Scientific paper

Simultaneous Determination of Phthalates, their Metabolites, Alkylphenols and Bisphenol A Using GC-MS in Urine of Men With Fertility Problems

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Received: 02-10-2013

Abstract

A GC-MS method was successfully applied to measure simultaneously the concentrations of endocrine disrupting compounds (5 dialkyl phthalates, 9 phthalate monoesters, 3 alkylphenols and bisphenol A) in 136 male urine samples. In the present study the method was validated and concentrations of EDCs were determined. The results were compared with results from other studies. Correlations between endocrine disrupting compounds and also correlations of endocrine disrupting compounds with two semen quality parameters are presented and evaluated. Significant positive correlations were found between almost all the endocrine disrupting compounds. The parameter sum of DEHP (SUM DEHP) was positively correlated to all the endocrine disrupting compounds but negatively to two semen quality parameters. Negative correlations between the endocrine disrupting compounds and the semen quality parameters could indicate that endocrine disrupting compounds could cause reproductive problems by decreasing the semen count and quality. This research will have helped to evaluate human exposure to endocrine disrupting compounds.

Keywords: Endocrine disrupting compounds, fertility, phthalates, bisphenol A, alkylphenols, human urine.

1. Introduction

Endocrine disrupting compounds (EDCs) such as phthalates, alkylphenols and bisphenol A are exogenous environmental chemicals that can interfere with human's or animal's normal hormone functions, and pose a potential threat to the environment and human health. Phthalates, alkylphenols (AP) and bisphenol A (BPA) are such endocrine disrupting compounds, being industrially manufactured and widely used.[1]

According to the US Environmental Protection Agency, an endocrine disrupting chemical is defined as "an exogenous agent that interferes with the production, release, transport, metabolism, binding, action, or elimination of those natural hormones in the body responsible for the maintenance of homeostasis and the regulation of developmental processes".[2]

Over the last decade, studies have shown that humans are exposed to endocrine disrupting compounds (phthalates, alkylphenols, bisphenol A, etc.) through various routes: air, soil, sediment, water, food, drink and even skin contact.[3–6] Meanwhile, phthalate metabolites, alkylphenols and bisphenol A have already been detected in human urine, blood and breast milk,[7–14] and BPA was even found in amniotic fluid, follicular fluid, placental tissue, semen, umbilical cord blood, fetal serum and adipose tissues.[14–16]

For the most part, few information exists about the extent of human exposure to these chemicals, and the potential toxic effects of these compounds are largely unknown.

In our previous study we established that the urinary BPA concentration is associated with lower natural logarithm transformed sperm count.[17]

1. 1. Production and Human Exposure

Dialkyl phthalates, alkylphenols and bisphenol A are important components of many industrial products and processes. Although exact quantities are difficult to estimate, it is reckoned that around 6 million tons of phthalates are produced worldwide every year.[18] The annual production of alkylphenols has been estimated to be 75,000 tons in the EU and 154,000 tons in the USA.[19] Since these data were published, the use of alkylphenols has been restricted in the EU, but they are still found in considerable concentrations in the environment.[19] With regard to bisphenol A, the estimated production range is 2.2–4.7 million tons, of which around 1.2 million tons are produced in the EU, and these amounts are rising by about 6–8% annually.[20] All these substances or their metabolites have been detected in human urine, serum, in breast milk, in saliva, and even in semen.[21–23]

1. 2. Urine

Urine is considered to be the most appropriate matrix for biomonitoring dialkyl phthalates, their metabolites, alkylphenols and bisphenol A.[27] The levels of these compounds have been studied in several countries, such as Japan,[13] Korea,[28] U.S.A,[10–11,26] and Germany.[30] Over recent years various analytical methods have been developed for analyzing phthalates, alkylphenols, and bisphenol A in human urine. Most of the reports used the liquid chromatography–tandem mass spectrometry (LC–MS/MS) method.[24,31–32] To our knowledge, there are only a few reports of studies on dialkyl phthalates, their metabolites, alkylphenols, and bisphenol A and their determination in urine using gas chromatography–mass spectrometry (GC–MS).[29,33] Kondo et. al. developed an analytical method for the determination of 5 monoalkyl phthalates (MEP, MBP, MEHP, MiNP and MBzP). The extracted phthalate monoesters were methylated with diazomethane and purified on a Florisil column.[33] GC/MS offers better separation of compounds and possibility for simultaneous determination of DAP, MAP, AP and BPA.

In our study, a GC-MS method was successfully applied for the simultaneous analysis of phthalate metabolites (MAP), phthalate diesters (DAP), alkylphenols (AP) and bisphenol A (BPA) in 136 human urine samples. To our knowledge, there have been no studies on the simultaneous determination of all the above-mentioned analytes in human urine until now. Samples were analyzed in order to investigate any significant correlations amongst the concentrations of the endocrine disrupting compounds (dialkyl phthalates, their metabolites, alkylphenols and bisphenol A).

