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Research Section

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Abstract—*n*-Butyl benzyl phthalate (BBP), a plasticizer used in polyvinylchloride (PVC) and other polymers, has been orally administered to female Wistar rats with four doses (150, 475, 780 and 1500 mg/kg body weight/day) for 3 consecutive days. Metabolites recovered in urines were analysed by gas chromatography–mass spectrometry (GC–MS) after 24, 48 and 72 hours. Six metabolites were identified. Mono-*n*-butyl phthalate (MBuP) and mono-*n*-benzyl phthalate (MBeP) represented respectively 29–34% and 7–12 % of the total recovered metabolites. Hippuric acid, the main metabolite of benzoic acid, represented the second major metabolite (51–56%). Phthalic acid, benzoic acid and an ω-oxidized metabolite of MBuP were also recovered in urine but in small quantities. BBP was never identified in urines. Total urinary metabolites recovery represented 56% of the dose administered in the first 24 hours. However, total recovery decreased when the dose increases (43% at 780 mg/kg body weight/day, only 30% at 1500 mg/kg body weight/day). Whatever the time was, BBP metabolites recovered in urines were all present and in the same proportions for the two lowest doses. Discrepancy in metabolites quantities expressed as percentages of the dose observed in urine of rat treated with the highest BBP dose disappeared with time as MBuP, MBeP and hippuric acid recovery has significantly increased at day 3. Metabolic profile of BBP in female rats has been established. The aim of the present study is to identify further the active(s) agent(s) involved in the BBP malformations and teratogenic effects.

Keywords: *n*-butyl benzyl phthalate; metabolism; metabolites; female rat.

Abbreviations: BBP = *n*-butyl benzyl phthalate; CI = chemical ionization; DBP = di-*n*-butyl phthalate; DEHP = di(2-ethylhexyl) phthalate; EI = electronic impact; GC = gas chromatography; GC–MS = -coupled gas chromatography–mass spectrometry; IS = internal standard; MBeP = mono-*n*-benzyl phthalate; MBuP = mono-*n*-butyl phthalate; MBuP ω-ox = mono-*n*-butyl phthalate ω-oxidized; 4-MHP = 4-methylhexyl phthalate; MW = molecular weight; m/z = mass/charge ratio; RT = retention time.

INTRODUCTION

n-Butyl benzyl phthalate (BBP) is a phthalic acid diester used extensively as plasticizer in the production of vinyl flooring, synthetic leather and adhesives, and is also a component of materials used in contact with food products (PVC food-wrap film, adhesives and coating for paperboard products used in food packaging; IARC, 1982). In the Fisher 344 male rat, BBP reduced accessory sex organ weight and caused generalized tissue degeneration atrophy (Agarwal *et al*., 1985). BBP is also developmentally toxic in rats (Ema *et al*., 1992a, 1993, 1995a; Field *et al*., 1989) and mice (Price *et al*., 1990). In previous studies, complete resorption of all implanted embryos was found in all pregnant rats given dietary BBP at 2% during the whole period (Ema *et al*., 1990) and the first half (Ema *et al*., 1992b) of pregnancy. Significantly increased incidences of foetuses with deformity of the cervical and thoracic vertebrae were found in pregnant rats given BBP on days 7–9, and significantly increased incidences of foetuses with cleft palate and fusion of the sternebrae were observed in pregnant rats given BBP on days 13–15 (Ema *et al*., 1993). Mono-*n*-butyl phthalate (MBuP), the major BBP metabolite identified

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in male rat urine (Eigenberg *et al.*, 1986) when administered throughout organogenesis produced maternal toxicity, increased incidence of foetuses with malformations and increased the number of resorptions and dead foetuses (Ema *et al.*, 1995b). Mono-*n*-benzyl phthalate (MBeP), the second BBP monoester, and/or its metabolites, are also teratogenic in Wistar rats (Ema *et al.*, 1996a,b). BBP metabolism has been studied by Eigenberg *et al.* (1986) in male F344 rats but we lack information about BBP metabolism in female rats. The aim of this study is to investigate the *in vivo* metabolism of this phthalate diester in the female rats in order to identify further the active agents involved in the malformations and teratogenic effects of BBP. Repeated oral doses of BBP were administered to female Wistar rats at levels of 150, 475, 780 and 1500 mg/kg body weight/ day for 3 consecutive days. Recoveries of BBP metabolites in female Wistar rat urines with time and dose of BBP are presented and metabolic map of BBP is suggested.

MATERIALS AND METHODS

Chemicals

n-Butyl benzyl phthalate (BBP) (purity >99%) was obtained from Monsanto Laboratory (Belgium). 4-Methylhexyl phthalate (4-MHP) (purity >95%) was synthetized (Dijon, France) in our laboratory (Paitry, 1995). For extractions of urinary metabolites, diethyl ether and methanol were purchased from Prolabo (Fontenay-sous-Bois, France) and for further experiment analyses (GC–MS) all solvents were utilized with a purity higher than 99%. *Helix pomatia* juice was purchased from IBF (Villeneuve-la-Garenne, France).

