Oral Propylparaben Administration to Juvenile Male Wistar Rats Did Not Induce Toxicity in Reproductive Organs

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Parabens are in widespread use as preservatives in drugs. In the late 1990s, concerns were raised about their capacity to disrupt endocrine function based on in vitro data and in vivo uterotrophic tests. Studies in juvenile male rats provided conflicting results on postpubertal sperm production. In an exploratory pharmacokinetic study, Wistar male rats received a single dose of propylparaben (PP) at 3, 10, 100, or 1000 mg/kg, orally on postnatal day (PND) 31. Plasma PP concentrations were quantifiable up 8 h after dosing with a mean T½ value of 15 min. Distribution was 4.8 l/kg, the plasma elimination half-life was 47 min, and clearance was 4.20 (l/h)/kg at 10 mg/kg. A sulfoconjugated metabolite was detected. In the juvenile toxicology study, PP was orally administered by gavage to 20 Wistar male rats at doses of 3, 10, 100, or 1000 mg/kg/day in 1% hydroxyethylcellulose for 8 weeks starting on PND21. A first subgroup of 10 males/dose was necropsied immediately after the 8-week exposure period; a second subgroup of 10 males/dose was necropsied after a 26-week washout period. Blood samples were taken from additional satellite animals after dosing on PND21 and PND77 for toxicokinetic analysis. There was no evidence of an effect of PP on the weight of the male reproductive organs, epididymal sperm parameters, hormone levels, or histopathology. The dose of 1000 mg/kg/day was the no-observed adverse effect level, corresponding to a maximum plasma concentration of 12 030 ng/ml and exposure to 47 760 ng·h/ml (AUC0–8 h) at the end of the treatment.

Key Words: propylparaben; parahydroxybenzoic acid ester; spermatogenesis; endocrine disruptor; puberty; sulfotransferase.

Parabens are esters of parahydroxybenzoic acid (PHBA), which have long been in widespread use as preservatives in drugs, foodstuffs, and cosmetics. Combinations of parabens afford a broad antimicrobial spectrum (Charnock and Finsrud, 2007). A combination of methylparaben (0.015%–0.2%) and propylparaben (PP) (0.02%–0.06%) is commonly used in oral pharmaceutical formulations (Rowe et al., 2006). Parabens used to be considered as safe but, in the late 1990s, concerns were raised about possible effects on endocrine function, based on in vitro data and in vivo uterotrophic tests. Parabens were shown to bind to estrogen receptors (ERs) in vitro with variable but low affinity, 10- to 2500-fold lower than that of the natural ligand estradiol-17β. Affinity increased with paraben chain length (Routledge et al., 1998). In vitro transactivation and cellular proliferation were also demonstrated (Blair et al., 2000; Byford et al., 2002; Okubo et al., 2001; Vo and Jeung, 2009).

The metabolite PHBA bound ERs weakly or not at all in vitro (Routledge et al., 1998). Various parabens were investigated in uterotrophic tests on immature or ovariecetomized female rodents with variable results. Effects were observed with all parabens with the most variable results for methylparaben (ie, the paraben with the shortest chain). For PHBA, results were inconsistent (Darbre et al., 2002; Hossaini et al., 2000; Kodà et al., 2005; Lemini et al., 1997, 2003, 2004; Routledge et al., 1998; Vo and Jeung, 2009, reviewed by Boberg et al., 2010).

Taken together, data suggested that parabens may disrupt endocrine function with this activity decreasing with chain length and barely detectable with the metabolite PHBA. The question is as to the relevance of these in vitro results and the data from uterotrophic tests in the context of prepubertal male genital tract development. In vivo studies in juvenile male rats showed some effects on the genital tract, namely decreased spermazoon production with PP and butylparaben (but not with methyl- or ethylparaben) (Oishi, 2001, 2002a,b, 2004). This is consistent with previously described in vitro data showing increasing binding and activation of ERs as the paraben carbon chain lengths so propyl- and butylparaben would be more potent. However, the mechanism is uncharacterized, and a direct spermicidal effect could not be ruled out (Song et al., 1989; Tavares et al., 2009).

In the Oishi studies, exposure of prepubertal male rats to propyl- or butylparaben in the diet from weaning to adulthood at nominal dose levels of 10, 100, or 1000 mg/kg/day inhibited spermazoon production after puberty. Testosterone levels were significantly decreased at the highest dosage, but because
no data were available for LH or FSH, no conclusion could be drawn about whether the decrease in testosterone level was due to a central or a peripheral effect. Although direct testicular toxicity could not be ruled out, endocrine disruption was suspected to be the primary cause in the light of previously described in vitro and in vivo data. In these studies, no data were presented on sperm recovery or plasma exposure, and the maximum nontoxic dose was not determined, so comparison with human exposure, if any, levels is impossible. No data in any other animal species are available. In 2008, a new study with butylparaben in a larger sample of male juveniles failed to confirm the Oishi data on sperm production although the same strain and protocol were used (Hoiberg et al., 2008), thereby casting doubt on the capacity of butylparaben to induce effects in juvenile males.