2. Experimental

In the presented work a GC-MS method was applied for the determination of di-methyl phthalate (DMP), di-ethyl phthalate (DEP), di-butyl phthalate (DBP), benzyl-butyl phthalate (BzBP) and di(2-ethylhexyl) phthalate (DEHP), their metabolites monoalkyl phthalates: mono-ethyl phthalate (MEP), mono-iso-butyl phthalate (MiBP), mono-n-butyl phthalate (MnBP), mono-(2-ethylhexyl) phthalate (MEHP), mono-benzyl phthalate (MBzP), mono-iso-nonyl phthalate (MiNP), mono-n-octyl phthalate (MnOP), mono-(2-ethyl-5-oxohexyl) phthalate (MEOHP) and mono-(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP) and alkylphenols: 4-tert-octylphenol (4tOP), 4-n-octylphenol (4nOP), 4-nonylphenol (4nNP) and bisphenol A (BPA) in human urine samples.

2. 1. Chemicals and Reagents

Monoalkyl phthalates: MEP, MnBP, MiBP, MEHP, MiNP, MnOP, MBzP, MEOHP, and MEHHP (>95.0%), their ($^{13}C_4$ for MAP and $^{13}C_{12}$ for BPA)-labelled internal standards (>95.0%) were purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA). Dialkyl phthalates: BzBP, DEHP, DnBP, DEP and DMP from Supelco (Bellefonte, PA, USA). Alkylphenols: 4nNP, 4tOP and 4nOP from Supelco (Bellefonte, PA, USA). Bisphenol A (>98.0%) and its $^{13}C_{12}$-labelled internal standards (>98.0%), were obtained from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA). β-Glucuronidase (Helix Pomatia) and β-Glucuronidase (Patella vulgata) from Sigma-Aldrich (Saint Louis, MO, USA). Glass wool was purchased from Supelco (Bellefonte, PA, USA). Hydrochloric acid was obtained from Riedel-de Haën (Seelze, Germany), dichloromethane, hexane and silica gel were obtained from Fluka, acetonitrile and sodium chloride were purchased from J. T. Baker, Pentafluoropyridine was purchased from (Aldrich) and MSTFA was purchased from (Ultra scientific).

2. 2. Sample Collection and Preparation

Our research was part of a prospective observational study that was designed with the aim to evaluate the effect of endocrine disruptor compounds' exposure on embryo development after medically-assisted reproduction (MAR).[23] The participants were couples seeking infertility treatment at the Department of Reproductive Medicine and Gynaecologic Endocrinology, University Medical Centre, Maribor, Slovenia. Couples in their 1st or 2nd MAR attempts were requested to participate in the study. They underwent routine infertility evaluation according to clinical practice prior being invited to the study. In order to avoid possible female negative embryo development factors, only couples with a female partner younger than

36 years were included. The male partners also underwent clinical examination and routine semen analysis before inclusion, and patients with histories of cryptorchism or the presence of varicocoele were unconsidered for recruitment to the study.

At the recruitment, informed consents were signed. At the same time, the patients completed questionnaires collecting data on medical histories, occupations and lifestyles. The study was approved by the National medical ethics committee of Slovenia.

Spot-urine samples of the male partners were collected after sperm collections on the morning of follicular aspiration. Sterile polypropylene cups were used for urine collection after being previously tested to be EDC free. All the samples were collected during the morning hours, between 7 to 9 A.M. The aliquot of each sample was separated in order to determine urinary creatinine concentration and the samples were immediately frozen to –80 °C, at which they remained until EDC testing. Previous studies had demonstrated that urinary BPA is stable at –20 °C for one year and that phthalate metabolites are stable at –70 °C for one year also.[29,34] Testing was conducted 2–6 months after the samples were obtained. The frozen samples were shipped to Institute of Public Health, Maribor, Slovenia for analyse. Before analysis, the samples were left to thaw for 24 h at 4 °C.

2. 3. Analytical Method

All the glassware was washed carefully to remove any phthalates, then dried and extracted with dichloromethane just before usage. A reagent blank was analyzed before sample analysis in each batch. 1 mL of urine was transferred into a 12 mL tubes and spiked with isotopically-labelled internal standards (50 μL, concentration 1 μg mL^{-1}). After β-glucuronidase (50 μL of ammonium acetate buffer, 50 units mL^{-1}) had been added, the sample was incubated at 40 °C for 90 min. The sample solution was then acidified with 75 μL hydrochloric acid, a concentration of 6 mol L^{-1}, that was previously extracted with dichloromethane and hexane. After adding sodium chloride (150 mg) and 12 mL acetonitrile, the sample solution was mixed and then centrifuged at 2500 rpm for 5 min. The next step was drying the sample using azeotropic distillation. The dry residue with analytes was extracted using dichloromethane and then the sample was loaded onto a SiO_2 column (10% HCl, sodium sulphate was added for water elimination), which had been preconditioned with dichloromethane (5 mL). A fraction of the endocrine disrupting compounds was eluted with dichloromethane (3 × 10 mL). The dichloromethane was then evaporated to dryness. Derivatisation was performed with MSTFA (*N*-methyl-*N*-trimethylsilyl trifluoroacetamide) and pen-

Table 1. Selected ions for quantification and for conformation.