Animal experiments

Female Wistar rats (IOPS) weighing 180–200 g were purchased from Iffa-Credo (L'Arbresle, France) and were acclimatized for 3 days before use. All animals were allowed free access to standard diet and water at all time throughout the experiment with a 12-hr light/dark cycle. Animals were housed in metabolism cages (Nalgene), for the first day of treatment until the end of the study, allowing urine and faeces collection. BBP was dissolved in corn oil and administered (5 ml/kg body weight) by gavage, daily, for 3 consecutive days at doses of 150, 475, 780 and 1500 mg/kg body weight (five rats per dose). The control rats were treated only with the vehicle (corn oil). Rats were weighed every day throughout the experiment. Urine samples were collected 24 hr after each gavage. The urines were diluted to 20 ml and the pH adjusted to 2 with chlorhydric acid (1 N). Samples were stocked at –40°C.

Extraction of metabolites and derivatization

Urinary metabolites were extracted according to Daniel and Bratt method (1974) with minor modifications. Urinary metabolites were extracted four times with ether, in order to get the best extraction recovery. 100 μl internal standard (5 mg/ml): 4-methylhexyl phthalate (4-MHP) was added before extraction for quantification. Figure 1 illustrates the procedure used.

In order to investigate conjugated metabolites of BBP, two modes of hydrolysis were used: alkaline and enzymatic hydrolysis.

Alcaline hydrolysis breaks down ester bonds of conjugates. The pH of urinary aliquots (1 ml) was adjusted to 11 with NaOH 1 M and incubated at ambiant temperature for 2 hr. The reaction was stopped by 1 M HCl and the pH adjusted to about 2. Then, metabolites were extracted according to the Fig. 1.

Enzymatic hydrolysis was realised on conjugated metabolites with *Helix pomatia* juice, a mixture of purified enzymes: 100000 U β-glucuronidase/ml and 1000000 U sulfatase/ml. This reaction liberates glucuronides and sulfo-conjugates. Urinary aliquots (1 ml) were adjusted to pH 4.8 with sodium acetate buffer (1 M) and incubated at 45°C with 300 μl enzyme mixture for 48 hr. The enzymatic reaction was stopped with 1 M chlorhydric acid and the pH of the mixture urines enzyme was adjusted to 2. Then, extraction of metabolites was realised according to the Fig. 1.

In order to analyse the urinary extracts in gas chromatography (GC), the carboxylic functions of free metabolites were methylated by treatment with a solution of diazomethane in ether (Deboer and Baker, 1954).

Urinary metabolite profile

Metabolites were quantified, after derivatization, on a Chrompack CP9000 gas chromatograph equipped with a flame-ionization detector (hydrogen 30 ml/min, air 300 ml/min) and a split injector. The capillary column was a Chrompack OV 1701 (length 25 m, internal diameter 0.32 mm). Nitrogen was used as the carrier gas (80 kPa). Operating temperatures were: injection port 250°C, detection port 280°C, oven temperature program was from 90°C to 250°C at 4°C/min.

Metabolites were characterized by coupled gas chromatography–mass spectrometry (GC-MS) performed at Fournier Laboratories (Dijon, France). Mass spectra were obtained on a PSQ7000 Finnigan Mat instrument. Chemical ionization mass spectra were obtained using methane as reagent gas. Electron impact mass spectra were taken with helium as carrier gas at an ionization energy of 70 eV.

Quantitation of metabolites

4-MHP was used as internal standard (IS). 100 μl IS at 5 mg/ml was added before each extraction.