This study was undertaken to resolve the conflicting published evidence on the in vivo effects of PP on the male reproductive tract in juvenile rats and to investigate other parameters, including low-dose exposure, recovery, exposure, and hormone analysis. The protocol was based on those of the published studies designed to investigate the effects of parabens on the male reproductive system with male rats exposed to comparable oral doses from weaning to adulthood. Additional endpoints were included to characterize other possible effects. This study was conducted in compliance with OECD Good Laboratory Practices (GLP) guidelines.

MATERIALS AND METHODS

PP. PP (propyl 4-hydroxybenzoate, CAS number 94-13-3) was supplied as Nipasol (100% pure from Clariant, Trosly-Breuil, France). It was suspended in 1% hydroxyethylcellulose 80–125 centipoises (Sigma-Aldrich) at 2% in water for injection at concentrations of 0.3, 1, 10, and 100 mg/ml, corresponding to dose levels of 3, 10, 100, and 1000 mg/kg/day when given to animals at a dosing volume of 10 ml/kg. Concentrations were determined by HPLC-UV (linearity established in the range 20–200 μg/ml). Homogeneity of the formulations was demonstrated with deviations within the acceptable limits of ± 15% of the nominal value for samples taken from the top, middle, or bottom of the preparation. Formulations were stable for 24 h at room temperature or 15 days at +2–8°C (except 100 mg/ml: 9 days at +2–8°C). Consequently, suspensions were prepared on a weekly basis and kept refrigerated. pH readings were between 6 and 7. On the first day of administration—1 of the 2 days of pharmacokinetic sampling—routine analysis showed that the first preparations were between 6 and 7. On the first day of administration—1 of the 2 days of the preparation. Formulations were stable for 24 h at room temperature or 15 days at +2–8°C (except 100 mg/ml: 9 days at +2–8°C). Consequently, suspensions were prepared on a weekly basis and kept refrigerated. pH readings were between 6 and 7. On the first day of administration—1 of the 2 days of pharmacokinetic sampling—routine analysis showed that the first preparations were between 6 and 7.

Experimental design. Wistar rats: Crl:WI (Han) were obtained from Charles River Laboratories France (Domaine des Oncins, 92910 Saint Germain sur l’Arbresle, France). At least 36 lactating female rats, each with a constituted litter of at least 5 male pups, arrived on PND14. In order to start dosing on PND21, the pups were weaned at PND20 and randomly allocated to groups using software that took litter origin into account (to avoid more than 2 siblings in the same group). One hundred males were allocated to the main study groups and 77 males to the toxicokinetics satellite groups. The allocated males were housed in groups of 2 (main study animals) or 2 or 3 (satellite animals for toxicokinetics) in disposable plastic cages with autoclaved sawdust and an isocap filter system. Cages and bedding were replaced at least once weekly. Rat pellets (diet reference A04C-10—Aston Pharma) and softened, filtered (0.2 μm) drinking water were provided ad libitum. Treatment was begun on day P21 and continued for 8 weeks. Animals were allocated to 5 dosage groups, which were given 0 (group 1, control, vehicle alone) and 3, 10, 100, or 1000 mg/kg/day (groups 2–5) PP by gavage at a dose volume of 10 ml/kg. Each group consisted of 20 rats divided into subgroups of 10 animals: subgroup 1 animals were necropsied at the end of the 8-week treatment period and subgroup 2 animals were necropsied after a 26-week washout period (covering 3 spermatogenic cycles). Satellite toxicokinetics groups had 17 animals per group (9 animals in the control group). Special measures (including staff training) were implemented to prevent contamination by parabens from products such as cleaning fluids, shampoos, moisturizers, and topical pharmaceuticals.