		Analyte	Selected ions (m/z)		Retention
			Quantification	Conformation	time(min)
Endocrine disrupting compounds	Dialkyl phthalates	DMP	163	194	10.8
		DEP	177	278, 149	15.3
		DBP	205	223, 149	28.2
		BzBP	206	238, 149	40.5
		DEHP	167	279, 149	46.3
	Monoalkyl phthalates	MEP	223	251, 221	17.0
		MEP-ISTD	227	255, 270	17.0
		MnBP	223	149, 163, 221	23.3
		MnBP-ISTD	227	153, 166, 225	23.3
		MiBP	221	279, 149	21.6
		MEHP	221	239, 223, 149	33.3
		MEHP-ISTD	227	243,153, 225	33.3
		MiNP	221	223, 239, 149	34.8
		MiNP-ISTD	227	243, 153, 225	34.8
		MnOP	221	239, 149	36.4
		MBzP	222	179	36.3
		MBzP-ISTD	226	182	36.3
		MEOHP	221	239, 149	39.1
		MEOHP-ISTD	225	243, 153	39.1
		MEHHP	221	295, 149	40.7
	Alkylphenols	4tOP	207	278, 263	16.9
		4nOP	179	278, 263, 180	23.5
		4nNP	292	277	27.0
		4nNP-ISTD	185	285, 300	26.9
	Bisphenol A	BPA	357	372	36.8
		BPA-ISTD	369	384	36.8

tafluoropyridine at 60 °C for 30 min. An aliquot of the sample solution was injected into a GC-MS system.

2. 4. Instrumental Analysis

The extracts were analyzed on a HP 6890 Gas Chromatograph with a 5973 Mass Selective Detector (Agilent Technologies, CA, USA). The detector was used in selected ion monitoring mode (SIM). A 30 m Capillary column DB-5 MS (Agilent) of i.d. 0.25 mm and a film thickness of 0.25 μm was used. The oven temperature was initially held at 105 °C for 0.75 min, then programmed to 120 °C at a rate of 30 °C min^{-1} and then to 320 °C at a rate of 2.7 °C min^{-1}, and held there for 5 min. Helium was used as carrier gas at a constant flow-rate of 0.9 mL min^{-1}. The amount injected was 1 L and the splitless technique was used. The ion source temperature was 230 °C. The injection port and transfer line were kept at 290 °C. The ions used for the selected ion monitoring (SIM) are summarized in Table 1. The ions observed as the base peak were used for quantification and the second, third or fourth most abundant ions were used for confirmation. The concentrations of five dialkyl phthalates, nine monoalkyl phthalates, three alkylphenols, and bisphenol A in the urine were calculated using a ^{13}C-labelled internal standard compound (ISTD).

Table 2. Recoveries and LOQ.

Analyte (μg L^{-1})	Recovery (%)	CV (%)	LOQ (μg L^{-1})
DMP	91.5	4.2	0.3
DEP	113.8	5.6	5
DBP	112.4	8.1	7
BzBP	101.3	4.7	6
DEHP	118.4	7.1	12
MEP	88.5	9.7	1.3
MEP-ISTD	95.5	1.7	/
MnBP	93.0	4.8	1.3
MnBP-ISTD	97.9	1.2	/
MiBP	99.0	6.7	1.2
MEHP	105.4	3.2	1.4
MEHP-ISTD	101.1	2.1	/
MiNP	101.8	1.2	0.3
MiNP-ISTD	99.4	0.9	/
MnOP	98.9	1.7	0.2
MBzP	105.8	2.8	0.8
MBzP-ISTD	102.5	1.6	/
MEOHP	93.8	5.4	0.2
MEOHP-ISTD	98.0	1.0	/
MEHHP	95.9	6.6	0.2
4tOP	75.0	9.1	1.4.
4nOP	80.7	8.3	1.3
4nNP	84.4	9.5	0.2
4nNP-ISTD	96.2	3.1	/
BPA	98.0	0.8	0.1
BPA-ISTD	98.8	0.6	/

Note: Results are means of six replicate determinations (inter-day).

2. 5. Validation

The recoveries from the urine spiked with 50 μg L^{-1} of each of the endocrine disrupting compounds were examined by calculating the ratio of the amount of analytes recovered after SiO_2 column purification to the amounts originally added. The overall recoveries and coefficients of variation were found to be satisfactory. Analyses were performed through under reproducibility conditions (inter-day CV). The recoveries were 75.0%–118.4% and CV were 0.6%–9.7%. (Table 2).

2. 6. 1. Linearity of Calibration Standards

The matrix-matched calibration curves were linear over the range from 0.1 μg L^{-1} for bisphenol A, 1 μg L^{-1} for monoalkyl phthalates, and alkylphenols and 5 μg L^{-1} for dialkyl phthalates to 200.0 μg L^{-1}. Linearity, expressed as the correlation coefficients (R^2) provided values all above 0.9980 for the linear range, as shown in Table 3.

Table 3. Linear range and correlation coefficients for each endocrine disrupting compound.

