Altered Semen Quality in Relation to Urinary Concentrations of Phthalate Monoester and Oxidative Metabolites

Russ Hauser, John D. Meeker,† Susan Duty,‡ Manori J. Silva,§ and Antonia M. Calafat§*

Background: Phthalates are multifunctional chemicals used in a variety of consumer, medical, and personal care products. Previously, we reported dose–response associations of decreased semen quality with urinary concentrations of monobutyl phthalate (MBP) and monobenzyl (MBzP) phthalate, which are metabolites of dibutyl phthalate and butylbenzyl phthalate, respectively. The present study extends our work in a larger sample of men and includes measurements of di(2-ethylhexyl) phthalate (DEHP) oxidative metabolites.

Methods: Between January 2000 and May 2004, we recruited 463 male partners of subfertile couples who presented for semen analysis to the Massachusetts General Hospital. Semen parameters were dichotomized based on World Health Organization reference values for sperm concentration (<20 million/mL) and motility (<50% motile) and the Tygerberg Kruger Strict criteria for morphology (<4% normal). The comparison group was men with all 3 semen parameters above the reference values. In a single spot urine sample from each man, phthalate metabolites were measured using solid-phase extraction coupled to high-performance liquid chromatography isotope-dilution tandem mass spectrometry.

Results: There were dose–response relationships of MBP with low sperm concentration (odds ratio per quartile adjusted for age, abstinence time, and smoking status = 1.00, 3.1, 2.5, 3.3; *P* for trend = 0.04) and motility (1.0, 1.5, 1.5, 1.8; *P* for trend = 0.04). There was suggestive evidence of an association between the highest MBzP quartile and low sperm concentration (1.00, 1.1, 1.1, 1.9; *P* for trend = 0.13). There were no relationships of monoethyl phthalate, monomethyl phthalate, and the DEHP metabolites with these semen parameters.

Conclusion: The present study confirms previous results on the relationship of altered semen quality with exposure to MBP at general population levels. We did not find associations between semen parameters and 3 DEHP metabolites.

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Phthalates are a class of multifunctional chemicals used in a variety of consumer and personal care products. High-molecular-weight phthalates (eg, di[2-ethylhexyl] phthalate [DEHP] and butylbenzyl phthalate [BBzP]) are primarily used as plasticizers in the manufacture of flexible vinyl, which is used in consumer products, flooring and wall coverings, food contact applications, and medical devices.[1] Manufacturers use low-molecular-weight phthalates (eg, diethyl phthalate [DEP] and dibutyl phthalate [DBP]) in personal care products (eg, perfumes, lotions, cosmetics), as solvents and plasticizers for cellulose acetate, and in making lacquers, varnishes, and coatings, including those used to provide timed release in some pharmaceuticals.[2,3]

Experimental studies in laboratory animals have shown that some phthalates are reproductive and developmental toxicants. Although most studies have focused on prenatal or perinatal exposure windows, there is some evidence that pubertal and adult exposure to DBP, BBzP, and DEHP can cause testicular toxicity.[4–6] Pubertal animals are more sensitive than sexually mature animals to the testicular toxicity of phthalates.[7,8] Testicular toxicity with germ cell loss was primarily induced through effects on Sertoli cells, the cell type responsible for initiation and maintenance of spermatogenesis.[9]

Phthalates metabolize quickly, do not accumulate, and are primarily excreted in urine.[1–3] Urinary concentrations of phthalate metabolites have been used as a biomarker of exposure to the precursor phthalate diesters. Scientific and public concern about potential health risks of phthalates was heightened after studies showing that a large proportion of the U.S. general population is exposed to phthalates.[10,11] However, the human evidence on the potential testicular toxicity of phthalates is very limited. We published preliminary reports on the relationship between urinary concentrations of phthalate metabolites and semen quality among male partners of infertile couples evaluated in an infertility clinic.[12,13] In the first report, among 168 men, we found dose–response relationships between urinary concentrations of monobutyl phthalate (MBP), a metabolite of DBP, and below World Health Organization (WHO) reference value sperm motility (odds ratio per tertile after adjusting for age, abstinence time, and smoking

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From the *Harvard School of Public Health/Massachusetts General Hospital, Boston, MA; the †University of Michigan, Ann Arbor, MI; ‡Simmons College, Boston, MA; and the §Centers for Disease Control and Prevention, Atlanta, GA.
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Correspondence: Russ Hauser, Occupational Health Program, Harvard School of Public Health, Building 1, Room 1405, 665 Huntington Ave., Boston MA 02115. E-mail: rhauser@hohp.harvard.edu.

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status = 1.0, 1.8, 3.0; *P* for trend = 0.02) and sperm concentration (1.0, 1.4, 3.3; *P* for trend = 0.07). There was also a dose–response relationship between urinary levels of monobenzyl phthalate (MBzP), a metabolite of BBzP, and low sperm concentration (1.0, 1.4, 5.5; *P* for trend = 0.02). In the second publication, among 220 men, high levels of MBP, MBzP, and mono(2-ethylhexyl) phthalate (MEHP), the hydrolytic metabolite of DEHP, were associated with reduced sperm motion parameters, VSL (straight line velocity), VCL (curvilinear velocity), and LIN (linearity = VSL/VCL 100) measured by computer-aided semen analysis (CASA).[14]

Not all of our previous results were consistent with experimental animal studies. For instance, in rodent studies, MEHP, MBP, and MBzP adversely affected semen production and quality.[4–6] We found associations between semen quality and MBP and MBzP but no associations with MEHP.

In the present report, we used a larger sample of men (n = 463) to explore the relationship of MBP and MBzP with semen quality. In addition, we extended the study to include urinary measurements of 2 oxidative metabolites of DEHP.

METHODS

The study was approved by the Harvard School of Public Health and Massachusetts General Hospital Human Subjects Committees, and all subjects signed an informed consent. Of the 463 subjects in the present study, 168 were subjects in our previous publication on the relationship between phthalates and conventional semen parameters, namely sperm concentration, motility, and morphology.[13] Study subjects were male partners in subfertile couples who presented to the Vincent Burnham Andrology laboratory at Massachusetts General Hospital between January 2000 and May 2004 for semen analysis as part of an infertility workup. Approximately 60% of eligible men between 20 and 54 years of age agreed to participate. Men presenting for postvasectomy semen analysis were excluded. Height and weight were measured and a questionnaire was used to collect information on medical history and lifestyle factors.

A semen sample was produced on-site by masturbation into a sterile plastic specimen cup. After collection, the sample was liquefied at 37°C for 20 minutes before analysis. Men were instructed to abstain from ejaculation for 48 hours before producing the semen sample and to complete a questionnaire that included information on the length of the sexual abstinence period. All semen samples were analyzed for sperm concentration and motion parameters by CASA (Hamilton-Thorn Version 10HTM-IVOS) as previously described.[13,14] Sperm morphology was determined using the strict criteria by Kruger et al.[15] Results were expressed as the percentage of normal spermatozoa.