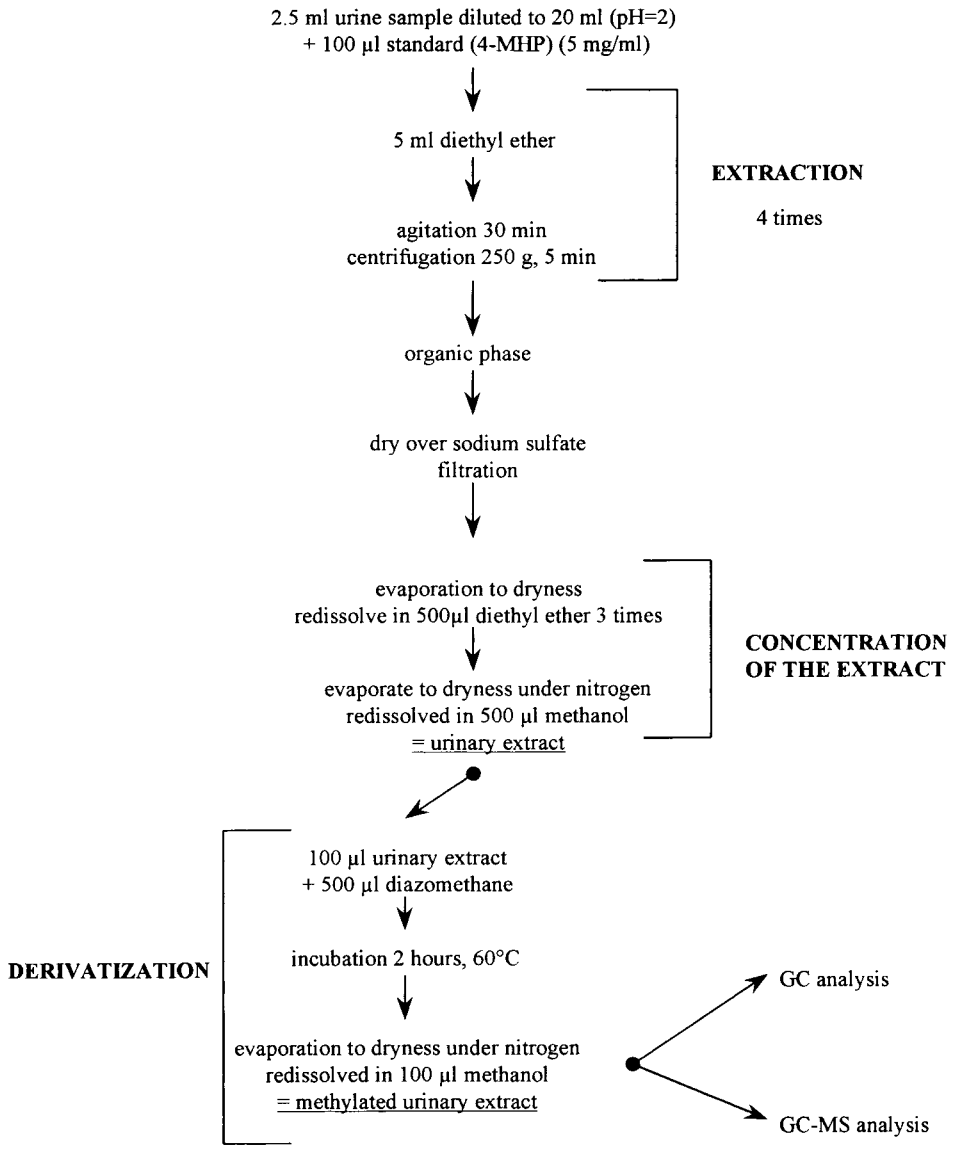

Fig. 1. Extraction and derivatization of BBP urinary metabolites.

The extraction recovery of each molecule was calculated with standards and was 100% for the 4-MHP, BBP and phthalic acid, 98% for the MBuP and MBeP and 95% for hippuric acid. The amount of each metabolite was determined by the peak area ratios of the metabolite and IS, reported to the quantity of the standard injected in GC. Detector response coefficient of each metabolite in comparison with IS was 1 for the hippuric acid, 0.85 for the BBP, 0.80 for the MBeP, MBuP and MBuP ω-oxidized and 0.68 for the phthalic acid. Then, the metabolite quantity excreted in urines can be determined.

Statistical analysis

All data are presented as means $\pm$ standard deviation. Data were analysed using a one-way analysis of variance (ANOVA) followed by Duncan's test with a preselected level of significance of $P < 0.05$.

RESULTS

Identification of metabolites

The metabolites excreted in urines were characterized by GC–MS. The chromatographic profile of the metabolites of BBP recovered in the urines is shown Fig. 2. The metabolites are numbered according to their retention time (RT). Metabolic products of BBP were investigated using positive methane chemical ionization (CI) and electronic impact (EI) mass spectrometry. The mass spectra of the major metabolites corresponding to the peaks no. 3, 4 and 6 are reported in Figs 3–5, respectively.

The CI was informative about the molecular weight (MW) of the metabolite generating ions at MW + 1 $(MH)^+$, MW + 29 $(M + C_2H_5)^+$ and MW + 41 $(M + C_3H_5)^+$ (Albro *et al.*, 1973). The fragment m/z = 163 was also present, as in the EI mass spectrum.

The structure of the metabolites was determined by fragmentation with EI. Some characteristic fragments were present. The mass spectrum of many dialkyl phthalates is characterized by intense peaks at m/z 149 and 163 arising respectively from protonated and methylated phthalic anhydride (Harvan *et al.*, 1980). The peak at m/z = 181 is due to a double hydrogen rearrangement involving a long side-chain (Budzikiewicz *et al.*, 1967).

Ion at m/z = 163 was recovered for compounds corresponding to the peaks no. 2, 3, 5, 6, m/z = 149 to the peaks no. 3, 5, 6 and m/z = 181 were represented in mass spectra of metabolites no. 3 and 5. The fragments m/z = 76 and 77 were represented in mass spectra of all metabolites of BBP and correspond to the benzene ring. The presence of these ions establishes that there has no further aromatic ring metabolism (Harvan *et al.*, 1980). The other fragments arise from the cleavage of the side-chain and determine, in addition to CI mass spectra, the molecule structure. The six metabolites of BBP recovered in female Wistar rats urines are reported Fig. 8.