Observations and hormone analysis. Animals were examined for clinical signs and weighed: on a daily basis prior to dosing (PND21), twice weekly during the 8-week treatment period, and then weekly during the washout period (subgroup 2 only). All animals were examined from PND38 to determine the day of balanopreputial separation (BPS). After 8 weeks of treatment, blood samples were taken from all animals (except those in the satellite groups) for hormone analysis at a set time in the morning. Blood samples of approximately 2 ml were withdrawn from a retro-orbital sinus under isoflurane anesthesia (AErrane, Baxter) from animals fasted for at least 14 h. Samples were analyzed for testosterone (kit ref. testosterone RIA RK-61M, Institut des Isotopes Ltd, Budapest, Hungary; Sensitivity, 0.2 nmol/l). LH (kit ref. rat LH RIA AHR002, IDS, Liège, Belgium; Sensitivity, 0.14 ng/ml), and FSH (kit ref. rat FSH IRMA, IDS, Liège, Belgium; Sensitivity, 0.2 ng/ml). Radioactivity was measured using a Packard RIA Star solid scintillation counter.

Tissue collection. On the day after the last dose (subgroup 1) or after the end of the washout period (subgroup 2), animals were weighed, euthanized by carbon dioxide inhalation, and given a macroscopic pathological examination. Any gross lesions were recorded, and the tissue was preserved in 10% neutral formalin. Testes and epididymides were weighed separately, and the seminal vesicles and prostate were weighed together (to avoid ligation of the seminal vesicles before weighing). Organs were weighed after dissection of fat or other contiguous tissues. The right testis, right epididymis, and left caput epididymis of all males were fixed in modified Davidson’s fluid. Histopathological examinations of the right testis and epididymis (caput, corpus, and cauda) were performed on all animals except satellite animals. The epididymis was stained with hematoxylin and eosin. From a single paraffin block of the testis, 2 transversal sections were taken at 2 separate levels, and for each level, 1 slide was stained with hematoxylin and eosin and a second was stained with Periodic acid–Schiff (in order to perform qualitative testicular staging).

Testicular spermatid count. The testis was removed immediately after sacrifice and then trimmed and weighed. The albuginea was carefully removed and the testis homogenized in 25 ml of distilled water. From this mix, a sample of 100 μl was added to 100 μl of distilled water in a HTR IDENT tube (which selectively stains condensed spermatid chromatin). A sample of 8 μl of the mixture was then deposited on a standard count Leja slide and counted in a Hamilton Thorne Research (HTR) IVOS version 12.1M computer-assisted sperm analyzer. Fields were counted to include at least 100 spermatids or a
maximum of 5 fields. The spermatozoon count per gram of testis (in millions per gram of testis) was calculated as follow: \((D \times H \times N) / (F \times V \times W)\), where \(D\) is the dilution factor with the nuclear stain (factor 2), \(H\) is the volume of homogenization \((25 \text{ cm}^3)\), \(N\) is the number of spermatozoa counted, \(F\) is the number of fields analyzed (5 fields), \(V\) is the volume of 1 analysis field \((3.825 - \text{10–6} \text{ cm}^3)\), and \(W\) is the testis weight \((g)\).

**Epidydimal sperm analysis.** Immediately after sacrifice, the cauda epididymis was removed and immersed in 5 ml of 199 medium with 250 \(\mu\)l of 1% bovine serum albumin at 37°C for 8–30 min (the analysis was performed within 30 min. This allowed the spermatozoa to diffuse into the medium. A Leja slide with a 100 \(\mu\)m cavity was then filled by capillary action and counted in a Hamilton Thorne Research (HTR) IVOS version 12.1M computer-assisted sperm analyzer. Five appropriate fields (excluding those containing debris) were selected for analysis by the system. Enough fields were counted to include at least 200 spermatozoa. Progressive velocity (VSL, \(\mu\)m/s), track speed (VCL, \(\mu\)m/s), and path velocity (VAP, \(\mu\)m/s) were recorded for measurement of sperm motility and amplitude of lateral head displacement (ALH, \(\mu\)m). Linearity (LIN = VSL/VCL) and straightness (STR = VSL/VAP) were derived from the recorded parameters as percentages.

**Bioanalytical and toxicokinetics.** Liquid chromatography–mass spectrometry (LC-MS/MS) over the calibration range of 20–200 ng/ml and 200–5000 ng/ml was validated (LLOQ, 20 ng/ml). The Mass spectrometer API4000 in negative Electrospray (ESI) mode monitored ions at \(m/z\) 179.1–92 for the Propylparaben. Sulfate PP of mass \(m/z\) 259.4 was observed in samples of PP-treated animals in proportion to dose, so this species was assimilated to metabolized PP. Blood samples were incubated with a sulfatase enzyme (Sulfatase from Helix pomatia, Sigma-Aldrich, ref. S9626) to release PP from this sulfate metabolite. Enzymatic cleavage was qualitatively monitored by LC-MS. Toxikokinetics results were expressed as free PP and total PP (free + sulfate metabolite).