Endocrine disrupting chemicals	Linear range (μg L^{-1})	Correlation coeficients (R^2)
DMP	5–200	0.9990
DEP	5–200	0.9990
DBP	5–200	0.9980
BzBP	5–200	0.9980
DEHP	5–200	0.9980
MEP	1–200	0.9981
MnBP	1–200	0.9988
MiBP	1–200	0.9980
MEHP	1–200	0.9981
MiNP	1–200	0.9975
MnOP	1–200	0.9980
MBzP	1–200	0.9980
MEOHP	1–200	0.9994
MEHHP	1–200	0.9980
4tOP	1–200	0.9990
4nOP	1–200	0.9980
4nNP	1–200	0.9960
BPA	0.1–200	0.9996

In order to determine the background levels of the endocrine disrupting compounds originating from analytical sample preparation, a blank test was carried out using hexane-extracted water instead of urine. The blanks (n = 25) were obtained from every batch during the whole study and the results are presented in Table 4. In order to verify the absence of analytes, representative specimen cups, tubes, pipette tips, and autosampler vials were prescreened and found to be endocrine disrupting compounds free (≥ LOD).

The limit of quantification (LOQ) for each of the endocrine disrupting compounds is summarized in Table 2. The LOQ for each of the five dialkyl phthalates, nine phthalate monoesters, three alkylphenols, and bisphenol A

Table 4. Concentrations and standard deviations of blank samples (N=25).

	N	Mean	
		Concentration (μg L^{-1})	Std. Deviation (μg L^{-1})
DMP	25	0.091	0.045
DEP	25	1.668	0.693
DBP	25	2.546	1.594
BzBP	25	2.093	1.172
DEHP	25	4.205	2.281
MEP	25	0.444	0.134
MnBP	25	0.422	0.133
MiBP	25	0.403	0.199
MEHP	25	0.479	0.104
MiNP	25	0.116	0.031
MnOP	25	0.075	0.038
MBzP	25	0.284	0.085
MEOHP	25	0.073	0.014
MEHHP	25	0.084	0.039
4tOP	25	0.469	0.345
4nOP	25	0.420	0.191
4nNP	25	0.084	0.033
BPA	25	0.077	0.042

were calculated from six replicates of a spiked blank sample. By careful control of any possible contamination, sample analysis was achieved with a low-level background, that allowed us to precisely evaluate the concentrations of the endocrine disrupting compounds.

The chromatogram of the spiked endocrine disrupting compounds mixture at 50.0 μg L^{-1} concentration level is shown in Figure 1 with recoveries included in Table 2.

2. 7. Method Performance

2. 7. 1. Daily Operation and Quality Control Procedure Quality Control.

QC samples were prepared from urine samples with low content of EDCs. The urine sample were then fortified with standards at concentration level of 50 μg L^{-1} and were stored at –20 °C until used. QC characterisation involved 30 discrete measurements for each endocrine disrupting compound on 30 separate days. A typical daily sample batch included 2 reagent blanks, 10 unknown samples, 1 QC sample. A water reagent blank was processed throughout the entire procedure along with unknown urine samples for monitoring for possible contamination. All the calibration standards injected on the same day were then used to generate a daily calibration curve for each analyte with correlation coefficients typically greater than 0.99 (concentration vs analyte/internal standard ratio). If the analytes in the reagent blank exceeded the limit of detection (LOD), we rejected the batch and repeated the samples.

2. 8. Statistical Analysis

Statistical data treatment was performed using program SPSS v. 21.0; Microsoft EXCEL was applied for the data preparation and generation of the results' outputs.

The direct examination of any inter-relation between the two measured analytes was mostly realized by the correlation analysis when determining the extent to which the