Phthalate metabolites were measured in urine because of potential sample contamination from the parent diester and because the metabolites, as opposed to the parent diesters, are believed to be the active toxicants.[9,16] The concentrations of phthalate metabolites were measured in a single spot urine sample collected on the same day as the semen sample. The urine and semen samples were collected in a sterile specimen cup prescreened for phthalates. The analytic approach for the measurement of the urinary phthalate monoester metabolites (ie, MEHP, MBP, MBzP, monoethyl phthalate [MEP], monomethyl phthalate [MMP]) and 2 oxidative metabolites of DEHP (ie, mono[2-ethyl-5-hydroxyhexyl] phthalate [MEHHP] and mono[2-ethyl-5-oxohexyl] phthalate [MEOHP]) involved enzymatic deconjugation of the metabolites from their glucuronidated form, solid-phase extraction, separation with high-performance liquid chromatography (HPLC), and detection by isotope-dilution tandem mass spectrometry.[17–19] Detection limits were in the low nanogram per milliliter range. Isotopically labeled internal standards and conjugated standards were used to increase precision of measurements. Along with the unknown samples, each analytic run included calibration standards, reagent blanks, and quality control materials of high and low concentration to monitor for accuracy and precision. Analysts at the Centers for Disease Control and Prevention, Atlanta, GA, were unaware of all information concerning subjects. Urinary phthalate metabolite concentrations were normalized for dilution by specific gravity. Unless otherwise noted, SG-adjusted concentrations were used in all statistical analyses.

Among the 463 samples, MEP, a metabolite of DEP, was detected in 100% of the samples, whereas MBP, MBzP, and MMP, a metabolite of dimethyl phthalate, were detected in 97%, 94%, and 76% of the samples, respectively. Eighty-three percent of samples had detectable levels of MEHP. The sample size for MEOHP and MEHHP was 230 because analytic methods for the quantification of these analytes were only recently implemented in this study. Over 95% of these samples had detectable levels of MEHHP and MEOHP.

Using the urinary concentrations of the 3 DEHP metabolites (MEHP, MEHHP, and MEOHP), we calculated the percent of these DEHP metabolites excreted as the hydrolytic monoester. We refer to this as %MEHP and consider it a phenotypic marker of the proportion of DEHP excreted in the urine as MEHP. The greater the %MEHP, the larger the percentage of DEHP excreted as MEHP relative to the excretion of the 2 oxidative metabolites. To calculate %MEHP, we converted MEHP, MEHHP, and MEOHP to nanomoles per milliliter, divided MEHP (nmol/mL) by the sum of MEOHP (nmol/mL), MEHHP (nmol/mL) and MEHP (nmol/mL), and multiplied by 100. Urine samples below the limit of detection for MEHP, MEHHP, or MEOHP were assigned a value of 1/2 LOD. To our knowledge, the use of %MEHP as a phenotypic marker of DEHP metabolism and excretion is novel and has not been used in human health studies.

Statistical Analysis

We used Statistical Analysis Software (SAS), version 9.1 (SAS Institute Inc., Cary, NC) for data analysis. To account for the known intraindividual variability in semen parameters, we performed analyses using categorized semen quality parameters. Semen parameters were dichotomized based on the WHO[20] reference values for sperm concentration (less than 20 million/mL) and motility (less than 50% motile sperm) and the Tygerberg Kruger Strict criteria for morphology (less than 4% normal sperm). We defined the comparison group as men with all 3 semen parameters at or above the reference values.

To explore dose–response relationships between below WHO reference value semen parameters and urinary levels of phthalate metabolites, we categorized phthalate concentrations into quartiles. In the primary analyses, using specific gravity-adjusted phthalate monoester concentrations, azoospermic men (n = 16) were excluded to prevent undue influence from extreme sperm counts (ie, zero sperm) and because the mechanism responsible for azoospermia may be related to an obstructive mechanism or Y-chromosome deletions. In addition, men with diabetes (n = 4) were excluded because diabetes may alter metabolism and excretion of phthalates. Therefore, after these exclusions, there were 443 men in the specific gravity-adjusted phthalate monoester analyses and 222 in the adjusted MEHHP, MEOHP, and %MEHP analyses. In sensitivity analyses, samples that were too dilute (SG $<$ 1.010) or concentrated (SG $>$ 1.030)[21] were excluded.

The Mantel-Haenszel χ^2 test was used to assess the relationship between categorical variables and the categorized semen parameters. We used multivariate logistic regression analysis to explore the relationship between low values for each semen parameter and quartiles of urinary levels of phthalate metabolites adjusting for covariates. Covariates considered included smoking status, race, age, body mass index, and abstinence time. Their inclusion in the multivariate models was based on statistical and biologic considerations.[22] Age was modeled as a continuous independent variable. Abstinence time was modeled as an ordinal 5-category variable (2 or fewer days, 3, 4, 5, and 6 or more days). Smoking status was included as a dummy variable (current and former vs never).

RESULTS

The demographic distribution of the 463 men, by semen parameter, is summarized in Table 1. Men were primarily white (84%) with a mean ± standard deviation age of 36.3 ± 5.5 years. Seventy-six men (16%) had a sperm concentration less than 20 million/mL, 221 men (48%) had less than 50% motile sperm, and 114 men (25%) had less than 4% normally shaped sperm. There were 210 men (45%) with values above reference values on all 3 semen parameters. The semen parameter categories were not mutually exclusive; a man could contribute data to one, 2 or all 3 of the reference value groups.

Men were primarily never smokers (72%) with 9% current smokers and 19% former smokers. Sperm concentration was lower among ever smokers (includes current and former smokers) than among never smokers. Men who had undergone a previous examination for infertility had lower sperm concentration, motility, and morphology than other men.

There was a wide distribution of the phthalate metabolite concentrations (Table 2). Except for a moderate correlation between MBP and MBzP (Spearman r = 0.50), the correlations among MBP, MBzP, MMP, MEP, and MEHP were all weak (less than 0.3). As expected, MEHP was strongly correlated with MEHHP and MEOHP with Spearman correlations of 0.74 and 0.71, respectively. MEHHP and MEOHP were strongly correlated to each other (Spearman correlation = 0.98).