Quantification of urinary metabolites

Oral administration of BBP to female Wistar rats led to at least six metabolites. The parent molecule was not recovered in the urines. Chromatographic profiles of urinary extracts were qualitatively and quantitatively identical with or without alkaline or enzymatic treatments of samples, suggesting the absence of glucuronides.

The analysis by GC–MS of urinary extracts demonstrated the presence of five free metabolites and of hippuric acid, a conjugate of benzoic acid with glycine by a peptide bond. At the administered doses, when data are expressed in % of total recovered metabolites, the major metabolites were hippuric acid (51–56%) and BBP monoesters: mono-*n*-butyl phthalate (MBuP) (29–34%) and mono-*n*-benzyl phthalate (MBeP) (7–12%). Mono-*n*-butyl phthalate ω-oxidized (MBuP ω-ox), phthalic acid were also present (1–2% and 2–3%, respectively) and benzoic acid was also recovered but in very small quantities.

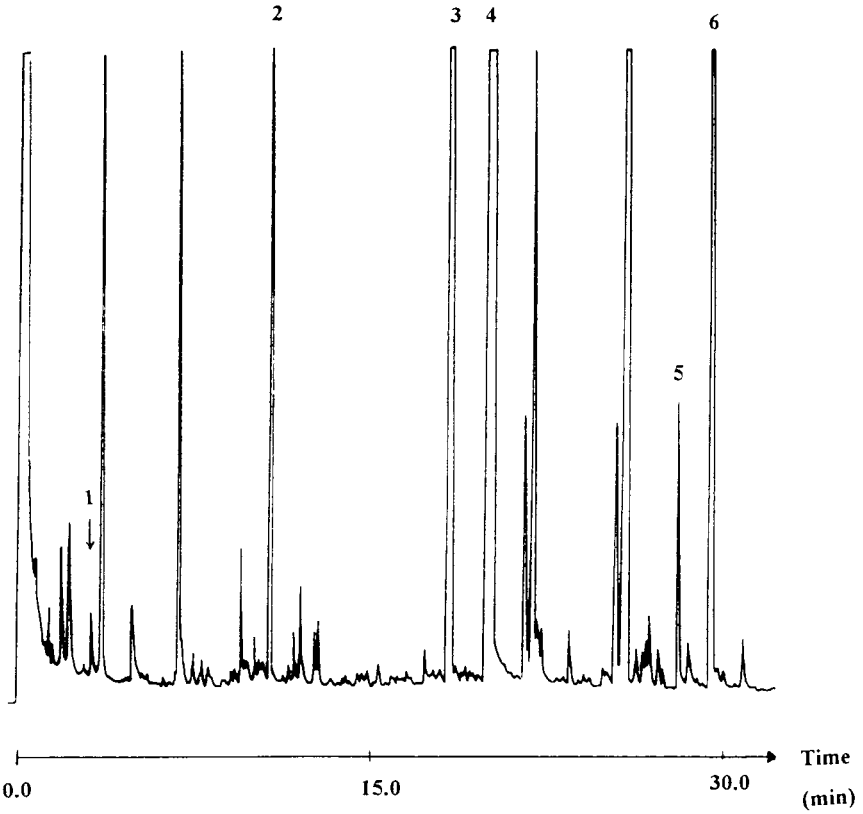

Fig. 2. Gas chromatographic profile of urinary extract after single oral administration of BBP to female Wistar rats. 1: benzoic acid (RT: 4.34) 2: phthalic acid (RT: 13.51) 3: MBuP (RT : 20.39) 4: hippuric acid (RT: 22.16) 5: MBuP ω-ox (RT: 28.56) 6: MBeP (RT: 30.09)