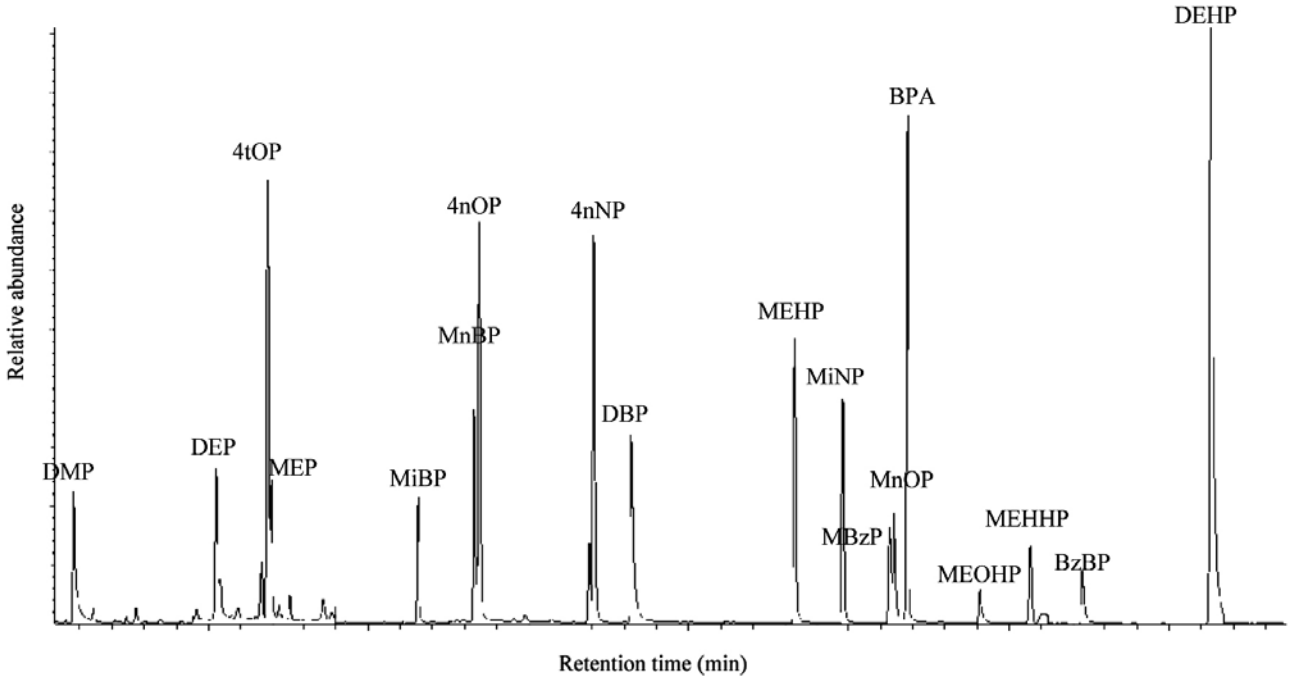

Figure 1. GC-MS chromatogram of 5 dialkyl phthalates, 9 silylated phthalate monoesters, 3 silylated alkylphenols and silylated bisphenol A.

values of the two variables were mutually dependent. The distribution of our results had already been checked and determined to be log-normal. The more common Pearson correlation analysis is a parametric method. The Pearson (pair) correlation coefficient values of +1 or –1 indicated a perfect linear relationship between the two considered variables. If there were violations of the data's normality and constant variability assumptions, the Spearman correlation coefficient is an optimal equivalent, because it is the rank-based robust statistical characteristic, and also works well for nonlinear correlations.[40] Non-parametric Spearman correlation analysis was performed due to the deviation from the normal distribution exhibited by several of the measured analytes.

3. Results and Discussion

The GC-MS method was applied for the quantitative determination of dialkyl phthalates, phthalate monoesters, alkylphenols, and bisphenol A in the human urine samples. This selective method allows for rapid determination of the endocrine disrupting compounds with the limits of detection in the low microgram per litre range. This method was applied to 136 human urine samples in order to prove influence on the fertility problem in human males. Detectable levels of some endocrine disrupting compounds were found in all the tested urine samples. This rapid, selective, and sensitive method would help to elucidate the human health relevance of phthalate exposure.

The ages of the men participating in the presented study ranged from 25 to 54 years (66% were between 30 and 40). Three quarter of the men were classified as overweight (BMI, ≥25 kg/m^2). (Table 5)

Endocrine disrupting compounds were determined in the urine of all the samples, MiNP and MnOP were only detected in 14 % of the samples, and BPA was detected in 88 % of the samples. Other dialkyl phthalates, monoalkyl phthalates and alkylphenols were detected in 65–75 % of the samples.

The highest median concentrations in the human male urine were observed for MEP, MnBP, MiBP, MEOHP, and MEHHP amongst the monoalkyl phthalates, and DBP amongst the dialkyl phthalates. Median concentrations of all three alkylphenols and BPA were at low concentration levels. (Table 6)

Table 5. Characteristics of the male subjects.

	Unit	Mean	Minimum	Maximum
Age	years	36.2	25.0	54.0
Height	m	1.79	1.68	1.96
Weight	kg	88.4	65.0	140.0
BMI	kg/m^2	27.5	22.0	39.2

We evaluated the suitability of the method for the determination endocrine disrupting compound levels in human urine samples and we compared our results to the NHANES study. Similarly to our research, the NHANES study showed few nondetectable samples for MEP, MBP, MBzP, and MEHP (81–100% detectable) whilest MiNP and MnOP were not detected in most samples (19–22% detectable).[25]

The endocrine disrupting compound levels in human urine are presented in Table 6; and the urinary creatinine adjustment results in Table 7. In our research, the phthalate monoesters with the highest urinary levels were MEP (3611.7 μg L^{-1}, 2901.9 μg g^{-1} creatinine), MnBP (199.8 μg L^{-1}, 104.7 μg g^{-1} creatinine), and MiBP (161.8 μg L^{-1}, 119.2 μg g^{-1} creatinine), which reflect exposure to DEP, DBP. DEP and DBP and are used extensively in products with volatile components such as perfumes, nail polishes, and hair sprays, possibly leading to inhalation and efficient absorption through the lungs. Dermal absorption also occurs at a significant rate for phthalates with short side-chains such as DEP, DBP.[35] In any event, these data on monoesters indicate that the internal dose of MEP and MBP is probably much higher than that of MEHP, MINP, MnOP, and MBzP.