Blood samples were taken from satellite animals treated with PP on the first and last days of dosing (PND21 and PND77, respectively) at 0.25, 0.5, 1, 2, 4, 8 (3 animals/time point), and 24 h (2 animals/time point) after dosing. Control animals were sampled only at the 0.25-, 0.5-, and 1-h time points. Blood samples of approximately 0.4–1 ml were taken from a retro-orbital sinus under isoflurane anesthesia and immediately placed on ice. Animals were sacrificed after the last time point.

**Statistical analysis.** For body and organ weights, statistical analyses were performed by the Provantis data acquisition system as follows: The best transformation for the data (none, log, or rank) was determined depending upon its kurtosis, the probability of Bartlett’s test for homogeneity of variances, and similarity of the group sizes. The homogeneity of means was assessed by ANOVA. Data were then analyzed to test for a dose-related trend to detect the lowest dose at which there was a significant effect based on the Williams test for parametric data or Shirley’s test for nonparametric data. If no trend was found and the means were not homogeneous, the data were analyzed by a stepwise parametric or nonparametric Dunnett’s test to look for significant differences vis-a-vis the control group (\(\alpha < 5\%\)). Hormone analysis, sperm analysis, and sexual maturation data were analyzed using a SAS software package. Levene’s test was used to test the equality of variance across groups, and Shapiro-Wilk’s test was used to assess the normality of the data distribution in each group. Data with homogeneous variances and normal distribution in all groups were analyzed using ANOVA followed by Dunnett’s test. Data showing nonhomogeneous variances or a nonnormal distribution in at least 1 group were analyzed using Kruskal-Wallis test followed by Wilcoxon’s rank sum test. Outliers were tested using a Dixon test.

**RESULTS**

**Pharmacokinetics**

In the preliminary pharmacokinetic study performed in 31-day-old Wistar rats, PP was rapidly absorbed following oral administration with \(T_{\text{max}}\) reached between 5 and 30 min after dosing (Fig. 1). Plasma PP concentrations were generally quantifiable up to 4 or 8 h after dosing. The increase in systemic exposure between 3 and 1000 mg/kg was less than dose proportional. Volume of distribution (Vd/F) readings were 4.8 l/kg at 10 mg/kg and 9.4 l/kg and 100 mg/kg. PP was eliminated quite rapidly following oral administration with respective half-lives of 47 and 58 min at 10 and 100 mg/kg. The clearance (Cl/F) values were 4.20 and 6.68 (l/h)/kg at 10 and 100 mg/kg, respectively.

In the juvenile toxicity study, no PP was detected in plasma from the control group. Total PP exposures were significantly lower on PND77 (dosing day 56) compared with PND21 (dosing day 1) (Fig. 2): for example, the total PP AUC \(0-24 h\) was 148 840 ng·h/ml at 1000 mg/kg/day on PND21 and decreased to 47 760 ng·h/ml by PND77 (Table 1). The highest value for total PP, expressed as AUC \(0-24 h\), was 243 348 ng·h/ml (PND21, 1000 mg/kg). Free plasma PP concentrations were low and quantifiable only at 100 mg/kg for up to 8 h with decreased exposure by PND77. The highest value for free PP \(C_{\text{max}}\) was 1727 ng/ml (PND21; 1000 mg/kg; \(T_{\text{max}} = 15\) min).

**Clinical Signs and Sexual Maturation**

Hypersalivation (and in some cases abnormal foraging) was observed in the high dose animals through the end of the treatment period. There were no other treatment-related clinical signs. There was no effect on mean body weight gain in any group, with mean weights of 199 g at onset of puberty, 343 g at the end of treatment, and 528 g at the end of recovery (Fig. 3).

No effect was observed on sexual maturation. Mean days of BPS were 44 ± 1 for control and 44 ± 2, 44 ± 2, 43 ± 2, and 43 ± 2 in the 3, 10, 100, and 1000 mg/kg/day groups, respectively (Fig. 4). The slight earliness (PND43) in the 100 and 1000 mg/kg/
kg dose groups was not statistically significant. Similar variability was documented in the historical control data of the testing facility (from mean day 43 ± 2 to mean day 47 ± 2 in 6 postweaning development studies in Wistar:Crl-WI rats, n = 25–32 per study) and in the literature (Lewis et al., 2002). Thus, the earlier BPS observed in the higher dose groups was not considered to be related to the treatment.