Although our study spanned only 5 years, we observed suggestive time trends in MEHP and MMP concentrations (Table 3). In regression analysis, there was a 0.20 (standard

TABLE 1. Demographic and Medical History by Semen Parameters (n = 463*)

	Comparison Subjects† (n = 210)	Sperm Concentration (<20 million/mL) (n = 76)‡	Sperm Motility (<50% motile) (n = 221)‡	Sperm Morphology (<4% normal) (n = 114)‡
Age; mean ± SD	35.7 ± 5.2	36.4 ± 6.2	36.8 ± 5.8	36.5 ± 5.8
Abstinence time; %				
≤2 d	25	31	25	18
3 d	31	27	30	30
4 d	18	16	20	21
5 d	12	13	10	10
≥6 d	14	13	15	21
Race; %				
White	88	80	82	86
Black	4	5	5	4
Hispanic	3	4	6	4
Other	6	11	7	6
Smoking status; %				
Never smoker	76	63	69	70
Ever smoker	24	37	31	30
Current smoker	7	11	9	9
Former smoker	17	27	21	21
Previous examination for infertility; %	29	51	42	42

*2 missing race, 3 missing smoking, 4 missing abstinence, and 3 missing previous infertility examination information.
†Men with sperm concentration ≥20 million/mL, motility ≥50% motile, and morphology ≥4% normal.
‡A man may contribute data to more than 1 category.

TABLE 2. Specific Gravity-Adjusted Urinary Phthalate Monoester and DEHP Oxidative Metabolite Concentrations (ng/mL urine)*

Phthalate Metabolite	No.	Minimum	Percentile					Maximum	Geometric Mean
			5th	25th	50th	75th	95th		
MEP	463	8.7	21.8	58.7	158	535	2214	11,371	180
MBzP	463	<LOD	0.9	4.2	8.0	15.5	40.6	540	7.4
MBP	463	<LOD	3.2	10.6	17.7	31.7	69.9	14,459	17.3
MMP	462	<LOD	<LOD	1.5	3.8	9.1	28.6	278	3.6
MEHP	463	<LOD	<LOD	3.1	7.9	20.9	127	876	8.0
MEOHP	230	<LOD	7.1	15.8	32.1	73.0	497	3063	38.0
MEHHP	230	<LOD	10.2	23.4	48.1	113	786	4806	57.4
%MEHP	230	0.2	2.4	5.1	9.6	16.1	30.5	60.2	8.8

*LODs for phthalates (ng/mL) are as follows; MEP = 1.0; MBzP = 0.8; MBP = 0.6; MMP = 0.71; MEHP = 1.2.; MEOHP = 1.07; and MEHHP = 0.95. Values below LOD were assigned a value of ½ LOD.
LOD indicates limit of detection.

error [SE] = 0.06) increase in the log MEHP per year. For MMP, there was a 0.27 (SE = 0.05) decrease in the log MMP per year. For MBP, MBzP, and MEP, there were yearly fluctuations in the medians with no consistent upward or downward trend.

The adjusted odds ratios (ORs) and their 95% confidence intervals (CIs) for the associations between low semen parameters and phthalate metabolites are shown in Table 4; crude results were similar. There were dose–response relationships between MBP and both below WHO reference value sperm concentration (OR per quartile adjusted for age, abstinence, and smoking = 1.0, 3.1, 2.5, 3.3; *P* value for trend = 0.04) and motility (1.0, 1.5, 1.5, 1.8; *P* value for trend = 0.04) (Figs. 1 and 2). There was suggestive evidence of a relationship between the highest quartile of MBzP and below WHO reference value sperm concentration (1.0, 1.1, 1.1, 1.9; *P* value for trend = 0.13). There were no relationships between MMP and MEP and semen parameters.

In Table 4, we also present results for DEHP metabolites, namely MEHP, MEHHP, MEOHP, and %MEHP. Note that the sample size for MEHHP, MEOHP, and %MEHP was only 222. There was no evidence of dose–response relationships for MEHP, MEHHP, and MEOHP with the 3 semen parameters. However, there was weak evidence of a relationship between %MEHP and low sperm motility. The odds ratio for each %MEHP quartile compared with the reference (adjusted for age, abstinence, and smoking) was elevated (1.0, 1.3, 1.6, 1.5; *P* for trend = 0.27). In analyses in which MEHP was in the same model with %MEHP, MEHHP, or MEOHP, the relationship between semen parameters and MEHP, MEHHP, MEOHP, and %MEHP remained qualitatively similar. Finally, there was also no evidence of statistical 2-way interactions of MEHP with %MEHP or with the oxidative metabolites.

Associations between CASA parameters and phthalate metabolites were mostly unremarkable (Table 5). There were no strong or consistent associations between any of the phthalate metabolites and CASA parameters. We previously saw an overall pattern of inverse associations of the CASA parameters VSL (straight line velocity), VCL (curvilinear velocity), and LIN (linearity = VSL/VCL × 100) with tertiles of MBP, MBzP, and MEHP.[14]

Sensitivity Analyses

To explore the robustness of our results, a reanalysis was performed after excluding urine samples that were highly dilute or concentrated based on extreme specific gravity values.[21] Sample size for analysis was reduced to 372 for most analytes and 182 for MEOHP and MEHHP. In the reanalysis, the odd ratios remained essentially unchanged (data not shown).

TABLE 3. Specific Gravity-Adjusted Urinary Phthalate Monoester Metabolite Concentrations by Year (ng/mL urine; 25th, 50th, and 75th percentiles)

Year	No.	MEP			MBzP			MBP			MMP			MEHP		
		25th	50th	75th	25th	50th	75th	25th	50th	75th	25th	50th	75th	25th	50th	75th
2000	141	66.0	153	443	4.96	10.5	16.2	10.0	16.3	29.8	2.13	4.32	9.72	2.75	6.23	18.8
2001	124	56.3	180	545	3.22	6.89	16.6	9.63	18.0	29.9	1.70	6.43	13.6	1.87	5.66	19.5
2002	91	58.2	137	639	3.46	5.47	8.93	8.35	13.4	23.4	2.40	5.28	11.3	2.09	7.58	21.0
2003	67	42.6	125	405	5.52	10.1	17.8	14.3	21.7	35.1	1.15	2.17	3.90	7.20	14.1	26.7
2004	40	98.7	309	1137	5.71	10.7	20.5	12.4	17.5	27.6	0.59	1.20	2.35	6.98	11.7	18.2

TABLE 4. Association of Below-Reference Quartiles of Specific Gravity-Adjusted Phthalate Monoester and DEHP Metabolite Levels With Sperm Concentration, Motility, and Morphology*

