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

Ph.D. Thesis

Emese Solymos

Supervisor: Dr. Kornél Torkos, C.Sc., habil. assoc. prof.

Institute of Chemistry, Eötvös Loránd University

Chemistry Doctoral School, Eötvös Loránd University

Director: Prof. Dr. György Inzelt, D.Sc.

Analytical, Colloid, Environmental and Electrochemical Doctoral Program

Program Leader: Prof. Dr. Gyula Záray, D.Sc.

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2. PREFACE

Patients receiving medical treatments such as blood transfusion, haemodialysis or nutritional support may be exposed to high amounts of plasticisers. 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) [1-3]. PVC medical devices contain up to 40% of DEHP by weight [4-6]. 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 [5-8].

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 [9-18]. 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 [19]. 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 [20-22].

Since increased urinary amounts of DEHP metabolites can result from blood transfusion, monitoring of concentration levels can be utilised as a marker for blood doping. Taking into consideration that for anti-doping testing mainly urinary sampling is conducted and existing methods are based on blood analysis and cover only homologous blood transfusion, the detection of blood transfusion is limited [23-28]. Accordingly, it would be a great leap forward to detect both homologous and autologous blood transfusions from urine samples. Monitoring urinary concentrations of DEHP metabolites in order to test for blood transfusion in athletes was proposed by Monfort *et al.* [29]. 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 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.

3. ABBREVIATIONS

$^{13}C_4$-5oxo-MEHP	$^{13}C_4$-mono(2-ethyl-5-oxohexyl) phthalate
$^{13}C_4$-MEHP	$^{13}C_4$-mono(2-ethylhexyl) phthalate
2cx-MMHP	mono[2-(carboxymethyl)hexyl] phthalate
5cx-MEPP	mono(2-ethyl-5-carboxypentyl) phthalate
5OH-MEHP	mono(2-ethyl-5-hydroxyhexyl) phthalate
5OH-MEHP-gluc	glucuronidated mono(2-ethyl-5-hydroxyhexyl) phthalate
5oxo-MEHP	mono(2-ethyl-5-oxohexyl) phthalate
5oxo-MEHP-gluc	glucuronidated mono(2-ethyl-5-oxohexyl) phthalate
ACD	acid-citrate-dextrose
AICAR	5-amino-4-imidazolecarboxyamide ribonucleoside
ASE	alkyl sulphonic acid phenyl ester
ATBC	acetyl tri-*n*-butyl citrate
ATP	adenosine triphosphate
BTHC	butyril trihexyl citrate,
CE	collision energy
CID	collision-induced dissociation
CPD	citrate-phosphate-dextrose
cps	counts per second
CV	coefficient of variation
DEHA	di(2-ethylhexyl) adipate
DEHP	di(2-ethylhexyl) phthalate
DEHT	di(2-ethylhexyl) terephthalate
DIDP	diisodecyl phthalate
DINCH	1,2-cyclohexanedicarboxylic acid diisononyl ester
DINP	diisononyl phthalate
DP	declustering potential
EAS-61	experimental additive solution-61
ECD	electron capture detector
ECMO	extracorporeal membrane oxygenation
EI	electron ionisation

ESI	electrospray ionisation
EU	European Union
EVA	ethylene vinyl acetate
FID	flame ionisation detector
GC	gas chromatography
Hb	haemoglobin
HPLC	high performance liquid chromatography
IC	in-competition
ICC	intraclass correlation coefficient
ISTD	internal standard
IV	intravenous
LC-MS/MS	liquid chromatography/tandem mass spectrometry
LIT	linear ion trap
LOD	limit of detection
LOQ	limit of quantification
MEHP	mono(2-ethylhexyl) phthalate
MRM	multiple reaction monitoring
MS	mass spectrometer / mass spectrometry
MS/MS	tandem mass spectrometry
NHANES	National Health and Nutrition Examination Survey
OOC	out-of-competition
PCI	positive chemical ionisation
PE	polyethylene
PEL	permissible exposure limit
PEO	polyethylene oxide
PO	polyolefin
PP	polypropylene
PU	polyurethane
PVE	plasma volume expander
QC	quality control
QQQ	triple quadrupole mass analyser
QTOF-MS	quadrupole time-of-flight mass spectrometry
QTrap	hybrid triple quadrupole/linear ion trap

RBC	red blood cell
RT	retention time
S/N	signal to noise ratio
SAGM	saline-adenine-glucose-mannitol
SPE	solid phase extraction
TEHTM	tri-(2-ethylhexyl) trimellitate
TOF	time-of-flight mass analyser
TPE	thermoplastic elastomers
TPN	total parenteral nutrition
TWA	time weighted average
UHPLC	ultra-high performance liquid chromatography
WADA	World Anti-Doping Agency

4. LITERATURE OVERVIEW

4.1. DI(2-ETHYLHEXYL) PHTHALATE

Phthalate ester plasticisers are used to impart flexibility, softness and extensibility to inherently rigid thermoplastic and thermoset resins [30]. The most commonly used phthalate ester plasticiser is di(2-ethylhexyl) phthalate (DEHP, Figure 4.1), which is mainly added to polyvinyl chloride (PVC), but also compatible with ethyl cellulose, cellulose nitrate and polystyrene [31]. More than 95% of the total amount of DEHP produced is used as a plasticiser in polymer products, while the rest is utilised for non-polymer applications. The content of DEHP in flexible polymer materials usually varies between 10 and 40% by weight. DEHP plasticised PVC is used in various products e.g. in building materials, in consumer products as well as in medical devices.
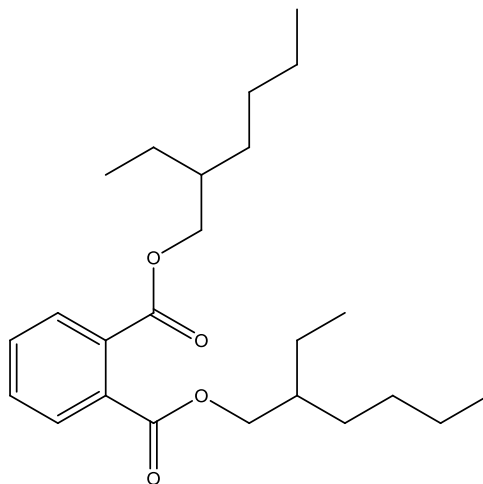
Figure 4.1: Chemical structure of di(2-ethylhexyl) phthalate (DEHP).

According to Annex I of Directive 67/548/EEC, DEHP is classified as a substance toxic to reproduction (Repr. Cat. 2; R60-61; May impair fertility; May cause harm to the unborn child) [32,33]. Since it is known that DEHP can be leached out from the polymer products the toxic effects of DEHP and its metabolites were studied to evaluate the human exposure and the possible sources of the plasticiser [1-4,7,34-37]. The main source was found to be food products, which can be contaminated through the manufacturing process or from the packaging material. Therefore, there are certain restrictions on the use of DEHP in food contact materials. It is not permitted for use in single-use applications, such as cap seals or gaskets. However, it can be safely and

legally used in non-fatty food contact materials for repeated use and as a technical support agent in concentrations up to 0,1 % in the final product, provided the migration of the plasticiser does not exceed the Substance Migration Limit (SML) of 1.5 mg/kg food [38,39]. Other possible sources are consumer products or indoor air and house dust contaminated from building materials. The EU Directive 2005/84/EC took effect in January 2007 and set the limit of DEHP in toys and childcare products at 0.1% by mass [40,41]. Other countries (US, Canada, Argentina, Brazil) have also set the same limit of DEHP in toys and children's products. DEHP is also prohibited in cosmetics in the European Union (EU) due to its classification as a reproductive toxicant [42,43].

4.1.1. Production and uses of di(2-ethylhexyl) phthalate

The market for DEHP has been decreasing over the last ten years. In 1997, the total Western European production of DEHP was 595,000 t/y and in the early 1990s DEHP represented about 51% of the total phthalate plasticiser market in the EU. Of the 341,000 tonnes produced in 2007, 187,000 tonnes were produced in Western Europe corresponding to 31% of the 1997 level [35]. The use of diisononyl phthalate (DINP) and diisodecyl phthalate (DIDP) have increased during the same period, indicating that DEHP has been replaced by DINP and DIDP in several applications. No data has been available for estimating the global production of DEHP. The content of DEHP is not known, but a rough estimate can be obtained assuming a phthalate content of the polymer of 30% and that DEHP accounts for 18% of the phthalates (EU manufacturing average). DEHP may be traded in end-product preparations such as sealants, adhesives and paint, but no information is available for estimating the DEHP content of these product groups [36].

The fate of the DEHP sent into circulation in the EU in 2007 is illustrated in Figure 4.2, where release from the use of end-products and disposal represent the total life-time emission of the articles produced in 2007 instead of total DEHP emission from end-products in the EU in 2007 [36]. The latter would depend on the total amount of DEHP accumulated in society and would probably be higher, as the amount of DEHP sent into circulation has been decreasing in recent years.
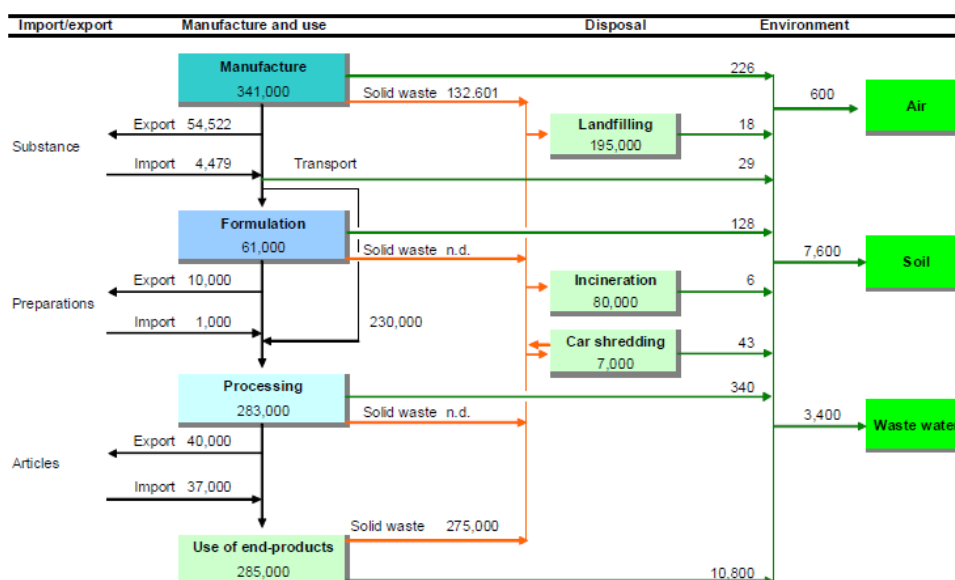
Figure 4.2: Overall flow of DEHP sent into circulation in the EU in 2007. Tonnes DEHP/year [36].

The manufactured DEHP is further processed in different formulation and processing steps, through which a wide range of end-products are produced as illustrated in the overview flow chart in Figure 4.3 [36].
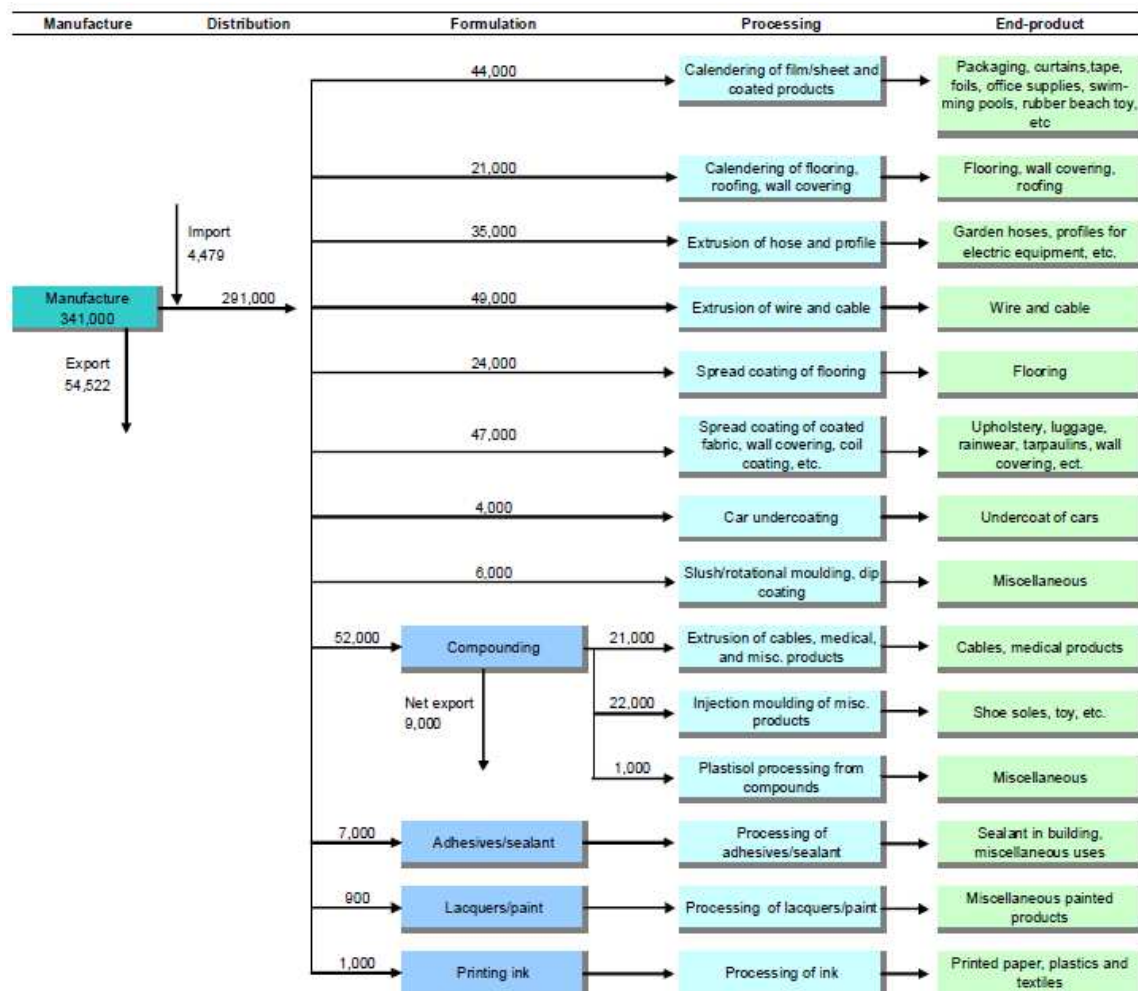
Figure 4.3: Overall flow of DEHP through manufacturing processes in the EU (tonnes DEHP/year) [36].

The main end-product uses of DEHP are as follows [35-37]:

Polymer applications:
- Flooring;
  - PVC flooring (with PVC surface);
  - Carpets with PVC back-coating;
  - Cork with PVC top-coating or back-coating;
- Wall covering;
- Roofing;
- Film/sheet and coated products:
  - Curtains, blinds, table linen, etc.;
  - Packaging;
  - Tape and self-adhesive foils;
  - Office supplies (ring binders, files, slip cases, etc.);
  - Toys (swimming pools, rubber beach toy, beach balls, etc.);
  - Medical bag/sheet devices;
  - Bottom sheets for hospitals;
- Wires and cables;
- Hoses and profiles;
  - Garden hoses and tubes;
  - Hoses and tubes in industry;
  - Profiles of windows and electrical products;
  - Medical tubing;
- Coated fabric;
  - Upholstery and car seats (synthetic leather);
  - Luggage;
  - Rainwear;
  - Tarpaulins;
  - Water beds;
- Moulded product;
  - Footwear;
  - Adult toys (DEHP is not permitted in toys for children);
- Car undercoating.

Non-polymer applications:
- Adhesives;
- Lacquers and paints;
- Printing inks (see comment below);
- Sealants (glass insulation, construction);
- Ceramics.

The estimated amount of DEHP in end-products marketed in the EU based on EU manufacture, import, export data is summarised in Table 4.1.

In the European Union, DEHP is not permitted for use in toys and childcare articles [40,41] or in cosmetics [42,43]. Additionally, there are certain restrictions on the use of DEHP in food contact materials [38,39].

Table 4.1: The estimated DEHP tonnage in end-products marketed in the EU based on EU manufacture, import and export data in 2007 (n.d.: no data) [36].

End-product use area	Tonnage (t/year)				% of total use
	EU Manufacture	Import	Export	End-product use	
Indoor uses					
Polymer applications					
Flooring	33,000	2,000	4,800	30,200	10.6
Wall covering	11,000	700	1,600	10,100	3.5
Film/sheet and coated products made by calendering	44,000	13,600	16,400	41,200	14.5
Wires and cables	52,000	6,200	5,600	52,600	18.5
Hoses and profiles	31,000	1,600	3,000	29,600	10.4
Coated fabric and other products from plastisol	31,000	2,200	1,400	31,800	11.2
Moulded products	3,000	2,700	700	5,000	1.8
Other polymer applications	12,300	10,900	3,100	20,100	7.1
Non-polymer applications					
Adhesives and sealant	4,000	n.d.	n.d.	4,000	1.4
Lacquers and paints	500	n.d.	n.d.	500	0.2
Printing ink	1,000	n.d.	n.d.	1,000	0.4
Other non-polymeric applications	20	n.d.	n.d.	20	0.0
Outdoor uses					
Polymer applications					
Calendered roofing material	600	n.d.	n.d.	600	0.2
Coil coated roofing material	3,000	n.d.	n.d.	3,000	1.1
Wire and cables – air	2,400	n.d.	n.d.	2,400	0.8
Wire and cables – soil	9,700	n.d.	n.d.	9,700	3.4
Coated fabric	12,800	n.d.	n.d.	12,800	4.5
Car undercoating	4,000	n.d.	n.d.	4,000	1.4
Hoses and profiles	3,700	n.d.	n.d.	3,700	1.3
Shoe soles	19,400	n.d.	n.d.	19,400	6.8
Non-polymer applications					
Lacquers and paints	400	n.d.	n.d.	400	0.1
Adhesives and sealant	3,300	n.d.	n.d.	3,300	1.2
Total end-product use (round)	282,000	40,000	37,000	285,000	100

The use in medical devices is estimated at 0.5% of the total DEHP production, of which the major use (more than 95%) is soft medical grade PVC in containers, flexible tubing and medical gloves [2]. The typical concentration of DEHP in plasticised PVC is approximately 30% (w/w).

The estimated releases from all activities are summarised in Table 4.2 [36]. The main releases are to soil and waste water. The use of end-products gives rise to the largest releases to the environment with washing of flooring, releases from underground cables and abrasive releases and pieces lost in the environment as the largest single sources. The releases from landfill may in fact be six times higher than indicated if total releases until the DEHP is ultimately degraded is considered, but no data on the long term fate of DEHP in landfills have been made available.

Table 4.2: Tonnage handled and releases of DEHP from manufacturing, formulation, processing, end-products use and disposal in the EU in 2007 [36].

Activity	Tonnage handled (t/year)	Emission to (t/year)		
		Air	Soil	Waste water
EU manufacture of DEHP	341,000	1	4	220
Transportation of substance from manufacturing [a]	345,479	0	0	29
Formulation	61,000	30	1	97
Processing	283,000	174	41	125
End-product uses, indoor	223,000	380	0	1,240
End-product uses, outdoor, non-abrasive	33,000	30	3,980	500
End-product uses, outdoor, abrasive leakages	33,000	5	3,500	1,200
Disposal and recycling operations	275,133	9	48	10
Total releases (round)		600	7,600	3,400

[a] The tonnage handled is the sum of EU production and import.

4.1.2. Occurrence of di(2-ethylhexyl) phthalate

4.1.2.1. Di(2-ethylhexyl) phthalate in the environment

Release of DEHP to the environment occurs during the production, transport, storage, formulation and processing of PVC. Since DEHP is not chemically bound to the polymer in flexible PVC the plasticiser will become released from the finished article during its use and after its final disposal.

DEHP enters the environment mainly via direct releases to air and waste water, from sewage sludge and from solid waste. In air, DEHP may occur both in the vapour phase and as solid particles. The nature of these particles can be either aggregated pure DEHP or polymer particles containing DEHP. Particles formed by weathering of polymer products probably represent an important route of DEHP distribution. It is estimated that around 800 industrial sites in the EU use DEHP or preparations containing DEHP [35]. Releases from these sources are expected to cause higher local exposure.

Table 4.3: Contribution to the total emissions of DEHP from different life-cycle stages [35].

Source	Emission contribution	Uncertainty in estimate	Emission type
Production	~2.5%	low	Point sources
Industrial uses	~2.5%	medium	Point sources
End-product uses	~32%	medium	Wide dispersive (and point sources)
Waste handling [a]	~63%	high	Wide dispersive (and point sources)

[a] Car shredding, incineration, landfills and waste remaining in the environment

The release of DEHP from industrial point source is low compared to the releases during end-product uses (Table 4.3). The primary recipients during production, formulation and processing are air and wastewater; while surface water and soil are exposed from outdoor use. Emission from car shredding, municipal landfills and incineration stations seem to be low and the main recipients are soil (car shredding), water (municipal landfills) and air (incineration). The low dissipation rate of DEHP in the municipal landfill environment will probably cause accumulation. The emissions from landfills may therefore increase in the future. Dominating sources are shoe soles, outdoor use of coil and fabric coatings and indoor floors. The primary recipients are expected to be soil followed by the aquatic compartment (mainly sediment) and to a lesser extent air. DEHP in waste formed from buried cables and demolished building material is assumed to

cause emission to ground water in urban areas. However, the uncertainty of these estimations is, high due to limited data. The overall distribution of DEHP is 2% to air, 21% to water and 77% to urban/industrial soil [35].

4.1.2.1.1. *Environmental fate of di(2-ethylhexyl) phthalate*

Photodegradation of DEHP (reaction with OH radicals) is important in the atmosphere ($t_{1/2}$ = 1 day) but is assumed to be of little importance in water and soil [35]. DEHP does not hydrolyse in water. The biodegradation of DEHP varies in available studies. Based on the results of standard biodegradation tests, DEHP is readily biodegradable. Experimental data indicates a biodegradation half-life for DEHP in surface water of 50 days and 300 days in aerobic sediment. Anaerobic conditions and low temperature further reduce the degradation rate. The primary biodegradation product of DEHP is mono(2-ethylhexyl) phthalate (MEHP).

DEHP is expected to be strongly adsorbed to organic matter and expected to be found in the solid organic phase in the environment. It will be strongly adsorbed to sludge in sewage treatment plants. DEHP has a low evaporation rate from its pure state and a moderate evaporation rate from a pure water solution ('semi-volatile').

DEHP is found to bioaccumulate in aquatic organisms. This indicates that uptake via the food chain might be an important exposure route.

4.1.2.1. Di(2-ethylhexyl) phthalate in food

Food is generally regarded as a major source of phthalate exposure in the general population. Contamination of food can occur during processing, handling, transportation, packaging and storage. There is a considerable difference in the degree of phthalate contamination of foods depending on packaging and processing practices and the lipid content [44]. The most important food sources of exposure are beverages, excluding water, dairy products, fats and oils, grains, milk and meat (Table 4.4) [45].

Table 4.4: Food concentrations of DEHP [3,45].

Food	Median concentration, µg/kg (Range)
Beverages	0.043 (0.006–1.7)
Cereal	0.05 (0.02–1.7)
Dairy (excluding milk)	0.96 (0.059–16.8)
Eggs	0.12 (<0.01–0.6)
Fats and oils	2.4 (0.7–11.9)
Fish	0.001 (0.00005-not given, 90th percentile 0.02)
Fruits	0.02 (<0.02–0.11)
Grains	0.14 (<0.1–1.5)
Meat, not processed	0.05 (<0.01–0.8)
Milk	0.035 (<0.005–1.4)
Nuts and beans	0.045 (<0.08–0.8)
Poultry	0.9 (0.05–2.6)
Processed meat	0.45 (<0.1–4.32)
Vegetables	0.048 (0.0098–2.2)
Infant formula, powdered	0.12 (<0.012–0.98)
Infant formula, liquid	0.006 (<0.005–0.15)
Breast milk	0.062 (0.01–0.6)
Baby food	0.12 (0.01–0.6)
Other food	0.05 (<0.01–25)

4.1.2.2. Di(2-ethylhexyl) phthalate in medical devices

PVC is ubiquitous in the health care environment. Due to of its properties, processability and relatively low cost, PVC is used in a wide range of products in hospitals. Some of them impact directly on patient care, whilst others contribute to the overall environment of the patient, including floor and wall coverings and clinical equipment.

The flexibility and barrier properties of plasticised PVC have resulted in extensive uses as tubes, sheets, containers and coverings. One of the oldest medical use of plasticised PVC is the blood bag. It remains the material of choice for the storage of blood and blood products, including red cell preparations and platelet rich plasma. It is also used in intravenous tubing for blood collection and infusion and in bags and tubes for the delivery of liquid food products. A wide variety of components are used to assist patients in respiration, including oxygen masks and tubes, endotracheal and tracheostomy tubes, nasal cannulas, humidifier equipment and resuscitator and ventilator components. Some major medical procedures aimed at short, medium or long-term functional assistance to organs involve PVC tubes and components. This includes extracorporeal membrane

oxygenation and haemodialysis. It is also used in umbilical vessel catheters, wound drainage tubes and osteotomy shunts. Examination gloves are used very extensively in clinical and laboratory procedures and they employ a wide variety of materials, including plasticised PVC [7].

Wahl *et al.* tested different phthalates in medical plastic articles and DEHP was found mainly in soft pliable PVC plastics used for invasive applications, such as dialysis tubing, infusion and blood storage bags and tubing (Table 4.5) [46].

Table 4.5: Phthalates identified in medical plastic articles[a] [46].

Plastic article	DEP	DIBP	DBP	BEP	DEHP
Eppendorf pipette tips	x		(x)		
Eppendorf cup	(x)		(x)		
Urine container 100 ml	(x)		(x)		
Urine container 500 ml	(x)		(x)		
Urine container 2500 ml	(x)		(x)		
Urine bag 1500 ml	(x)		(x)		x
Syringe 60 ml	(x)		(x)		(x)
Insulin syringe	(x)		(x)		
Heparin syringe	(x)		(x)		x
Microfilter 40 µl	x		x	(x)	x
Serum monovette	(x)		(x)		
Butterfly	x			x	x
Luerlock obturator	(x)		(x)		x
Infusion tubing	(x)	x	x	x	xx
Infusion bag	(x)		x	(x)	xx
Blood storage bag					xx
Blood infusion tubing	(x)		(x)	x	xx
Intestinal tubing	x		x		x
Dialysis tubing	(x)		(x)		xx

[a] (x) < 1%, x < 20% and xx > 85% of total volatiles.
DEP: diethyl phthalate, DIBP: diisobutyl phthalate, DBP: dibutyl phthalate,
BEP: butyl 2-ethylhexyl phthalate, DEHP: di(2-ethylhexyl) phthalate.

Quantitative information of the amount of plasticised PVC used for medical devices is not available. Medical applications account for 0.5% of the total PVC volume used in Western Europe [47].

4.1.2.3. Di(2-ethylhexyl) phthalate in blood and blood products

4.1.2.3.1. *Accumulation of DEHP during storage of blood*

It is well established that DEHP could leach out from PVC bags and accumulate in the stored blood and blood products [48-59]. While plasticisers have very low solubility in water (0.01 mg/100 mL at 25°C), DEHP is accumulated in whole blood (stored in PVC bag) at a rate of 0.25 mg/100 mL blood/day during storage at 4°C, to a level of 5-7 mg/100 mL of blood at the end of 21 days [51,52,58].

The extraction of DEHP into blood products is biphasic [60]. The rate is maximal during the first few hours when the blood is handled at a higher temperature and is moved over new surfaces in handling. After this, when the blood is stored at refrigerated temperatures and the surfaces area of the plastic is held constant, the rate of extraction becomes much lower.