Organ Weights and Histopathology

No effect was observed on the weight of the reproductive organs (epididymis, prostate and seminal vesicle, and testis) at the end of treatment (Fig. 5a), except in 1 animal in 10 given 10 mg/kg/day that sporadically presented severe diffuse tubular atrophy in the testis (animal no. 49). Absolute weight of the left testis was 1.12 g compared with a mean of 1.78 g in the mid-low-dose group and 1.81 g in the control group (Table 3). At the end of the recovery period (rats aged 37 weeks), minimal focal or multifocal tubular atrophy/hypoplasia was recorded in the testis of 3 animals on 10 given 3 mg/kg/day, not correlated to any difference in the mean testis weight compared with control group (Fig. 5b). One animal in 10 in the 1000 mg/kg/day dose group presented severe testicular atrophy/hypoplasia with a left testis weighing 0.68 g and a right testis weighing 0.70 g compared with mean of 1.93 g in the control group.

### TABLE 1

<table>
<thead>
<tr>
<th>Dose (mg/kg/day)</th>
<th>C_max (ng/ml)</th>
<th>T_max (min)</th>
<th>t (h)</th>
<th>AUC_0–8 h (ng·h/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>First day of treatment (PND21)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>786</td>
<td>15</td>
<td>1</td>
<td>408</td>
</tr>
<tr>
<td>1000</td>
<td>25 003</td>
<td>30</td>
<td>8</td>
<td>148 840</td>
</tr>
<tr>
<td>Last days of treatment (PND77–78)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>500</td>
<td>15</td>
<td>4</td>
<td>538</td>
</tr>
<tr>
<td>1000</td>
<td>12 030</td>
<td>15</td>
<td>4</td>
<td>47 760</td>
</tr>
</tbody>
</table>

**Note:** t, time of the last quantifiable plasma concentration; AUC_0–8 h, area under the plasma concentration-time curve from 0 to 8 h after administration; C_max, maximum plasma concentration observed after dosing; T_max, time to reach C_max.

FIG. 2. Plasma PP exposure at the beginning and end of the treatment period. Blood sampling was performed following the first (on PND21) and the last (on PND77) oral administrations of PP. Time points were 15 min (n = 3), 30 min (n = 3), 1 h (n = 3), 4 h (n = 3), 8 h (n = 3), and 24 h (n = 2) post dosing. Exposure is quantified as AUC_0–8 h (ng·h/ml). Nominal dose levels were adjusted to actual dose levels based on the results of the formulation analysis for achieved concentration: on PND21 (dosing day 1), the nominal dose of 100 mg/kg was adjusted to 47 mg/kg for the mid-high-dose group. This preparation did not meet the acceptance criteria. Accordingly, a new preparation was formulated for the remaining treatment days. On PND77 (dosing day 56), an adjusted dose of 7.8 mg/kg was used instead of the nominal dose of 10 mg/kg. On PND21, no AUC_0–8 h could be calculated for the 10 mg/kg dose group as individual concentrations for the 30 min and 1 h time point were outside the validated range and thus considered BLQ. Abbreviations: AUC, area under the curve; PND, postnatal day; PP, propylparaben.
GAZIN, MARSDEN, AND MARGUERITE

In 100 or 1000 mg/kg/day animals, testis weights were slightly but significantly higher, only for the right testis, at the end of the recovery period with no dose relationship (Fig. 5b). As it could be assumed that the animal presenting severe testicular atrophy in the 1000 mg/kg/day group could hide a possible relationship between dose and an increase of testis weight, value for this animal was deleted and the remaining data were reanalyzed. This did not reveal any increase in testis weight in the 1000 mg/kg/day dose group compared with the 100 mg/kg/day dose group, suggesting the absence of any dose relationship. A slightly but significantly higher epididymis weight was also recorded at the end of the recovery period, only for the left epididymis, in all the treated groups compared with the control group. No dose relationship was shown. Taking these results together coupled with the lack of either histological correlates or effects at the end of dosing, lack of consistency between left/right organs, differences in testis, or epididymis weight at the end of the recovery period, effects were considered to correspond to normal variability. No consistent histopathological findings of note were observed.

**Sperm Count and Motility**

PP did not affect mean testicular spermatid counts (Fig. 6) nor epididymal sperm counts or mean motility parameters (Table 2) in any group at the end of either treatment or recovery. There was a drop in testicular sperm count between the end of treatment (77 days) and the end of recovery (258 days), but age probably accounts for this difference as this was observed in all groups, including the control group. More specifically, 1 animal given 10 mg/kg/day, sacrificed at the end of treatment period, and another given 1000 mg/kg/day, sacrificed at the end of the recovery period, had extremely low sperm counts, and their sperm motility parameters were recorded as zero. These changes were associated with severe testicular atrophy/hypoplasia or hypospermatogenesis (above mentioned animals no. 49 and no. 99—Table 3). These isolated findings were not considered as related to treatment, based on sporadic occurrence. At the end of the recovery period, the mean testicular spermatid count was slightly lower in the 1000 mg/kg/day group compared with the concurrent control (95 ± 38 vs 110 ± 13 million per gram of testis, n = 10) (Fig. 6), but the difference is not statistically significant. There was no histological correlate in the male reproductive organs (apart from the animal mentioned above with incidental testicular lesions). In addition, a semi-quantitative count was carried out on the epididymal sperm used for the motility evaluation. Although the mean value was slightly lower in the 1000 mg/kg/day recovery group compared with control (641 ± 254 vs 806 ± 229, n = 10) (Table 2), the ranges of individual values were similar in the control and treated groups.