	Sperm Concentration		Sperm Motility		Sperm Morphology	
Phthalate Quartile	**No.**	**Adjusted OR (95% CI)**	**No.**	**Adjusted OR (95% CI)**	**No.**	**Adjusted OR (95% CI)**
MBP (n = 443†)						
1	7	1.0	40	1.0	30	1.0
2	19	3.1 (1.2 to 8.1)	49	1.5 (0.8 to 2.6)	23	0.8 (0.4 to 1.6)
3	15	2.5 (0.9 to 6.7)	54	1.5 (0.8 to 2.6)	25	0.9 (0.5 to 1.7)
4	19	3.3 (1.2 to 8.5)	60	1.8 (1.1 to 3.2)	20	0.8 (0.4 to 1.6)
P for trend		0.04		0.04		0.59
MBzP (n = 443†)						
1	13	1.0	44	1.0	29	1.0
2	13	1.1 (0.4 to 2.6)	53	1.3 (0.7 to 2.3)	18	0.7 (0.3 to 1.4)
3	13	1.1 (0.4 to 2.5)	53	1.3 (0.8 to 2.3)	24	0.9 (0.4 to 1.7)
4	21	1.9 (0.8 to 4.3)	53	1.3 (0.7 to 2.3)	27	1.1 (0.6 to 2.1)
P for trend		0.13		0.36		0.76
MMP (n = 442†)						
1	18	1.0	60	1.0	28	1.0
2	13	0.5 (0.2 to 1.3)	44	0.5 (0.3 to 0.9)	26	0.7 (0.3 to 1.3)
3	19	1.0 (0.5 to 2.3)	57	0.9 (0.5 to 1.6)	19	0.7 (0.3 to 1.4)
4	9	0.4 (0.1 to 0.9)	41	0.5 (0.3 to 0.9)	25	0.7 (0.3 to 1.3)
P for trend		0.15		0.12		0.26
MEP (n = 443†)						
1	12	1.0	46	1.0	29	1.0
2	19	1.5 (0.7 to 3.6)	55	1.1 (0.6 to 1.9)	27	0.8 (0.4 to 1.6)
3	14	1.0 (0.4 to 2.5)	48	0.8 (0.5 to 1.5)	25	0.7 (0.3 to 1.3)
4	15	1.2 (0.5 to 3.0)	54	1.0 (0.6 to 1.8)	17	0.5 (0.3 to 1.1)
P for trend		0.94		0.84		0.07
MEHP (n = 443)						
1	16	1.0	42	1.0	28	1.0
2	15	1.0 (0.4 to 2.3)	55	1.4 (0.8 to 2.5)	26	1.1 (0.5 to 2.1)
3	14	0.9 (0.4 to 2.0)	56	1.3 (0.7 to 2.3)	22	0.8 (0.4 to 1.5)
4	15	0.8 (0.4 to 1.8)	50	1.1 (0.6 to 1.9)	22	0.7 (0.4 to 1.5)
P for trend		0.58		0.89		0.30
MEOHP (n = 222‡)						
1	4	1.0	31	1.0	13	1.0
2	10	3.1 (0.8 to 11.7)	26	0.9 (0.4 to 2.0)	16	1.4 (0.5 to 3.7)
3	5	1.1 (0.3 to 4.6)	25	0.6 (0.3 to 1.3)	8	0.5 (0.2 to 1.5)
4	9	1.6 (0.4 to 6.3)	27	0.8 (0.3 to 1.6)	10	0.7 (0.3 to 2.0)
P for trend		0.97		0.32		0.23
MEHHP (n = 222‡)						
1	7	1.0	29	1.0	12	1.0
2	6	1.5 (0.4 to 5.5)	28	1.3 (0.6 to 2.9)	18	2.1 (0.8 to 5.6)
3	5	0.7 (0.2 to 2.6)	25	0.7 (0.3 to 1.6)	8	0.5 (0.2 to 1.6)
4	10	1.1 (0.4 to 3.6)	27	0.8 (0.4 to 1.8)	9	0.7 (0.3 to 2.0)
P for trend		0.91		0.39		0.17
%MEHP (n = 222‡)						
1	8	1.0	23	1.0	14	1.0
2	8	1.3 (0.4 to 4.5)	26	1.3 (0.6 to 2.8)	12	1.0 (0.4 to 2.8)
3	7	1.2 (0.3 to 4.3)	30	1.6 (0.7 to 3.5)	6	0.4 (0.1 to 1.3)
4	5	0.8 (0.2 to 3.1)	30	1.5 (0.7 to 3.4)	15	1.2 (0.5 to 3.2)
P for trend		0.86		0.27		0.90

For MMP, N = 442†.

*Adjusted for age (continuous), abstinence time (5 categories: ≤2 d, 3, 4, 5, and 6+) and smoking (current, former, and never). Comparison group is men at or above reference value for sperm concentration (≥20 million/mL), motility (≥50% motile), and morphology (≥4% normal morphology).

†Excludes 16 men with azoospermia and 4 men with diabetes. One man was missing MMP.

‡Excludes 7 men with azoospermia and one man with diabetes.

Quartile cut points (ng/mL): MBP 0.3–10.6, 10.3–17.7, 17.8–31.7, 31.7–14,459; MBzP 0.04–4.2, 4.2–8.0, 8.0–15.3, 15.5–540.2; MMP 0.1–1.5, 1.5–3.8, 3.8–9.1, 9.2–278.1; MEP 8.7–58.7, 59.6–157.6, 157.9–534.3, 535.0–11,371; MEHP 0.01–3.1, 3.1–7.9, 7.9–20.7, 20.9–875.8; MEOHP 0.4–15.8, 16.1–31.9, 32.1–69.6, 73.0–3063; MEHHP 0.5–23.4, 23.4–48.0, 48.2–109.5, 112.8–4806.

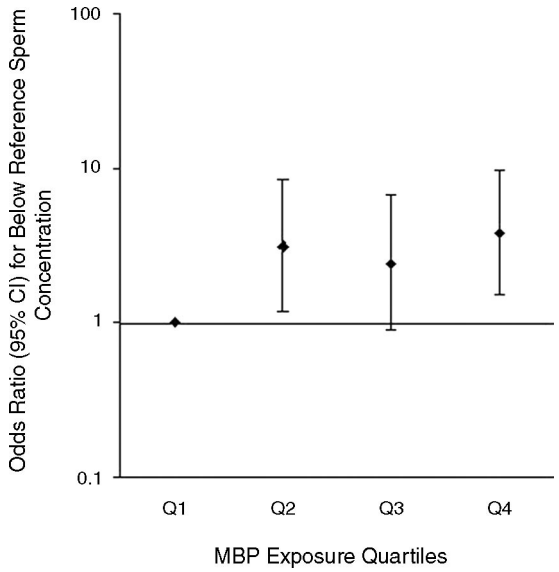

FIGURE 1. Adjusted odds ratios (95% confidence intervals) for below reference sperm concentration associated with quartiles of SG-adjusted monobutyl phthalate urinary concentration (adjusted for age, abstinence time, and smoking status).