Essentially, all DEHP in blood is protein bound with about three-quarters being bound to lipoprotein [61]. Fractionation of the blood indicated that the plasticiser was located almost exclusively in the plasma fraction and was specifically associated with the lipoprotein fraction of plasma [51]. Proteins such as albumin, fibrinogen, plasminogen and immunoglobulin G have very low affinities for DEHP in comparison with plasma [62]. Several early reports indicated that the amount of DEHP that leaches from transfusion packs correlated with triglyceride concentrations in the plasma [63-65]. DEHP added to the plasma tended to migrate to the areas of low and very low density lipoproteins, thus supporting the suggestion that DEHP may be solubilised and transported in blood in a manner similar to the triglycerides. DEHP is also found in platelet concentrates stored in PVC bags (approximately 11% in the platelet pellet and 89% in the supernatant) [54].

Rock *et al.* demonstrated that there is a constant hydrolysis of DEHP to MEHP in stored whole blood [56]. The accumulation of DEHP and MEHP continued throughout the storage of whole blood, platelet-rich plasma, platelet concentrates and platelet-poor plasma. The levels of DEHP were much higher than those of MEHP and the highest concentrations were detected in platelet-poor plasma. The authors concluded that the accumulation of MEHP was due to the hydrolysis of DEHP by plasma proteins rather than by leaching from the blood bags. Similar conclusions were reached by Peck *et al.* [57]. In addition, they observed that the storage temperature affects not only the extent of

leaching but activity of the enzyme, however, DEHP continues to be hydrolysed to MEHP even in the frozen state [66].

The distribution of MEHP in the plasma protein fractions was different from that of DEHP. MEHP is bound principally to non-lipoprotein constituents in the serum and this binding distribution is unaffected by lipid concentration [65]. The supernatant contained more MEHP and measurable amounts of MEHP were found in the albumin fraction [62]. Miripol *et al.* reported that irrespective of whether whole blood or packed cells were stored in acid-citrate-dextrose (ACD)-adenine or citrate-phosphate-dextrose (CPD)-adenine solution in PVC bags, there was a continuing and increasing level of DEHP observed [67]. However, in packed cells the levels of DEHP were lower by a factor of about three. Given that plasma proteins and not erythrocytes provide the major binding sites for DEHP. Similar data were obtained by Peck *et al.* [57]. They also found that the accumulation of DEHP may be related to the increase in ratio of the plastic surface area to the volume of plasma in red blood cell (RBC) concentrates. Gulliksson *et al.* studied the effect of decreased plasma residue and replacement solutions on the extraction of materials from plastic storage bags into RBC concentrates [68]. Significantly less accumulation of DEHP per unit was observed in RBC components resuspended in saline-adenine-glucose-mannitol (SAGM) medium compared to CPD-adenine solution. It was observed that the final level of DEHP appeared to be related to the amount of residual plasma, rather than to the volume of SAGM added.

Although, earlier reports had not found significant amounts of DEHP taken up by RBCs, Rock *et al.* reported that labelled DEHP was incorporated into RBC concentrates stored at 4°C [69]. There was an immediate binding of 28% of the available ^{14}C-DEHP into the RBCs on day 0, with approximately equal amounts being incorporated into the cytosol and membrane fractions. The total amount and relative distribution of the ^{14}C-DEHP did not change significantly over 7 days. Since earlier studies examined RBCs stored as whole blood, not RBC concentrates and DEHP which is taken up by the lipid fraction of plasma, much of the DEHP in the former experiments may have preferentially bound to the plasma lipoproteins. In the case of RBC concentrates, limited plasma is available, thus favouring association of the DEHP with RBCs. This difference has important implications since in the majority of blood banks RBC concentrates are stored rather than whole blood.

Rock *et al*. also found that DEHP leaches also into platelet concentrates and that 6-10% of the labelled DEHP added might bind to the platelets [70]. They also suggested that

DEHP might be involved in the loss of the platelet function during storage and affect the viability of stored platelets. DEHP bound to the platelets was found to be 90% in the membrane and 10% in the cytosol. The binding was reversible – DEHP was lost on resuspension of the platelets into new plasma [71].

4.1.2.3.2. *Effect of plasticisers on erythrocytes*

It was subsequently shown that the DEHP improves RBC storage by reducing haemolysis and membrane loss by microvesiculation [72,73]. Other plastics, such as polyolefins (PO), which are used in platelet storage bags because of their high gas permeability, were associated with greater RBC haemolysis and microvesiculation when used in conjunction with standard storage solutions. However, the addition of DEHP or similar plasticisers to the RBCs stored in PO bags resulted in a better survival rate of the erythrocytes [69,74,75]. Similar results were found using glass storage tubes supporting the idea that DEHP has a beneficial influence on the RBCs. This excludes the possibility that PO bags might adversely affect them [69]. Without the protection afforded by the plasticiser, RBC storage would be limited to 3 weeks by using conventional storage solutions [76,77].

Hess *et al.* developed an additive storage solution, Experimental Additive Solution-61 (EAS-61), in which RBCs can be stored for 9 weeks in PVC bags [78]. EAS-61 works, in part, by suppressing RBC microvesiculation and haemolysis. The increased volume of additive solution also seemed to be important for raising adenosine triphosphate (ATP) levels in RBC concentrates, improving RBC morphology and reducing haemolysis [79]. To evaluate whether EAS-61 might suppress RBC haemolysis during storage in a different plastic and to learn more about cellular mechanisms important for blood storage, Hill *et al.* conducted a study comparing RBCs stored in PVC and PO containers in 100 and 200 ml volumes of EAS-61 [80]. Although haemolysis of RBCs stored in EAS-61 was below conventional limits for at least 6 weeks, RBCs stored in PO bags had four times more haemolysis and lower RBC ATP concentrations than those stored in PVC. When DEHP was added to RBC stored in the PO bags, the degree of shape change, microvesiculation and haemolysis were reduced. Exposure of warm blood to PO bags caused a rapid initial shape change which persisted but progressed at a slower rate after the RBCs were transferred to PVC bags. This result suggests that DEHP can suppress further shape change and haemolysis, even after the processes have been initiated.

Several studies investigated the possible preservation role of butyril trihexyl citrate (BTHC) in RBC concentrates stored in BTHC-plasticised PVC bags [81-83]. Furthermore, it was found to be suitable for the storage of platelets [84,85]. Chemically, BTHC is esterified citric acid. The central hydroxyl group is esterified with butyrate, while the three carboxyl groups are esterified with hexanol. During metabolism it is hydrolysed to hexanol, butyric acid and citric acid. Straight-chain alcohols (such as hexanol) were found to expand RBCs ghost membrane areas sufficiently to inhibit haemolysis [86-88]. It was also reported that hexanol (144.6 µg/mL) found to suppress haemolysis and vesiculation of RBCs during storage, which supports that the hexanol is a key component of BTHC [89].
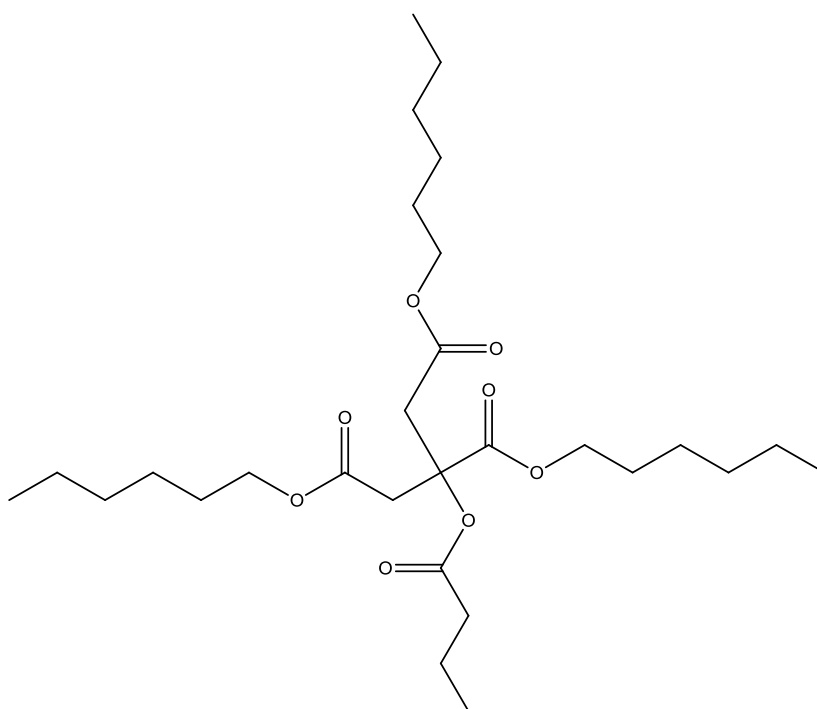
Figure 4.4: Chemical structure of butyril trihexyl citrate (BTHC).

4.1.3. Pharmacokinetics of di(2-ethylhexyl) phthalate

The metabolism and excretion of DEHP was extensively studied in rats whereas only a limited number of studies are available on humans.

After oral uptake, DEHP is enzymatically hydrolysed into its primary monoester, mono(2-ethylhexyl) phthalate (MEHP), partially in the mouth by salivary esterase [90], then mainly in the gastrointestinal tract through phase-I biotransformation [91,92]. Therefore, the majority of DEHP is rapidly adsorbed as MEHP in the gut [2]. DEHP hydrolysing lipases can be found in many tissues (especially in the pancreas, intestinal mucosa, liver) and in blood plasma [92,93]. Following a multistep oxidative pathway MEHP is further metabolised in the liver [94]. The hydroxylation of the alkyl chain at various positions results in the formation of primary (ω-oxidation) and secondary alcohols (ω-1 and ω-2 oxidation) which can be further oxidised to ketones and carboxylic acids. The carboxylated alkyl chain can undergo α- or β-oxidation yielding shorter carboxylated alkyl chains [91,95-97].

Most of the orally administered DEHP is systemically absorbed in humans and excreted in urine in the form of the five major metabolites (mono(2-ethyl-5-hydroxyhexyl) phthalate (5OH-MEHP), mono(2-ethyl-5-carboxypentyl) phthalate (5cx-MEPP), mono(2-ethyl-5-oxohexyl) phthalate (5oxo-MEHP), mono(2-ethylhexyl) phthalate (MEHP) and mono[2-(carboxymethyl)hexyl] phthalate (2cx-MMHP) as illustrated in Figure 4.7. The major metabolite in urine detectable up to 12 h after oral administration of D^4-DEHP was 5OH-MEHP, followed by 5oxo-MEHP and MEHP; after 12 h it was 5cx-MEPP and after 24 h it was 2cx-MMHP (Figure 4.5) [22,98]. Excretion in urine follows a multiphase elimination pattern. After an absorption and distribution phase of 4 to 8 h the half-lives of excretion in the first elimination phase (until 14-16 h after administration) were approximately 2 h for MEHP with slightly higher half-lives for 5OH-MEHP and 5oxo-MEHP. The elimination half-lives of 5cx-MEPP and 2cx-MMHP were between 15 and 24 h. In the second phase, beginning 14 to 18 h post dose, half-lives were 5 h for MEHP and 10 h for 5OH-MEHP and 5oxo-MEHP. In the time window 36 to 44 h, no decrease in the excreted concentrations of 5OH-MEHP and 5oxo-MEHP was observed.

In the first elimination phase (8 to 14 h post dose), the mean excretion ratios of MEHP to 5oxo-MEHP and MEHP to 5OH-MEHP were 1 to 1.8 and 1 to 3.1, respectively. In the

second elimination phase (up to 24 h post dose) mean excretion ratios of MEHP to 5oxo-MEHP to 5OH-MEHP changed to 1 to 5.0 to 9.3, respectively. The excretion ratio of 5OH-MEHP to 5oxo-MEHP remained constant throughout at 1.7. The respective half-lives of the metabolites in serum were estimated to be less than 2 h except for 2cx-MMHP, for which the half-life was at least 5 h. In contrast to urine the major DEHP metabolite in serum was MEHP.

After 24 h, 67.0% (range: 65.8-70.5%) of the DEHP dose was excreted in urine, comprising 5OH-MEHP (23.3%), 5cx-MEPP (18.5%), 5oxo-MEHP (15.0%), MEHP (5.9%) and 2cx-MMHP (4.2%) [98]. An additional 3.8% of the DEHP dose was excreted on the second day, comprising 2cx-MMHP (1.6%), 5cx-MEPP (1.2%), 5OH-MEHP (0.6%) and 5oxo-MEHP (0.4%). In total, about 75% of the administered DEHP dose was excreted in urine within two days. No dose dependency in metabolism and excretion was observed. 5OH-MEHP and 5oxo-MEHP in urine reflect short-term and 5cx-MEPP and 2cx-MMHP long-term exposure.

First-morning urine samples collected from children showed decreasing ratios of the oxidative metabolites 5OH-MEHP, 5oxo-MEHP relative to the monoester (MEHP) with increasing age [10,99]. This might indicate an enhanced oxidative metabolism in children.
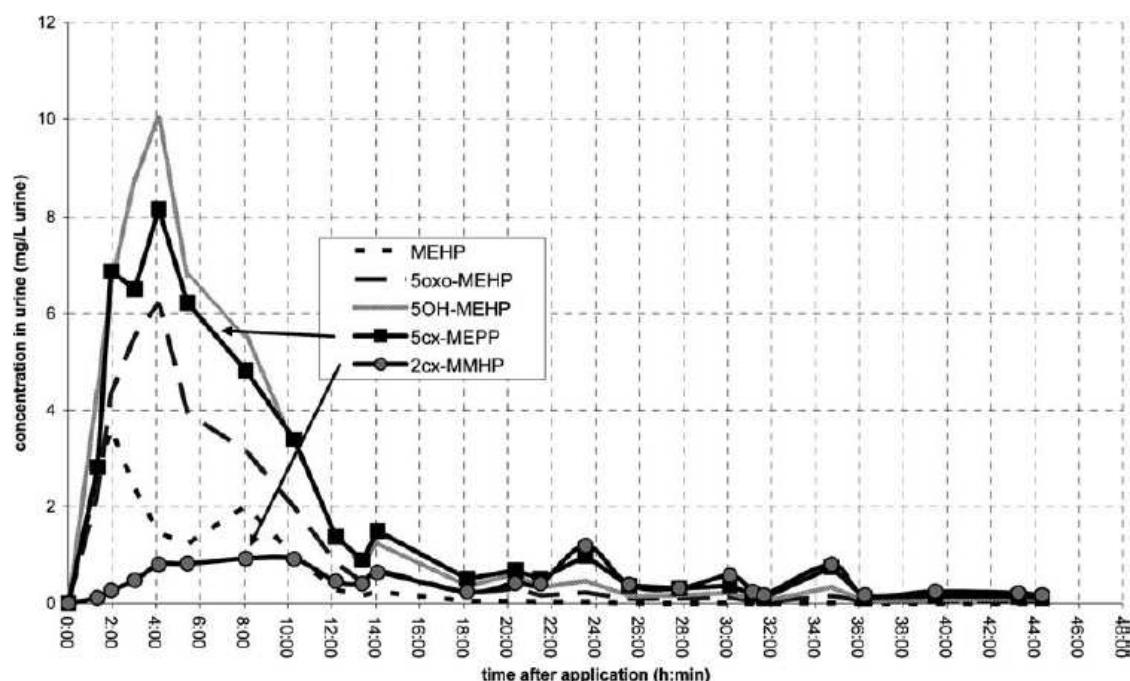
Figure 4.5: Time course of the excretion of the DEHP metabolites in urine (mg/L) after a high dose of D$_4$-DEHP administered orally [98].

After intravenous exposure to DEHP a sharp increase in urinary DEHP metabolite concentrations were observed [100-102]. Maximum concentrations of 5OH-MEHP, 5oxo-MEHP, 5cx-MEPP and MEHP were observed 4 h after the procedure. 2cx-MMHP was excreted at highest concentrations after 8 h (Figure 4.6). As an indication of longer elimination half-lives, the major metabolites excreted in urine 24 h after the exposure were 5cx-MEPP and 2cx-MMHP [103]. The elimination characteristics and relative distribution of the DEHP metabolites in urine were found to be similar to that after oral administration, which indicates that the toxicokinetic behaviour of DEHP in humans appears to be qualitatively unaffected by the route of administration [2,103].
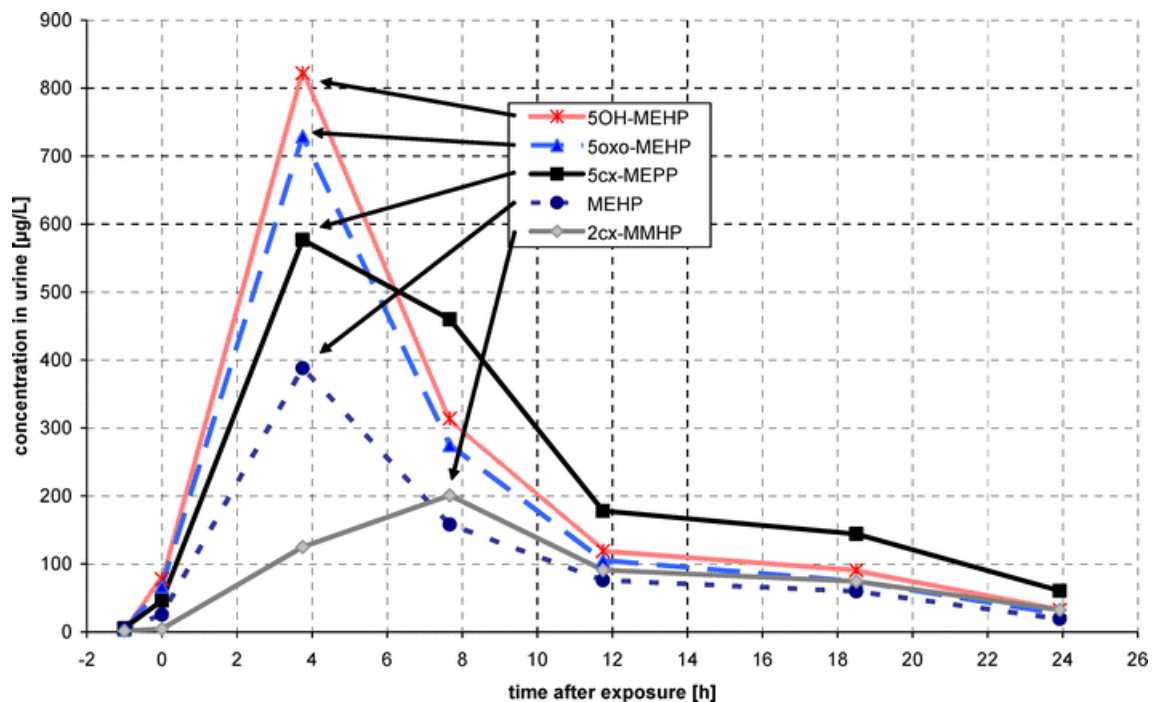
Figure 4.6: Time course of excretion of the DEHP metabolites in urine (µg/l) after intravenous exposure to DEHP [103].

Athletes are thought to have blood transfusions only a short time before competition, therefore the measurement of DEHP metabolites provides a sufficient detection window to test for blood doping. The most suitable metabolites are the oxidative secondary metabolites, such as 5OH-MEHP and 5oxo-MEHP. Although the elimination rate of 5cx-MEPP is higher than the elimination rate of 5oxo-MEHP, its elimination is slower, therefore the concentration of 5oxo-MEHP is higher in spot urine samples collected after administration.

25

In humans, at least 65% of metabolites are eliminated as conjugates following phase-II glucuronidation [91,97,20]. While the carboxylic acid metabolites were found to be excreted only partially in their glucuronidated form, the alcohol and ketone metabolites are excreted mainly as glucuronic acid conjugates [2,104].

The data regarding metabolism and bioavailability following inhalation and dermal exposure are limited. It can be assumed that only a fraction of the amount inhaled will be available to the lungs while the majority will probably be swallowed and become orally bioavailable. The dermal absorption appears to be poor in human [2,35].
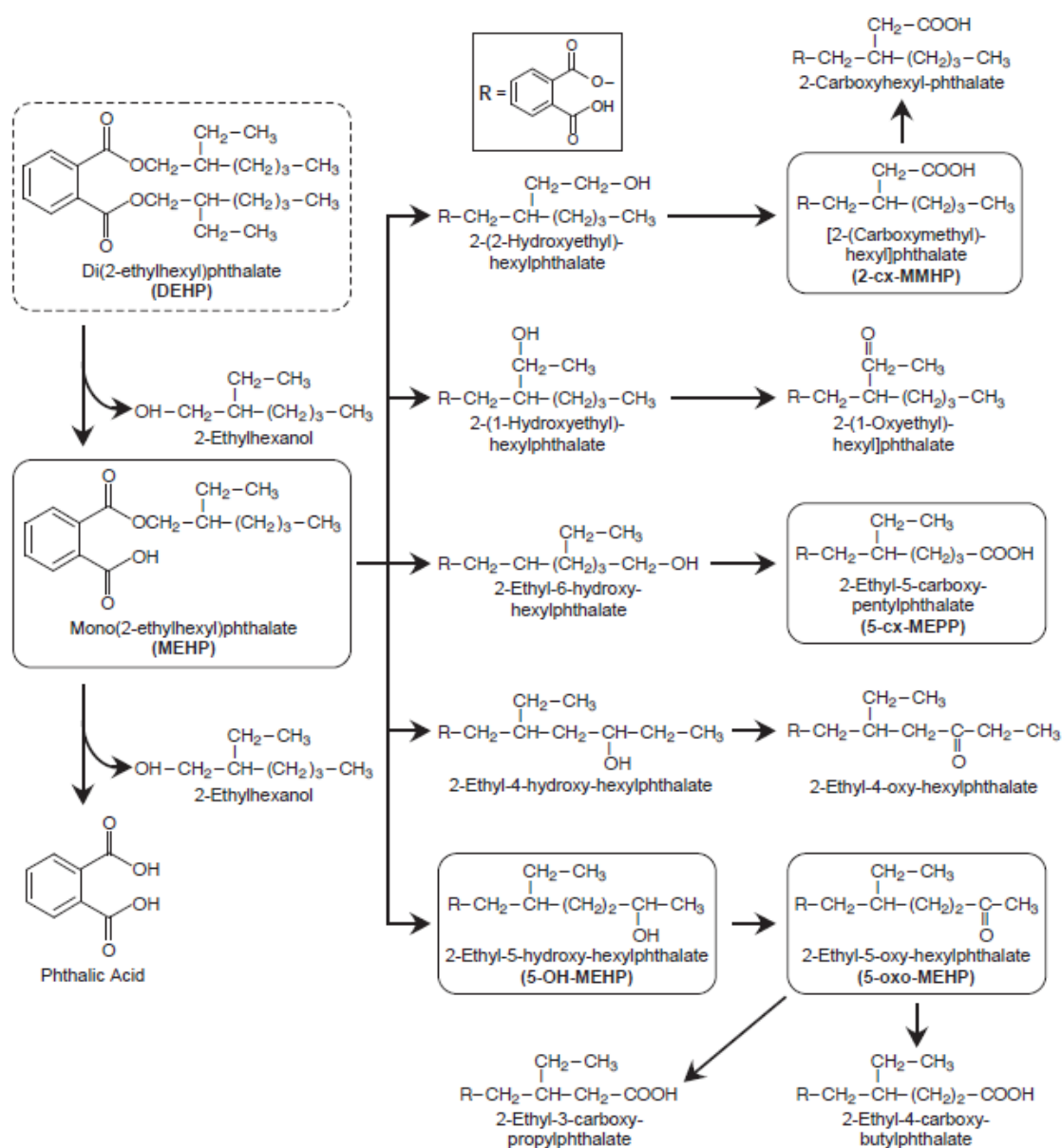
Figure 4.7: DEHP metabolism in humans [3,15,91,97].

4.1.4. Di(2-ethylhexyl) phthalate exposure

4.1.4.1. Di(2-ethylhexyl) phthalate exposure of the general population

Humans are exposed to DEHP via oral, dermal and inhalation routes of exposure. General population intake estimates for DEHP have been developed using probabilistic analysis [45]. The average level of exposure to DEHP from all sources in the general population has been estimated to be in the range of 3 to 30 μg/kg body weight/day [7]. Dietary intake has been identified as an important route of exposure [45,105]. More than 90% of estimated daily DEHP intake in people over the age of 6 months is from food. Estimated DEHP intakes by age group and from different sources of exposure are shown in Table 4.6 and Table 4.7. It was noted that exposure estimates, back-calculated based on measurements of urinary metabolites, gave lower estimates of daily intake compared to probabilistic analysis. They suggested that the current study may have overestimated food exposure to DEHP due to the use of outdated food measurements or due to failure to account for cooking-associated loss of DEHP in food. Nevertheless, important differences exist among populations and individuals associated with various dietary habits and lifestyles; therefore, in certain cases daily intakes can reach the order of a few mg/kg [7,45].

Table 4.6: Estimated DEHP intake by age group [45].

Age group	Median DEHP intake (μg/kg/day)
Adult (20-70 years)	8.2
Teen (12-19 years)	10
Child (5-11 years)	18.9
Toddler (7 months-4 years)	25.8
Infant (0-6 months)	
Formula-fed	5.0
Breast-fed	7.3

DEHP has also been shown to be a constituent of dust in households. Ingested dust represents the most important non-food source of exposure, accounting for 4.3% of total exposure to DEHP. Inhalation of indoor air represents approximately 1% of exposure. Fromme *et al.* found 775.5 mg/kg DEHP in dust collected from 30 apartments in Germany [105]. Bornehag *et al.* investigated 346 dust samples from children's bedrooms

in Denmark and the geometric mean of DEHP concentration was 789 mg/kg of dust [106].

Table 4.7: DEHP Intake (µg/kg/day) from environmental and food sources [45].

Source	Adult (20-70 years)	Teen (12-19 years)	Child (5-11 years)	Toddler (7 months-4 years)	Infant (0-6 months)	
					Formula-fed	Breast-fed
Outdoor air	0.0	0.0	0.0	0.0	0.1	0.0
Indoor air	1.0	0.9	1.0	0.9	1.5	1.1
Drinking water	0.1	0.1	0.1	0.1	0.7	0.0
Ingested soil	0.0	0.0	0.0	0.0	0.0	0.0
Ingested dust	4.3	4.2	5.0	6.6	54.1	39.3
Beverages [a]	11.2	5.2	3.3	2.2	0.0	0.0
Cereals	2.4	2.0	3.5	5.5	0.0	0.0
Dairy products [b]	13.2	11.7	12.2	12.9	0.0	0.0
Eggs	1.1	0.7	0.8	1.3	0.0	0.0
Fats and oils	16.9	19.1	16.5	11.1	0.0	0.0
Fish	1.6	0.8	0.7	0.4	0.0	0.0
Fruit products	0.9	0.8	1.1	1.4	0.0	0.0
Grains	13.4	16.6	18.1	11.1	0.0	0.0
Meats	5.5	5.2	3.7	3.3	0.0	0.0
Milk	3.1	6.7	8.6	12.6	0.0	0.0
Nuts and beans	1.0	1.0	0.9	0.8	0.0	0.0
Other foods	10.3	11.2	11.3	18.9	0.0	0.0
Poultry	3.9	3.5	3.5	3.6	0.0	0.0
Processed meats	3.4	3.4	3.4	2.5	0.0	0.0
Vegetable products	6.6	6.1	6.1	4.9	0.0	0.0
Formula/breast milk	–	–	–	–	43.7	59.6

[a] Excluding water.
[b] Excluding milk.

The EU Scientific Committee on Food (SCF) has recommended a tolerable daily intake (TDI) for DEHP of 50 µg/kg/day [107]. Likewise, for the exposure of children to DEHP from PVC toys a TDI of 50 µg/kg/day was estimated by the EU Scientific Committee on Toxicity, Ecotoxicity and the Environment [108]. The Reference Dose (RfD) for DEHP recommended by the US Environmental Protection Agency (EPA) is 20 µg/kg/day [109]. DEHP and its metabolites can be measured in the blood and urine to confirm recent exposures. For monitoring purposes urine samples are preferred since they can be collected by using noninvasive techniques. Being the major urinary metabolites, 5oxo-MEHP and 5OH-MEHP may be sensitive predictors of DEHP exposure due to their relatively high concentration in urine and their lack of susceptibility to

contaminants in the sample collection process or during the analytical procedure [14,3,15,21].

Calculations of population exposure based on urinary metabolites are generally within the range of 3 to 30 μg/kg/day, but estimates made from the upper 95th percentile of measured ranges in some studies exceed this range by up to a factor of 2 [14,15]. Contrarily, alternative estimates have been calculated using different methods as much as 5-fold lower [110].