**Hormone Analysis**

PP had no effect on hormone levels (LH, FSH, and testosterone) at the end of the treatment period (Table 2). One animal in each of the control and 1000 mg/kg groups sacrificed at the end of the washout period presented outlier testosterone values (140.4 and 96.3 nmol/l, respectively) compared with mean group values of 23.0 and 22.8 nmol/l (Table 3). These animals were sacrificed at the end of the recovery period, and no histopathological evidence—or any other sign—of any effect of treatment was detected.

**DISCUSSION**

Repeated daily oral administration of PP to juvenile rats starting on PND21 did not induce any effect on body weight or general health. There was no unscheduled death. Hypersalivation was noted occasionally in the high-dose males throughout the treatment period. This effect was also reported at high doses.
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with methyl paraben in male rats (Anonymous, 2005). No other treatment-related clinical signs were observed. Treatment had no impact on the age of sexual maturation of the males: BPS, an endpoint for sexual maturation, is an androgen-dependent developmental landmark that could be affected by endocrine disruptors. For example, a delay of 5 days in BPS could be observed with an ER modulator such as raloxifene (Pinilla et al., 2001). In our study, no significant effect was observed on BPS. The only findings of note were minimal or severe tubular atrophy/hypoplasia in the testis in 5 animals associated with aspermia in 2 cases. Given the distribution of these cases in the groups and the absence of any effect on spermatogenesis, these were considered to be spontaneous in origin and not related to treatment with PP. The only statistically significant finding was an increase in testis weight at the end of the recovery period. There was no evidence of any treatment-related effect on sperm motility in any of the treated groups. Similarly, treatment did not affect levels of the measured hormones (LH, FSH, and testosterone). Plasmatic exposure to PP was demonstrated in PP-treated animals. The increase in exposure to PP was markedly less than dose proportional between 3 and 1000 mg/kg/day. PP was mainly sulfoconjugated, and only very low levels of free plasma PP were measured. It is not known whether sulfoconjugated PP can bind and activate ER, and it is possible that sulfoconjugated PP might get converted to free PP in tissues. This type of process occurs with catecholamines (Kauffman, 2004) and estradiol, which circulates as estradiol sulfate and is locally converted to active estradiol by tissue sulfotransferases (SULT) (Harris et al., 2005). Furthermore, it has been shown that parabens inhibit SULT activity in liver cytosol extracts and that SULT inhibition increased with increasing paraben ester chain length (Prusakiewicz et al., 2007). Above and beyond concerns about endocrine disruption, this may indicate that parabens interact with SULTs and affect sulfonation, as observed in our study. However, this requires further investigation as the mechanism of inhibition has not been determined.

The results of our study indicate that, after oral administration by gavage to juvenile rats, PP was absorbed relatively
TABLE 2
Epididymal Sperm Count, Motility, and Plasma Hormone Levels at the End of Treatment

<table>
<thead>
<tr>
<th>Dose Level (mg/kg/day)</th>
<th>Epididymal sperm count</th>
<th>Epididymal sperm motility</th>
<th>Plasma hormone levels</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number of sperm</td>
<td>Motile sperm ratio (%)</td>
<td>Testosterone (nmol/l)</td>
</tr>
<tr>
<td>0</td>
<td>428±202</td>
<td>81.1±12.5</td>
<td>16.9±13.9</td>
</tr>
<tr>
<td>3</td>
<td>501±204</td>
<td>88.2±5.4</td>
<td>17.6±10.5</td>
</tr>
<tr>
<td>10</td>
<td>449±271</td>
<td>71.4±28.8</td>
<td>21.2±11.9</td>
</tr>
<tr>
<td>100</td>
<td>473±212</td>
<td>85.5±9.5</td>
<td>22.9±14.0</td>
</tr>
<tr>
<td>1000</td>
<td>547±198</td>
<td>85.8±9.6</td>
<td>18.9±11.1</td>
</tr>
</tbody>
</table>

Note: Epididymal sperm count and motility were investigated at the end of the treatment period. Results are presented as arithmetic mean ± standard deviation (n = 10). Motile sperm ratio, ratio to counted sperm; VAP, average path velocity; VSL, straight line velocity; VCL, curvilinear velocity; ALH, amplitude of lateral head displacement; STR, straightness = VSL/VAP × 100; LIN, linearity = VSL/VCL × 100. Plasma hormone levels, results are presented as arithmetic mean ± standard deviation (n = 20). Two outlier testosterone values (140.4 and 96.3 nmol/l) in the control and 1000 mg/kg group, respectively) were removed with α risk 1% using Dixon test. LOQ testosterone = 0.2 nmol/l; LLOQ FSH = 0.2 ng/ml; LLOQ LH = 0.14 ng/ml.