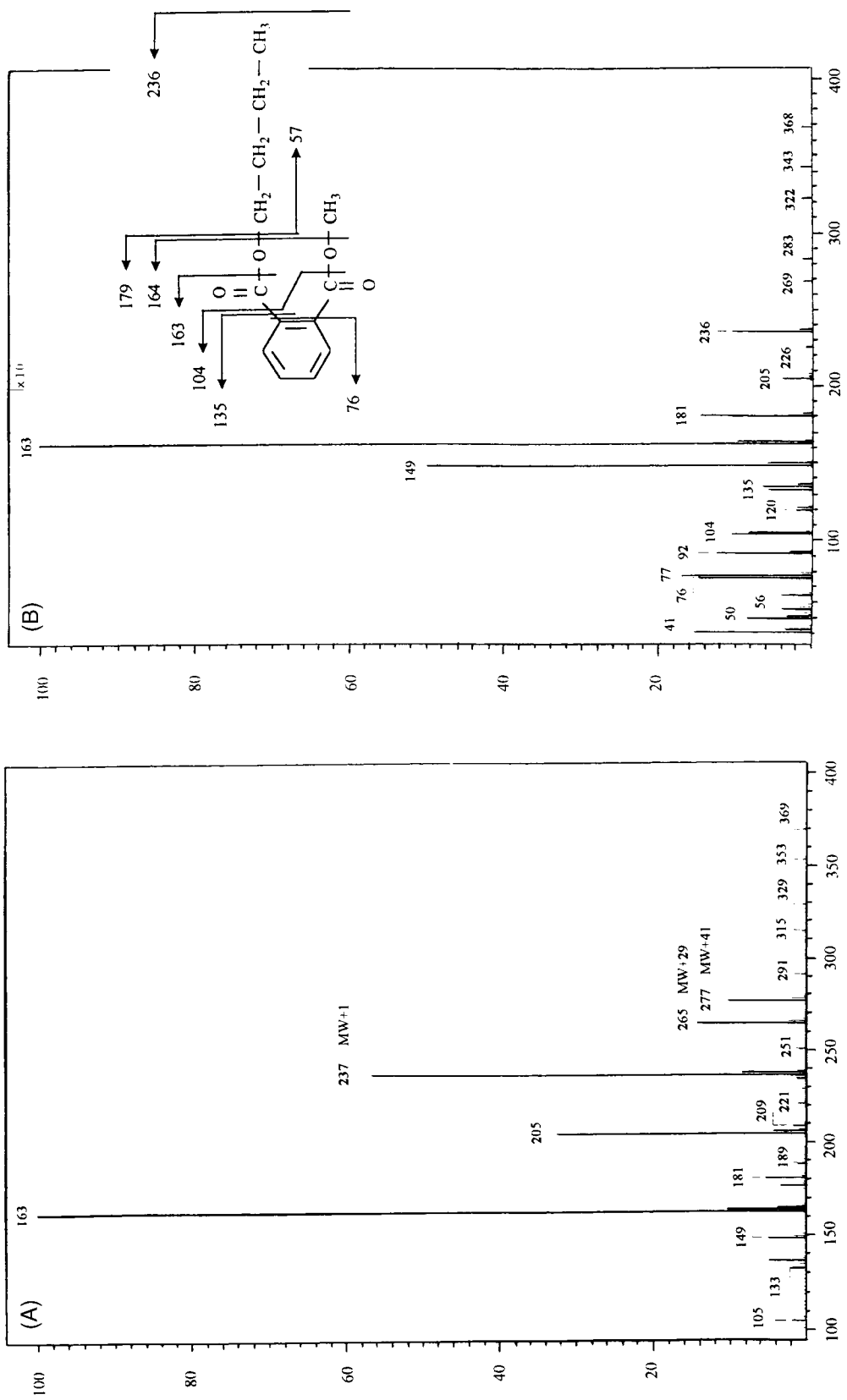

Fig. 3. Mass spectra of mono-*n*-butyl phthalate (peak no. 3 on the chromatographic profile), after derivatization with diazomethane obtained by positive chemical ionization (A) and electronic impact (B).