Despite the fact that DEHP is the most widely produced and used phthalate, we found higher urinary concentrations of MEP, MiBP, and MnBP than of MEHP. Also the concentrations of DEHP were not the highest (20.6 μg L^{-1}), amongst the dialkyl phthalates, the highest values were for DBP (58.6 μg L^{-1}). The lower MEHP concentrations may be due to lower exposure, absorption, metabolism, or excretion. Metabolism studies of DEHP show that MEHP undergoes further oxidative metabolism in order to produce additional metabolites.[45] Studies suggest that the urinary concentrations of two of these oxidative metabolites, MEOHP and MEHHP are several-fold higher than those for MEHP.[36,43] Therefore, the relatively low concentrations of MEHP may result, at least in part, from alternative metabolic pathways. A similar metabolism may be important for other long-alkyl-chain phthalates such as dioctyl phthalate[42] and di-isononyl phthalate[37] and might explain the lower frequency and magnitude of detection of their respective monoesters compared with the monoesters of short-alkyl-chain phthalates (e.g., MEP, MBP).

The high levels of MEP across the population are most likely to be associated with the everyday usages of consumer products that commonly contain DEP,[42] such as detergents, soaps, cosmetics, shampoos, and perfumes. Furthermore, the higher concentrations of MEP in adults and adolescents than in children are consistent with the known behavioural uses of phthalate containing consumer products (e.g., adults are more likely to use cosmetics than are children).[38] Dialkyl phthalates had the highest concentrations in the urine samples, between 100 and 500 μg L^{-1}.

Table 6. Total endocrine disrupting compound concentrations (μg L^{-1}) in 136 urine samples of men with fertility problems.

Analytes	N	Range	Minimum	Maximum	Geometric mean	Median
MEP	136	3610.7	1.3	3611.7	192.9	184.8
tOP	136	61.2	1.4	62.2	6.4	6.9
nOP	136	49.8	1.3	50.8	5.5	5.8
4nNP	136	21.2	0.2	22.2	4.8	4.9
MnBP	136	198.8	1.3	199.8	18.7	18.3
MiBP	136	160.8	1.2	161.8	22.9	21.6
MEHP	136	46.1	1.4	47.1	6.6	6.6
MiNP	136	20.1	0.3	21.0	1.3	1.1
MnOP	136	34.3	0.2	35.2	1.5	1.5
MBzP	136	19.7	0.8	20.7	3.4	3.5
DMP	136	141.6	0.3	142.6	4.9	4.9
DEP	136	132.0	5	133.0	17.4	18.0
DBP	136	564.9	7	565.9	62.2	58.0
BzBP	136	546.2	6	547.2	22.0	22.4
DEHP	136	303.4	12	304.4	19.5	18.7
BPA	136	14.7	0.2	14.8	2.2	2.2
MEOHP	136	63.2	0.2	64.2	11.3	11.2
MEHHP	136	49.6	0.2	50.6	8.0	7.9

Table 7. Total endocrine disrupting compound concentrations (μg g^{-1}) in 136 urine samples of men with fertility problems (normalized to creatinine content).

Analytes	N	Range	Minimum	Maximum	Geometric mean	Median
MEP	136	2900.9	1.3	2901.9	173.6	181.8
tOP	136	75.9	1.4	76.2	6.1	5.9
nOP	136	73.4	1.3	73.7	6.5	5.6
4nNP	136	38.0	0.2	38.3	4.6	4.4
MnBP	136	104.4	1.3	104.7	15.1	14.9
MiBP	136	118.9	1.2	119.2	20.4	20.8
MEHP	136	55.6	1.4	56.0	6.1	6.3
MiNP	136	20.3	0.3	20.6	1.3	1.3
MnOP	136	13.3	0.2	13.6	1.3	1.3
MBzP	136	15.1	0.8	15.5	2.9	2.9
DMP	136	152.3	0.3	152.6	4.3	4.4
DEP	136	210.5	5	210.8	19.5	19.6
DBP	136	553.6	7	554.0	58.6	62.4
BzBP	136	271.6	6	271.9	18.0	18.7
DEHP	136	543.3	12	543.6	20.6	22.4
BPA	136	30.4	0.2	30.4	2.0	1.9
MEOHP	136	35.9	0.2	36.4	9.7	9.8
MEHHP	136	42.2	0.2	42.5	6.7	6.8

In Table 8 we compared our results to other research in USA and the Netherlands, where endocrine disrupting compounds have been measured in urine samples.[25,38,44] It is evident from the comparison that the MEP results (167.0, 179.0, 112.0 μg L^{-1}) are in the same range for all the studies. The MEHP levels were lower in the USA (3.0 and 3.68 μg L^{-1}), about half the value in comparison to Europe (6.1 and 6.9 μg L^{-1}). In the research[25] the results of MiNP and MnOP were not calculated, due to the high percentage of samples bellow LOD. The values of BPA are comparable and are in low range. The levels of secondary metabolites (MEOHP and MEHHP) were slightly lower in our study in comparison with the USA and the Netherlands' studies. The highest levels of MEHP in this study (47.1 μg L^{-1}, 56.0 μg/g creatinine) agreed with the levels found in the other research.[25,37] The median MEHP levels for this general reference population were 7-fold lower than the highest values. In the urine, more lipophilic phthalate monoesters, such as MEHP, MINP and MnOP were generally found at lower levels than other monoe-

Table 8. Phthalate concentrations in this study vs two NHANES studies (μg g^{-1}) and one European from Netherlands.