DEHP exposure levels have been shown to have high geographical variability. Estimates based on the urinary MEHP measurements obtained by Blount *et al.* [11] suggest that the average total daily ambient exposure of individuals in the United States to DEHP is likely to be <3.6 μg/kg/day [111,112]. Another regional exposure calculated by EUSES (The European Union System for the Evaluation of Substances) based on measured urinary excretion of DEHP metabolites in a German adult population [15] was estimated at 17 μg/kg/day (95th percentile) [35]. The chosen exposure estimates can be compared with measured DEHP concentrations in food from Denmark and Japan, indicating intake levels up to 16 μg/kg/day and 59.9 μg/kg/day, respectively [35]. Guo *et al.* compare urinary DEHP metabolite levels in humans from seven Asian countries during 2006-2007 indicating widespread exposure to phthalates [113]. Urine samples from Kuwait contained the highest concentrations of the metabolites of DEHP followed by samples from India, China, Japan, Vietnam Korea and Malaysia. The estimated exposure doses to DEHP in Kuwait exceeded the 20 μg/kg/day RfD value recommended by the EPA.

The National Health and Nutrition Examination Survey (NHANES) of the Centers for Disease Control and Prevention 2001–2002 measured monoester metabolites of various phthalate esters (including MEHP, 5OH-MEHP and 5oxo-MEHP) in 2782 urine samples from adults and children of the US population [114]. The NHANES database was updated regularly for the following periods: 2003–2004 ($n = 2605$), 2005-2006 ($n = 2548$) and 2007-2008 ($n = 2604$). The phthalate levels in these different periods were found to be similar (Table 4.8). Mean concentrations vary by age with younger age groups having higher urinary concentrations of the metabolites than older children and adults. In addition, a number of investigators have evaluated the concentrations of urinary DEHP metabolites in small populations (without known elevated exposure) for a variety of purposes and these results are summarized in Table 4.8.

Table 4.8: Urinary concentrations of DEHP metabolite in different populations without known elevated exposure.

Population	Gender	Origin	Year	*n*	Sample type	Urinary DEHP metabolite concentration (ng/mL)						References	References
						MEHP		5oxo-MEHP		5OH-MEHP			
						Mean	Max	Mean	Max	Mean	Max		
Adult (35 - 49 years)	women	Washington, DC, U.S.	1996–1997	46	first morning	16.5	143.9					Hoppin *et al.*, *Environ. Health Perspect.* 110:515-518 (2002)	[115]
Student and adult (7 – 64 years)	53 women, 32 men	Erlangen, Germany	2002	85	first morning	15.7	177	57.2	544	79.6	818	Koch *et al.*, *Environ. Res.* 93:177–185 (2003)	[15]
Adult	14 women, 5 men	Germany		19	first morning	9.0*	43.1	19.6*	55.1	32.1*	103	Koch *et al.*, *Int. J. Hyg. Environ. Health* 207:15-22 (2004)	[99]
Adult	11 men	MA, U.S.		369		5.7	110**	-	-	-	-	Hauser *et al.*, *Environ. Health Perspect.* 112:1734-1740 (2004)	[116]
Adult		U.S.	2001	127	spot	na	20.4**	14.8	243**	19.3	220**	Kato *et al.*, *Environ. Health Perspect.* 112 (3):327-330 (2004)	[13]
Adult (25 - 51 years)	men	Germany		5	spot	15.5	44.5	38.2	100.8	52.3	125.8	Koch *et al.*, *Int. J. Hyg. Environ.-Health* 208:489–498 (2005)	[102]
Adult	men	MA, U.S.	1999-2003	295		5.9	131**					Duty *et al.*, *Hum. Reprod.* 20 (3):604-610 (2005)	[117]
Adult		U.S.		43		<0.9*	11.84	5.68*	116.38	7.99*	134.90	Kato *et al.*, *Anal. Chem.* 77 (9):2985-2991 (2005)	[118]
Adult ad children		Germany		19	spot	14.0	49.9	41.3	72.5	52.1	96.1	Preuss *et al.*, *J. Chromatogr. B* 816 (1-2):269-280 (2005)	[16]
Student (20 - 29 years)	326 women, 308 men	Münster, Germany	1988-2003	634	24 h	7.6*	129	16.7*	251	21*	275	Wittassek *et al.*, *Int. J. Hyg. Environ.-Health* 210:319–333 (2007)	[18]
Adult (14 - 60 years)	27 women	Munich, Germany	2005	399	spot	5.6	206.5	17.3	439.9	22.0	674.3	Fromme *et al.*, *Int. J. Hyg. Environ. Health* 210 (1):21-33 (2007)	[12]
Adult (14 - 60 years)	23 men	Munich, Germany	2005	399	spot	5.4	55.4	15.6	215.4	23.0	309.3	Fromme *et al.*, *Int. J. Hyg. Environ. Health* 210 (1):21-33 (2007)	[12]
Adult	28 women	NY, U.S.		246	spot	4.8*	46.8**	18.2*	107.6**	20.2*	149.6**	Adibi *et al.*, *Environ. Health Perspect.* 116:467–473 (2008)	[119]
Adult (23 - 39 years)	women	Sweden	2001	38		13	57	19	83	25	126	Högberg *et al.*, Environ. Health Perspect. 116 (3):334-339 (2008)	[120]

Population	Gender	Origin	Year	*n*	Sample type	Urinary DEHP metabolite concentration (ng/mL)						References	References
						MEHP		5oxo-MEHP		5OH-MEHP			
						Mean	Max	Mean	Max	Mean	Max		
Adult (>20 years)		U.S.	2001-2002	1647	first morning	4.20	39.5**	12.0	116**	18.1	175**	NHANES 2001-2002	[114]
Adult (>20 years)		U.S.	2003-2004	1534	first morning	2.23	29.5**	12.9	139**	19.5	225**	NHANES 2003-2004	[114]
Adult (>20 years)		U.S.	2005-2006	1490	first morning	2.94	41.5**	14.7	182**	23.4	306**	NHANES 2005-2006	[114]
Adult (>20 years)		U.S.	2007-2008	1814	first morning	2.62	27.3**	11.1	108**	20.5	214**	NHANES 2007-2008	[114]
Adult	men	Giessen, Germany	2004-2005	349	spot	4.35*	175.43	9.02*	224.65	12.66*	325.73	Herr *et al.*, *Int. J. Hyg. Environ. Health* 212 (6):648-653 (2009)	[121]
Adult (18 - 26 years)	men	Copenhagen, Denmark	2006	60	spot	9.18	59.12	34.4	264.89	46.56	423.87	Frederiksen *et al.*, *J. Anal. Toxicol.* 34:400-410 (2010)	[122]
Adult (21 - 26 years)	15 women, 15 men	Barcelona, Spain		30		11.7*	47.7	34.7*	105.3	27.7*	90.9	Monfort *et al. Transfusion* 50 (1):145-149 (2010)	[29]
Athletes				127	spot	7.1	19.7***	18.4	49.8***	18.4	51.7***	Monfort *et al. Transfusion* 50 (1):145-149 (2010)	[29]
Adult (30 - 50 years)	men	Seoul, Korea		25	spot	2.14	83.50	2.56	72.4	3.45	154.00	Park *et al.*, J. *Prev. Med. Public. Healt.* 43:301-308 (2010)	[123]
Adult	women	Green Bay, WI, U.S.	1999-2005	45	first morning	3.6	30.8	13.9	164.3	20.9	268.2	Peck *et al.*, *J. Expo. Anal. Environ. Epidemiol.* 20:90-100 (2010)	[124]
All	women	China	2010	99	spot	5.9	56.9	12.3	111	20.4	299	Guo *et al.*, *Environ. Int.* 37 (5):893-898 (2011)	[125]
All	men	China	2010	84	spot	11.3	207	22.0	564	36.2	1120	Guo *et al.*, *Environ. Int.* 37 (5):893-898 (2011)	[125]
Adult (21 - 31 years)	15 women, 15 men	Barcelona, Spain		30	24 h	16.0*	26.4***	38.2*	111.7***	51.4*	112.7***	Monfort *et al.*, *J. Chromatogr. B* 908:113-121 (2012)	[126]
Athletes				464	spot	5.5*	15.3***	13.6*	39.8***	27.3*	76.0***	Monfort *et al.*, *J. Chromatogr. B* 908:113-121 (2012)	[126]

*Median value; ** 95th percentile value; *** 90th percentile value.

4.1.4.2. Occupational exposure to di(2-ethylhexyl) phthalate

Workers in industries manufacturing or using DEHP plasticisers might be frequently exposed to above average levels of this compound [1]. Those living near industrial facilities or hazardous waste sites, with higher than average levels of DEHP in water, might also be exposed to higher than average concentration of DEHP.

Maximum occupational exposures to DEHP, mainly by inhalation, are generally set at 0.7 mg/kg/day when the workplace air concentrations meet the Occupational Safety and Health Administration's Permissible Exposure Limit (PEL) expressed as a Time Weighted Average (TWA) standard for 8 h, usually 5 mg/m^3 [7,127]. Workplace air levels ranging from 0.02 to 4.1 mg/m^3 were reported at facilities using or manufacturing DEHP [128]. Exposures of phthalate and PVC production workers to DEHP are estimated to be typically less than 143 and 286 µg/kg/day, respectively [3]. Between 2003 and 2005, Hines *et al.* estimated DEHP exposure of 156 workers from eight industries handling DEHP. The combined estimates based on the urinary concentrations of three DEHP metabolites ranged from 0.6 to 850 µg/kg/day [129]. The external and internal exposure of DEHP in occupationally exposed population is summarised in Table 4.9.

Table 4.9: The external and internal exposure of DEHP in occupationally exposed adults [35].

Population	Inhalation		Dermal		Total multiple routes
	External (mg/m^3)	Internal (µg/kg/day)	External (mg/day)	Internal (µg/kg/day)	Internal (µg/kg/day)
Production of DEHP	5	530	650	460	990
Industrial use of DEHP	10	1060	420	300	1360
Industrial end-products containing DEHP	10	1060	1300	928	1988

4.1.4.3. Di(2-ethylhexyl) phthalate exposure from medical devices

DEHP is currently the primary plasticiser used in PVC-containing medical devices such as containers for blood or liquid nutrients, tubing and catheters [2]. Thus, patients undergoing medical treatment may be exposed to DEHP released from PVC medical devices. The exposure to DEHP varies depending on the medical procedure as it is a function of the lipophilicity of the fluid that comes into contact with the medical device, the relative surface area of the PVC material, the temperature, the flow rate and the contact time [130-136]. Substances such as blood [6], plasma [6,134,137], red blood cell [6,134,138] or platelet concentrates [6,134], intravenous lipid emulsion [134-136,139,140] or total parenteral nutrition solution [140,133] and formulation aids used to solubilise IV medications [130,131,134,141-143] can extract DEHP from PVC tubing and containers. In contrast, nonlipid-containing fluids, such as crystalloid IV solutions, saline priming solutions for extracorporeal membrane oxygenation (ECMO) and hemodialysis and peritoneal dialysis solutions extract relatively small amounts of DEHP from the PVC constituents of the devices [7].

The exposure can be long- or short-term. Long-term exposures in adults are haemodialysis, continuous ambulatory peritoneal dialysis (CAPD), transfusions of blood and blood products to patients with leukemia, aplastic anaemia, sickle cell anaemia, clotting disorders, administration of TPN and enteral nutrition of critically ill patients. Short-term DEHP exposures include blood transfusions, e.g. in trauma patients, patients undergoing surgical procedures or ECMO procedures, and intravenous infusion of drugs [2]. Estimated upper-bound doses of DEHP received by patients undergoing various medical procedures are summarised in Table 4.10 [3].

Transfusion of blood and its components is one of the major source of exposure to DEHP in infants and adults. DEHP leaches from the PVC blood bag during storage and from the tubing used during infusion. MEHP is formed through the hydrolysis of DEHP by plasma lipases in blood during storage [92,57]. The conversion was shown to increase with increasing storage time and temperature, while storage at low temperatures reduced the rate of hydrolysis [56]. Blood transfusion of trauma patients was found to be the short-term procedure that gives the highest acute DEHP exposure in adults up to 8.5 mg/kg/day. MEHP exposure due to exchange transfusion has been estimated to be in the range of 5 to 680 µg/kg/day [144,145].

Exposure to DEHP can also occur through voluntary medical treatments such as blood donation. Buchta *et al.* determined serum DEHP concentrations in donors after apheresis procedure [100]. The median amount of DEHP exposed during a single plateletpheresis procedure was estimated to be 6.5 mg/kg, with a broad inter-individual variation ranking from 1.8 to 20.3 mg/kg. Koch *et al.* estimated DEHP exposure of plasma donors, discontinuous-flow platelet donors and continuous-flow platelet donors by determining three DEHP metabolites in urine (5OH-MEHP, 5oxo-MEHP and MEHP). The highest exposure was observed for continuous-flow plateletpheresis with daily DEHP intakes of 28.2-38.1 μg/kg/day. For discontinuous-flow platelet donors values were lower (14-24 μg/kg/day), while the internal burden after plasma donation (3.1-9.6 μg/kg/day) was not elevated in comparison to controls (3.0-11.6 μg/kg/day). This may be due to the fact that the lipid-rich plasma may contain most of the DEHP which is removed from the body by the procedure [2].

Table 4.10: Estimated upper-bound dose of DEHP received by adult and neonatal patients undergoing various medical procedures [3].

Medical procedure	Estimated DEHP dose (mg/kg/day)	
	Adult (70 kg)	Neonate (4 kg)
Crystalloid intravenous (IV) solution infusion	0.005	0.03
Infusion of pharmaceuticals with solubilisation vehicles		
Administered according to manufacturer instructions	0.04	0.03
Mixed and stored at room temperature for 24 hours	0.15	
TPN administration		
Without added lipid	0.03	0.03
With added lipid	0.13	2.5
Administered via ethyl vinyl acetate bag and PVC tubing	0.06	
Blood transfusion		
Trauma patient	8.5	
Transfusion/extracorporeal membrane oxygenation (ECMO) in adult patients	3.0	
Exchange transfusion in neonates		22.6
Replacement transfusions in neonates in NICU		0.3
Replacement transfusions to treat anaemia in chemotherapy and sickle cells disease patients	0.09	
Replacement transfusions in patients undergoing coronary artery bypass grafting	0.28	
Treatment of cryodisorders with cryoprecipitate	0.03	
Cardiopulmonary bypass		
Coronary artery bypass grafting	1	
Orthotopic heart transplant	0.3	
Artificial heart transplant	2.4	
ECMO		14
Apheresis	0.03	
Haemodialysis	0.36	
Peritoneal dialysis	< 0.01	
Enteral nutrition	0.14	0.14
Aggregate exposures of NICU infants undergoing IV administration of sedatives, IV administration of TPN and replacement transfusion		2.83

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4.1.5. Alternatives to di(2-ethylhexyl) phthalate-plasticised poly(vinyl chloride)

After DEHP was classified as toxic to reproduction (Repr. Cat. 2), it has been replaced by alternative substances for many applications, which is reflected in the decline in the total consumption of the substance. In addition, for some applications the plasticised PVC has been replaced with other materials. The main alternatives to DEHP are diisononyl phthalate (DINP) and diisodecyl phthalate (DIDP). Since 2000 DINP and DIDP have been exclusively used in PVC flooring, wall covering and in carpets with PVC back-coating. DEHP was mainly replaced by DINP and DIDP in synthetic (PVC) leather for upholstery, in toys and childcare products and in soft PVC for medical applications. Non-phthalate alternatives are used in PVC for applications where there has been a concern regarding human exposure to the substance (e.g. toys, medical devices and food packaging). Alternatives marketed specifically for these product groups include adipates, citrates, carboxylates, alkylsulphonic acid ester and castor oil derivatives. The alternatives are in general more expensive than DEHP with DINP being the least expensive alternative at an incremental cost of about 10%. Applications for which the selected alternatives are specifically mentioned by certain suppliers are shown in Table 4.11, but the substances may be used for other applications as well [36].

Table 4.11: Applications specifically mentioned by suppliers of selected alternatives of DEHP [36].

	DINP[a]	DEHT[a]	BTHC[a]	DINCH[a]	ASE[a]
Polymer applications					
Flooring and wall covering	x	x			
Film/sheet and coated products	x	x		x	x
Medical products			x	x	
Wire and cable	x				
Coated fabric and footwear		x		x	x
Toys		x			x
Automotive	x				
Non-polymer applications					
Adhesives				x	x
Printing inks				x	x
Sealants (glass insulation, construction)	x				x

[a] DINP: diisononyl phthalate, DEHT: di(2-ethylhexyl) terephthalate, BTHC: butyril trihexyl citrate, DINCH: 1,2-cyclohexanedicarboxylic acid diisononyl ester, ASE: alkyl sulphonic acid phenyl ester.

4.1.5.1. Alternatives to di(2-ethylhexyl) phthalate-plasticised poly(vinyl chloride) in medical devices

There have been several attempts trying to eliminate or reduce the use of DEHP in medical devices. As it has been discussed above DEHP is beneficial for maintaining the viability and long-term storage of RBCs. Other components in blood, such as platelets, have a higher metabolic rate therefore suitable containers must have higher permeability to oxygen and carbon dioxide. This is achieved by using other plasticisers, such as trimellitates and citrates [146]. In medical tubing and parenteral infusion bags DEHP has been almost completely substituted. However, for RBCs only a limited number of DEHP-free products are available [147,148].

The most important factors influencing the viability of RBCs are the content of ATP, 2,3-diphosphoglycerate, haemoglobin (Hb), the integrity of RBC membrane lipids and proteins and the flexibility of the RBC membrane in the microcirculation. Metabolic changes and oxidative damage depend strongly on the storage medium, its pH and the temperature. The requirements necessary for a blood bag material are resistance to heat and chemicals, especially during the sterilisation, and permeability of gases to assure that the pH and the oxygen level are kept constant [147].

Other plasticisers have been considered to substitute DEHP in PVC medical devices, such as acetyl tri-n-butyl citrate (ATBC), 1,2-cyclohexanedicarboxylic acid diisononyl ester (DINCH), di(2-ethylhexyl) adipate (DEHA), butyril trihexyl citrate (BTHC) [89] and tri-(2-ethylhexyl) trimellitate (TEHTM, also known as TOTM) [73]. Possible alternatives for blood bags are ATBC, TEHTM and BTHC, the latter being the most satisfactory plasticiser [146]. It was found to be effective for the storage of blood, red cell concentrates and platelet rich plasma [81-85,89]. ATBC might be used in medical tubing, DINCH for tubing and nutrient solution bags and TEHTM in infusion equipment [148,149]. Since the leaching rates of these plasticisers are usually lower than that of DEHP they seem to be promising alternatives, however, insufficient information is available to assess the use and safety of these compounds in medical devices.

Another alternative to phthalate plasticisers is using polymeric plasticisers [2]. In order to minimise migration their molecular weight must be medium-high. In addition, polymeric plasticisers generally make processing of the plastic more difficult [150].

Another possibility is to replace PVC in blood bags with other polymers. Several polymer materials and their blends have been suggested, including ethylene vinyl acetate

(EVA), polyethylene (PE), polypropylene (PP), polyurethane (PU), thermoplastic elastomers (TPE) and fluoropolymers (e.g. polytetrafluoroethylene, PTFE) [2,151]. At the moment PVC is the most economical material for medical applications and unless other alternatives match in price, PVC will continue as the market leader in medical applications [146].

Recently, the addition of polyethylene oxide (PEO) to plasticised PVC bags has been suggested either as an additive or copolymer [152,153]. Furthermore, PEO was used as an inner coating of PVC bags in order to reduce the migration of DEHP into lipophilic solutions [154].

The main difficulty for the substitution of DEHP-plasticised PVC in blood bags is caused by the improved survival rate of erythrocytes facilitated by DEHP (see Section 4.1.2.3.2). These alternative materials and the elimination of DEHP can lead to shorter RBC storage time and further health risks for patients receiving blood transfusions. In order to improve RBC survival different additive solutions have been proposed which seem to be promising in combination with some of the alternative materials [80,155,156].

The safety evaluation of medical devices and their composing materials including material characteristics, leaching and toxicology is described in the EN ISO 10993 series on Biological Evaluation of Medical Devices (ISO, Geneva, Switzerland; CEN, Brussels, Belgium).

For some compounds sufficient toxicological data is available to indicate a lower hazard compared to DEHP. However, a risk assessment of these alternative materials could not yet be performed due to a lack of human exposure data. For others, information on the toxicological profile is inadequate to identify the hazard. This limits the proper evaluation of the potential to replace DEHP-plasticised PVC in medical devices. The risk and benefit should be carefully evaluated for each individual medical device and each medical procedure in which the alternative needs to be used [2].

4.2. BLOOD TRANSFUSION IN SPORTS DRUG TESTING

The World Anti-Doping Agency (WADA) defines blood doping as "the misuse of certain techniques and/or substances to increase one's red blood cell mass, which allows the body to transport more O_2 to muscles and therefore increase stamina and performance" [157]. The most widely known prohibited methods are the stimulation of erythropoiesis, the use of synthetic oxygen carriers and the transfusion of blood.

Blood transfusion is the process of transferring blood or a blood-based product into the circulatory system of a person [158]. Homologous blood transfusion is the transfusion of blood from another person compatible for ABO and Rhesus D blood groups, whereas autologous blood transfusion is the reinfusion of the individual's own stored blood [158]. Using blood transfusion in order to improve physical performance first appeared in the 1970s among elite endurance athletes [159,160]. It became less popular at the end of the 1980s due to the introduction of recombinant erythropoietin and the prohibition of blood transfusion methods by the International Olympic Committee for the 1988 Olympic Games [158]. The implementation of a direct test to detect exogenous erythropoietin in urine in 2001 [161] compelled cheating athletes to start using blood transfusion procedures again [162,163].

In order to further improve detection of abnormal blood profiles, WADA is leading the development of a strategy against doping in sport called the Athlete Biological Passport [164-166] which follows the athlete's biological variables over time. The objective of this strategy is to detect abnormal variations of determined biological parameters (e.g. haematocrit, haemoglobin, red blood cell count, percentage of reticulocytes and reticulocyte count) in order to better target testing and/or sanction those found with abnormal variations [157]. WADA's Athlete Biological Passport Operating Guidelines were approved by WADA's Executive Committee and took effect on December 1st, 2009 [167].

4.2.1. Methods for the investigation of blood transfusion

Detection of homologous blood transfusion was implemented by Nelson *et al.* using blood group antibodies to identify minor RBC populations in blood samples by fluorescence-based flow cytometry even 2-3 weeks after infusion [26,168]. The method was later validated by Giraud *et al.* analysing 140 blood samples containing different percentages (0-5%) of a minor RBCs population and most samples containing 1.5% minor RBC population were unambiguously detected, yielding 78% sensitivity [24]. An improved method was published by Voss *et al.* which allowed clear identification of mixed red blood cell populations in homologous blood transfusion samples containing 0.3-2.0% of donor blood [25].

However, as the direct detection of autologous blood transfusion is still not possible, indirect methods are in development evaluating blood parameters such as haematocrit value, haemoglobin concentration, percentage of reticulocytes and corresponding parameters (ratios or total number of cell types). With the combination of haemoglobin, bilirubin, iron and erythropoietin measurements in serum 50% of autologous blood transfusion was detectable during the first week after transfusion [169]. Examining haematological response to blood withdrawal and reinfusion, Damsgaard et el. found that individual variations in [Hb] exceeding 15% between samples obtained shortly before competition may be indicative of autologous blood manipulation [170]. However, these variables are mainly based on concentrations and therefore highly affected by fluctuations in plasma volume; consequently they may not adequately reflect the absolute changes of Hb mass induced by blood transfusions [28,27]. Thus, a more sensitive parameter is the determination of loss in total Hb mass through blood donation using CO rebreathing [171,172]. It is still unproven if Hb mass measurements are suitable to detect the absolute changes induced by blood transfusions. Furthermore, the individual variability of Hb mass and the impact of other biological factors need to be investigated [27,173]. The total Hb mass showed moderate variation (approximately 2%) in subjects undertaking regular exercise [174] and was not influenced by short periods of training [175,176]. In contrast, total Hb mass was found to be increased in elite athletes following exercise in combination with altitude exposure [177-179]. Prommer *et al.* reported the high stability of total Hb mass over a period of 1 year (variation <6%, suggesting that it should be included in the Athlete Biological Passport and analysed by probabilistic

inference techniques that define subject-based reference ranges [180]. Compared to the currently used indirect parameters, the ratio between the mass of Hb in the mature erythrocyte population and in the reticulocyte fraction (RBCHb/RetHb) is the best indicator of autologous blood doping [181,182].

Besides the difficulties related to the practical applicability of CO rebreathing method for detecting nonphysiologic increases in Hb mass, its integration into the Biological Passport may be problematic due to the lack of clear standardisation and harmonisation in the determination of Hb mass [183,184]. In addition, blood samples for doping control are available in some selected situations only and therefore they cannot be considered as a general screening approach.

In the recent years, research focused on the development of further indirect markers of autologous blood transfusion, such as a marker of degradation during storage (2,3-bisphosphoglycerate levels), markers of alteration of the red cell membrane structure, gene expression levels and transcriptional markers, proteomic and metabolomic approaches and markers of neocytolosis [158]. These markers may be included in the Biological Passport in the future.

Current research on direct testing of autologous blood transfusion is based on the detection of exogenous substances in the athletes blood or urine samples spread during the withdrawal, the storage or the reinfusion of the blood, such as plasticisers leaked from the blood bags and residues of solvents used for cryogenic storage of the blood [158]. Monfort *et al.* compared the urinary concentrations of DEHP metabolites in patients subjected to clinical care and to blood transfusion. It was noticed that urine samples collected up to two days after blood transfusion contained significantly higher amounts of DEHP metabolites compared to control samples, indicating that DEHP metabolites could be used in sports drug testing as alert markers for the potential misuse of blood transfusion [29].

4.3. METHODS USED FOR THE DETERMINATION OF URINARY DI(2-ETHYLHEXYL) PHTHALATE METABOLITES

Due to the increased use of plasticised PVC products in the last few decades and the toxic properties of phthalate plasticisers the need for testing phthalate esters and their metabolites in environmental, biological and food samples gained remarkable importance.

Monitoring the concentration of DEHP in biological samples might underestimate exposure since it is quickly and extensively metabolised *in vivo*. Therefore, for biomonitoring purposes mainly MEHP and the secondary metabolites 5oxo-MEHP and 5OH-MEHP have been tested in various matrices, such as urine, blood, plasma, serum, saliva, sweat and milk.

Since DEHP and its metabolites are present in environmental and biological samples at low level different techniques have been used for their extraction and concentration from the matrices. For DEHP measurement mainly gas chromatographic (GC) methods were used in combination with electron capture (ECD), flame ionisation (FID) or mass spectrometric (MS) detection. In some cases high performance liquid chromatographic (HPLC) methods were applied using UV spectrometric detection and found to be useful for the analysis of isomeric mixtures and metabolites of phthalates without derivatisation. Recently, several LC-MS and LC-MS/MS methods have been developed mainly applying electrospray ionisation in positive ionisation mode for DEHP and negative mode for the monoesters, 5oxo-MEHP and 5OH-MEHP.

4.3.1. Sample extraction and clean-up

Due to its highly apolar characteristic DEHP is mainly extracted using apolar organic solvents, such as dichloromethane, cyclohexane, hexane, isooctane or by non-polar solid phase extraction (SPE). The extraction is usually carried out by shaking the samples; however, there are examples for Soxhlet extraction, accelerated solvent extraction and ultrasonic or microwave assisted extraction procedures in solid matrices.

Since DEHP metabolites are less apolar and excreted in urine as glucuronide conjugates, the samples are usually hydrolysed using β-glucuronidase prior to extraction. In some

cases liquid-liquid extraction was applied using ethyl acetate as the solvent [29,185,126]. A more common procedure is the SPE technique using C8 [16,186,187], C18 [188,118,189] on other mixed-mode solid phase materials [190-193]. In order to avoid contamination from the laboratory environment automated SPE [191,194] and online-SPE, so-called column-switching techniques [16,186-188,118,195,196], have been developed as well.

Though the sample extract can be purified by gel permeation chromatography [197] or by SPE using sorbents, such as aluminium oxide [198], silica [199] or Florisil [200], it is most commonly analysed without any additional clean-up.