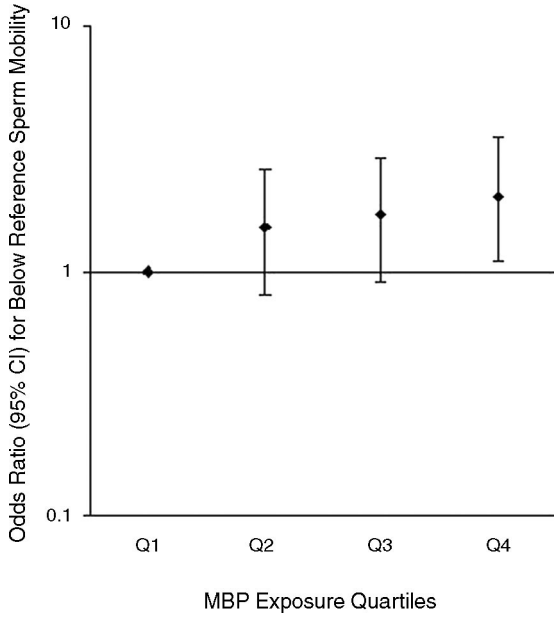

FIGURE 2. Adjusted odds ratios (95% confidence intervals) for below reference sperm motility associated with quartiles of SG-adjusted monobutyl phthalate urinary concentration (adjusted for age, abstinence time, and smoking status).

To further explore the robustness of our results, we excluded the 168 subjects from our previous publication on phthalates and semen quality[13] and performed a subset analysis on the 295 new recruits. Because there was suggestive evidence of a temporal trend in MEHP and MMP for the full set, we considered whether urinary concentrations of these monoesters differed between the 168 subjects in our original publication and the 295 new recruits. The median [25th and 75th percentiles] MEHP concentration (ng/mL) was higher in the new recruits (9.3 [3.6 and 22.3]) as compared with the original subjects (6.2 [2.7 and 17.6]). Likewise, MMP was lower in the new recruits as compared with the original subjects. Among the 295 new recruits, the dose–response relationships between MBP and low sperm concentration and motility were slightly weaker than among the original 168 men. However, the overall trend and interpretation remained the same. The association between MBzP and low sperm concentration became weaker and less stable in the new recruits. Results for the other metabolites remained essentially unchanged.

DISCUSSION

The present study confirms in a larger sample of men an association between MBP and below WHO reference value sperm concentration and motility. This result is consistent with toxicologic studies in laboratory rodents showing that MBP is a testicular toxicant.[6] We did not find dose–response relationships between poor semen quality and MEP and MMP consistent with our earlier results as well as with the toxicologic literature.[23] The suggestive relationship found in our earlier publication between MBzP and sperm concentration was weaker in the present study. There was evidence of an association between the highest MBzP quartile and low value sperm concentration.

Although laboratory studies in rats consistently find associations between MEHP and altered male reproductive function,[24] no associations between MEHP and semen parameters were observed either in our previous study[13] or in this larger and more powerful analysis. There are several potential explanations for the lack of an association. One is that, in adult men, environmental background levels of MEHP may not be associated with altered semen quality. Laboratory studies have shown that adult rats are less sensitive to MEHP than are rats exposed in utero or during puberty.[8,25]

An alternative explanation is that urinary levels of MEHP may not adequately reflect internal dose. Although the metabolism of the relatively low-molecular-weight phthalates (eg, DEP) ends with the hydrolytic monoester,[2,3,26] the metabolism of the higher-molecular-weight phthalates (eg, DEHP) continues with transformation of the hydrolytic monoester to oxidative products.[1,26–30] In humans, urinary concentrations of MEOHP and MEHHP are severalfold higher than that of MEHP.[31–34] A third oxidative metabolite of DEHP, mono(2-ethyl-5-carboxypentyl) phthalate, has also been found at higher concentrations in urine than MEHP,[35–38] suggesting that these metabolites may provide greater analytic sensitivity than does MEHP. Toxicologic studies show that MEHP is the toxic metabolite of DEHP.[1] The evidence on the toxicity of MEHHP and MEOHP is mixed.[39,40]

The present study did not find strong dose–response associations of semen quality with any of the DEHP metabolite indices. There was a suggestion of an association be-

TABLE 5. Adjusted* Regression Coefficients for a Change in Sperm Motion Parameters Associated With Quartiles of Specific Gravity-Adjusted Phthalate Monoester and DEHP Metabolite Levels

		Sperm Motion Parameters†		
Phthalate Monoester	Quartile	Straight Line Velocity (VSL)‡ Coefficient (CI)	Curvilinear Velocity (VCL)§ Coefficient (CI)	Linearity (LIN)‖ Coefficient (CI)
MBP (n = 433)	2	−0.97 (−3.68 to 1.74)	−3.46 (−8.05 to 1.14)	1.11 (−0.80 to 3.02)
	3	−0.11 (−2.79 to 2.58)	−1.32 (−5.87 to 3.24)	0.84 (−1.06 to 2.73)
	4	−0.88 (−3.57 to 1.81)	−1.65 (−6.20 to 2.91)	0.38 (−1.52 to 2.27)
	P for trend	0.68	0.71	0.78
MBzP (n = 433)	2	0.66 (−2.01 to 3.34)	1.44 (−3.10 to 5.99)	−0.23 (−2.12 to 1.66)
	3	0.11 (−2.59 to 2.81)	1.29 (−3.29 to 5.88)	−1.13 (−3.04 to 0.77)
	4	−1.31 (−3.98 to 1.36)	−1.20 (−5.73 to 3.34)	−0.69 (−2.58 to 1.20)
	P for trend	0.29	0.60	0.33
MMP (n = 432)	2	0.06 (−2.61 to 2.74)	−1.29 (−5.83 to 3.25)	0.97 (−0.92 to 2.86)
		−0.70 (−3.37 to 1.97)	−2.37 (−6.88 to 2.15)	1.10 (−0.78 to 2.98)
	4	0.78 (−1.92 to 3.48)	1.11 (−3.47 to 5.68)	0.28 (−1.63 to 2.18)
	P for trend	0.73	0.71	0.85
MEP (n = 433)	2	0.02 (−2.66 to 2.70)	−0.28 (−4.82 to 4.25)	0.34 (−1.55 to 2.23)
	3	0.81 (−1.92 to 3.55)	−0.47 (−5.09 to 4.16)	1.67 (−0.25 to 3.60)
	4	2.11 (−0.61 to 4.83)	4.48 (−0.13 to 9.08)	−0.31 (−2.23 to 1.61)
	P for trend	0.10	0.07	0.93
MEHP (n = 433)	2	−1.52 (−4.20 to 1.16)	−1.93 (−6.49 to 2.62)	−0.68 (−2.58 to 1.21)
	3	−2.03 (−4.73 to 0.68)	−2.97 (−7.57 to 1.62)	−0.71 (−2.62 to 1.21)
	4	−1.06 (−3.77 to 1.65)	−2.05 (−6.65 to 2.56)	0.17 (−1.75 to 2.08)
	P for trend	0.41	0.34	0.87
MEOHP (n = 217)	2	1.26 (−2.69 to 5.21)	5.80 (−0.75 to 12.3)	−2.62 (−5.46 to 0.22)
	3	2.09 (−1.84 to 6.03)	3.93 (−2.60 to 10.5)	−0.26 (−3.09 to 2.57)
	4	0.17 (−3.72 to 4.06)	1.47 (−4.98 to 7.92)	−0.12 (−2.91 to 2.68)
	P for trend	0.83	0.78	0.70
MEHHP (n = 217)	2	0.67 (−3.32 to 4.67)	2.21 (−4.45 to 8.88)	−0.64 (−3.53 to 2.25)
	3	1.99 (−1.95 to 5.93)	1.92 (−4.65 to 8.48)	0.72 (−2.13 to 3.56)
	4	0.48 (−3.38 to 4.35)	0.64 (−5.81 to 7.08)	0.92 (−1.88 to 3.71)
	P for trend	0.66	0.86	0.38
%MEHP (n = 217)	2	2.43 (−1.50 to 6.36)	3.85 (−2.71 to 10.4)	−0.57 (−3.42 to 2.28)
	3	0.23 (−3.83 to 4.30)	0.22 (−6.56 to 7.00)	−0.21 (−3.16 to 2.74)
	4	−0.13 (−4.07 to 3.81)	1.23 (−5.35 to 7.81)	−1.42 (−4.28 to 1.44)
	P for trend	0.69	0.98	0.39