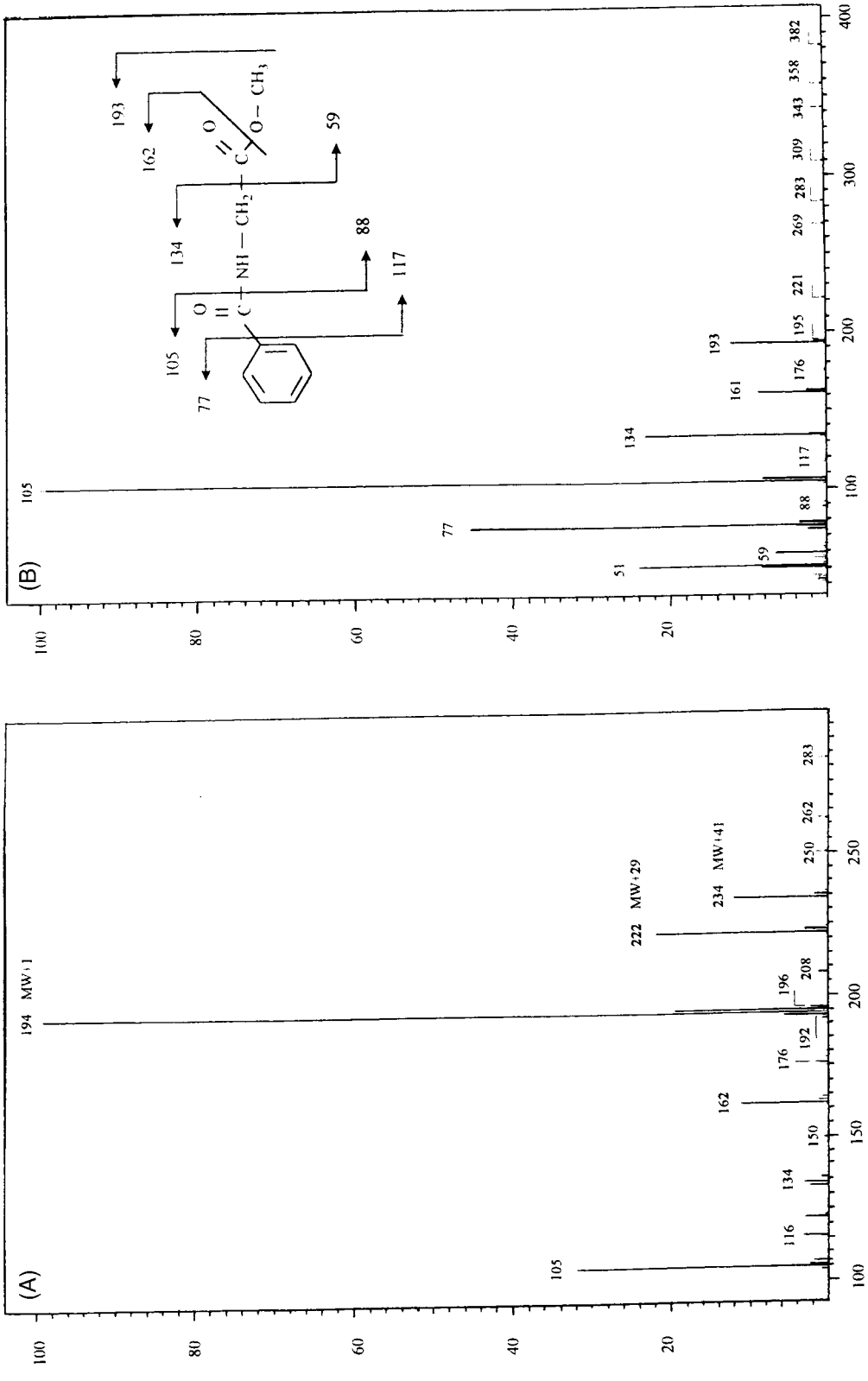

Fig. 4. Mass spectra of hippuric acid (peak no. 4) after derivatization with diazomethane obtained by positive chemical ionization (A) and electronic impact (B).

Dose dependency

Four doses of BBP: 150, 475, 780 and 1500 mg/kg body weight/day were tested in female Wistar rats by oral administration in order to test dose dependency on BBP metabolism. The recovered metabolites in urines represented respectively 58%, 54%, 43% of the doses 150, 475, 780 mg/kg body weight/day and only 30% of the dose 1500 after 24 hr. Metabolites quantities, expressed in percentage of the dose, are presented Fig. 6 (A,B). The first day (A), the quantities of metabolites (except MBuP) recovered in urines were similar for the doses 150 and 475 mg/kg body weight/day. At the dose 780 mg/kg body weight/day, hippuric acid is significantly in a lower amount. At the highest dose, all metabolites quantities decreased significantly compared to the doses 150 and 475 mg/kg body weight/day. The second day (B), the quantities of hippuric acid excreted started to decrease significantly at the dose 475 mg/kg body weight/day. Elimination of MBuP, MBeP and MBuPω-ox was similar for the doses 150 and 475 mg/kg body weight/day. Elimination of MBuP, MBeP and hippuric acid was similar for the two highest doses but in a lower amount compared to the first doses. In contrast, on day 3 (data not shown), the quantities of all metabolites excreted *via* the urinary pathway were constant with the dose of BBP.

Time dependency

Figure 7 (A,B) indicates the daily elimination of BBP metabolites following three consecutive administrations of two BBP doses: 475 (A) and 1500 mg/kg body weight/day (B) to female Wistar rats. Urines were collected 24 hr after each treatment corresponding to days 1, 2 and 3. Treatment with the low dose resulted in a steady-state level urinary excretion of metabolites within 72 hr, suggesting a lack of time dependency. Multiple dosing with the highest dose of BBP (1500 mg/kg body weight/day) showed identical levels of urinary excretion of all metabolites the first 48 hr, whereas the last day, the quantities of MBuP, MBeP and hippuric acid were respectively 1.9-, 2.4- and 1.4-fold increased, showing a slight time-dependency at the highest dose of BBP.