4.3.2. Measurement techniques

DEHP was primarily measured using gas chromatography coupled to mass spectrometry. In some cases flame ionisation or electron capture detection in combination with gas chromatography was applied. However, FID is not specific for phthalates and ECD detectors respond more sensitively towards halogenated compounds.

Using electron ionisation (EI) for GC-MS, the major fragment at m/z 149 corresponds to the protonated phthalic acid anhydride ion which is generally used for quantification. Besides the most abundant ion at m/z 149, the spectrum is rather poor and the molecular ion (m/z 390) is not detectable. Additional ions at m/z 279 and 167 confirm the identity of the peak (Figure 4.8) [201].
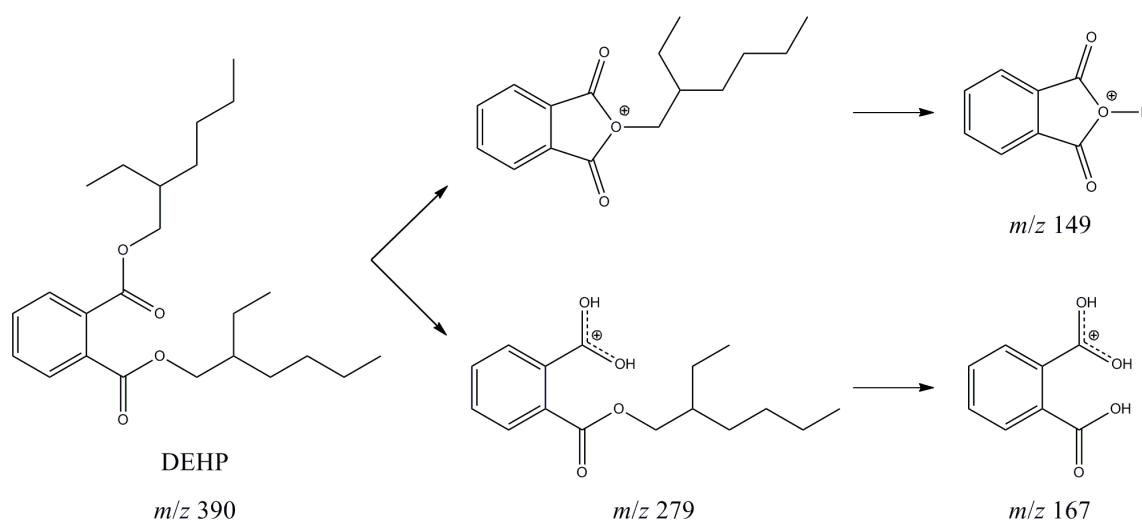
Figure 4.8: EI mass fragmentation of DEHP [201].

Positive chemical ionisation (PCI), applying methane or ammonia as reagent gas, provides a mass spectra containing more abundant peaks for high-mass ions allowing better identification and distinction of the different phthalates [202,203].

The free fatty acid group on the monoester metabolites can cause adsorption of the molecule on the column during gas chromatographic analysis. Therefore, derivatisation is necessary to allow quantitative analysis of trace level monoester by GC. This can be achieved by esterification [95] or oximation [204] and silylation of the acid group. However, derivatisation can lead to further hydrolysis with the formation of phthalic acid dimethyl ester or to the hydrolysis of the parent molecule causing false positive results for the monoesters [201].

Alternatively, phthalate monoesters were analysed in urine samples without derivatisation using HPLC coupled to UV detection. Separation of the DEHP metabolites were reported by using CN [205], C18 [29,126,187-189,193], phenyl [118,190-192,194] and phenyl-hexyl [16,186] columns showing reasonable separations.

Recently, several LC-MS or LC-MS/MS methods have been developed for the analysis of DEHP and its metabolites in biological samples. The ionisation of the compounds were achieved by either using electrospray ionisation (ESI) in negative [16,186-188,118,191,194] and positive mode [29,126] or atmospheric pressure chemical ionisation in negative mode [185,190,192].

The product ion spectra of MEHP produced by collision-induced dissociation (CID) was described by Blount *et al.* [190] and phthalate specific negative ions were found at *m/z* 77, 105, 121, and 147, corresponding to putative benzyl, benzaldehyde, benzoate and phthalic anhydride fragment anions (Figure 4.9). Later Koch *et al.* published ESI negative Q1 mass spectra of the secondary metabolites 5OH-MEHP and 5oxo-MEHP with the predicted structures of the fragment ions (Figure 4.10) [186].
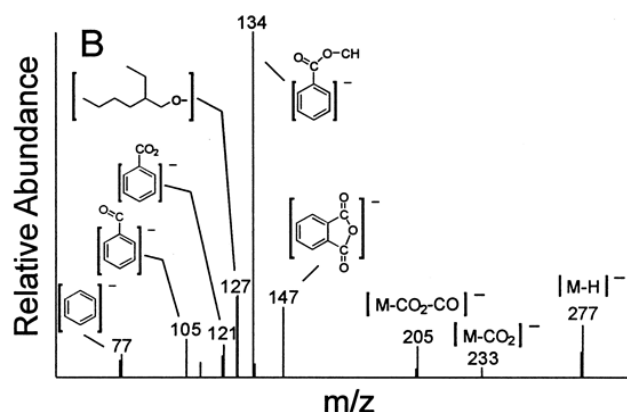
Figure 4.9: ESI negative product ion spectra of MEHP presented by Blount *et al.* [190].

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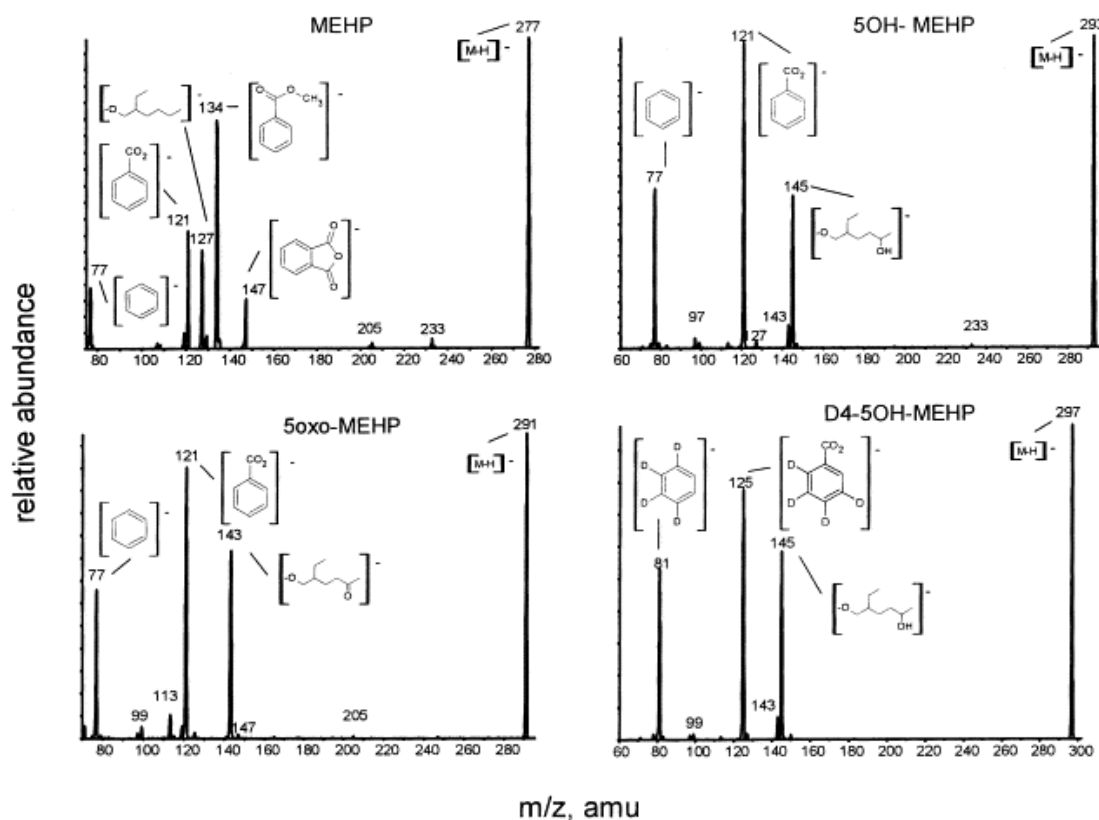
Figure 4.10: ESI negative Q1 mass spectra of MEHP, 5OH-MEHP, 5oxo-MEHP and D₄-5OH-MEHP with the predicted structures of the fragments published by Koch *et al.* [186].

4.3.3. Quality assurance and blank values

Due to their widespread use and physicochemical properties many phthalates are ubiquitous; therefore, their direct measurement is difficult. The samples can be contaminated during sample collection, transportation, storage and the analytical testing procedure. Consequently, caution should be taken to keep background concentrations at low levels, blank values should be controlled and a correction has to be made.

In bioanalytical applications mostly phthalate metabolites are analysed which are usually not subject to contamination [206]. In particular, the secondary metabolites of DEHP were found to be more sensitive biomarkers of exposure to DEHP than MEHP [207].

4.4. SCREENING METHODS IN SPORTS DRUGS TESTING

In sports drug testing the number of samples and doping agents constantly increases; thus, multi-target approaches, combining high-throughput, simplified sample preparation and a reliable detection for various classes of compounds are required. On the basis of modern and powerful analytical instruments, consisting of liquid chromatographs coupled to sensitive mass spectrometers, e.g. triple-quadrupole, time-of-flight (TOF) or Fourier transform (OrbiTrap) instruments, numerous new multi-target assays have been developed providing reliable detection of prohibited substances. Currently, different screening procedures are utilised for the analysis of diuretics [208-215], beta2-agonists [213,214,216], stimulants [211,213,214,217], narcotics [214,213,218-220] and plasma volume expanders (PVE) [221-223]. With the ongoing progress in the field of liquid chromatography and triple quadrupole mass spectrometry, new generation instruments have become accessible with the possibility of enhanced scan speed and scan-to-scan polarity switching. These achievements allow the development of new multi-target approaches by combining classical "stand-alone" screening procedures to detect several different categories of prohibited substances with versatile chemical structures. In contrast to TOF based approaches fast polarity switching allows the detection of acidic and basic compounds within a single run. Moreover, the high sensitivity of such instruments enables the identification of many prohibited substances without pre-concentration steps, resulting in very simple and fast "dilute-and-shoot" methods. In sports drug testing two assays have demonstrated its applicability for the combined detection of diuretics, stimulants and narcotics using direct injection of urine specimens based on ultra-high performance liquid chromatography/tandem mass spectroscopy (UHPLC-MS/MS) and ultra-high performance liquid chromatography/quadrupole time-of-flight mass spectrometry (UHPLC-QTOF-MS) [224,225].

4.5. STATISTICAL METHODS

4.5.1. Huber's statistics

Most estimates of central tendency (e.g. arithmetic mean) and dispersion (e.g. standard deviation) depend on an implicit assumption that the data comprises a random sample from a normal distribution. Analytical data often contains a higher than expected proportion of results far from the arithmetic mean, as well as outliers. In such cases statistical models which assume normal distribution of the data points are inappropriate to describe the dataset. As Figure 4.11 illustrated the mean has a high bias and the standard deviation is too large. In addition the actual values of the outliers have a great influence on the estimate values.
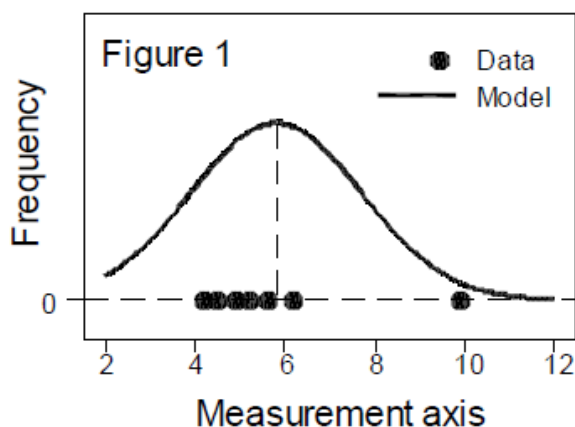
Figure 4.11: Illustration of the statistics using the model based on a normal distribution on data containing outliers [226].

A more reasonable interpretation of the dataset is to exclude the outliers from the calculations resulting in a plausible normal model for most of the data points (Figure 4.12). Although it provides us with no warning about the possible outliers, this model is often preferable in applications in analytical science [227].
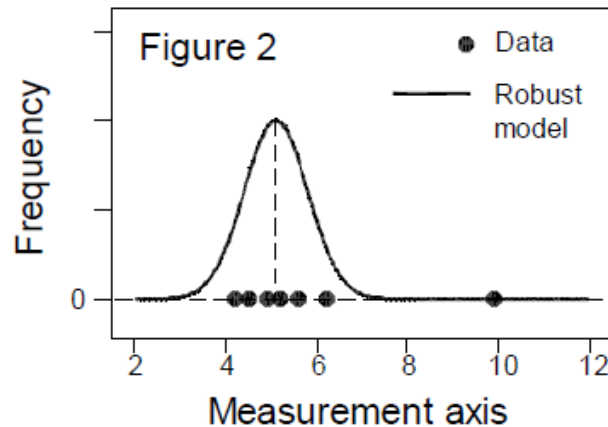
Figure 4.12: Illustration of the statistics using the model based on a normal distribution on data excluding outliers [227].

A typical solution to handle suspect values is the identification of the outliers at particular confidence levels by employing tests such as Dixon's or Grubbs'. These procedures are not necessarily straightforward, since the test may be misleading if two or more outliers are present and it raises the question of when it is justifiable to exclude outliers [227].

Robust statistical models provide alternative solutions as describing the dataset without excluding any outliers. Huber's method, for example transforms the original data by a process called Winsorisation, in which an outlying value is 'moved' so that its residual is reduced (i.e. the difference between the single value and the sample mean) [228].

Starting with initial estimates μ_0 as the median or arithmetic mean and σ_0 as the standard deviation, if a value x_i falls above $\mu_0 + 1.5\sigma_0$, it will be changed to $\tilde{x}_i = \mu_0 + 1.5\sigma_0$. Similarly, if a value x_i falls below $\mu_0 - 1.5\sigma_0$, it will be changed to $\tilde{x}_i = \mu_0 - 1.5\sigma_0$. All the other data remains unchanged ($\tilde{x}_i = x_i$). From the new data, improved estimates of the mean $\mu_1 = mean(\tilde{x}_i)$ and standard deviation $\sigma_1 = 1.134 \times stdev(\tilde{x}_i)$ are calculated. The factor 1.134 is derived from the normal distribution, given a value 1.5 for the multiplier most often used in the Winsorisation process. This procedure is then iterated using the improved estimates for the Winsorisation at each cycle. The process converges to an acceptable degree of accuracy and the resulting values are the robust estimates μ_H and σ_H [227].

4.5.2. Intraclass correlation

Intraclass correlation was introduced by Fisher *et al.* as descriptive statistic that can be used to describe how strongly values of the same group coincide with each other [229]. The coefficient (ρ) can be described as the proportion of the total variance which is attributed to variation between groups (σ_b^2):

$$\rho = \frac{\sigma_b^2}{\sigma_b^2 + \sigma_w^2},$$

where σ_w^2 is the within-group variability.

The intraclass correlation coefficient (ICC) approaches 0 in the case of low within-group homogeneity and it approaches 1 when the between-group variation is large compared to the within-group variation.

5. AIM OF THE THESIS

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 the three major DEHP metabolites (MEHP, 5oxo-MEHP and 5OH-MEHP) in urine samples.

- To determine the levels of the phthalate metabolites in control samples, in athletes' samples and in post-transfusion samples.

- To estimate an upper reference limit for the urinary concentrations of the metabolites in subjects without extraordinary DEHP exposure.

- To investigate the possible intra-individual variability of the metabolites over time.

- To integrate the DEHP metabolites into an existing screening procedure and apply the method for routine doping control samples.

6. MATERIALS AND METHODS

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

6.1.1. Materials

6.1.1.1. Chemicals and reagents

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 (Table 6.1). 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 AG, Goettingen, Germany).

Table 6.1: Reference materials

Analyte Name	Abbreviation	Formula	CAS No.	Molecular Weight
mono(2-ethylhexyl) phthalate	MEHP	$C_{16}H_{22}O_4$	4376-20-9	278.3435
mono(2-ethyl-5-oxohexyl) phthalate	5oxo-MEHP	$C_{16}H_{20}O_5$	40321-98-0	292.3270
mono(2-ethyl-5-hydroxyhexyl) phthalate	5OH-MEHP	$C_{16}H_{22}O_5$	40321-99-1	294.3429
$^{13}C_4$-mono(2-ethylhexyl) phthalate	$^{13}C_4$-MEHP	$*C_4C_{12}H_{22}O_4$	n.a.	282.3141
$^{13}C_4$-mono(2-ethyl-5-oxohexyl) phthalate	$^{13}C_4$-5oxo-MEHP	$*C_4C_{12}H_{20}O_5$	n.a.	296.2976

6.1.1.2. Reference solutions

A stock standard solution was prepared of each individual compound with a concentration of 100 µg/mL in acetonitrile and stored at +4°C. The working solutions of the compounds were prepared at the concentration level of 1 µg/mL in acetonitrile:water (1:1, *v:v*).

6.1.2. LC-MS/MS method development

6.1.2.1. Chromatographic parameters

For the chromatographic separation an Agilent 1100 HPLC system was used which consisted of a vacuum degasser, an autosampler and a binary pump. 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. The injection volume was 10 µL.

6.1.2.2. Mass spectrometric parameters

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. Nitrogen was used as curtain, nebuliser, and auxiliary gas delivered from a nitrogen generator (CMC Instruments, Eschborn, Germany). 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 (Table 6.3). Enhanced product ion spectra were acquired using linear ion trap (LIT) mode. Detailed method parameters are listed in Table 6.2. To evaluate the acquired data Analyst 1.5 software was used.

Table 6.2: LC-MS/MS method parameters.

Chromatographic Parameters			
HPLC system	Agilent 1100 Series LC System		
HPLC column	Phenomenex Gemini C6-phenyl (100 x 2.0 mm; 3 µm) column		
	Phenomenex Gemini C6-phenyl (4 x 2.0 mm) pre-column		
Mobile Phase	A: 5 mM ammonium acetate, 0.1% acetic acid (pH = 3.5)		B: acetonitrile
Gradient	0 min	100% A	0% B
	8 min	0% A	100% B
	10 min	0% A	100% B
	re-equilibration 4.5 min at 100% A		
Flow rate	0.25 mL/min		
Injection Volume	10 µL		

Mass spectrometric parameters	
Mass spectrometer	AB SCIEX 5500 QTrap
Ionisation	ESI in negative ionisation mode
Scan Mode	MRM
Interface Temperature	400°C
Nebulizer Gas	N_2, 40 psi
Auxiliary Gas	N_2, 20 psi
Curtain Gas	N_2, 20 psi
Ionspray Voltage	-4500 V
Entrance Potential	-10 V
Collision Gas	N_2, 3.5×10^{-3} Pa
Collision Cell Exit Potential	-10 V

Table 6.3: Chromatographic and mass spectrometric parameters of phthalate monoesters.

Urinary phthalate monoesters	Abbreviation	Retention time[a]	Precursor ion[b]	Product ion[c]	Collision energy	Declustering potential
		(min)	(m/z)	(m/z)	(eV)	(V)
Mono(2-ethylhexyl) phthalate	MEHP	10.96	277	**134**	-22	-70
				127	-24	-70
				77	-36	-70
Mono(2-ethyl-5-oxohexyl) phthalate	5oxo-MEHP	9.41	291	**143**	-20	-70
				121	-26	-70
				77	-46	-70
Mono(2-ethyl-5-hydroxyhexyl) phthalate	5OH-MEHP	9.25	293	**121**	-28	-70
				77	-46	-70
				145	-20	-70
Mono(2-ethylhexyl) phthalate-$^{13}C_4$	$^{13}C_4$-MEHP	10.95	281	**137**	-22	-70
				79	-38	-70
				127	-24	-70
Mono(2-ethyl-5-oxohexyl) phthalate-$^{13}C_4$	$^{13}C_4$-5oxo-MEHP	9.40	295	**143**	-20	-70
				124	-26	-70
				79	-46	-70

[a] Retention time was calculated as the mean value of six replicates.

[b] The analysis was performed in negative ionisation mode.

[c] The values in bold font indicate the quantifier ions.

6.1.3. Sample preparation

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.

6.1.4. Quantification of target compounds

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 $^{13}C_4$-MEHP, 5oxo-MEHP and 5OH-MEHP to $^{13}C_4$-5oxo-MEHP) were used. Calibration graphs were obtained by analysing spiked blank urine samples at concentration levels of 1, 10, 50, 100, 150, 200 and 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 of 1.020 g/mL according to WADA guidelines [230].

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.

6.1.5. Assay validation

For validation the following parameters were determined; specificity, linearity, ion suppression, accuracy, intra- and inter-day precision, limit of detection (LOD) and limit of quantification (LOQ). The validation was performed according to the guidelines of the International Conference on Harmonisation and WADA [231,232]. All calibration samples were prepared as described above, using 1 mL of blank urine spiked with respective amounts of reference standards and subsequent dilution with acetonitrile:water (1:1, *v:v*) to a volume of 5 mL.

6.1.5.1. Specificity

Evaluation of the specificity was carried out by analyzing six different spiked and blank urine samples collected from healthy volunteers to test for interfering signals in the selected MRM chromatograms at the expected retention times of the analytes.

6.1.5.2. Linearity

Calibration curves ($n = 6$) for the metabolites were generated using aliquots of a blank urine sample spiked at concentrations of 1, 10, 50, 100, 150, 200, 250 ng/mL. The ratio of peak areas for the analytes and the corresponding internal standards (ISTDs) were used to calculate the correlation coefficient, intercept and slope.

6.1.5.3. Ion suppression / enhancement

The extent of ion suppression or enhancement was investigated by analysing six different blank urine samples via post-column continuous infusion of a mixture of the reference compounds (100 ng/mL, 20 µL/min) [233].

6.1.5.4. Accuracy and precision

The accuracy and precision were determined using six replicates of spiked urine samples at the concentration levels of 10, 100 and 250 ng/mL (QC_{low}, QC_{medium}, QC_{high}). To test for accuracy the concentrations of the respective aliquots were calculated utilising an external calibration curve. To establish the inter-day precision the same samples were prepared and analysed on three consecutive days ($n = 6+6+6$). The precision of the

method was determined by calculation of the coefficient of variation (CV) of the area ratio of the quantifier ion transition of the analytes and the respective internal standards.

6.1.5.5. Stability

In earlier studies the decomposition of DEHP metabolites was noticed in urine above 4°C [234]. To ensure accurate analytical results the stability of the DEHP metabolites in the prepared samples was tested. Therefore, three different quality control (QC) samples were prepared at the concentration level of 100 ng/mL stored for up to 4 months at -20, 4°C and room temperature, respectively.

6.1.5.6. Limit of detection and limit of quantification

LOD and LOQ were defined as the lowest concentrations of the analyte in a sample that gave signal-to-noise ratios of 3:1 and 10:1 for the quantifier ion transition with a precision of less than 20% and an accuracy of 80 to 120%. Six blank urine samples were analysed to establish the noise intensity. Three parallel artificial urine samples spiked at a concentration of 0.5 ng/mL for LOD and 1 ng/mL for LOQ were prepared and analysed.

6.1.5.7. Uncertainty of the method

The uncertainty of the measurement (u_c) was calculated according to WADA guidelines [235]. It is based on the data obtained through the validation of the method.

$$u_c(y) = \sqrt{u(s_w)^2 + u(B)^2},$$

where s_w is the intermediate precision, B is the average recovery bias and $u(B)$ was calculated using the equation:

$$u(B) = \sqrt{(B)^2 + \left(\frac{s_B}{\sqrt{n}}\right)^2 + \left(c_{ref}\right)^2},$$

where s_B is the standard deviation of the average bias of n replicate analysis ($n = 6$) and c_{ref} is a random error contribution ($c_{ref} = 3$).

For determination of the expanded uncertainty ($U_{95\%}$) a coverage factor $k = 2$ can be applied if u_c has a 95% confidence level.

$$U_{95\%} = k \cdot u_{c\,(k=2)}.$$

6.1.6. Density and creatinine measurement

The density of the urine samples was measured using an Anton Paar DMA 38 density meter (Graz, Austria).

Creatinine measurements were carried out using a Creatinine Assay Kit (Cayman Chemical Company, Ann Arbor, MI, USA). The assay relies on the Jaffe' reaction [236]. The dynamic range of the kit is 0-15 mg/dL of creatinine. Since creatinine levels in human urine typically range from 25 to 400 mg/dL urine samples were diluted 1:20 with HPLC-grade water. The sample creatinine concentration was determined using a creatinine standard curve (0, 2, 4, 6, 8, 10, 12, and 15 mg/dL).

6.1.7. Study subjects

6.1.7.1. Reference populations

6.1.7.1.1. *Control samples*

In order to determine the physiological levels of DEHP metabolites, urine samples were collected from 100 healthy volunteers (68 male, 31 female, 1 unknown) from the German Sport University Cologne (control samples). The participants were between 20 and 59 years of age and regularly exercised.

6.1.7.1.2. *Athletes' samples*

In addition, 468 official doping control urine samples were analysed (374 male, 90 female, 4 unknown), received between January 2010 and June 2010 from national and international federations, taken in-competition ($n = 217$, IC) and out-of-competition ($n = 251$, OOC) covering different sporting disciplines.

6.1.7.2. Post-transfusion samples

6.1.7.2.1. *Excretion urine samples*

Urine samples of patients receiving autologous blood transfusion were collected between 0 to 24 hours after transfusion ($n = 10$, 2 samples from each volunteer after blood transfusion, 2 female and 3 male, age 44-79, Surface-Zentrum für Orthopädie, Neuss, Germany) and blank samples of each volunteer were taken before transfusion to estimate the individual reference values. Written consent was received from each volunteer.

6.1.7.2.2. *Post-transfusion samples with 14 and 28 days storage of transfused blood*

Urine samples were collected from 25 healthy volunteers, who partake in moderate training, and receive autologous RBC transfusions.

Blood collection, preparation, storage of RBCs and the autologous RBC transfusion were performed by a specialised laboratory according to usual clinical practice, using blood bags (Macopharma, Tourcoing, France) and leukoreduction filtration systems (Fenwal Inc., Lake Zurich, IL, USA) with the approval of the Ethics Committee of the Ruhr-University Bochum (Reg. No. 3200-08).

The subjects were separated into two groups in terms of transfusion. The first group consisted of 12 subjects (5 female, 7 male, aged 15-40 $n = 161$) who received the RBC transfusions (500 ml) 14 days after the phlebotomy, and the 13 subjects of the second group (5 female, 8 male, aged 21-28, $n = 220$) were reinfused after 28 days of RBC storage (500 ml). Urine samples were collected several days before blood transfusion (including the day of blood collection), on the day of the reinfusion and up to 21 days after transfusion (Figure 6.1). Samples from the first group were collected on days 3, 4 (blood collection), 5, 11, 17, 18 (blood transfusion), 19, 20, 21, 23, 25, 28, 32, 39 and from the second group on days 0, 3, 4 (blood collection), 5, 11, 18, 25, 31, 32 (blood transfusion), 33, 34, 35, 37, 39, 42, 46 and 53. On the day of the blood collection and the reinfusion samples were obtained from 1 to 3 hours after procedure.