Excludes 10 men without sperm motion measurements.
*Adjusted for age (continuous), smoking (current, former vs never), and abstinence time (5 categories: ≤2 d, 3, 4, 5, and 6+ days).
†Hamilton Thorne Integrated Visual Optic System Version 10 software was used to measure sperm motion parameters.
‡Straight line velocity (μm/s) is a measure of sperm progression; coefficient units are μm/s per quintile.
§Curvilinear velocity is a measure of sperm vigor; coefficient units are μm/s per quintile.
‖Linearity (%) is a measure of sperm swimming pattern; coefficient units are percent per quintile.

tween below reference sperm motility and %MEHP. However, the sample size was relatively small and thus the analysis was less powerful. We found that the higher the %MEHP, the greater the odds of low sperm motility. This may indicate that individuals who excrete DEHP primarily as MEHP have a lower ability to metabolize MEHP to the oxidative metabolites and may be at risk for low sperm motility as compared with individuals who excrete DEHP as MEHHP and MEOHP. We recognize that there are additional DEHP metabolites that we did not measure[37,38]; thus, %MEHP as calculated in the present study is not comprehensive, although MEHP, MEHHP, and MEOHP account for approximately 50% of the DEHP dose.[30,36]

To our knowledge, this is the first study to explore the associations of semen quality with DEHP oxidative metabolites and %MEHP. Further investigation is warranted on the potential use of %MEHP as a phenotypic marker of the proportion of DEHP excreted as MEHP and its oxidative metabolites. As for other phthalates, there may be interindividual variability in DEHP metabolism and urinary excretion of metabolites. Furthermore, the timing of collection of the urine sample may contribute to differences in urinary concentrations of MEHP and the oxidative metabolites among individuals because the oxidative metabolites have a longer half-life than MEHP.[36] For instance, a urine sample collected several hours after DEHP exposure would contain primarily

MEHP. Likewise, a urine sample collected 12 hours after DEHP exposure may have higher concentrations of MEHHP and MEOHP as compared with MEHP. The differences in half-lives of DEHP metabolites should be taken into account when interpreting the meaning of %MEHP after a single pulsed exposure to DEHP. However, the interpretation of %MEHP would be more straightforward if there were chronic exposure to DEHP, making the differences in half-lives less influential on urinary concentrations.

There are few studies on human exposure to phthalates and semen quality,[13,41] and we are not aware of any that have measured DEHP oxidative metabolites. In a recently published study, Jonsson and colleagues recruited 234 young Swedish men at the time of their medical conscript examination.[41] Each man provided a single urine sample for measurement of concentrations of MEP, MEHP, MBzP, MBP, and phthalic acid. Semen quality was assessed using traditional semen parameters. Urinary phthalate levels were divided into quartiles and were used to calculate the mean difference and 95% confidence interval between the lowest and highest quartiles. For MEHP, the 63% of men with urinary concentrations below the detection limit (15 ng/mL) were compared with the 18% of men who had the highest concentrations of MEHP. Because multivariate-adjusted results and unadjusted results differed by less than 15%, potential confounders such as abstinence time and smoking status were not kept in the models.

In contrast to the present study, Jonsson et al[41] found no relationships of MBP or MBzP with any of the semen parameters. In addition, MEHP was not associated with any of the semen parameters. Men in the highest quartile for MEP had less motile sperm (mean difference was 8.8%; 95% CI = 0.8 to 17) and more immotile sperm (8.9%; 0.3–18) than men in the lowest MEP quartile. Phthalic acid was actually associated with improved function as measured by more motile sperm and fewer immotile sperm. Phthalic acid, which can be formed from the hydrolysis of any phthalate, is a nonspecific marker of phthalate exposure.[42] Interactions between urinary phthalate levels and PCB 153 (measured previously in serum samples from these men) were assessed by including an interaction term in the models. There was no evidence of multiplicative interactions between PCB 153 and any of the phthalates with the reproductive markers (data not shown). This is in contrast to our previous study,[43] in which we found interactions of MBP and MBzP with PCB 153 in relation to sperm motility.

Although the Swedish study[41] and the present one were both cross-sectional in design and based on the collection of a single urine and semen sample from each adult male participant, there are several important differences. The population in the Swedish study consisted of young men (median age = 18 years; range = 18–21 years) who were undergoing a medical examination before military service. Because approximately 95% of young men in Sweden undergo the conscript examination, these young men reflected the general population of young Swedish males. In contrast, in the present study, the median age of the men recruited from an infertility clinic was 35.5 years and ranged from 22 to 54 years. It is possible that subfertile men (eg, those presenting to an infertility clinic) are more "susceptible" to reproductive toxicants, including phthalates, than men from the general population. Also, middle-aged men may be more susceptible to reproductive toxicants because of an age-related response to the toxicant.

However, there is no evidence to suggest that older or less fertile men are more susceptible to phthalates. Furthermore, although the men in our study were recruited from an infertility clinic, such a population is heterogeneous and includes both fertile and infertile men (because the female partner's infertility may be at least partially the cause of the couple's infertility). However, if there were a difference in susceptibility to phthalates of men in infertile relationships, the generalizability of our results to the general population may be limited. Further research is needed to better understand susceptibility factors in relation to phthalate exposure and semen quality.