FIG. 6. Testicular spermatid count. Two outlier values were removed following a Dixon test (n = 10). These included a value of 7 million/g of testis in the 10 mg/kg group at the end of the treatment (α < .05) and value of 0 in the 1000 mg/kg group at the end of the recovery period (α < .01). These values corresponded with 2 animals presenting severe but incidental testicular atrophy/hypoplasia (animals no. 49 and no. 99).

generally for up to 4 or 8 h after dosing. PP was eliminated from the plasma with a half-life of about 1 h. PP distribution volume reached 9.4 l/kg, which is substantially greater than the volume of blood in a rat (about 0.7 l/kg) (Lee and Blaufox, 1985), suggesting that it is sequestered in the tissues, extensively metabolized or rapidly cleared via the kidneys. Paraben metabolism has been shown to be largely driven by carboxylesterases that generate PHBA as the main metabolite. Excretion—predominantly in the urine—is rapid with more than 90% of the dose excreted within 24 h of administration (Aubert et al., 2012; Jones et al., 1956; Kiwada et al., 1980; Tsukamoto and Terada 1964). In our study, the decrease in plasma levels with time could be explained by maturation of the carboxylesterase system during the exposure period during which juvenile rats grew into adulthood (De Zwart et al., 2008; Karanth and Pope, 2000). It is difficult to relate these findings to paraben pharmacokinetics in humans about which little is known. In 1 study, paraben levels were measured in the urine, serum, and seminal plasma of 60 healthy Danish men (Frederiksen et al., 2011). Methylparaben and PP were the most frequently measurable, and the highest concentrations were found in urine. These parabens in the urine were conjugated so sample contamination could be ruled out as conjugation is proof of an internal metabolic process (Meeker et al., 2011). Another study detected parabens in tumor biopsies, but it was possible that the samples had been contaminated with laboratory and personal care products (Darbre et al., 2004).

The major published multiple-dose studies on parabens in rats (Hoberman et al., 2008; Oishi, 2001, 2002a,b) are controversial. For butylparaben, they provide divergent critical
### TABLE 3

**Individual Noteworthy Findings and Mean Data in Corresponding Groups**

<table>
<thead>
<tr>
<th>Dose Level, mg/kg/day</th>
<th>Testos. (SD)</th>
<th>Testis W. (SD)</th>
<th>Million sperm/g testis (SD)</th>
<th>Histopathology</th>
<th>End of Treatment</th>
<th>End of Recovery</th>
<th>Histopathology</th>
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<tr>
<td>0 animal no. 15</td>
<td>140.4 NP NP NP</td>
<td>Testosterone: Severe tubular atrophy/hypoplasia of the testis. Tubules lined by Sertoli cells only, no germ cells to be observed. Minimal diffuse Leydig cell hypertrophy/hyperplasia. Small epididymides: Atrophy and asperma in the epididymides.</td>
<td>1.83 101.5</td>
<td>Tests (right): Lymphoid cell infiltration; interstitial; perivascular; focal; minimal.</td>
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<tr>
<td>10 animal no. 49</td>
<td>8.7 1.12 7.0 NP NP NP</td>
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<tr>
<td>1000 animal no. 91</td>
<td>96.3 NP NP NP</td>
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<tr>
<td>1000 animal no. 99</td>
<td>19.9 NP NP NP</td>
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<td>Mean values</td>
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<tr>
<td>0</td>
<td>16.9 (13.9) 1.81 (0.17) 153.9 (24.7)</td>
<td>Testis weight and sperm count: 3/10 animals (including animal no. 15).</td>
<td>1.93 (0.15) 110.3 (13.4)</td>
<td>No noteworthy findings. Historical background data (Ricerca testing facilities—8 studies—10- to 12-week-old Wistar rats) show an incidence of 3/63 animals with minimal or slight tubular atrophy (1 animal in each of 3 out of 8 studies).</td>
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<tr>
<td>3</td>
<td>17.6 (10.5) 1.78 (0.11) 144.4 (34.8)</td>
<td>Minimal focal or multifocal tubular atrophy/hypoplasia was recorded in the testis of 3/10 animals.</td>
<td>2.03 (0.22) 103.1 (20.8)</td>
<td>No noteworthy findings.</td>
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<tr>
<td>10</td>
<td>21.2 (11.9) 1.76 (0.32) 145.2 (25.3)</td>
<td>No noteworthy findings (except animal no. 49).</td>
<td>1.96 (0.24) 106.0 (17.9)</td>
<td>No noteworthy findings.</td>
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<tr>
<td>100</td>
<td>22.9 (14.0) 1.74 (0.18) 151.3 (26.4)</td>
<td>No noteworthy findings.</td>
<td>2.11 (0.22) 111.2 (19.4)</td>
<td>No noteworthy findings.</td>
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<tr>
<td>1000</td>
<td>18.9 (11.1) 1.77 (0.14) 162.3 (30.9)</td>
<td>Minimal focal or multifocal tubular atrophy/hypoplasia was recorded in the testis of 1/10 animals.</td>
<td>2.14 (0.09) 105.4 (19.1)</td>
<td>No noteworthy findings (except animal no. 99).</td>
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</table>