DISCUSSION

Six metabolites have been identified in the female Wistar rat urines after oral BBP administration (150, 475, 780 and 1500 mg/kg body weight/day); MBuP, MBuP ω-ox, MBeP, benzoic acid, hippuric acid and phthalic acid. Total metabolites recovered in urines account for 56% of the dose the first 24 hr (150 and 475 mg/kg body weight/day) and 43% of the dose 780 mg/kg body weight/day. But BBP metabolites recovery in urines decreased significantly (30%) with the highest BBP dose (1500 mg/kg body weight/day). It could probably be due to a lower absorption as Eigenberg *et al*. (1986) have shown that BBP faecal excretion became the predominent pathway (57% of the dose) in the male rat at 2000 mg/kg body weight/day. Whatever the dose and the time, all metabolites of BBP were present. For the two first doses (150 and 475 mg/kg body weight/day) metabolites are recovered in the same proportion. Hippuric acid quantity started to decrease in urine of rat treated with 780 mg/kg body weight/day (23% at 780 and 18% at 1500 mg/kg body weight/day). Indeed, at 24 hr and with the highest dose, all BBP metabolites are in a smaller quantity compared with other doses. However, with time, this discrepancy disappeared since all metabolites excreted were constant with the dose by day 3. BBP, the parent molecule, is never recovered in the urines. In fact, the major route of BBP excretion is into the bile in male F344 rats (Eigenberg *et al*., 1986). BBP, like di-*n*-butylphthalate (DBP) (Tanaka *et al*., 1978), undergoes extensive enterohepatic circulation. The two major metabolites are MBuP (29–34%), the monoester issued from the hydrolysis of the methyl benzyl group, and hippuric acid (51–56%). MBeP, which represents the second monoester issued from the hydrolysis of the butanol group, is recovered in the rat urines in a smaller percentage (7–12%) compared to MBuP. These findings coincide with results obtained by Eigenberg *et al*. (1986) showing that orally ingested BBP is mainly excreted in urines, most of it as MBuP (44% of the dose). BBP is an asymmetric diester with the possibility forming an equal amount of MBuP and MBeP; the butyl group might be more resistant that benzyl group to the hydrolysis as we observed larger quantities of MBuP formed. Hydrolysis of phthalate esters has been reported to occur largely in the gastrointestinal tract (Kluwe, 1982). Antibiotics had no effect on hydrolysis of DEHP (di 2-ethylhexyl phthalate) indicating that the enzymes were endogenous and not derived from the gut flora. Also, experimental evidence suggests that pancreatic lipases probably play an important role in the hydrolysis in the phthalate diesters (White *et al*., 1983). Liver lipases are also able to hydrolyse phthalate diesters and are the most effective for DEHP (Albro and Thomas, 1973). Tanaka *et al*. (1978) have shown a very fast hydrolytic rate of DBP *in vitro* when using rat liver homogenates or microsomal preparation.

The second major metabolite recovered in the female rat urines is hippuric acid (51–56%); this molecule might be issued from a detoxication pathway of benzoic acid (a toxic compound) by elimination as a conjugate with the glycine (Bridges *et al*., 1970 ; Tremblay and Qureshi, 1993). Benzoic acid has been identified in our study by GC–MS in very small quantities in urines. Hippuric acid is also the ultimate metabolite of several aromatic compounds (Teuchy *et al*., 1971). Using ring-labelled [^{14}C]BBP,