Although only 14% of the young Swedish men, as compared with 65% of men in the present study, agreed to participate,[41] it is unlikely that the young Swedish men did so differentially in relation to reproductive function and phthalate levels. Therefore, selection bias as a result of the low participation rate is unlikely in the Swedish study. In our study, we have data supporting the assertions that recruitment of subjects through an infertility clinic is not likely to introduce selection bias. In a recent publication,[44] among a cohort of men that overlaps with the men in the present study, we reported no differences in semen characteristics among participants and nonparticipants, suggesting that men's participation was not based on semen quality. Furthermore, we believe it is unlikely that men participated based on their exposure to phthalates because the men would not have had this information.

Several other notable differences between the 2 studies include the collection, storage, and analysis of the urine samples. In the Swedish study,[41] it is unclear at what temperature urine samples were stored and the elapsed time between collection and analysis. More importantly, the analytic methods used for phthalate measurement differed between the Swedish and present study. The detection limits for MEP, MBP, MBzP, and MEHP in the Swedish study were 30, 15, 7, 15 ng/mL, which are manyfold higher than the detection limits (approximately 1 ng/mL) in the present study. In addition, the precision of comparisons of duplicate analysis on different days was low in the Swedish study, likely due to the lack of isotope-labeled standards for the phthalate metabolites measured. In the present study, we measured the phthalate metabolites using isotope-dilution high-performance liquid chromatography tandem mass spectrometry.[17–19] The method is precise (%RSDs from replicate measurements are $<15\%$). The higher limits of detection and lower analytical precision in the Swedish study may contribute to measurement error of urinary phthalate levels and may result in bias toward the null. However, by dividing the phthalate levels into quartiles for the statistical analysis, some of these measurement errors may be minimized. The Swedish study used urinary creatinine to adjust for urine dilution as compared with specific gravity in the present study. Based on

the medians in the tables from the Swedish study, the creatinine-adjusted values were quite different from the unadjusted values. In contrast, in the present study, medians between values adjusted and unadjusted for specific gravity were not markedly different.

The statistical methods used for the data analysis also differed between studies. We used multivariate logistic regression with categorized semen parameters as the primary outcome. Men with all 3 semen parameters above the reference range were used as comparison subjects. In contrast, in the Swedish study,[41] for the primary analysis, semen parameters were used as a continuous measurement and mean differences between men in the highest and lowest phthalate quartiles were calculated. In addition, the Swedish researchers performed logistic regression analyses and the results were reported to be consistent with their primary analyses. However, it is unclear whether the comparison group in the logistic regression analyses included only men with all 3 semen parameters above the reference range. Dilution of associations between phthalates and semen parameters may occur if the comparison group did not consist of a homogenous group of men with normal semen parameters.

In summary, although the present study and the Swedish study[41] have some similarities, there are important differences in the study population, study design, analytical methods and statistical analyses. These differences may partially account for the inconsistent results across the 2 studies. However, both studies should serve to help guide further work on the potential relationship between phthalates and male fertility.

A final observation from the present study was that during the 5 calendar years (2000–2004) of the study, we observed suggestive time trends in MEHP and MMP concentrations. MEHP increased, MMP decreased, and the others showed no trend. Whether the trends reflect changes in the exposure patterns of the study subjects or patterns in the commercial use of these phthalates is unclear. Alternatively, the smaller sample sizes in the later years may have contributed to instability in their distributions and may partially account for the apparent trends.

In conclusion, the results of the present study among men from an infertility clinic were consistent with our previous work on the relationship of urinary levels of MBP with low semen quality. Like in our earlier work,[13,14] there was a lack of an association between MEHP and semen quality. In the present study, we measured MEHHP and MEOHP, 2 oxidative metabolites of DEHP that are found in higher concentrations than MEHP in urine. We also did not find a relationship between the DEHP oxidative metabolites and semen parameters. However, the higher the %MEHP, the greater the odds of low sperm motility. We hypothesize that this may indicate that individuals who excrete higher concentrations of MEHP may be at risk for lower sperm motility as compared with individuals who primarily excreted DEHP as oxidative metabolites. Although this relationship was not strong, further confirmatory studies are warranted. Finally, our male reproductive health study is ongoing, and future analyses will include measures of additional oxidative metabolites of DEHP, allowing for more complex analyses.

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REFERENCES

1. Agency for Toxic Substances and Disease Registry, 2002. Toxicological profile for di(2-ethylhexyl)phthalate (DEHP). Available at: http://www.atsdr.cdc.gov/toxprofiles/tp9.htm. Accessed June 9, 2005.
2. Agency for Toxic Substances and Disease Registry, 1995. Toxicological profile for diethyl phthalate (DEP). Available at: http://www.atsdr.cdc.gov/toxprofiles/tp73.html. Accessed June 9, 2005.
3. Agency for Toxic Substances and Disease Registry, 2001. Toxicological profile for di-n-butyl phthalate (DBP). Available at: http://www.atsdr.cdc.gov/toxprofiles/tp135.html. Accessed June 9, 2005.
4. Agarwal DK, Maronpot RR, Lamb JC, et al. Adverse-effects of butyl benzyl phthalate on the reproductive and hematopoietic systems of male-rats. *Toxicology*. 1985;35:189–206.
5. Parmar D, Srivastava SP, Seth PK. Effect of di(2-ethylhexyl)phthalate (DEHP) on spermatogenesis in adult-rats. *Toxicology*. 1986;42:47–55.
6. Srivastava SP, Srivastava S, Saxena DK, et al. Testicular effects of di-n-butyl phthalate (DBP): biochemical and histopathological alterations. *Arch Toxicol*. 1990;64:148–152.
7. Dostal LA, Chapin RE, Stefanski SA, et al. Testicular toxicity and reduced Sertoli-cell numbers in neonatal rats by di(2-ethylhexyl) phthalate and the recovery of fertility as adults. *Toxicol Appl Pharmacol*. 1988;95:104–121.
8. Higuchi TT, Palmer JS, Gray LE, et al. Effects of dibutyl phthalate in male rabbits following in utero, adolescent, or postpubertal exposure. *Toxicol Sci*. 2003;72:301–313.
9. Li LH, Jester WF, Orth JM. Effects of relatively low levels of mono-(2-ethylhexyl) phthalate on cocultured Sertoli cells and gonocytes from neonatal rats. *Toxicol Appl Pharmacol*. 1998;153:258–265.
10. Centers for Disease Control and Prevention, National Center for Environmental Health, Division of Laboratory Sciences. Third National Report on Human Exposure to Environmental Chemicals, 2005. Available at: http://www.cdc.gov/exposurereport/3rd/pdf/thirdreport.pdf. Accessed August 1, 2005.
11. Silva MJ, Barr DB, Reidy JA, et al. Urinary levels of seven phthalate metabolites in the US population from the National Health and Nutrition Examination Survey (NHANES) 1999–2000. *Environ Health Perspect*. 2004;112:331–338.
12. Duty SM, Singh NP, Silva MJ, et al. The relationship between environmental exposures to phthalates and DNA damage in human sperm using the neutral comet assay. *Environ Health Perspect*. 2003;111:1164–1169.
13. Duty SM, Silva MJ, Barr DB, et al. Phthalate exposure and human semen parameters. *Epidemiology*. 2003;14:269–277.
14. Duty SM, Calafat AM, Silva MJ, et al. The relationship between environmental exposure to phthalates and computer-aided sperm analysis motion parameters. *J Androl*. 2004;25:293–302.
15. Kruger TF, Acosta AA, Simmons KF, et al. Predictive value of abnormal sperm morphology in in vitro fertilization. *Fertil Steril*. 1988;49:112–117.
16. Peck CC, Albro PW. Toxic potential of the plasticizer di(2-ethylhexyl) phthalate in the context of its disposition and metabolism in primates and man. *Environ Health Perspect*. 1982;45:11–17.
17. Silva MJ, Slakman AR, Reidy JA, et al. Analysis of human urine for fifteen phthalate metabolites using automated solid-phase extraction. *J Chromatogr B*. 2004;805:161–167.