An independent blind analysis was carried out on this set of samples using a different analytical procedure by the Bioanalysis Research Group, IMIM-Hospital del Mar (Barcelona, Spain). The correlation between the results obtained by the two laboratories was published by Monfort *et al.* [237]. In this study only our results are shown.
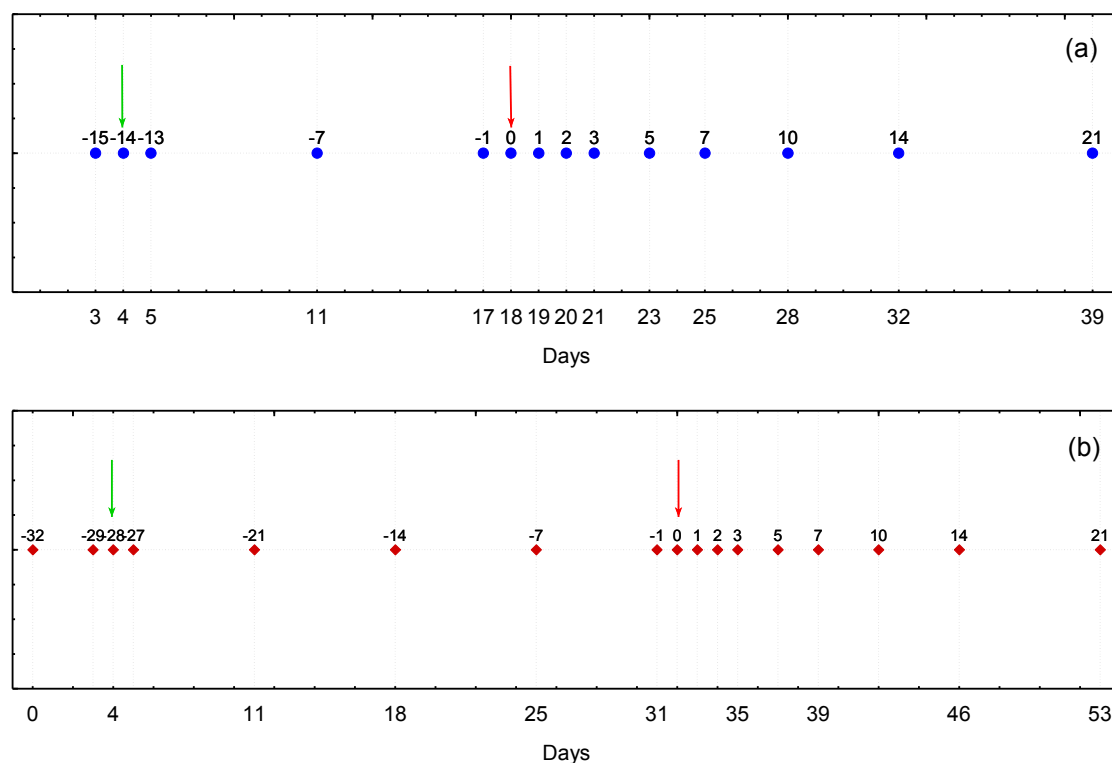
Figure 6.1: Sample collection protocol from 25 healthy volunteers, who partake in moderate training, and receive autologous RBC transfusions: Group 1 (a), Group 2 (b). Green arrows indicate the days of phlebotomy and red arrows indicate the days of transfusion.

6.1.7.3. Longitudinal study

In order to determine the intra-individual variability of urinary concentrations of DEHP metabolites, urine samples were collected from seven healthy volunteers (3 female, 4 male; age: 27-37 years) during one week (29-45 samples per volunteer, $n = 253$). The samples were collected from a different starting date 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 at -20°C until analysis.
Informed consent was obtained from all medicated patients and volunteers.

6.1.8. Statistical evaluation

6.1.8.1. Reference populations

The values of all investigated compounds exhibited heavily skewed distributions. Log-transformation yielded nearly symmetric distributions which, however, still contained numerous high values. Therefore, calculation of mean and standard deviation to describe the population was inappropriate. Instead, Huber's method [227] was applied to the log-transformed values for descriptive statistics as well as for the calculation of reference limits. Huber's M-estimator yields robust estimates for the central tendency (μ_H) and for the dispersion (σ_H) of an empirical distribution. The distribution should be symmetrical but may contain a limited number of outliers. Ideally, μ_H will reflect the mean or median of the uncontaminated distribution. Similarly, σ_H approximates the standard deviation under ideal conditions. Therefore, these parameters were used in analogous fashion to mean and standard deviation in order to estimate the probability of given values falling outside a certain interval.

On a logarithmic scale, the σ_H of all compounds except MEHP was multiplied with the 0.999 quantile of the Gaussian distribution (3.09). This value was then added to the respective values of μ_H to obtain upper 99.9% reference limits. These resulting values were transformed back to concentrations. The data below LOQ were substituted with the value of the LOQ divided by two (LOQ/2). Statistical analysis was performed using the latest version of the software "R" [238] where the functions for Huber's statistics can be found in library "MASS" [239] and STATISTICA software version 7.0 (StatSoft Inc., Tulsa, OK, USA).

6.1.8.2. Longitudinal study

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.

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

6.2.1. Materials

6.2.1.1. Chemicals and reagents

Internal standard mefruside was acquired from Bayer AG (Leverkusen, Germany). Glacial acetic acid and ammonium acetate were purchased from Sigma (Steinheim, Germany). All references and 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 GmbH., Göttingen, Germany).

6.2.1.2. Reference solutions

A stock standard solution of the internal standard was prepared at a concentration of 1 mg/mL in methanol and stored at -20°C. The reference working solution of the compound was prepared at a concentration level of 10 µg/mL in methanol.

6.2.1.3. Quality Control samples

For the phthalate glucuronides, quantified post-transfusion samples were analysed as QCs.

6.2.2. Sample preparation

An aliquot of 90 µL of urine sample was fortified with 50 ng of the internal standard mefruside (corresponding to 10 µL of a 5 µg/mL solution of the ISTD in methanol) the samples were then mixed, and an aliquot of 5 µL was injected into the instrument.

6.2.3. LC-MS/MS analysis

Chromatographic separation of target analytes was achieved on an Agilent 1100 Series HPLC system equipped with a Nucleodur C_{18} Pyramid analytical column (50 x 2 mm, 3 µm particle size; Macherey-Nagel, Düren, Germany) 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. The injection volume used was 5 µL.

Tandem mass spectrometry was carried out using a hybrid triple quadrupole / linear ion trap mass spectrometer (AB Sciex 5500 QTrap; Darmstadt, Germany) controlled by Analyst Software 1.5 (AB Sciex). Fast polarity switching (50 msec) electrospray ionisation was used with the following conditions: ionspray voltage +5500 V (positive) and -4500 V (negative), ion source temperature 450°C, nitrogen was used as curtain, nebuliser, and auxiliary gas. The analytes and the ISTD (mefruside) were detected utilising multiple reaction monitoring (MRM) of diagnostic ion transitions at dwell times of 10 msec. For optimisation of the orifice potential and the collision energy solutions of pure reference compounds for each analyte were directly injected using a 1 mL syringe at a flow rate of 10 µL/min. Nitrogen was used as the collision gas (3.5 x 10^{-3} Pa) delivered from a nitrogen generator (CMC Instruments, Eschborn, Germany). Detailed method parameters are listed in Table 6.4. Target ion transition of MRM experiments are listed in Table 6.5.

Table 6.4: LC-MS/MS screening method parameters.

Chromatographic Parameters			
HPLC system	Agilent 1100 Series LC System		
HPLC column	Macherey-Nagel Nucleodur C18 Pyramid analytical column (2 x 50 mm, 3 µm)		
	Phenomenex Gemini C6-phenyl (4 x 2.0 mm) pre-column		
Mobile Phase	A: 5 mM ammonium acetate, 0.1% acetic acid (pH = 3.5)		B: acetonitrile
Gradient	0 min	100% A	0% B
	4.5 min	10% A	90% B
	10 min	0% A	100% B
	6.25 min post-run equilibration time (using 0.5 mL/min flow rate)		
Flow rate	0.35 mL/min		
Injection Volume	5 µL		

Mass spectrometric parameters	
Mass spectrometer	AB SCIEX 5500 QTrap
Ionisation	ESI in polarity switching mode (50 msec)
Scan Mode	MRM
Interface Temperature	450°C
Nebulizer Gas	N_2, 60 psi
Auxiliary Gas	N_2, 30 psi
Curtain Gas	N_2, 30 psi
Ionspray Voltage	+5500 V, -4500 V
Entrance Potential	+/-10 V
Collision Gas	N_2, 3.5 x 10^{-3} Pa
Collision Cell Exit Potential	-10 V (for DEHP metabolites)

Table 6.5: Chromatographic and mass spectrometric parameters of phthalate glucuronide conjugates.

Compound	RT[a] (min)	RRT[b]	Ionisation Mode[c]	Precursor ion (m/z)	Product ion (m/z)	Collision Energy (eV)	Declustering Potential (V)
mefruside (ISTD)	5.43	1.00	-	381	78	-80	-100
5OH-MEHP-gluc	5.15	0.95	-	469	293	-20	-70
5oxo-MEHP-gluc	5.18	0.95	-	467	291	-20	-70

[a] RT: retention time
[b] RRT: relative retention time
[c] – negative ionisation mode

6.2.4. Validation of the method

For validation the following parameters were determined; specificity, ion suppression, intra- and inter-day precision, limit of detection (LOD) and robustness. Additionally, to test for linearity and accuracy correlation between the conjugated metabolites and the unconjugated analogues were investigated. The validation for identification of target analytes was performed according to the guidelines of the International Conference on Harmonisation and WADA [240,241]. All calibration samples were prepared and analysed as described above.

6.2.4.1. Specificity

Evaluation of specificity was carried out by analysing six different spiked and six different blank urine samples collected from healthy volunteers (5 female, 1 male) to test for interfering signals in the selected MRM chromatograms at expected retention times of the analytes.

6.2.4.2. Ion suppression / enhancement

The extent of ion suppression or enhancement was investigated by analysing six different blank urine samples via post-column continuous infusion of a mixture of the reference compounds (1 µg/mL, 20 µL/min) [233].

6.2.4.3. Accuracy and linearity

To ensure the ability of the method for semi-quantitative purposes the correlation of 5OH-MEHP and 5oxo-MEHP concentrations and the relative intensities of the corresponding 5OH-MEHP-gluc and 5oxo-MEHP-gluc were determined.

6.2.4.4. Precision

Intra-day precision was determined at three concentration levels for each compound (QC_{low}, QC_{medium}, QC_{high}) using six replicates of spiked urine samples. The corresponding inter-assay precision was calculated from samples prepared and analysed on three different days. The precision of the method was determined by calculation of the coefficient of variation (CV) of the area ratio of the ion transition of the analytes and the internal standard.

6.2.4.5. Limit of detection

LOD was estimated via the signal to noise ratio (S/N) of the respective ion traces using ten blank samples and ten fortified samples at concentration levels of 50 ng/mL.

6.2.4.6. Robustness

Robustness was tested by comparison of the relative retention times of the analytes in the QC samples over a month.

6.2.5. Post-transfusion samples

Post-transfusion samples provided by Surface-Zentrum für Orthopädie (Neuss, Germany) were analysed for 5oxo-MEHP-gluc and 5OH-MEHP-gluc. Urine samples were collected from 5 volunteers between 0 to 24 h after blood transfusion (2 female, 3 male, age 44-79). Informed consent was obtained from all medicated patients and volunteers.

6.2.6. Routine samples

Approximately 13,000 samples were tested for the glucuronide conjugates of DEHP metabolites using the screening procedure (Section 6.2.2 and 6.2.3) within a year. Samples showing higher relative intensities (5oxo-MEHP-gluc and 5OH-MEHP-gluc related to the internal standard) than the relative intensities corresponding to the 99.9% reference limits were tested using the original method (Section 6.1.2 and 6.1.3) in order to confirm the results and determine the accurate concentrations of the DEHP metabolites.

7. RESULTS AND DISCUSSION

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

7.1.1. Chromatographic and mass spectrometric parameters

Good chromatographic separation was achieved using a Phenomenex Gemini phenyl-hexyl column. Phenyl-hexyl columns have the structure of a phenyl column in which the hexyl group acts as a spacer. It shows high selectivity for aromatic compounds due to the $\pi - \pi$ interactions which can lead to increased retention of polar aromatic compounds compared to an alkyl-bonded phase [242].

After optimization, electrospray ionisation of all DEHP metabolites produced negatively charged molecular ions [M-H]⁻ in suitable abundance. Product ion mass spectra generated after collision-induced dissociation (CID) of deprotonated molecules yielded phthalate-specific fragment ions for all DEHP monoesters using appropriate collision energies (Figure 7.1). All metabolites produced common ions at *m/z* 77 and 121 corresponding to benzyl and benzoate fragments. The most abundant fragments were observed at *m/z* 127, 134 and 147 for MEHP corresponding to the anions of 2-ethylhexanol, methylbenzoate and phthalic anhydride, respectively, as demonstrated in earlier studies [186,190]. The stable isotope-labelled internal standard of MEHP dissociated similarly showing ions at *m/z* 79, 124, 127, 137 and 151 consistent with the presence of two, three or four ^{13}C-atoms. Furthermore, a characteristic ion was found at *m/z* 143 and *m/z* 145 in the case of 5oxo-MEHP and 5OH-MEHP corresponding to deprotonated analogues of 5-(hydroxymethyl)heptane-2-one and 2-ethylhexane-1,5-diol.
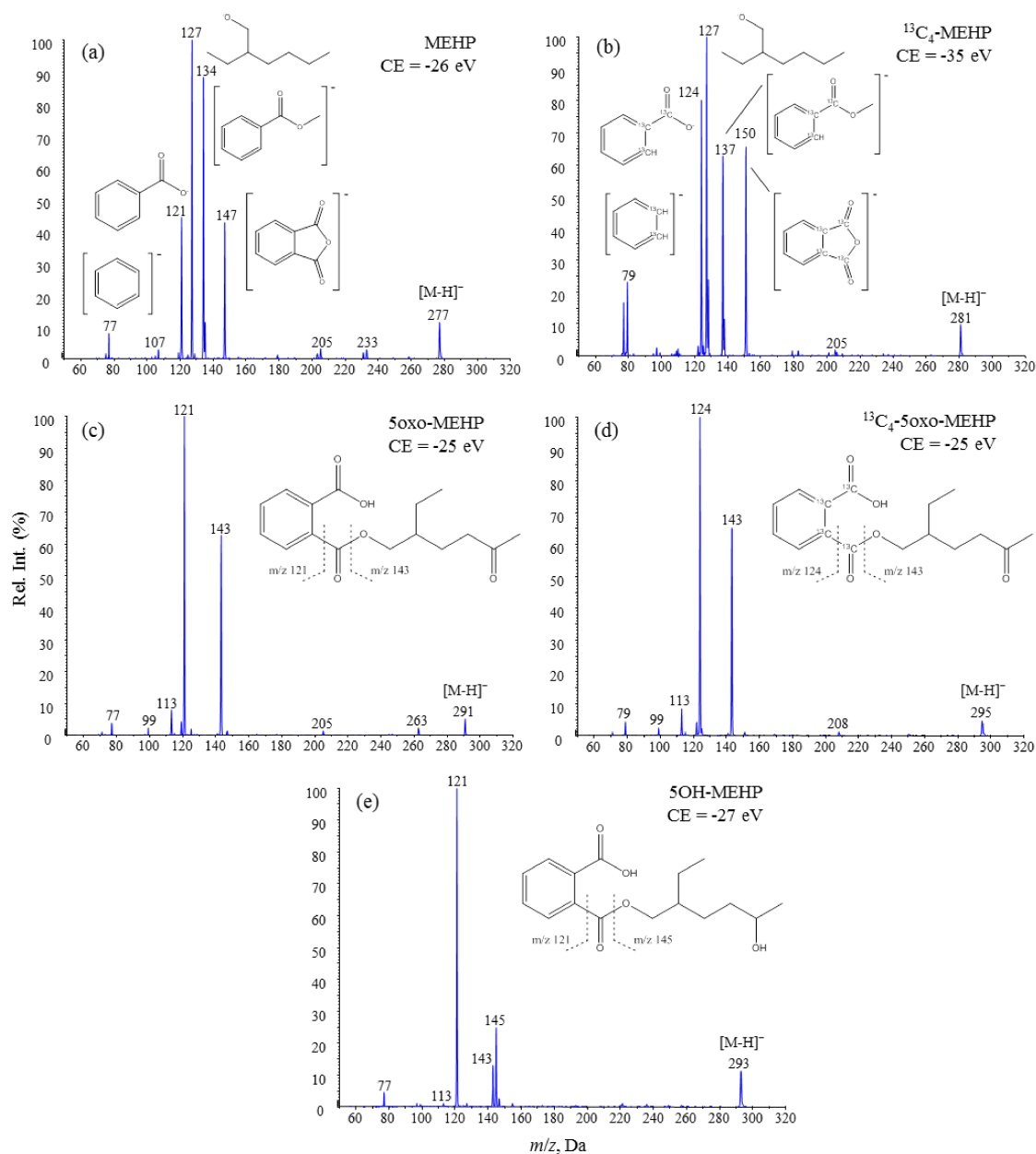
Figure 7.1: Negative ESI enhanced product ion spectra of the deprotonated molecules [M-H]⁻ of MEHP (a), ^{13}C$_4$-MEHP (b), 5oxo-MEHP (c), ^{13}C$_4$-5oxo-MEHP (d) and 5OH-MEHP (e) obtained in linear ion trap mode via direct injection of 1 μg/mL reference solutions employing collision energies from -25 to -35 eV.

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. For identification two additional qualifier ion transitions were acquired to ensure maximum selectivity (*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). The most relevant mass spectrometric parameters are summarized in Table 6.3.

The relative retention time and the relative ratios of three ion transitions of each compound were used to confirm the identity of the analyte (Table 7.1). The average retention times of the target compounds under the chosen conditions were 10.99 min for MEHP, 9.41 min for 5oxo-MEHP and 9.25 min for 5OH-MEHP (Table 7.1, Figure 7.2).
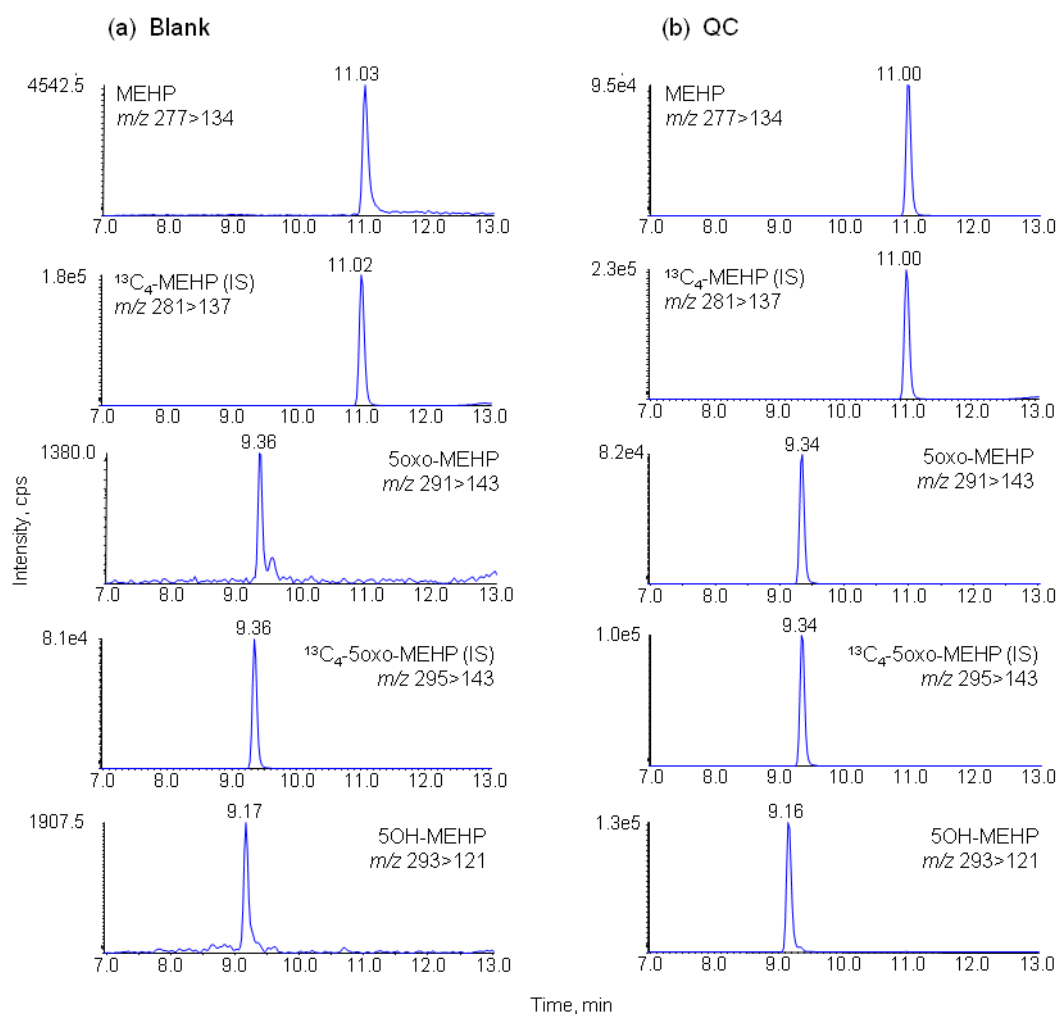
Figure 7.2: Extracted ion chromatograms of human urine samples: (a) blank urine sample; (b) quality control (QC) sample (urine sample fortified with 100 ng/mL of phthalate monoesters). Only quantifier ion transitions of analytes are shown.

7.1.2. Sample preparation aspects

Phthalate metabolites are excreted into urine mainly as glucuronides. Within the present study, the effect of incubation time on enzymatic hydrolysis was tested by determining deconjugated phthalate metabolites released after 2, 10, 20, 30 and 60 minutes of incubation at room temperature and also at 50°C for 30 minutes. Enzymatic hydrolysis of the analytes was found to be complete after 10 minutes at room temperature for all analytes (Figure 7.3). No improvement was observed in the case of incubation at 50°C from 30 minutes onwards. Further dilution yielded the final solution that was injected into the LC-MS/MS system resulting in a very simple and rapid sample preparation.
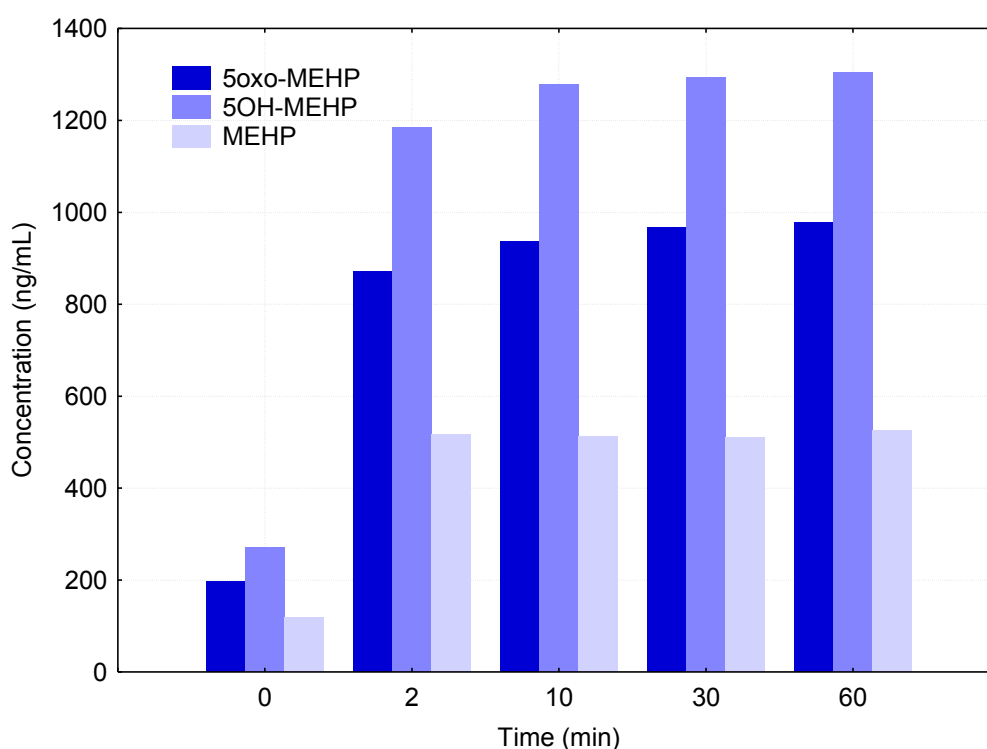
Figure 7.3: Optimization of enzyme deconjugation time of phthalate monoester glucuronides in urine at room temperature.

7.1.3. **Validation results**

In order to test for assay suitability, the following parameters were determined; specificity, linearity, ion suppression, accuracy, intra- and inter-day precision, LOD and LOQ.

7.1.3.1. Specificity

In terms of specificity, no significant interfering signals of the blank samples were detected at the expected retention times of the analytes. As expected, low basal levels of the analytes were always detectable with stable relative ion ratios identical to the reference compound.

7.1.3.2. Linearity

The assay demonstrated a linear correlation between analyte concentration and response within the given concentration range (1-250 ng/mL) with the correlation coefficients (R^2) above 0.99. The slope and the intercept of the calibration curves were 0.004 and 0.009 for MEHP, 0.008 and 0.001 for 5oxo-MEHP, 0.014 and -0.015 for 5OH-MEHP, respectively.

7.1.3.3. Ion suppression / enhancement

With regard to ion suppression or enhancement there was no significant decrease or increase of the electrospray response at the expected retention times of the analytes while the urinary matrix was injected.

7.1.3.4. Accuracy and precision

For accuracy, the relative recoveries were determined at three concentration levels (10, 100, 250 ng/mL) by means of comparison of theoretical and calculated concentrations ranging from 84% to 106% as shown in Table 7.1. The intra- and inter-day precisions were determined at three concentration levels with coefficients of variation less than 10% for low (10 ng/mL), medium (100 ng/mL), and high (250 ng/mL) concentration levels.

7.1.3.5. LOD and LOQ

At the required signal-to-noise ratio of 3, the LODs were estimated at 0.2-0.3 ng/mL and the corresponding LOQs at 1 ng/mL for MEHP, 5oxo-MEHP and 5OH-MEHP (Table 7.1) with CVs below 6.2% and accuracies ranging from 92.1 to 106.6%. The analytes were identified at the concentration level of the LOD using two and at the LOQ using three ion transitions. This assay demonstrated good sensitivity, although samples were diluted (1:5) and injected directly without any preconcentration steps.

7.1.3.6. Stability

Within this study the stability of the DEHP metabolites in the prepared samples (QCs) was tested. No significant decrease of the phthalate concentrations was observed in the quality control samples stored at -20, 4°C and room temperature for up to 4 months ensuring accurate quantification. Although in earlier studies the decomposition of DEHP metabolites was observed in urine samples stored above 4°C [234], quality control samples in this study were found to be stable, this was probably influenced by sample dilution (acetonitrile/water).

7.1.3.7. Uncertainty of the measurement

The uncertainty of the measurement is 12.88% for MEHP, 11.15% for 5oxo-MEHP and 19.84% for 5OH-MEHP which represents the expanded uncertainties at 95% confidence level using a coverage factor of $k = 2$.

Table 7.1: Summary of assay validation results.

	Accuracy			Precision											LOD	LOQ
	QC_{low}	QC_{medium}	QC_{high}	QC_{low}		QC_{medium}		QC_{high}		Retention Time		MRM ratio[c]		(S/N>3)	(S/N>10)
	Relative Recovery (%)[a]			CV (%)		CV (%)		CV (%)		Mean[a]	CV[a]	Mean[a]	CV[a]	Conc.	Conc.
				intra-day[a]	inter-day[b]	intra-day[a]	inter-day[b]	intra-day[a]	inter-day[b]	(min)	(%)	(%)	(%)	(ng/mL)	(ng/mL)
MEHP	94.8	99.4	97.7	6.10	2.89	3.61	2.63	3.33	2.70	10.99	0.32	34.35	0.88	0.2	1.0
5oxo-MEHP	96.1	105.2	97.9	3.41	3.92	3.17	3.07	0.71	3.10	9.41	0.25	30.31	1.45	0.3	1.0
5OH-MEHP	84.9	91.8	89.0	1.69	4.26	3.99	2.38	2.92	3.66	9.25	0.28	16.13	0.35	0.2	1.0

[a] Intra-day precision was calculated as the mean value of six replicates (n=6).

[b] Inter-day precision was calculated as the mean value of six replicates measured on three different days (n=18).

[c] MRM ratio: Ratio of the peak area detected at the quantifier and the qualifier ion transitions. The quantifier and qualifier ion transitions are *m/z* 277/134 and 277/127 for MEHP, 291/143 and 291/121 for 5oxo-MEHP, 293/121 and 293/77 for 5OH-MEHP, respectively.