**Note:** Testos., testosterone value; Testis W., testis weight (left testis), g; NP, not performed. Testosterone: n = 20. Testis weight and sperm count: n = 10. Noteworthy individual findings are in bold character. Several data were excluded from mean calculation as concluded outliers following Dixon test: 1 euthanized at the end of the recovery period; 2 euthanized at the end of the treatment; 3 animal no. 15 excluded for testosterone mean; 4 animal no. 49 excluded for mean sperm count; 5 animal no. 91 excluded for testosterone mean; 6 animal no. 99 excluded for mean relative testis weight and mean sperm count.
effect levels ranging from a LOAEL of 10 mg/kg bw/day to a NOAEL of 1000 mg/kg bw/day, respectively. For PP, our study did not confirm results reported in the Oishi (2002b) study. Indeed, Oishi’s results indicated that daily sperm production assessed on frozen tissues (epididymis and testis) was significantly decreased at all dose levels after oral PP exposure from weaning to adulthood. Otherwise no treatment-related effects on the weights of the testes, epididymides, prostate, seminal vesicles, and preputial gland were observed. The serum testosterone concentration decreased in a dose-dependent manner with statistical significance attained at the highest dose. Certain differences in experimental conditions might account for these inconsistencies. Our protocol design was expanded to characterize changes and included a low dose of 3 mg/kg/day to try to establish a NOAEL. The high dose level of 1000 mg/kg/day was maintained in order to induce a toxic effect and evaluate recovery. Gavage rather than dietary administration was chosen to ensure controlled exposure and mimic exposure to PP through oral pharmaceuticals. Plasma exposure was determined using specific satellite groups, and the exposure duration was extended from 4 to 8 weeks. Previous studies were conducted with an 8-week exposure period starting on PND21 (Hoferman et al., 2008; Oishi, 2001, 2004) apart from PP that was only administered for 4 weeks (Oishi, 2002b). We assessed sexual maturation (BPS), performed testicular spermatid counts and epididymal sperm analysis on fresh samples using a computer-assisted sperm analyzer, and included histopathological analysis. Although only plasma testosterone was measured by Oishi, we also measured LH and FSH. At the initiation of treatment (weaning—PND21), the testis had descended (PND19 in rats compared with gestation day 245 in 91% of humans). The treatment period covered the male rat’s juvenile (PND21–35), peripubertal (PND35–55), pubertal (PND55–70), and early adult stages, covering postweaning androgen secretion, prepubertal testosterone surges, the start of spermatogenesis, and postpubertal stabilization of sperm production (Klonisch et al., 2004; Marty et al., 2003) in order to ensure a sensitive assessment of effects on male reproductive development. Larger numbers of animals were studied to provide better statistical power (up to 20 animals per group compared with just 8 in the Oishi study). Additional animals were added in order to evaluate recovery over a 26-week washout period (covering 3 spermatogenic cycles, 10 animals per group). In addition, toxicokinetics groups were included to assess plasma levels of PP.

In conclusion, although exposure to PP was observed following gavage administration to rats, there was no evidence of any effect on male rat reproductive organs. This new toxicity study conducted according to GLP in an appropriate and statistically robust manner failed to reproduce the effects on reproductive function observed by Oishi (2002b). The dose of 1000 mg/kg/day was the NOAEL, corresponding to a maximum plasma concentration of 12 030 ng/ml and an exposure of 47 760 ng·h/ml (AUC_{0→t}) at the end of the treatment.

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REFERENCES


