainly as conjugates following phase-II glucuronidation. To ensure compatibility with direct injection screening procedures and enable the comprehensive monitoring of concentration levels in routine doping control samples the implementation of glucuronidated DEHP metabolites as target analytes into a multi-target approach was required. The assay is based on LC-(ESI)-MS/MS using direct injection of urine specimens to screen for various classes of prohibited substances (diuretics, beta2-agonists, narcotics, stimulants and their sulfo-conjugates, plasma volume expanders, selective androgen receptor modulators, etc.)

An aliquot of 90 µL of urine sample was fortified with 50 ng of the internal standard mefruside the samples were then mixed, and an aliquot of 5 µL was injected into the instrument.

Chromatographic separation of target analytes was achieved on a Nucleodur C$_{18}$ Pyramid analytical column (50 x 2 mm, 3 µm particle size) connected to a Phenomenex Gemini C$_{6}$-phenyl (2 x 4 mm) pre-column. The mobile phase consisted of 5 mM ammonium acetate buffer containing 0.1% glacial acetic acid (pH = 3.5, mobile phase A) and acetonitrile
(mobile phase B). A linear gradient at a flow rate of 0.35 mL/min was employed starting at 0% B, increasing to 90% B within 4.5 min and then re-equilibrating (0.5 mL/min) at 0% B for 6.25 min. The overall runtime was 10.75 min injection-to-injection.

Tandem mass spectrometry was carried out using a hybrid triple quadrupole / linear ion trap mass spectrometer (AB Sciex 5500 QTrap) in MRM mode. Fast polarity switching electrospray ionisation was used with the following conditions: ionspray voltage +5500 V (positive) and -4500 V (negative), ion source temperature 450°C.

Due to the lack of reference material the glucuronidated DEHP metabolites were characterised and identified using liquid chromatography coupled to high resolution / high accuracy mass spectrometry (Exactive OrbiTrap®, Thermo Fisher). A post-transfusion sample was analysed containing known amounts of 5OH-MEHP and 5oxo-MEHP, in which the glucuronidated conjugates of 5OH-MEHP and 5oxo-MEHP were identified as deprotonated molecular ions [M-H]⁻ at m/z 469.1708 Da and 467.1559 Da with the calculated error of 1.48 ppm and 0.03 ppm, respectively.

For optimisation of the declustering potential and the collision energy solutions of pure reference compounds for each analyte were directly injected. For screening purposes the ion transitions at m/z 469/293 for 5OH-MEHP-gluc and at m/z 467/291 for 5oxo-MEHP-gluc were monitored.

### 3.2.2. Routine samples

Approximately 13,000 samples were tested for the glucuronide conjugates of DEHP metabolites over a year. From these samples, 160 samples were tested in order to quantify the unconjugated metabolites. In 44 cases the concentrations exceeded the reference limits calculated from the athletes’ population and the results were reported to the federations.
4. SCIENTIFIC RESULTS OF THE THESIS

1. Development and validation of an analytical procedure based on direct injection and LC-MS/MS determination for the identification and quantification of three main DEHP metabolites (MEHP, 5oxo-MEHP and 5OH-MEHP) in urine samples.

2. Comparison of DEHP metabolite concentrations in different reference populations and estimation of upper 99.9% reference limits for two DEHP metabolites (5oxo-MEHP and 5OH-MEHP).

3. Determination of intra-class correlation coefficients (ICC) to demonstrate the intra-individual variability of the three DEHP metabolites calculated from the urinary metabolite concentrations in seven volunteers in samples collected during a week.

4. Demonstration of the applicability of the procedure to detect abnormally high urinary DEHP metabolite concentrations as markers for autologous and homologous blood transfusions with the quantification of DEHP metabolites in post-transfusion samples and comparison of the metabolite concentrations and their ratios with the data calculated from the reference populations.

5. Integration of glucuronidated conjugates of the DEHP metabolites into existing screening procedure and the validation of the method for these analytes.

6. Demonstration of the suitability of the screening procedure for sports drug testing by analysing post-transfusion and routine doping control samples.
5. PUBLICATIONS RELATED TO THE THESIS

5.1. SCIENTIFIC PAPERS


5.2. PRESENTATIONS

E. Solymos, S. Guddat, H. Geyer, A. Thomas, M. Thevis, W. Schänzer
Intra-individual variability of urinary concentrations of di(2-ethylhexyl) phthalate metabolites (poster presentation)
29th Manfred Donike Cologne Workshop on Dope Analysis, 2011, Cologne, Germany

High-throughput screening for various classes of doping agents using a new „dilute-and-shoot” LC-(ESI)-MS/MS multi-target approach (oral presentation)
29th Manfred Donike Cologne Workshop on Dope Analysis, 2011, Cologne, Germany