7.1.4. Correlation of the results corrected to creatinine and specific gravity

In this study, creatinine was assayed in samples collected from healthy volunteers of the control group ($n = 100$) and of the longitudinal study ($n = 253$). In addition, the density was measured in all of the samples. Pearson correlation showed high correlation between the density and creatinine values (Figure 7.4).
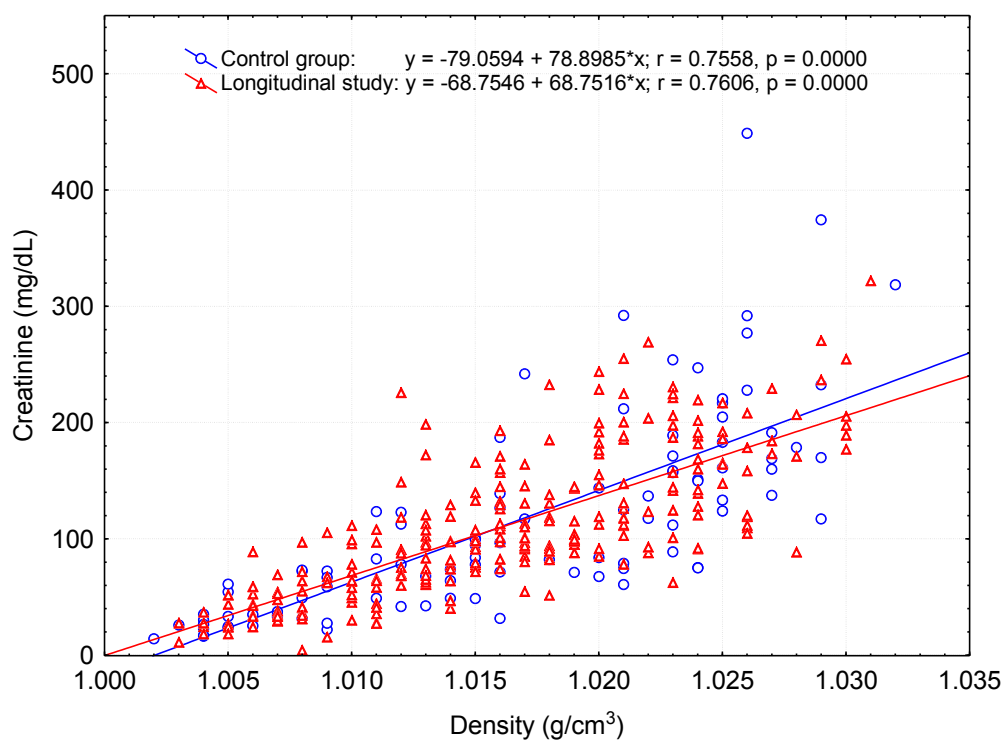
Figure 7.4: Correlation of density and creatinine values in urine samples from healthy volunteers: control group (blue circles), longitudinal study (red triangles).

However, Lorber *et al.* found that creatinine may not be suitable for the correction of DEHP metabolite concentration due to the short elimination time of the metabolites. Creatinine correction predicted higher concentrations when the ratio of the analytes suggested that the DEHP exposure was close in time and it began to underpredict the intake after approximately 8 hours [243]. Therefore, correction to specific gravity was used for statistical evaluation and routine purpose.

7.1.5. Reference populations

7.1.5.1. Control samples

In order to determine physiological levels of DEHP metabolites, urine samples from 100 healthy volunteers were analysed. The primary metabolite MEHP was quantifiable in 100% of the control samples ($n = 100$), the secondary metabolites 5OH-MEHP and 5oxo-MEHP could be quantified in 93% and 91% of the control specimens, with concentrations of MEHP, 5oxo-MEHP and 5OH-MEHP ranging from 2.1 ng/mL to 50.4 ng/mL, from < LOQ to 36.2 ng/mL and from < LOQ to 65.5 ng/mL in the control samples (Table 7.2, Figure 7.5), respectively.

Table 7.2: Results of statistical evaluation of phthalate monoester levels in the control urine samples ($n = 100$).

Urinary phthalate monoester	μ_H[a]	Min	Max	Upper reference limit, 99.9%[a]	Frequency of quantification
	(ng/mL)	(ng/mL)	(ng/mL)	(ng/mL)	%
5oxo-MEHP	6.2	< LOQ	36.2	54.4	93
5OH-MEHP	12.5	< LOQ	65.5	94.8	91
MEHP	9.3	2.1	50.4	74.6	100

[a] Estimators for the central tendency (μ_H) and upper reference limit (99.9%) of the control group (ng/mL). Estimates based on Huber's robust statistical method (see 6.1.8.1 for the precise meaning of μ_H).

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 (Table 7.4). Thus, 5oxo-MEHP and 5OH-MEHP represented more appropriate markers to indicate blood transfusion.

7.1.5.2. Athletes' samples

For the determination of the concentration levels of secondary DEHP metabolites in elite athletes, 468 official doping control samples covering different kind of sport and origin were investigated. It is stressed that the athletes' samples cannot fulfil the criteria of a controlled study, since medical treatment cannot be excluded.

MEHP, 5OH-MEHP and 5oxo-MEHP were quantifiable in 58%, 95% and 80% of the samples, respectively. The highest concentration in the doping control samples was found for 5OH-MEHP, followed by 5oxo-MEHP and MEHP (Table 7.3). Figure 7.5 shows the histogram and the density plot for log-normal distribution of urinary 5oxo-MEHP and 5OH-MEHP monoester levels quantified in 468 athlete specimens. 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. Within the investigated athletes' group there were 4 outliers (0.85%) identified, yielding concentrations in the range found after blood transfusion (Figure 7.6). Since information regarding the medical treatment of these athletes was not available, increased levels of secondary DEHP metabolites may indicate an unusual increased environmental exposure or blood transfusion. Three out of four outliers were taken out-of competition at the same time, originating from one cycling team. As recommended by Monfort *et al.* the basal levels of DEHP metabolites may be implemented to athletes' Biological Passport [164-167] to suspect the misuse of blood transfusions [29].

Table 7.3: Results of statistical evaluation of phthalate monoester levels in athletes' urine samples (*n* = 468).

Urinary phthalate monoester	μ_H[a]	Min	Max	Upper reference limit, 99.9%[a]	Frequency of quantification
	(ng/mL)	(ng/mL)	(ng/mL)	(ng/mL)	%
5oxo-MEHP	6.1	< LOQ	590.7	157.3	80
5OH-MEHP	18.9	< LOQ	1125.0	193.0	95
MEHP	2.1	< LOQ	203.3	96.1	58

[a] Estimators for the central tendency (μ_H) and upper reference limit (99.9%) of the athletes group (ng/mL). Estimates based on Huber's robust statistical method (see 6.1.8.1 for the precise meaning of μ_H).
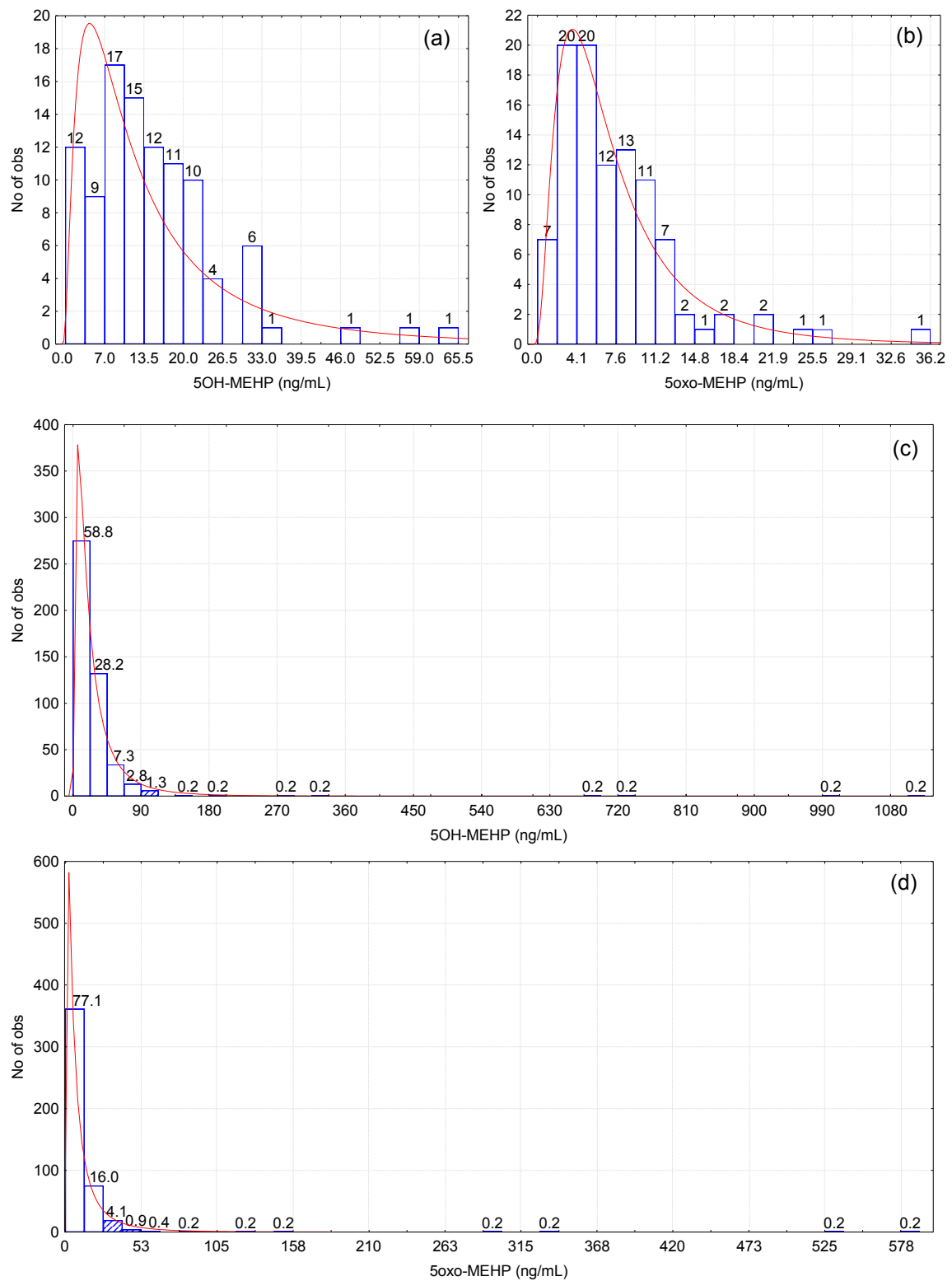
Figure 7.5: Histograms and density plots for log-normal distributions of urinary DEHP monoester values in 100 urine samples of the control group: 5OH-MEHP (a), 5oxo-MEHP (b); and in 468 urine samples from athletes: 5OH-MEHP (c), 5oxo-MEHP (d). Labels represented above bars indicate the percentage values.

7.1.6. Post-transfusion samples

7.1.6.1. Excretion study

Urine samples of the excretion study showed elevated DEHP metabolite concentrations up to 24 hours after blood transfusion. In comparison to the control group (Table 7.2) significantly higher concentrations of the secondary DEHP metabolites were observed in urine samples collected after blood transfusion (Table 7.4). 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.

Table 7.4: Concentrations of phthalate monoesters in the urine samples of transfused patients.

Patient	Time after transfusion	Concentration (ng/mL)		
	(hours)[a]	5oxo-MEHP	5OH-MEHP	MEHP
	12	634.4	908.2	239.7
1	24	484.5	540.8	204.3
	Blank	24.7	41.6	15.2
	6	740.9	876.1	351.8
2	12	250.4	279.6	116.6
	Blank	24.7	41.6	15.2
	6	1098.0	2122.0	46.2
3	12	980.2	2333.3	29.8
	Blank	0.5	25.3	10.7
	6	1374.4	1720.8	139.8
4	12	390.0	532.0	39.6
	Blank	31.2	67.8	4.5
	5	4995.0	8605.0	471.3
5	14	940.4	1692.4	98.0
	Blank	9.8	68.0	15.8

[a] Blank samples were taken before blood transfusion.

The 99.9% upper reference limits of the control group were determined as 54.4 ng/mL for 5oxo-MEHP and 94.8 ng/mL for 5OH-MEHP which are 12- and 9-times lower than the lowest value measured after blood transfusion. The chromatogram of a control sample and a post-transfusion sample are shown in Figure 7.7.
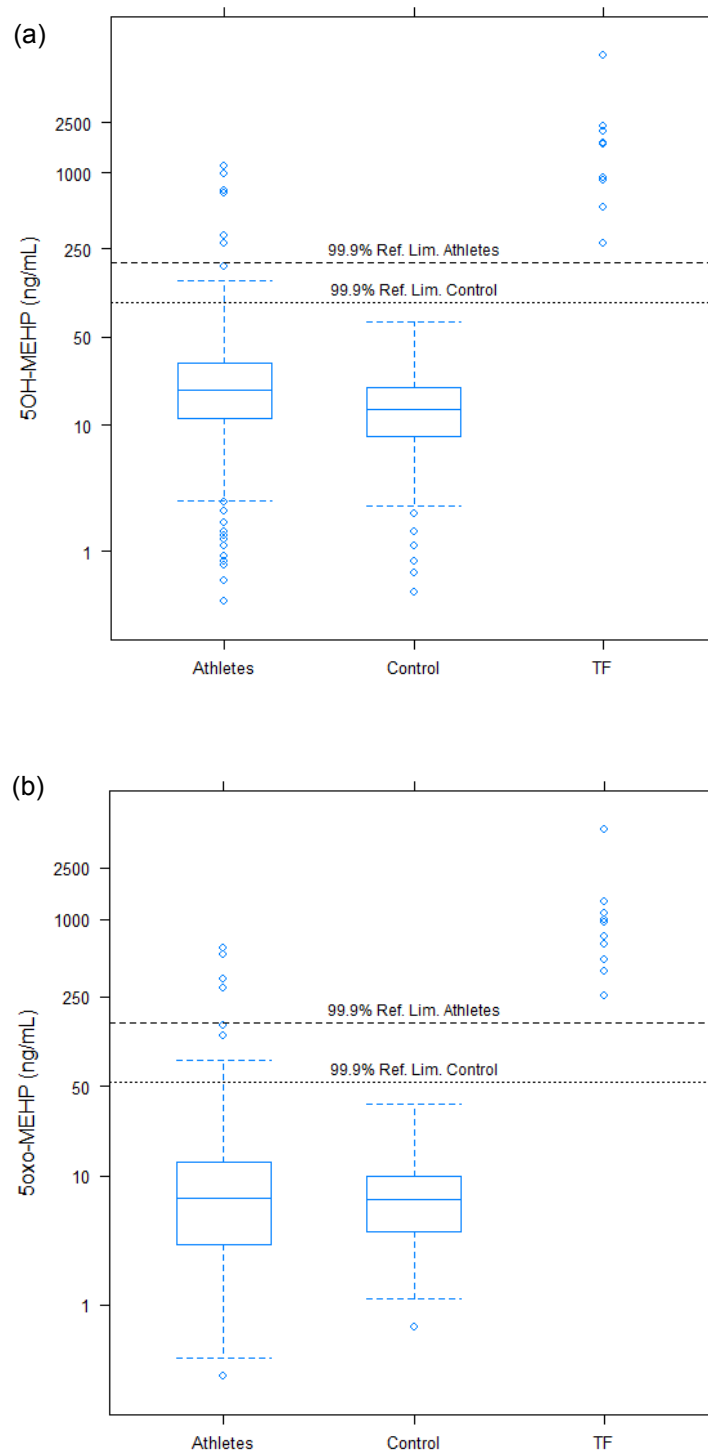
Figure 7.6: Concentrations of urinary DEHP metabolites 5OH-MEHP (a) and 5oxo-MEHP (b) in athletes' samples, in the control group and in transfused patients (TF). Horizontal bars indicate the medians, boxes enclose the 25th and 75th percentiles, whiskers indicate the minimum and maximum data values excluding the outliers and circles illustrate the outside values. The 99.9% reference limits are calculated from the log-transformed concentrations.

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. Within the investigated athletes' group there were 4 outliers (0.85%) identified, yielding concentrations in the range found after blood transfusion (Figure 7.6).
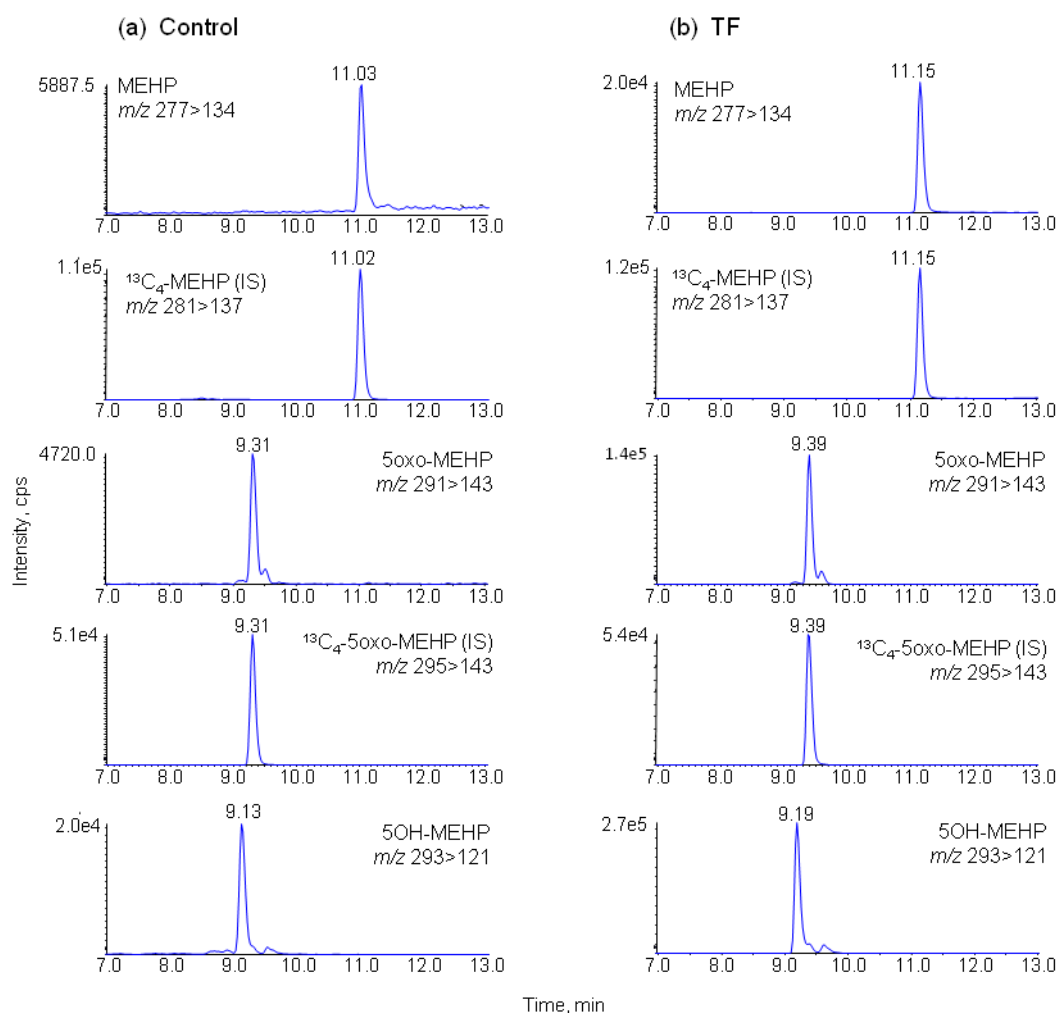
Figure 7.7: Extracted ion chromatograms of human urine samples: (a) control sample containing 13 ng/mL of 5oxo-MEHP, 32.8 ng/mL of 5OH-MEHP and 5.1 ng/mL of MEHP and (b) undiluted sample of transfused patient containing 1374.4 ng/mL of 5oxo-MEHP, 1720.8 ng/mL of 5OH-MEHP and 139.8 ng/mL of MEHP. Only quantifier ion transitions of analytes are shown.

7.1.6.2. Urinary concentrations of the metabolites related to the storage time of the transfused blood

Samples collected at the Ruhr-University Bochum were divided into three groups: control samples (collected from Group 1 and Group 2 excluding the day of blood transfusion), samples collected on the day of transfusion from Group 1 (TF-1, blood stored for 14 days before transfusion) and from Group 2 (TF-2, blood stored for 28 days).

5oxo-MEHP, 5OH-MEHP and MEHP were quantified in 80%, 89% and 57% of the specimens of the control group, with concentrations ranging from $<$ LOQ to 175.0 ng/mL, from < LOQ to 496.5 ng/mL and from < LOQ to 9.5 ng/mL, respectively. The 99.9% upper reference limits of the control group were determined as 198.5 ng/mL for 5oxo-MEHP and 341.6 ng/mL for 5OH-MEHP (Table 7.5). The urinary levels of the metabolites significantly increased on the day of transfusion compared to the day before with maximum concentrations of 8440 ng/mL and 11200 ng/mL for 5oxo-MEHP and 5OH-MEHP respectively (Table 7.6 , Figure 7.8).

Table 7.5: Results of statistical evaluation of phthalate monoester levels in the control samples collected at the Ruhr-University Bochum (n = 356).

Urinary phthalate monoester	μ_{H}[a]	Min	Max	Upper reference limit, 99.9%[a]	Frequency of quantification
	(ng/mL)	(ng/mL)	(ng/mL)	(ng/mL)	%
5oxo-MEHP	5.9	$<$ LOQ	175.0	198.5	80
5OH-MEHP	12.1	$<$ LOQ	496.5	341.6	89
MEHP	2.5	$<$ LOQ	9.5	60.8	57

[a] Estimators for the central tendency (μ_{H}) and upper reference limit (99.9%) of the hospitalized patients (ng/mL). Estimates based on Huber's robust statistical method (see 6.1.8.1 for the precise meaning of μ_{H}).
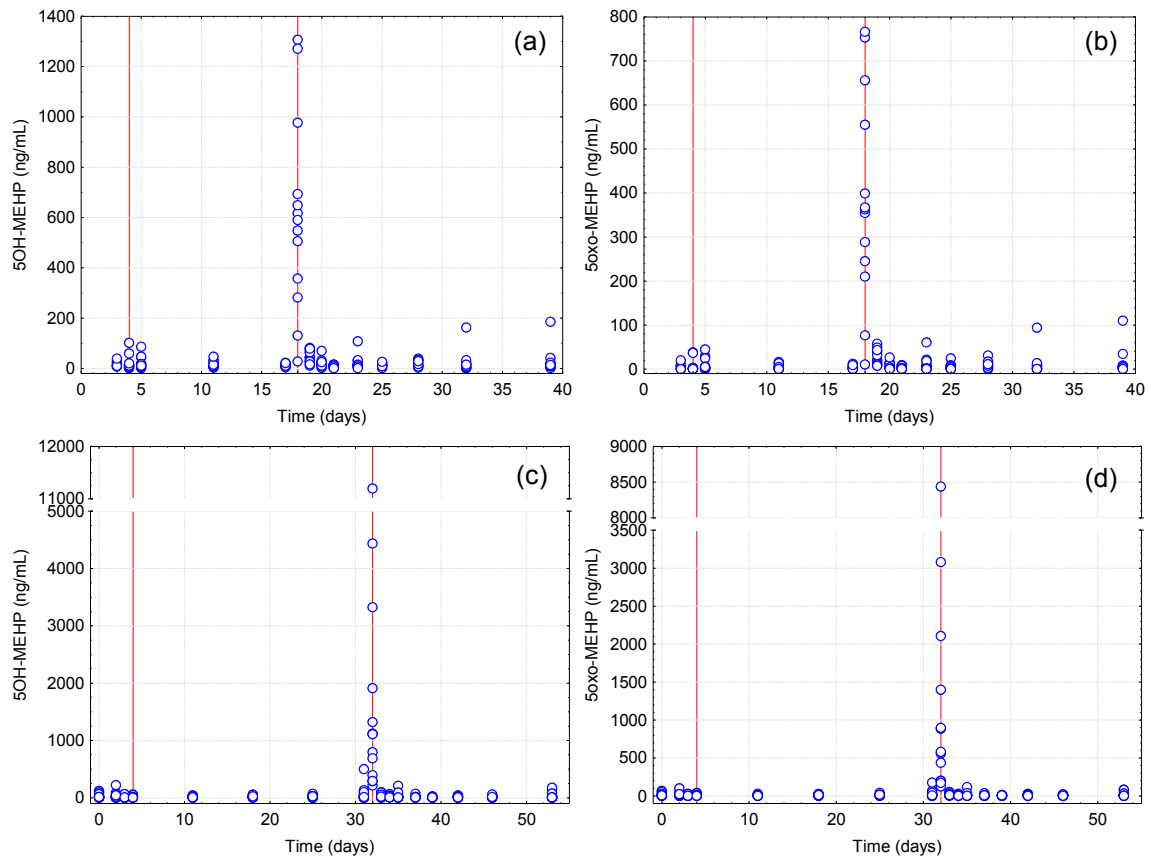
Figure 7.8: Concentrations of urinary DEHP metabolites 5OH-MEHP (a,c) and 5oxo-MEHP (b,d) in samples collected from subjects receiving blood transfusions at the Ruhr-University Bochum. Group 1: transfusion of blood stored for 14 days (a,b), Group 2: transfusion of blood stored for 28 days (c,d). Vertical red lines indicate the day of phlebotomy (4th day) and the day of blood transfusion (18th day (a,b) and 32nd day (c,d)).

Median (μ_H) values of the control group were in agreement with the other reference populations (Section 7.1.5). However, the 99.9% reference limits were higher than the ones calculated from the athletes' populations. A possible reason for the higher values is that the concentration of the phthalate metabolites can be elevated up to 24 hours after transfusion [103] and the values from the day after transfusion were included in the control group. The concentrations on the day after transfusion in some cases were slightly but not significantly higher than the median values of the control group. Some samples from TF-2 showed higher levels of the metabolites than samples from TF-1 (Figure 7.9) which is in agreement with data showing increasing DEHP concentration over time in blood stored in PVC bags [6].

Table 7.6: Concentrations of phthalate monoesters in urine samples collected on the day of reinfusion at the Ruhr-University Bochum (*n* = 25).

Subjects	Concentration (ng/mL)		
	5oxo-MEHP	5OH-MEHP	MEHP
Group TF-1			
1	354.7	506.0	80.0
2	656.0	976.0	295.6
3	76.6	131.3	42.0
4	753.3	1306.7	458.7
5	554.7	693.3	211.3
6	288.8	548.0	44.4
7	210.0	282.0	112.6
8	362.9	617.1	228.9
9	245.0	356.7	78.0
10	766.0	1270.0	324.0
11	366.9	590.0	318.5
12	399.3	648.7	174.0
Average	**419.5**	**660.5**	**197.3**
Min	**76.6**	**131.3**	**42.0**
Max	**766.0**	**1306.7**	**458.7**
Group TF-2			
1	886.0	1320.0	396.0
2	2105.0	3325.0	1550.0
3	559.1	796.4	750.0
4	3080.0	4433.3	2050.0
5	894.4	1122.2	498.9
6	126.8	213.3	100.0
7	8440.0	11200.0	5860.0
8	580.0	1110.0	193.5
9	436.3	687.5	393.8
10	200.0	288.3	118.0
11	195.7	392.2	149.6
12	170.0	293.0	185.0
13	1400.0	1914.3	654.3
Average	**1467.2**	**2084.3**	**992.2**
Min	**126.8**	**213.3**	**100.0**
Max	**8440.0**	**11200.0**	**5860.0**

Figure 7.9: Concentrations of urinary DEHP metabolites 5OH-MEHP (a) and 5oxo-MEHP (b) in samples collected from subjects receiving blood transfusions at the Ruhr-University Bochum; in the control samples (Control), after transfusion of blood stored for 14 days (TF-1), after transfusion of blood stored for 28 days (TF-2). *Horizontal bars* indicate the medians, *boxes* enclose the 25th and 75th percentiles, *whiskers* indicate the minimum and maximum data values excluding the outliers and circles illustrate the outside values.

Independent analysis was performed on this set of samples by the Bioanalysis Research Group, IMIM-Hospital del Mar (Barcelona, Spain) using a different analytical method. The results obtained by the two laboratories were compared by Monfort *et al.* showing high correlation between the two sets of data [237].