18. Blount BC, Milgram KE, Silva MJ, et al. Quantitative detection of eight phthalate metabolites in human urine using HPLC-APCI-MS/MS. *Anal Chem*. 2000;72:4127–4134.
19. Silva MJ, Malek NA, Hodge CC, et al. Improved quantitative detection of 11 urinary phthalate metabolites in humans using liquid chromatography–atmospheric pressure chemical ionization tandem mass spectrometry. *J Chromatogr B*. 2003;789:393–404.
20. World Health Organization. *WHO Laboratory Manual for the Examination of Human Semen and Sperm–Cervical Mucus Interaction*, 4th ed. New York: Cambridge University Press; 1999.
21. Teass AW, Biagini RE, DeBord G. In: Eller PM, ed. *NIOSH Manual of Analytical Methods*, 4th ed. Cincinnati: US Department of Health and Human Services, Centers for Disease Control and Prevention, National Institute for Occupational Safety and Health, Division of Physical Sciences and Engineering; 1998.
22. Hosmer DW Jr, Lemeshow S, eds. *Applied Logistic Regression*. New York: John Wiley & Sons; 1989.
23. Gray LE Jr, Ostby J, Furr J, et al. Perinatal exposure to the phthalates DEHP, BBP, and DINP, but not DEP, DMP, or DOTP, alters sexual differentiation of the male rat. *Toxicol Sci*. 2000;58:350–365.
24. Park JD, Sultan SMH, Klaassen CD. Testicular toxicity of di-(2-ethylhexyl)phthalate in young Sprague-Dawley rats. *Toxicology*. 2002;171:105–115.
25. Sjoberg P, Lindqvist NG, Ploen L. Age-dependent response of the rat testes to di(2-ethylhexyl) phthalate. *Environ Health Perspect*. 1986;65:237–242.
26. Albro PW, Moore B. Identification of the metabolites of simple phthalate diesters in rat urine. *J Chromatogr*. 1974;94:209–218.
27. Albro PW, Thomas RO. Enzymatic hydrolysis of di-(2-ethylhexyl) phthalate by lipases. *Biochim Biophys Acta*. 1973;306:380–390.
28. Albro PW, Lavenhar SR. Metabolism of di(2-ethylhexyl)phthalate. *Drug Metab Rev*. 1989;21:13–34.
29. McKee RH, El Hawari M, Stoltz M, et al. Absorption, disposition and metabolism of di-isononyl phthalate (DINP) in F-344 rats. *J Appl Toxicol*. 2002;22:293–302.
30. Koch HM, Bolt HM, Angerer J. Di(2-ethylhexyl)phthalate (DEHP) metabolites in human urine and serum after a single oral dose of deuterium-labelled DEHP. *Arch Toxicol*. 2004;78:123–130.
31. Barr DB, Silva MJ, Kato K, et al. Assessing human exposure to phthalates using monoesters and their oxidized metabolites as biomarkers. *Environ Health Perspect*. 2003;111:1148–1151.
32. Kato K, Silva MJ, Reidy JA, et al. Mono(2-ethyl-5-hydroxyhexyl) phthalate and mono-(2-ethyl-5-oxohexyl) phthalate as biomarkers for human exposure assessment to di-(2-ethylhexyl) phthalate. *Environ Health Perspect*. 2004;112:327–330.
33. Koch HM, Rossbach B, Drexler H, et al. Internal exposure of the general population to DEHP and other phthalates—determination of secondary and primary phthalate monoester metabolites in urine. *Environ Res*. 2003;93:177–185.
34. Kato K, Silva MJ, Needham LL, et al. Determination of 16 phthalate metabolites in urine using automated sample preparation and on-line preconcentration/high-performance liquid chromatography/tandem mass spectrometry. *Anal Chem*. 2005;77:2985–2991.
35. Preuss R, Koch HM, Angerer J. Biological monitoring of the five major metabolites of di-(2-ethylhexyl)phthalate (DEHP) in human urine using column-switching liquid chromatography-tandem mass spectrometry. *J Chromatogr B*. 2005;816:269–280.
36. Koch HM, Bolt HM, Preuss R, et al. New metabolites of di(2-ethylhexyl)phthalate (DEHP) in human urine and serum after single oral doses of deuterium-labelled DEHP. *Arch Toxicol*. 2005;79:367–376.
37. Silva MJ, Samandar E, Preau JLJ, et al. Urinary oxidative metabolites of di(2-ethylhexyl) phthalate in humans. *Toxicology*. 2006;219:22–32.
38. Silva MJ, Reidy JA, Samandar E, et al. Measurement of eight urinary metabolites of di(2-ethylhexyl) phthalate as biomarkers for human exposure assessment. *Biomarkers*. 2006;11:1–13.
39. Gray TJB, Gangolli SD. Aspects of the testicular toxicity of phthalate-esters. *Environ Health Perspect*. 1986;65:229–235.
40. Stroheker T, Cabaton N, Nourdin G, et al. Evaluation of anti-androgenic activity of di-(2-ethylhexyl)phthalate. *Toxicology*. 2005;208:115–121.
41. Jonsson BAG, Richthoff J, Rylander L, et al. Urinary phthalate metabolites and biomarkers of reproductive function in young men. *Epidemiology*. 2005;16:487–493.
42. Kato K, Silva MJ, Needham LL, et al. Determination of total phthalates in urine by isotope-dilution liquid chromatography-tandem mass spectrometry. *J Chromatogr B*. 2005;814:355–360.
43. Hauser R, Williams P, Altshul L, et al. Evidence of interaction between polychlorinated biphenyls and phthalates in relation to human sperm motility. *Environ Health Perspect*. 2005;113:425–430.
44. Hauser R, Godfrey-Bailey L, Chen Z. Does the potential for selection bias in semen quality studies depend on study design? Experience from a study conducted within an infertility clinic. *Hum Reprod*. 2005;20:2679–2583.