Metabolites μg g^{-1} creatinine	Subjects n=136 GM[a]	NHANES 1999–2006[25] n=10000 GM[a]	NHANES 1999–2000[38] n=100 GM[a]	NETHERLANDS[44] n=100 GM[a]
MEHP	6.1	3.0	3.68	6.9
MEP	173.6	167	179	112
MBzP	2.9	13.0	16.2	8.9
MiNP	1.3	nc	–	–
MnOP	1.3	nc	–	–
MnBP	15.1	99.1	22	43.2
MEOHP	9.7	13.9	–	15.0
MEHHP	6.7	21.2	–	14.3
BPA	2.0	–	–	1.1

a = GM = Geometric mean. nc = not calculated due to high percentage of samples < LOD

sters. The relatively low median MEHP, MINP and MnOP levels suggested either low exposures to DEHP, DiNP, and DnOP storage in adipose tissue, or metabolism and excretion through another pathway.[39]

3. 1. Correlation Between Analytes

Numerous significant correlations were found amongst the studied analytes when using Spearman correlation analysis, which was accepted as the decisive correlation tool for this work (Table 9). Statistically significant correlations were found for numerous pairs of analytes (Table 9) as determined in all 136 urine samples ($n = 136$).

Mutual correlation were investigated for all pairs of the investigated endocrine disrupting compounds, which may stress the most important relationships amongst them. The relationships among the key groups of descriptors are especially important, mainly DEHP, MEHP, MEOHP, MEHHP, DMP, and DBP.

There were positive correlations between almost all the endocrine disrupting compounds. The highest correlation coefficients were observed between MEOHP and MEHHP (0.792), and between MBzP and both secondary metabolites (0.545). Very high correlation was found between MiNP and MnOP. This was expected because too many values were below LOD. The highly significant correlations were expected between DEHP and its metabolites (MEHP and both secondary metabolites) as well as other similar compounds.

The sum of the diethylhexyl phthalates (SUM DEHP) was a parameter calculated from four endocrine disrupting compounds (DEHP, MEHP, MEOHP and MEHHP). The parameter SUM DEHP was positively correlated to all the endocrine disrupting compounds and negatively to the clinical parameters.

Statistically significant (p-values<0.01) correlations were found between the three evaluated DEHP metabolites: MEOHP, MEHHP, and MEHP ($p < 0.01$), and between three of the alkylphenols: 4nNP, tOP and nOP ($p < 0.01$). We also observed statistically significant correlation between the concentrations of phthalate metabolites and alkylphenols. It is noteworthy, that the concentrations of phthalates with longer chain were correlated to alkylphenols; tOP, nOP and 4nNP were positively correlated to MiNP and MnOP with $p < 0.01$. MBzP was correlated to similar compounds, MnBP, MiBP, MEHP, MiNP, MnOP and 4nNP, as well.

We discovered that the concentrations of BzBP, MBzP, MnBP, MiBP, MEHP and both secondary metabolites were correlated (Spearman correlation coefficients of all were higher than $R = 0.3$, $p < 0.01$). BzBP, the parent phthalate that provides metabolic product MBzP, can also be metabolized to MBP, namely < 10% of the total BzBP in the humans was metabolized to MBP.[41] Furthermore, we observed significant correlations between the concentrations of MEHP and both DBP ($R = 0.523$, $p < 0.01$) and between MEHP and DEHP ($R = 0.466$, $p < 0.01$). BPA was positively correlated to almost all endocrine disrupting compounds, the highest correlations were between BPA and MEOHP (0.383), MBzP (0.326), MEP (0.302) and SUM DEHP (0.293).

Semen concentration and Semen motility were determined by clinical analyses and represented parameters of the semen quality. There was a positive correlation between these two parameters. We can see from the correlation table, negative correlation of these two parameters to all endocrine disrupting compounds. The highest negative correlation was between Semen concentration and SUM DEHP (–0.302). The correlation results could show that the endocrine disrupting compounds can cause reproductive problems by decreasing sperm count and quality.

4. Conclusions

The presented study determined endocrine disrupting compounds with GC-MS in 136 human male urine samples collected in the year 2012. Participants were couples seeking infertility treatment. They underwent routine

Table 9. Correlation coefficients between urinary biomarkers of exposure.