The 99.9% reference limits calculated from the control samples of this study were found to be 198.5 ng/mL and 341.6 ng/mL for 5oxo-MEHP and 5OH-MEHP, respectively (Table 7.5). The values are comparable with the reference limits established by Monfort *et al.* at 158.5 ng/mL for 5oxo-MEHP and 338.8 ng/mL for 5OH-MEHP [126]. The lower reference limit calculated for 5oxo-MEHP by Monfort *et al.* is possibly due to the use of a different analytical procedure or different statistical calculation. The higher value for 5OH-MEHP compared to the reference limit originating from the athletes' population (Table 7.3) is probably due to the different origin of the samples.

Urinary concentrations of DEHP metabolites obtained from transfused patients were in agreement with the results of other publications testing urine samples of plasma and platelet donors shortly after apheresis [103,102] and hospitalized patients receiving autologous blood transfusion [29].

7.1.7. Longitudinal study

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

Intra-individual variability of urinary DEHP metabolites was tested 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 e.g. residential, dietary or environmental exposure.

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 56.0 ng/mL, 77.7 ng/mL and 154.3 ng/mL, respectively (Table 7.7). The calculated median values for each volunteer 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.

Table 7.7: Results of statistical evaluation of phthalate monoester levels in the samples collected for the longitudinal study ($n = 253$).

Urinary phthalate monoester	μ_H[a]	Min	Max	Upper reference limit, 99.9%[a]	Frequency of quantification
	(ng/mL)	(ng/mL)	(ng/mL)	(ng/mL)	%
5oxo-MEHP	6.0	< LOQ	77.7	86.2	87
5OH-MEHP	14.6	< LOQ	154.3	103.6	96
MEHP	1.9	< LOQ	56.0	48.3	57

[a] Estimators for the central tendency (μ_H) and upper reference limit (99.9%) of the longitudinal study group (ng/mL). Estimates based on Huber's robust statistical method (see 6.1.8.1 for the precise meaning of μ_H).
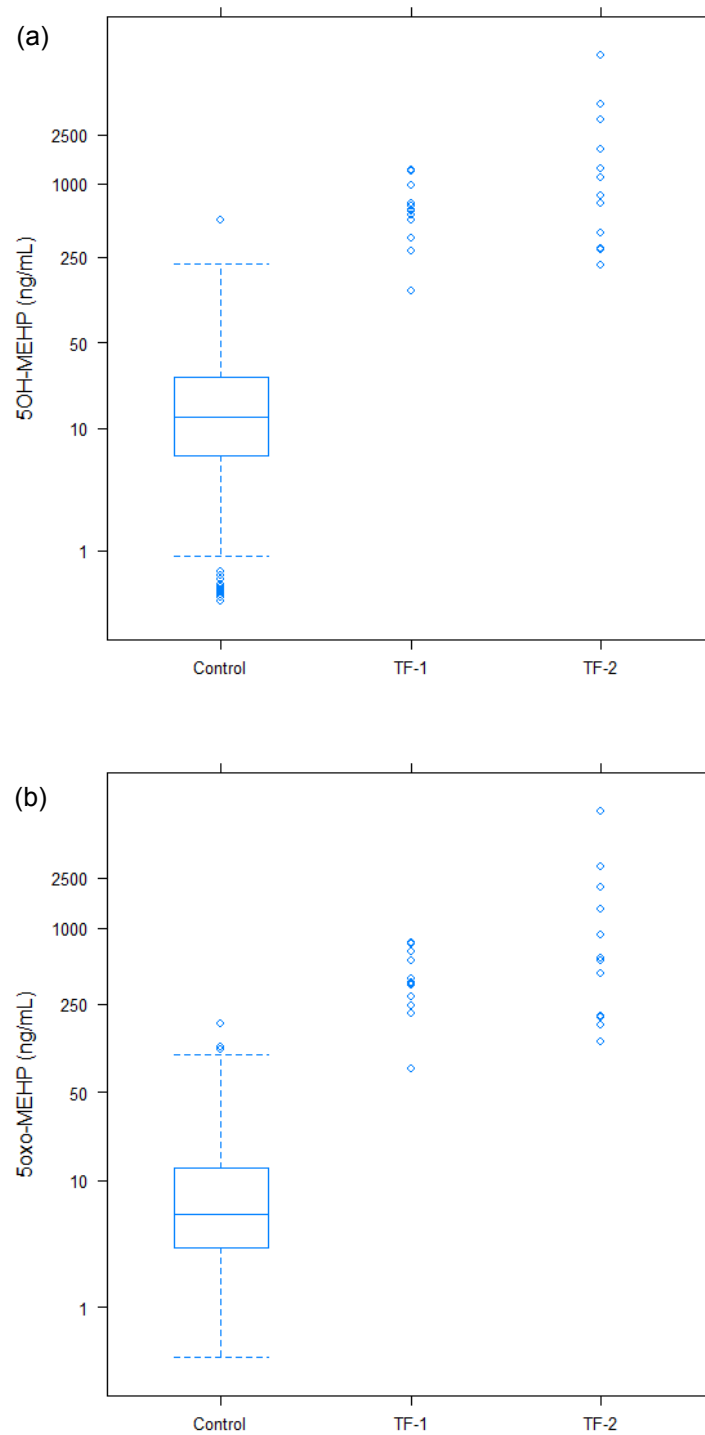
The urinary concentrations of MEHP, 5oxo-MEHP and 5OH-MEHP corrected to specific gravity are plotted for each subject in Figure 7.10.

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 7.10, some of the studied subjects demonstrated only weak variability (volunteers 1-5) while others showed considerable variance (volunteers 6 and 7). However, no correlation between the elevated values of volunteers 6 and 7 could be identified (Figure 7.10 (b)).

Generally, the median values within this study were in agreement with earlier results [29,244,245,123,119], although slightly higher median concentrations were observed in some cases [12,15,246,124]. Regarding the maximum concentrations, the values were consistent with the maximum concentrations found in the reference populations (Section 7.1.5.1 and 7.1.5.2). However, some studies reported significantly higher maximum concentrations. In 2007, Fromme *et al.* demonstrated a substantial within-subject variability of urinary DEHP metabolites by investigating 50 healthy volunteers (Munich, Germany) on 8 consecutive days, with maximum concentrations for 5oxo- and 5OH-MEHP ranging from 439.9 to 674.3 ng/mL for women and from 215.4 to 309.3 ng/mL for men [12]. Inter-individual variability of urinary DEHP metabolites was also studied by Preau *et al.* analysing samples collected from 8 volunteers (Atlanta, 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 [246]. Analysis of samples from 25 men working in dental laboratories (Seoul, Korea), with possible occupational exposure to DEHP, showed a significant difference 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 [123]. Contrarily, when analysing first morning urine samples the level of DEHP metabolites was observed to be more reproducible over time [124,115].
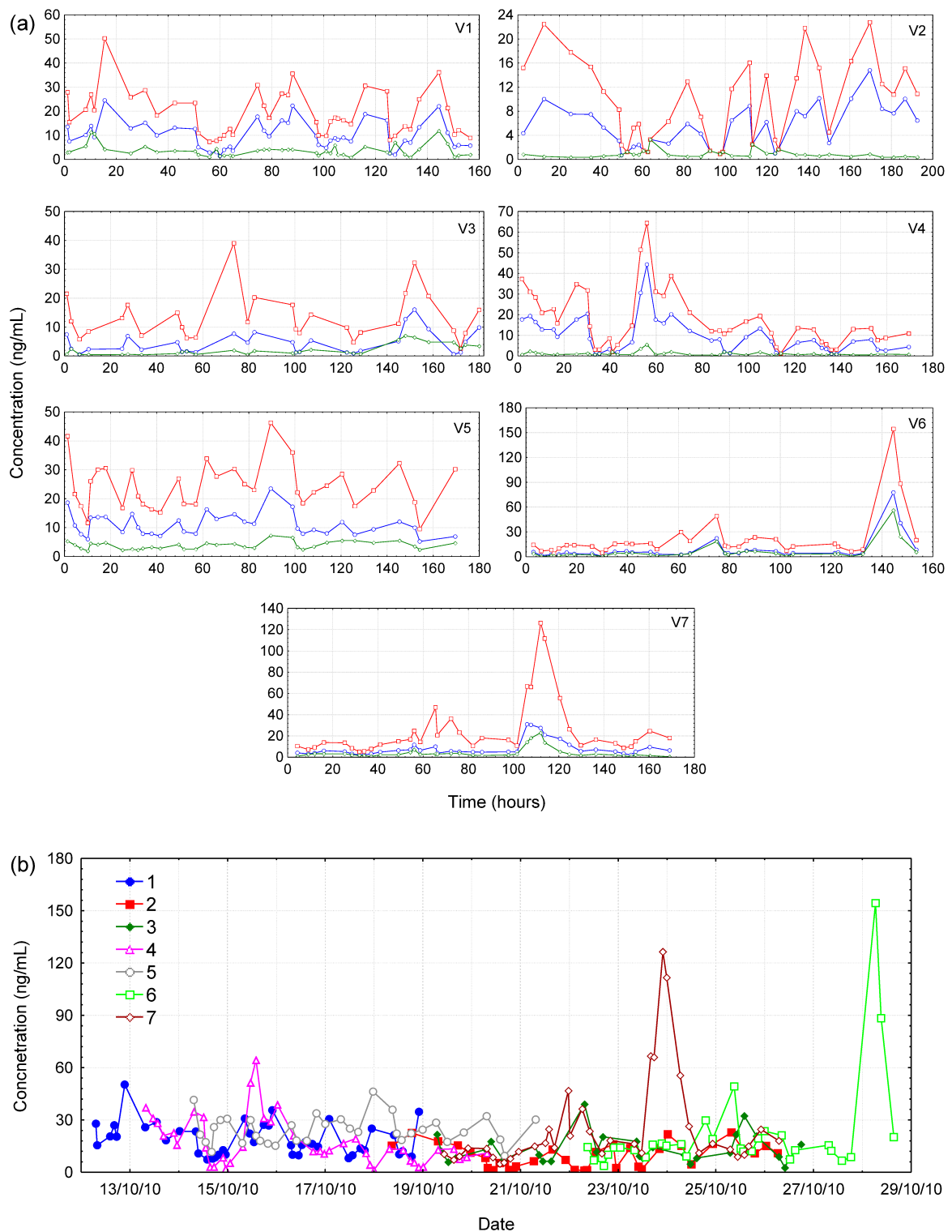
Figure 7.10: (a) Concentrations of 5oxo-MEHP (blue line), 5OH-MEHP (red line) and MEHP (green line) corrected to specific gravity in urine samples collected from 7 volunteers (V1-V7). (b) Gravity-adjusted concentrations of 5OH-MEHP represented in uniform-scale for all volunteers.

As described above, samples of transfused patients and reference populations were investigated, and the determined 99.9% upper reference limits of an athletes' population ($n = 468$) provided values of 157.3 ng/mL for 5oxo-MEHP and 193.0 ng/mL for 5OH-MEHP (Section 7.1.5.2). In this work, the determined concentrations of each subject did not exceed this upper reference limits. Although urinary concentrations of the metabolites showed considerable intra-individual variation, no increased values have been observed comparable to the concentrations measured in urine specimens collected after blood transfusion (Section 7.1.6). The results confirm our findings that determination of the urinary concentration of DEHP metabolites has a high potential to indicate homologous or autologous blood transfusion, and may provide supporting evidence to prove blood doping. Additionally, longitudinal studies would present valuable data that can be utilised for interpretation of abnormally high DEHP metabolite concentrations in athletes.

7.1.8. Comparison of reference populations

Control samples were classified into 4 groups: samples collected from healthy volunteers at the German Sport University Cologne (Control-1), samples collected from healthy volunteers for the longitudinal study (Control-2), samples collected from subjects receiving blood transfusions at the Ruhr-University Bochum (Control-3) and the official doping-control urine samples (Athletes).

All control populations tested in this study gave consistent results (Figure 7.11). Higher values were found only in the athletes' samples which were considered as outliers (Section 7.1.5.2). Compared to the athletes' population (Table 7.3) the slightly increased concentration of MEHP in the control samples (Control-1, Table 7.2) is probably caused by contamination of urine specimens since MEHP is also present ubiquitously in the environment (e.g. light induced conversion of DEHP) [10,22,190]. The basal concentration of the metabolites in the reference populations were on the lower side of the usual range reported earlier [12,15,245,246,122] probably due to the more homogenous nature of these populations. The mean values were in accordance with most of the studies [12,13,18,29,118,237,244,245,123,119,246,124,115,126], only slightly higher values were found in some cases [15,16,99,122,125]. Other studies showed considerably higher maximum urinary concentrations of DEHP metabolites [12,15,246,122,125] probably due to higher occupational or environmental exposure or the different origin of the samples.

It is noteworthy that in most studies of DEHP exposure first morning urine samples were collected which are known to have slightly higher phthalate levels than spot urine samples collected throughout the day [246,247].

No correlation was observed between the concentrations of the metabolites and the gender or the age of the subjects. In the case of the athletes' population no significant difference in the urinary DEHP metabolite levels was found for samples collected in-competition and out-of competition or regarding the sport type.

Since in different countries there is a trend in substituting DEHP according to its toxicity. This may result in lower general exposure in humans, and thus, decreasing urinary concentrations over time [18].
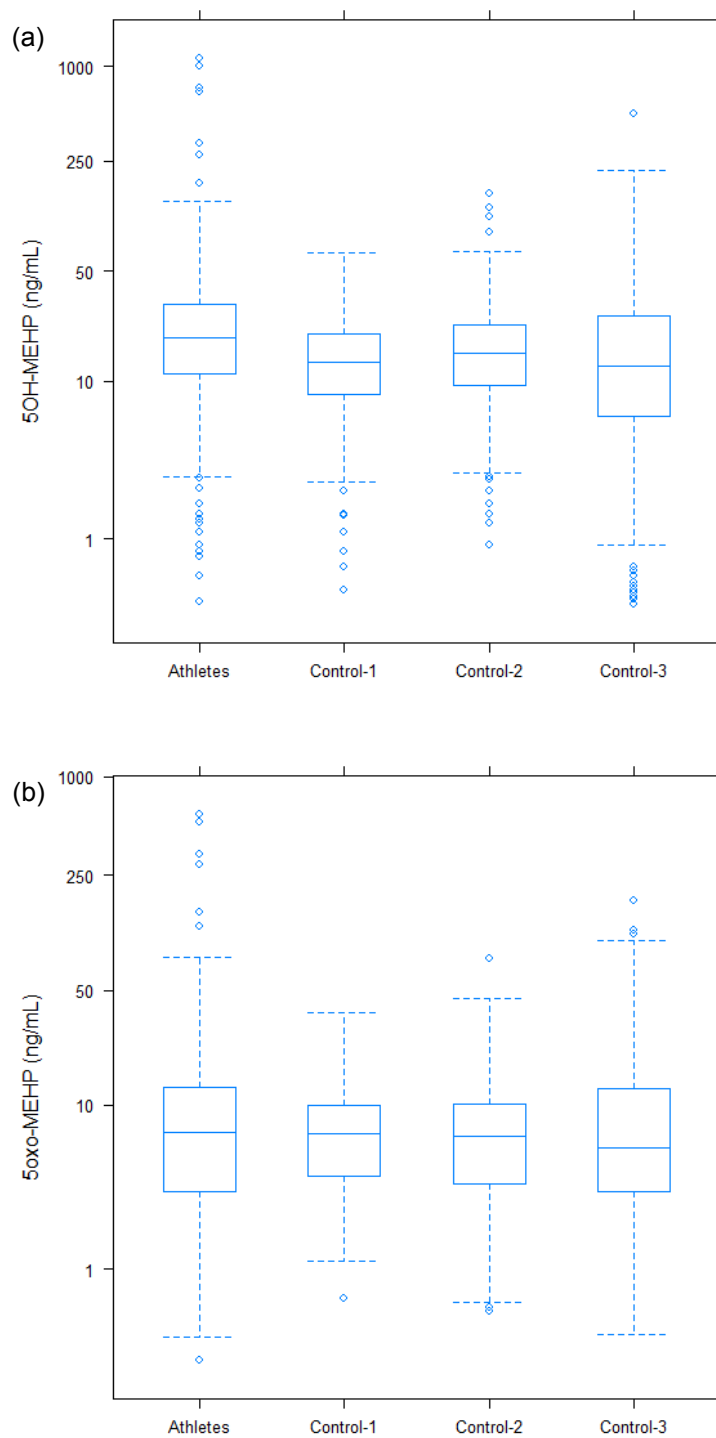
**Figure 7.11: Concentrations of urinary DEHP metabolites 5OH-MEHP (a) and 5oxo-MEHP (b) in athletes'
samples and in different control groups. 100 samples collected from healthy volunteers (Control-1), samples
collected from healthy volunteers for the longitudinal study (Control-2), and control samples collected from
subjects receiving blood transfusions at the Ruhr-University Bochum (Control-3).** *Horizontal bars* **indicate the
medians,** *boxes* **enclose the 25th and 75th percentiles,** *whiskers* **indicate the minimum and maximum data values
excluding the outliers and circles illustrate the outside values.**

7.1.9. Comparison of reference populations and post transfusion samples

Post-transfusion samples were classified into 3 groups: the samples collected at the Ruhr-University Bochum from volunteers receiving transfusion of blood stored for 14 days (Group TF-1) and for 28 days (Group TF-2, Section 7.1.6.2), and the excretion urine samples collected in Neuss (Group TF-3, Section 7.1.6.1).

92% of the samples from Group TF-1 showed higher levels of 5oxo-MEHP and 5OH-MEHP than the 99.9% reference limit of 157.3 ng/mL and 193.0 ng/mL, respectively. Regarding TF-2, 92% of 5oxo-MEHP and all 5OH-MEHP concentrations were found to be higher than the reference limits. Levels of both metabolites exceeded the corresponding reference limits in all post-transfusion samples of Group TF-3 (Figure 7.12).

The findings of Monfort *et al.* suggest that transfusion of DEHP seems to promote an increase in the rate of oxidative metabolism of MEHP to secondary metabolites, or a rapid elimination of MEHP [237]. In contrast, in this study the ratios of the secondary metabolites to MEHP did not show significant difference in the post-transfusion samples compared to the control groups (Table 7.8). Earlier studies showed that the relative distribution of the metabolites excreted in urine after intravenous exposure was similar to the distribution after oral administration [98,103]. Due to the rapid metabolism of DEHP and the different elimination half-life times of the metabolites, the main factor influencing the ratios of the metabolites is the time between the exposure and the collection of the urine specimens.

The ratios of both oxidative metabolites relative to MEHP were consistent which is in agreement with earlier studies showing that the kinetic profiles of these two metabolites are similar [103]. The data may suggest a lower ratio of 5OH-MEHP to 5oxo-MEHP in post-transfusion samples compared to their ratio in the control samples. This could be a result of different pharmacokinetic behaviour of the metabolites after parenteral exposure, however, due to the limited amount of data it needs to be investigated further.

Table 7.8: Average ratios of DEHP metabolite concentrations in different study groups.

Sample Group	Average ratio of the metabolites		
	5oxo-MEHP / MEHP	5OH-MEHP / MEHP	5OH-MEHP / 5oxoMEHP
Athletes	5.74	15.84	3.76
Control-1	0.90	1.84	2.04
Control-2	4.92	10.62	2.68
Control-3	3.01	6.16	2.28
TF-1	2.64	4.21	1.57
TF-2	1.58	2.47	1.56
TF-3	9.49	18.06	1.71
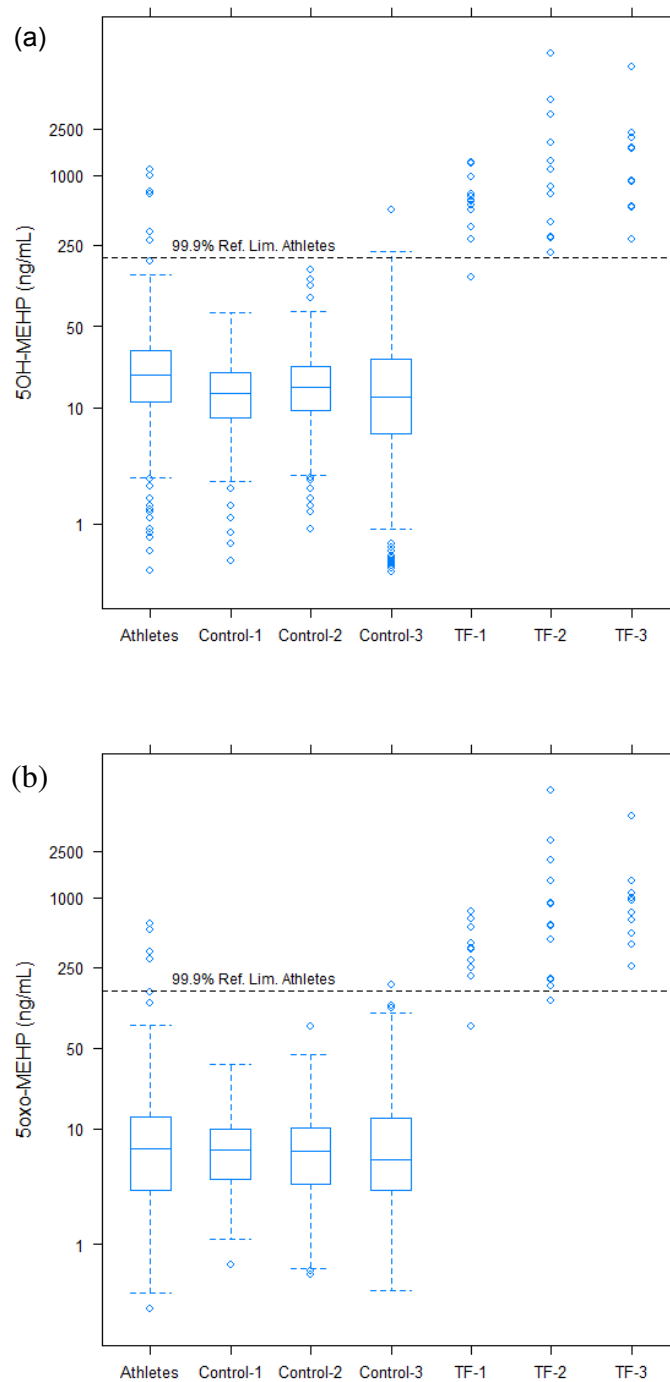
Figure 7.12: Concentrations of urinary DEHP metabolites 5OH-MEHP (a) and 5oxo-MEHP (b) in athletes' samples, in different control groups and in transfused patients. 100 samples collected from healthy volunteers (Control-1), samples collected from healthy volunteers for the longitudinal study (Control-2), control samples collected from subjects receiving blood transfusions at the Ruhr-University Bochum (Control-3), samples from subjects receiving transfusions after 14 days blood storage (TF-1), after 28 days blood storage (TF-2) and samples from transfused patients of the excretion study (TF-3). *Horizontal bars* **indicate the medians,** *boxes* **enclose the 25th and 75th percentiles,** *whiskers* **indicate the minimum and maximum data values excluding the outliers and circles illustrate the outside values.**

7.1.10. Conclusion

A direct injection HPLC-(ESI)-MS/MS method is presented for the quantification of three DEHP metabolites in human urine as a marker for illicit blood transfusion in sports drug testing. The straightforward assay provides good sensitivity and reliability for the determination of phthalate monoesters using ^{13}C-labelled internal standards without purification, concentration or further sample preparation steps resulting in a high-throughput method, that ensures compatibility to existing "dilute-and-shoot" screening methods in doping control. Currently, the presented assay covers only DEHP. However, an implementation of alternatively used plasticisers in blood bags or other medical devices seems to be promising assuming that the substances used have comparable physical properties [147]. As a future prospect the applicability of the method on different plasticisers has to be investigated.

Within this study it was clearly demonstrated that urinary concentration levels of the secondary DEHP metabolites 5OH-MEHP and 5oxo-MEHP after blood transfusion significantly differs from concentrations found in a control group. The reference limits (99.9%) obtained from the investigation of control and athletes' specimens enables the identification of abnormally high concentrations of DEHP metabolites indicating homologous or autologous blood transfusion. Nevertheless, to prosecute blood doping this assay provides valuable data that can be used as supporting evidence in interpretation of conspicuous Biological Passport data. Based on this study, the abnormally high concentrations of DEHP metabolites analysed at the Laboratory for Doping Analysis at the Institute of Biochemistry of the German Sports University Cologne are reported to federations which adopted the biological passport program.

7.2. INTEGRATION OF DI(2-ETHYLHEXYL) PHTHALATE METABOLITES INTO SCREENING PROCEDURE

The phthalate metabolites are excreted into urine mainly as conjugates following phase-II glucuronidation [20]. Commonly, these conjugates are enzymatically hydrolysed and determined with LC-MS/MS [29]. 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.

7.2.1. Chromatographic and mass spectrometric parameters

The assay is based on LC-(ESI)-MS/MS using direct injection of urine specimens to screen for various classes of prohibited substances. Using a highly sensitive new generation hybrid mass spectrometer enables a combined screening of diuretics, beta2-agonists, narcotics, stimulants and their sulfo-conjugates, plasma volume expanders, selective androgen receptor modulators and 5-amino-4-imidazolecarboxyamide ribonucleoside (AICAR). The possibility of fast polarity switching (50 ms) ensures an optimized ionisation, regardless of differences in the acidic or basic character of the molecules. Therefore, the detection of a wide range of doping agents can be accomplished in one analytical run. Analysing native urine specimens using direct injection provides the ability to screen for many different compounds and their metabolites without time-consuming sample preparation steps.

The chromatographic run was optimised taking into account the chemical versatility of the analytes, resulting in a wide range of polarities. A gradient starting at 100% aqueous buffer (5 mM ammonium acetate, 0.1% glacial acetic acid, pH = 3.5) was required to ensure sufficient retention for hydrophilic compounds. To avoid column blockage a pre-column was used. Over 2000 analyses were conducted with the same analytical column without any loss in chromatographic performance. As presented (Table 7.10) the relative retention times of the analytes proved to be stable with CVs of less than 2%, fulfilling the recommended identification criteria [248].

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) (for method see Table 11.1). A post-transfusion sample was analysed containing known amounts of 5OH-MEHP (665 ng/mL) and 5oxo-MEHP (421 ng/mL), 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 calculated errors of 1.48 ppm and 0.03 ppm, respectively (Table 7.9, Figure 11.1, Figure 11.2).

Table 7.9: Results of high accuracy mass measurement of urinary phthalate monoester glucuronides.

Urinary phthalate monoester	RT[a] (min)	Theoretical Mass (Da)	Measured Mass (Da)	Mass Accuracy (ppm)
5oxo-MEHP-gluc	7.91	467.1559	467.1559	0.0328
5OH-MEHP-gluc 1	7.64	469.1715	469.1708	-1.4811
5OH-MEHP-gluc 2	7.98	469.1715	469.1710	-1.1201
5OH-MEHP-gluc 3	8.40	469.1715	469.1716	0.2181

[a] RT: retention time

Additionally, the glucuronides of 5OH-MEHP and 5oxo-MEHP (5OH-MEHP-gluc and 5oxo-MEHP-gluc) were investigated with liquid chromatography-tandem mass spectrometry in enhanced (linear ion trap) product ion mode (Figure 7.13). The most abundant product ions at *m/z* 291 and 293 were generated by the loss of glucuronic acid (-176 Da) from the conjugated molecules and consequently the ion corresponding to glucuronic acid was observed at *m/z* 175. The fragment of 5oxo-MEHP-gluc at *m/z* 143 and the corresponding fragment of 5OH-MEHP-gluc at *m/z* 145 were identified as the deprotonated analogues of 5-(hydroxymethyl)heptane-2-one and 2-ethylhexane-1,5-diol resulting from the loss of 2-formylbenzoic acid from the unconjugated molecules. The fragment at *m/z* 113 is assigned as the product of elimination of formaldehyde (CH_2O, -30 Da) or methanol (CH_3OH, -32 Da) from the fragments at *m/z* 143 or 145, respectively, or by elimination of water (H_2O, -18 Da) and carbon-dioxide (CO_2, -44 Da) from the product ion at *m/z* 175 (glucuronic acid) [249]. The product ion at *m/z* 85 was characterized as a specific fragment of the glucuronic acid [249]. 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. As depicted in Figure 7.14 and Figure 11.2, the peak

shapes suggested more glucuronide forms of the analytes. This was proven with optimised chromatographic conditions (Table 7.9, Table 11.1, Figure 11.2) indicating the different locations of the glucuronic moiety.
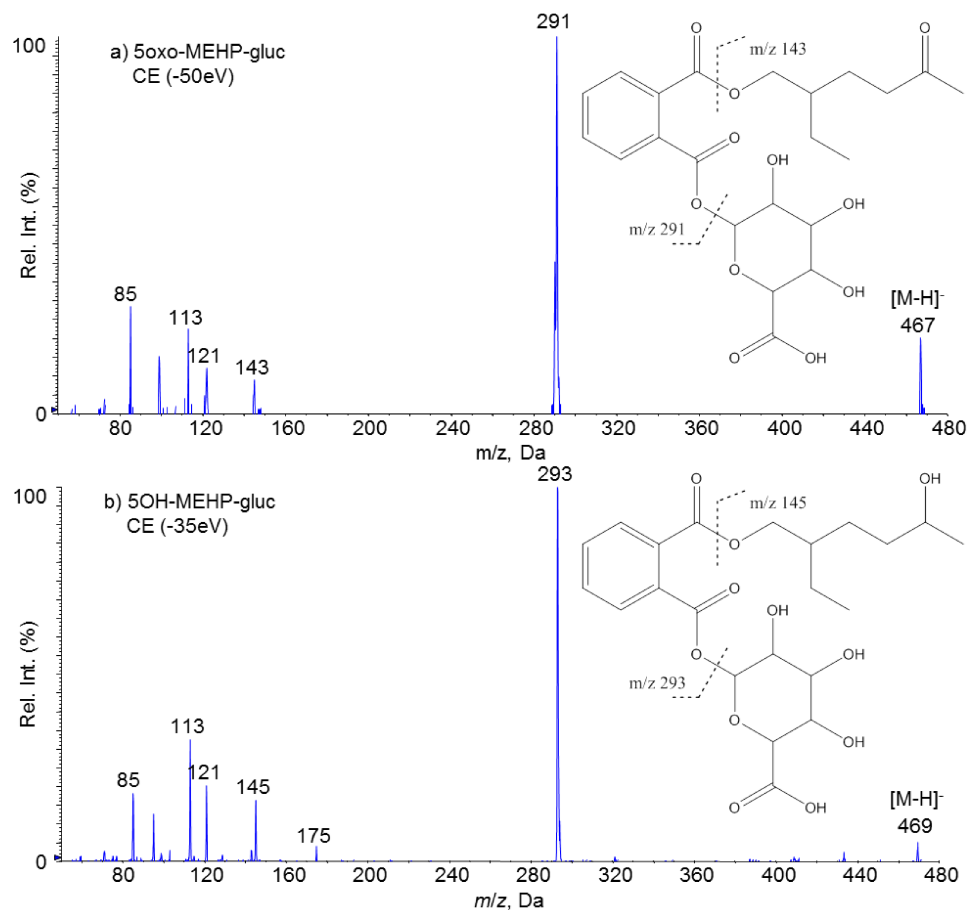
Figure 7.13: Negative ESI enhanced product ion spectra of the deprotonated molecules [M-H]⁻ of 5oxo-MEHP-gluc (a) and 5OH-MEHP-gluc (b) obtained by analysing a post-transfusion urine sample (5oxo-MEHP: 421 ng/mL, 5OH-MEHP: 665 ng/mL) in linear ion trap mode.
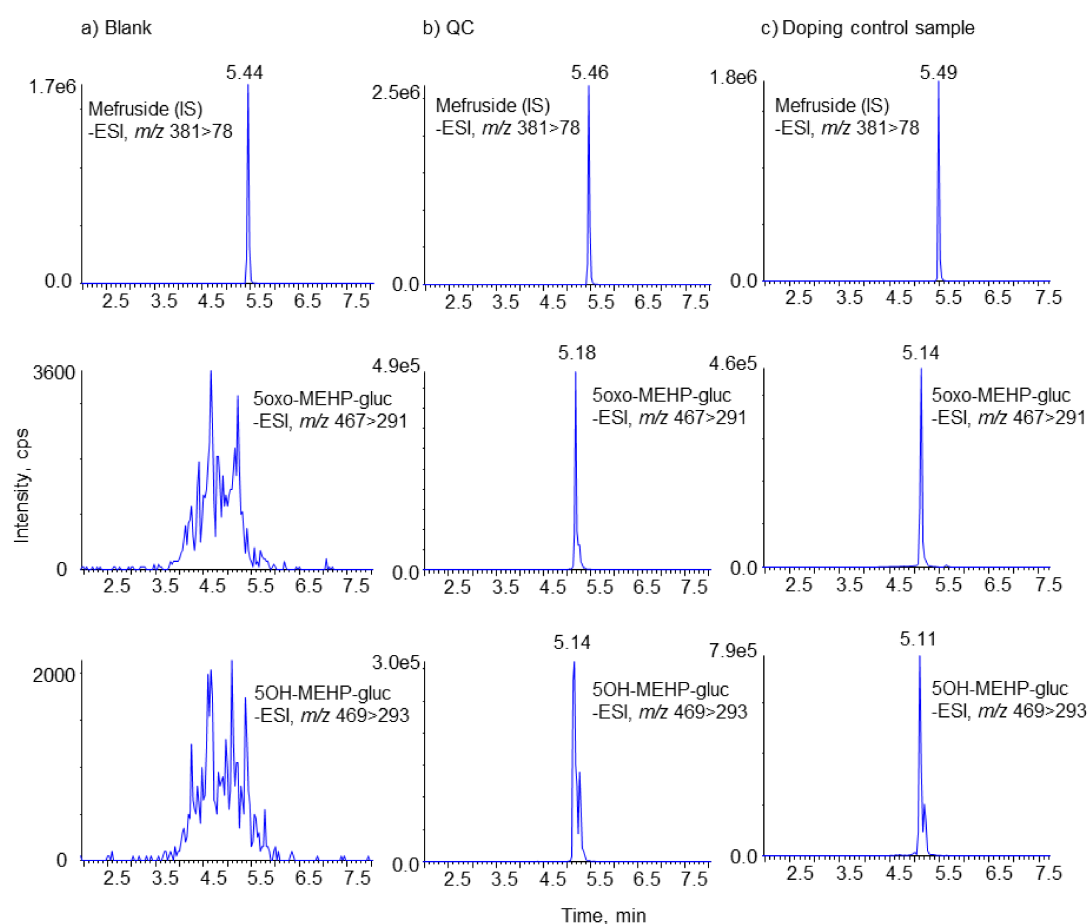
Figure 7.14: Extracted ion chromatograms of 5oxo-MEHP-gluc and 5OH-MEHP-gluc in a blank sample (a), a quality control sample (QC, post-transfusion sample: 5oxo-MEHP: 421 ng/mL, 5OH-MEHP: 667 ng/mL) (b) and in a suspicious doping control sample (5oxo-MEHP: 410 ng/mL 5OH-MEHP: 905 ng/mL) (c).

7.2.2. Sample preparation aspects

Direct injection of native urine specimens provides a wide applicability of the assay as well as a very simple and rapid sample preparation without hydrolysis, purification or further sample preparation steps. Allowing the new target analytes, 5oxo-MEHP-gluc and 5OH-MEHP-gluc, phase-II metabolites of DEHP, to be analysed avoiding time-consuming hydrolysis steps.

7.2.3. Validation results

In order to test for assay suitability, the following parameters were determined; specificity, ion suppression / enhancement, intra- and inter-day precision, LOD, accuracy and linearity. The method was validated for 5OH-MEHP-gluc and 5oxo-MEHP-gluc using post-transfusion samples.

7.2.3.1. Specificity

In terms of specificity, no interfering signals of the matrix were detected at the expected retention times of the analytes except for low basal levels of phthalate metabolites which was also found in earlier studies [29].

7.2.3.2. Robustness

Stable retention times are of the utmost importance for reliable evaluation, especially if native urine is injected. Analysis of QC samples over 4 weeks yielded stable retention times (CV <2%) for all compounds (Table 7.10).

7.2.3.3. Ion suppression / enhancement

There was no evidence of ion suppression or enhancement as no significant decrease or increase of the electrospray response was observed at the expected retention times of the analytes when urinary matrix was injected.

7.2.3.4. Precision

Intra-and inter-day precisions were determined at three concentration levels with coefficients of variation of less than 20% for all analytes.

7.2.3.5. LOD

At the required signal-to-noise ratio of 3, the LODs were estimated to be 20 and 30 ng/mL for 5oxo-MEHP-gluc and 5OH-MEHP-gluc respectively (Table 7.10).

7.2.3.6. Accuracy and linearity

Correlation of 5OH-MEHP and 5oxo-MEHP concentrations and the relative intensities of the corresponding 5OH-MEHP-gluc and 5oxo-MEHP-gluc were determined to demonstrate the suitability of the method for semi-quantitative purposes. The unconjugated phthalate concentrations of the samples were measured according to the method described earlier (Section 6.1.2 and 6.1.3). The relative intensities of phthalate glucuronides were highly correlated to the corresponding phthalate concentrations ($r = 0.993$ for 5oxo-MEHP, $r = 0.990$ for 5OH-MEHP) suggesting that the method provides reliable data to screen for abnormally high concentrations of DEHP metabolites indicating homologous or autologous blood transfusion (Figure 7.15).

(a)

(b)
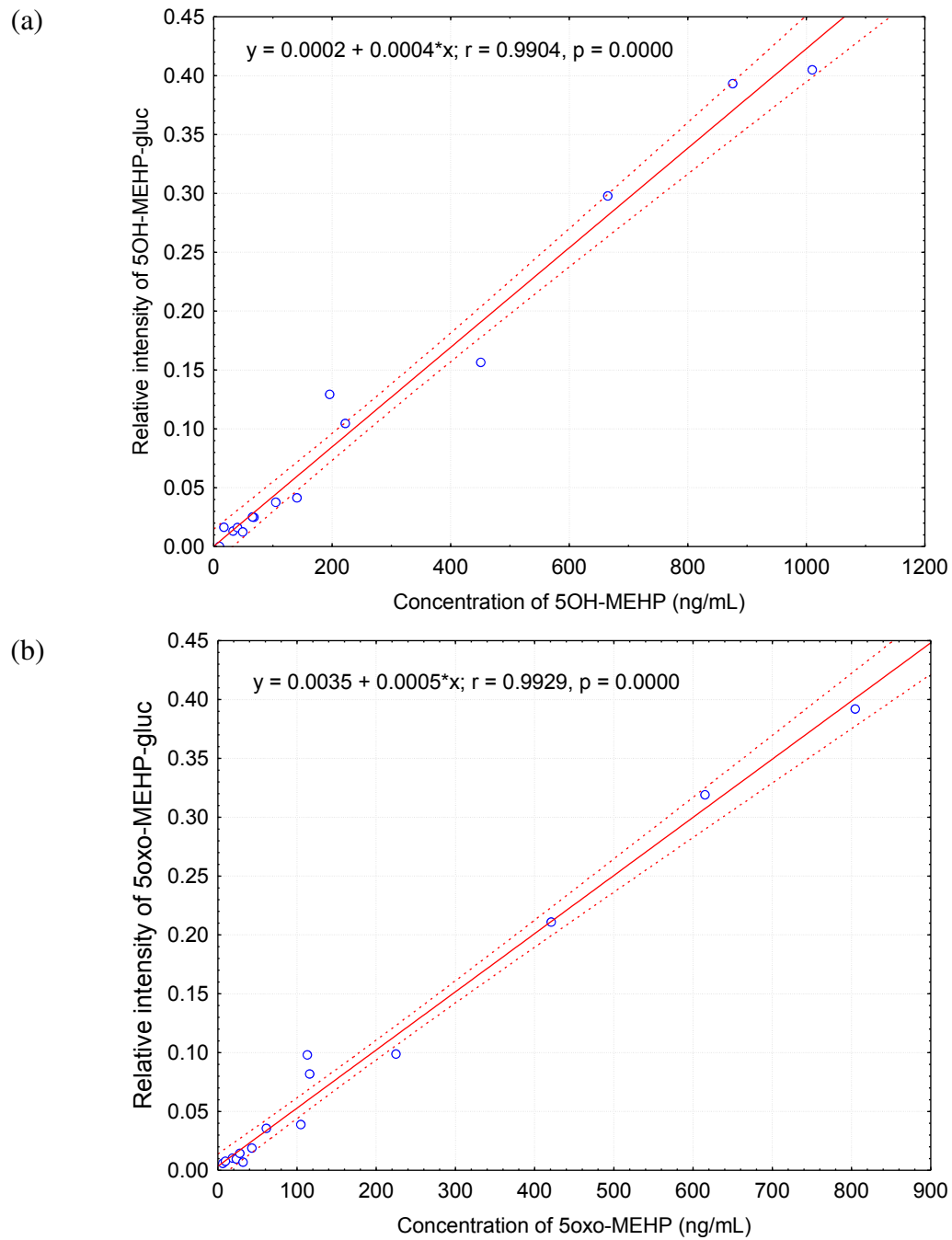
Figure 7.15: Correlation of 5OH-MEHP (a) and 5oxo-MEHP (b) concentrations and the relative intensities of the corresponding glucuronide conjugates.

Table 7.10: Summary of screening validation results.

Compound	LOD	Precision RRT[a] (*n*=6)		Concentration (ng/mL)			Intra-day precision CV (%) (*n*=6/6/6)			Inter-day precision CV (%) (*n*=18/18/18)		
	(ng/mL)	CV (%)	QC_{low}	QC_{middle}	QC_{high}	QC_{low}	QC_{middle}	QC_{high}	QC_{low}	QC_{middle}	QC_{high}
5OH-MEHP-gluc	30	1.36	100	550	1010	10.88	7.85	7.33	10.09	5.51	6.35
5oxo-MEHP-gluc	20	1.10	100	550	1010	10.89	8.91	7.45	10.91	6.57	4.65

[a] RRT: relative retention time

7.2.4. Routine samples

Approximately 13,000 samples were tested for the glucuronide conjugates of DEHP metabolites over a year period. 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.

From the 44 cases 13 samples originated from athletes doing indoor sports and 31 from athletes exercising outdoor; 27 were taken in-competition and 17 out-of-competition. 15 were cyclists' samples, 12 were taken in-competition (IC) and 3 were taken out-of-competition (OOC). In addition, there were adverse analytical findings for hydroxyethyl starch in 3 of the cyclists' samples.

It is noteworthy, that samples from certain countries outside of the European Union showed slightly higher mean urinary DEHP concentrations.

7.2.5. Conclusion

The ongoing increase of the number of prohibited compounds and samples in sports drug testing forces doping-control laboratories to develop multi-target assays that combine high-throughput, simplified sample preparation and a reliable detection for different classes of compounds. Glucuronidated DEHP metabolites were integrated into a comprehensive, simple and robust method based on LC-(ESI)-MS/MS enabling the monitoring of concentrations far below the required limits after direct injection of urine specimens. Using the presented method, screening for such markers in each doping control sample is straightforward and provides a valuable tool in the fight against blood doping.

8. SUMMARY

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.

A promising approach to indicate homologous or autologous blood transfusion is the quantification of increased urinary levels of di(2-ethylhexyl) phthalate (DEHP) metabolites found after blood transfusion.

The commonly used plasticiser for flexible PVC products, such as blood bags, is DEHP which is known to diffuse into the stored blood. Therefore, a straightforward, rapid and reliable assay was developed for the quantification of the main metabolites mono(2-ethyl-5-oxohexyl) phthalate, mono(2-ethyl-5-hydroxyhexyl) phthalate and mono(2-ethylhexyl) phthalate that can easily be implemented into existing multi-target methods used for sports drug testing.

Quantification of the DEHP metabolites was accomplished after enzymatic hydrolysis of urinary glucuronide conjugates and direct injection using isotope-dilution liquid-chromatography / tandem mass spectrometry.

The method was fully validated for quantitative purposes considering the following parameters; specificity, linearity (1-250 ng/mL), inter- (2.4-4.3%) and intra-day precision (0.7-6.1%), accuracy (85-105%), limit of detection (0.2-0.3 ng/mL), limit of quantification (1 ng/mL), stability and ion suppression effects.

Urinary DEHP metabolites were measured in control groups without special exposure to DEHP ($n = 100$ from Cologne and $n = 356$ from Bochum), in athletes ($n = 468$) being subject to routine doping control and in hospitalised patients receiving blood transfusions ($n = 10$ from Neuss and $n = 25$ from Bochum). This investigation demonstrates that significantly increased levels of secondary DEHP metabolites were found in urine samples collected 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. Although urinary concentrations of DEHP metabolites showed considerable intra-individual variation, no increased values have been observed comparable to the concentrations measured in urine specimens collected after blood transfusion.

Additionally, the conjugates of secondary DEHP metabolites were integrated into an existing multi-target screening procedure based on liquid chromatography / electrospray ionisation tandem mass spectrometry (LC-(ESI)-MS/MS).

The assay was fully validated for qualitative purposes considering the following parameters; specificity, intra- (3.2-16.6%) and inter-day precision (0.4-19.9%) at low, medium and high concentration, robustness, limit of detection (20 and 30 ng/mL for 5oxo-MEHP-gluc and 5OH-MEHP-gluc respectively) and ion suppression/enhancement effects.

Analysis of post-transfusion and routine doping control samples demonstrated the applicability of the method for sports drug testing. This straightforward and reliable approach accomplishes the combination of different screening procedures resulting in a high-throughput method that requires only a small volume of urine sample in the µl range and increases the efficiency of the laboratories' daily work.

9. ÖSSZEFOGLALÁS

Az autológ és a homológ vértranszfúzió kimutatása a doppinganalitika legnagyobb kihívást jelentő feladatai közé tartozik. Míg a homológ vértranszfúzió kimutatása 2004 óta lehetséges minor vér antigéneken keresztül, az autológ transzfúzió kimutatása jelenleg nem lehetséges.

Egy ígéretesnek tűnő módszer a homológ és autológ vértranszfúzió kimutatására a di(2-ethil-hexil)ftalát (DEHP) metabolitok megemelkedett koncentrációjának vizeletből történő meghatározása.

A módszer alapja, hogy a vér tárolására és szállítására használt PVC zsákok ftalát lágyítókat tartalmaznak, amelyek idővel a vérbe diffundálnak. Vértranszfúzió után ezen lágyító molekulák metabolitjai meghatározhatóak a vizeletben, lehetővé téve a vértranszfúzió kimutatását. A legáltalánosabban alkalmazott lágyító a DEHP, amelynek metabolitjai – mono(2-etil-hexil)ftalát (MEHP), mono(2-etil-5-hidroxi-hexil)ftalát (5OH-MEHP) és mono(2-etil-5-oxo-hexil)ftalát (5oxo-MEHP) – nagyrészt glükuronid-konjugátumként ürülnek a vizelettel.

Munkám során kifejlesztettem és validáltam egy megbízható és gyors analitikai módszert a fent említett DEHP metabolitok (MEHP, 5oxo-MEHP és 5OH-MEHP) vizeletből történő meghatározására. A komponensek meghatározását a glükuronid-konjugátumok enzimatikus hidrolízisét követő direkt injektálással és LC-MS/MS detektálással végeztem.

A DEHP metabolitok koncentrációját vizsgáltam sportolóktól származó rutin doppinganalitikai mintákban ($n = 468$), különböző kontrollcsoportokban ($n = 100$, Köln és $n = 356$, Bochum), valamint vértranszfúzión átesett betegektől származó vizeletmintákban ($n = 10$, Neuss and $n = 25$, Bochum). A vértranszfúziót követő 24 órában gyűjtött vizelet mintákban lényegesen magasabb oxidatív DEHP metabolit koncentrációkat mértem a kontrollcsoportokhoz képest. A módszer kiegészítő adatokat nyújt a sportolók biológiai útleveléhez (Athlete Biological Passport), így egyéb rendellenes vérparaméterekkel együtt (pl.: hematokrit, hemoglobin, retikulocita szám, vörösvértest szám stb.) a vérdopping megerősítő bizonyítékául szolgál.

A DEHP metabolit koncentrációk lakóhelyi, étrendi vagy környezeti kitettség hatására esetlegesen bekövetkező egyéni időbeli variabilitását hét önkéntestől egy héten keresztül gyűjtött vizeletminták ($n = 253$) elemzésével határoztam meg. Bár az eredmények

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jelentős egyéni változékonyságot mutatnak, a vértranszfúzió után gyűjtött mintákban mért koncentrációkat megközelítő értéket egy mintában sem mértem. Továbbá, a longitudinális vizsgálatok hasznos információval szolgálnak az emelkedett DEHP metabolit koncentrációk értelmezéséhez.

Az oxidatív DEHP metabolitok glükuronid-konjugátumait beépítettem egy LC-MS/MS szűrőmódszerbe, amely jelenleg használatban van számos tiltott szer együttes vizsgálatára. A módszert kvalitatív és szemi-kvantitatív szempontok szerint validáltam az általam vizsgált komponensekre. Az eljárás doppinganalitikai alkalmasságát igazoltam vértranszfúzión átesett önkéntesektől, valamint hozzávetőleg 13000 sportolótól származó vizeletminta elemzésével.

10. PUBLICATIONS RELATED TO THE THESIS

SCIENTIFIC PAPERS

E. Solymos, S. Guddat, H. Geyer, U. Flenker, A. Thomas, J. Segura, R. Ventura, P. Platen, M. Schulte-Mattler, M. Thevis, W. Schänzer, (2011) 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.* 401 (2) 517-528

E. Solymos, S. Guddat, H. Geyer, A. Thomas, M. Thevis, W. Schänzer, (2011) Di(2-ethylhexyl) phthalate metabolites as markers for blood transfusion in doping control - intra-individual variability of urinary concentrations *Drug Test. Anal.* 3 (11-12) 892–895

S. Guddat, E. Solymos, A. Orlovius, A. Thomas, G. Sigmund, H. Geyer, M. Thevis, W. Schänzer, (2011) High-throughput screening for various classes of doping agents using a new „dilute-and-shoot" liquid chromatography / tandem mass spectrometry multi-target approach *Drug Test. Anal.* 3 (11-12) 836-850

ORAL PRESENTATION

S. Guddat, E. Solymos, A. Orlovius, A. Thomas, H. Geyer, M. Thevis, W. Schänzer
High-throughput screening for various classes of doping agents using a new „dilute-and-shoot" LC-(ESI)-MS/MS multi-target approach
29th Manfred Donike Cologne Workshop on Dope Analysis, 2011, Cologne, Germany

POSTER PRESENTATION

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

11. APPENDICES

11.1. TABLES

Table 11.1: LC-MS method parameters for high accuracy mass spectrometry measurement of phthalate glucuronides.

Chromatographic Parameters			
HPLC system	Thermo Accela UPLC system		
HPLC column	Hypersil Gold, 50 mm x 2.1 m, 1.9 µm		
Mobile Phase	A: 0.1% formic acid		B: acetonitrile
Gradient	0 min	100% A	0% B
	2 min	100% A	0% B
	8 min	0% A	100% B
	9 min	0% A	100% B
	2.5 min post-run equilibration time		
Flow rate	0.25 mL/min		
Injection Volume	10 µL		
Mass spectrometric parameters			
Mass spectrometer	Thermo Fisher Exactive OrbiTrap® with heated electrospray ionisation (HESI-I-probe)		
Ionisation	ESI in negative ion mode (50 msec)		
Scan Mode	full scan		
Resolution	30000 FWHM		
Capillary Temperature	275°C		
Sheat Gas	N_2, 40 psi		
Auxiliary Gas	N_2, 4 psi		
Ionspray Voltage	-3500 V		

11.2. FIGURES
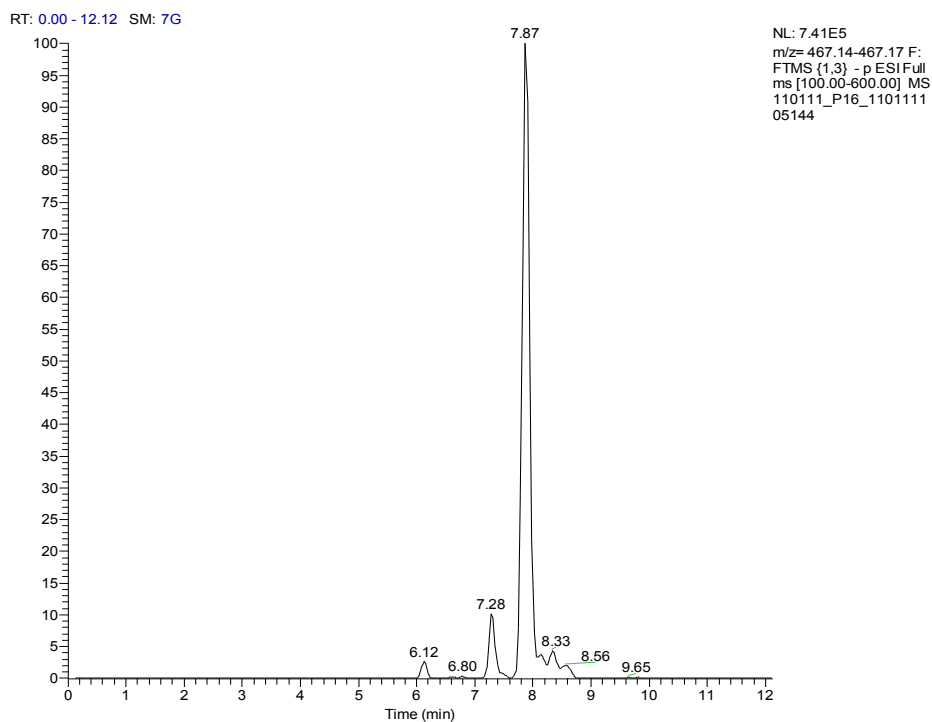
Figure 11.1: Chromatogram of 5oxo-MEHP-gluc (see method in Table 11.1).
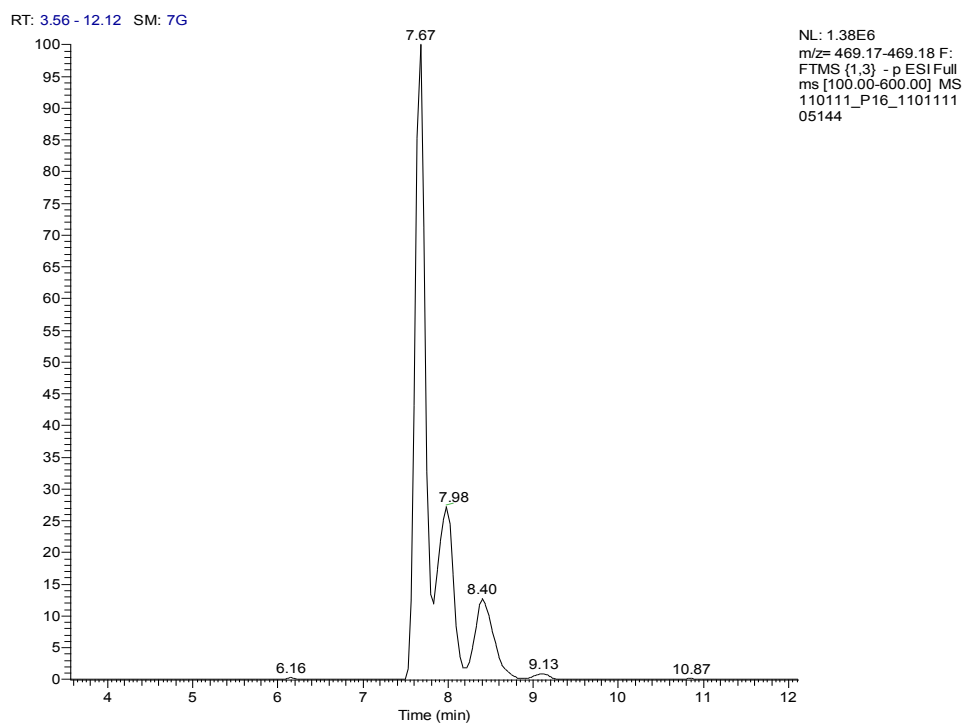
Figure 11.2: Chromatogram of 5OH-MEHP-gluc indicating different locations of the glucuronic moiety (see method in Table 11.1).

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