	MEP	tOP	nOP	4nNP	MnBP	MiBP	MEHP	MiNP	MnOP	MBzP	DMP	DEP	DBP	BzBP	DEHP	BPA	MEOHP	MEHHP	SUM DEHP	CONC.
tOP	0.051																			
nOP	–0.020	**0.411**																		
4nNP	0.017	**0.353**	**0.327**																	
MnBP	**0.228**	–0.089	–0.086	**0.199**																
MiBP	0.071	–0.051	–0.028	0.151	**0.488**															
MEHP	0.157	–0.053	0.026	0.098	**0.325**	**0.303**														
MiNP	0.162	**0.405**	**0.526**	**0.350**	0.160	**0.281**	**0.339**													
MnOP	0.130	**0.391**	**0.414**	**0.336**	**0.179**	**0.284**	**0.326**	**0.918**												
MBzP	0.131	0.121	0.003	**0.228**	**0.298**	**0.287**	**0.299**	**0.263**	**0.262**											
DMP	**0.231**	0.108	0.012	**0.259**	**0.405**	**0.370**	**0.411**	**0.343**	**0.377**	**0.450**										
DEP	0.161	**0.291**	**0.373**	0.099	0.134	0.080	**0.214**	**0.379**	**0.303**	0.124	0.138									
DBP	**0.192**	0.004	0.095	0.073	**0.216**	0.125	**0.525**	**0.223**	**0.183**	0.073	**0.269**	**0.357**								
BzBP	**0.222**	**–0.220**	–0.075	0.167	**0.417**	**0.266**	**0.327**	**0.225**	**0.246**	**0.477**	**0.528**	0.089	**0.198**							
DEHP	**0.172**	–0.041	0.037	–0.059	**0.276**	**0.325**	**0.460**	**0.291**	**0.230**	**0.276**	**0.441**	**0.282**	**0.479**	**0.411**						
BPA	**0.302**	0.022	0.149	0.029	**0.212**	**0.251**	0.131	**0.243**	**0.234**	**0.326**	**0.263**	**0.241**	0.130	**0.261**	**0.235**					
MEOHP	0.064	0.107	–0.021	**0.230**	**0.458**	**0.346**	**0.452**	**0.339**	**0.338**	**0.545**	**0.485**	0.150	**0.270**	**0.389**	**0.411**	**0.383**				
MEHHP	0.120	0.108	–0.067	**0.275**	**0.412**	**0.333**	**0.432**	**0.257**	**0.273**	**0.545**	**0.470**	0.121	0.138	**0.462**	**0.296**	**0.276**	**0.792**			
SUM DEHP	0.138	0.008	–.0080	0.088	**0.391**	**0.385**	**0.675**	**0.301**	**0.297**	**0.500**	**0.549**	**0.189**	**0.466**	**0.470**	**0.747**	**0.293**	**0.819**	**0.708**		
CONC.	–0.096	–0.014	0.092	0.004	–0.006	–0.044	**–0.188**	–0.011	–0.062	–0.014	**–0.181**	0.097	**–0.214**	–0.115	**–0.266**	–0.075	**–0.190**	–0.169	**–0.302**	
MOTILITY	–0.111	0.081	0.055	0.017	–0.127	–0.075	**–0.224**	0.018	–0.019	0.058	–0.133	–0.028	**–0.204**	–0.086	–0.167	–0.083	**–0.172**	–0.152	**–0.234**	**0.5017**

The significant Spearman correlation are denoted with bold ($p<0.05$) and the highly significant coefficients ($p<0.01$) are denoted with bold and underlined.

infertility evaluation according to clinical practice prior to being invited to the study. The male partners also underwent clinical examinations and routine semen analyse.

The GC-MS method was successfully applied to the analysis of 5 dialkyl phthalates, 9 phthalate monoesters, 3 alkylphenols, and bisphenol A, in human urine. Using this method we were able to simultaneuosly measure the level of endocrine disrupting compounds in human urine. Endocrine disrupting compounds were detected in the urine samples of all the men. The highest median concentrations in the human males' urine were observed for MEP, which is most likely associated with the everyday use of consumer products that commonly contain DEP.[24]

Numerous significant correlations were found amongst the studied analytes when using nonparametric Spearman correlation analysis. There were positive correlations between almost all the endocrine disrupting compounds. The relationships amongst the key groups of descriptors were especially important , mainly DEHP, MEHP, MEOHP, and MEHHP. Semen concentration and Semen motility were determined by clinical analyses and represent the parameters of sperm quality. The highest negative correlation was between Semen concentration and SUM DEHP (–0.302). The correlation results could indicate that the endocrine disrupting compounds can cause reproductive problems by decreasing sperm count and quality. Research will help to evaluate human exposure to dialkyl phthalates, alkylphenols, and bisphenol A.

5. Acknowledgements

The authors thank to the Slovenian Research Agency and the Ministry of Higher Education, Science and Technology of the Republic of Slovenia, contract number P2-006 for financial support and appreciate the technical and scientific assistance of Nino Javernik, and Matej Domanjko from the Public Health Institute, Maribor.

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Povzetek

S plinsko kromatografijo in masno spektrometrijo smo simultano določali koncentracije endokrinih motilcev (5 dialkil ftalatov, 9 monoalkil ftalatov, 3 alkilfenole in bisfenol A) v 136 vzorcih moških urinov. Rezultate smo primerjali z ostalimi študijami. Ovrednotili smo korelacije med endokrinimi motilci in korelacije med endokrinimi motilci ter parametri semena. Signifikantne pozitivne korelacije obstajajo med skoraj vsemi endokrinimi motilci. Parameter vsote DEHP (SUM DEHP) pozitivno korelira z vsemi endokrinimi motilci in negativno s paramateri kvalitete semena. Negativne korelacije med endokrinimi motilci ter parametri kvalitete semena nakazujejo, da lahko povzročajo probleme s plodnostjo z zmanjšanjem koncentracije in gibljivosti semena. Raziskava bo pripomogla k ovrednotenju izpostavljenosti ljudi vplivom endokrinih motilcev.