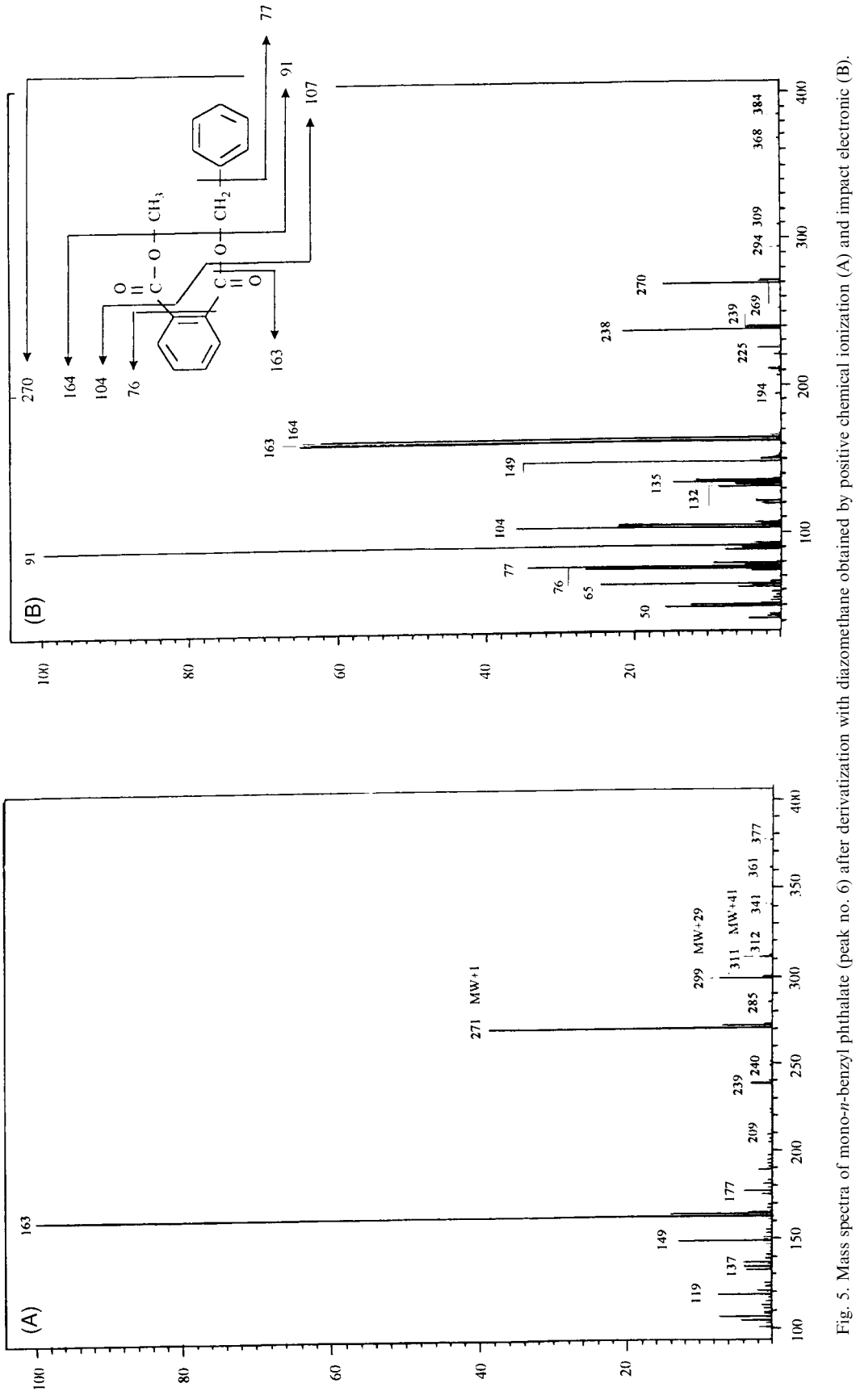

Fig. 5. Mass spectra of mono-*n*-benzyl phthalate (peak no. 6) after derivatization with diazomethane obtained by positive chemical ionization (A) and impact electronic (B).

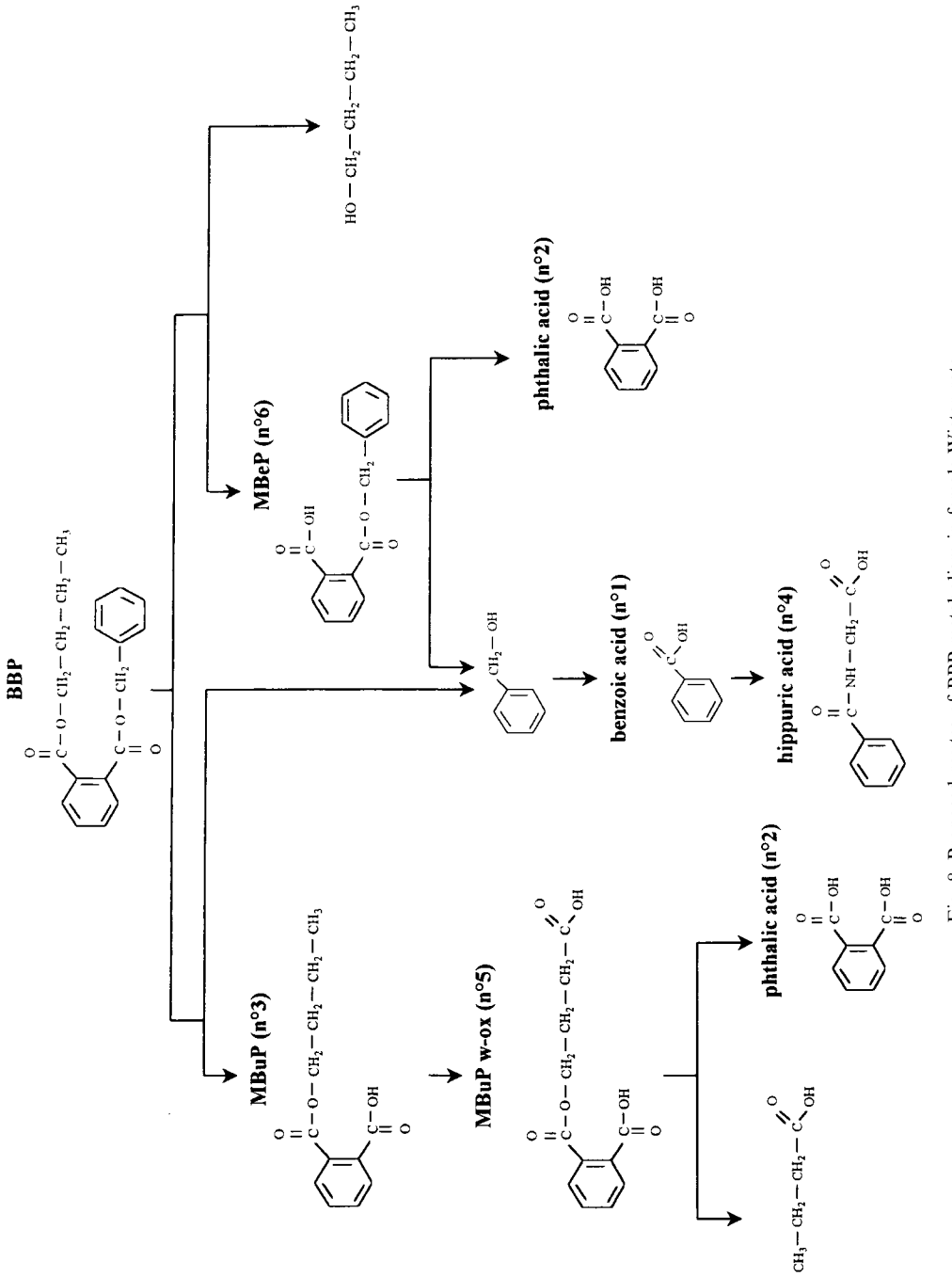

Fig. 8. Proposed routes of BBP metabolism in female Wistar rats.

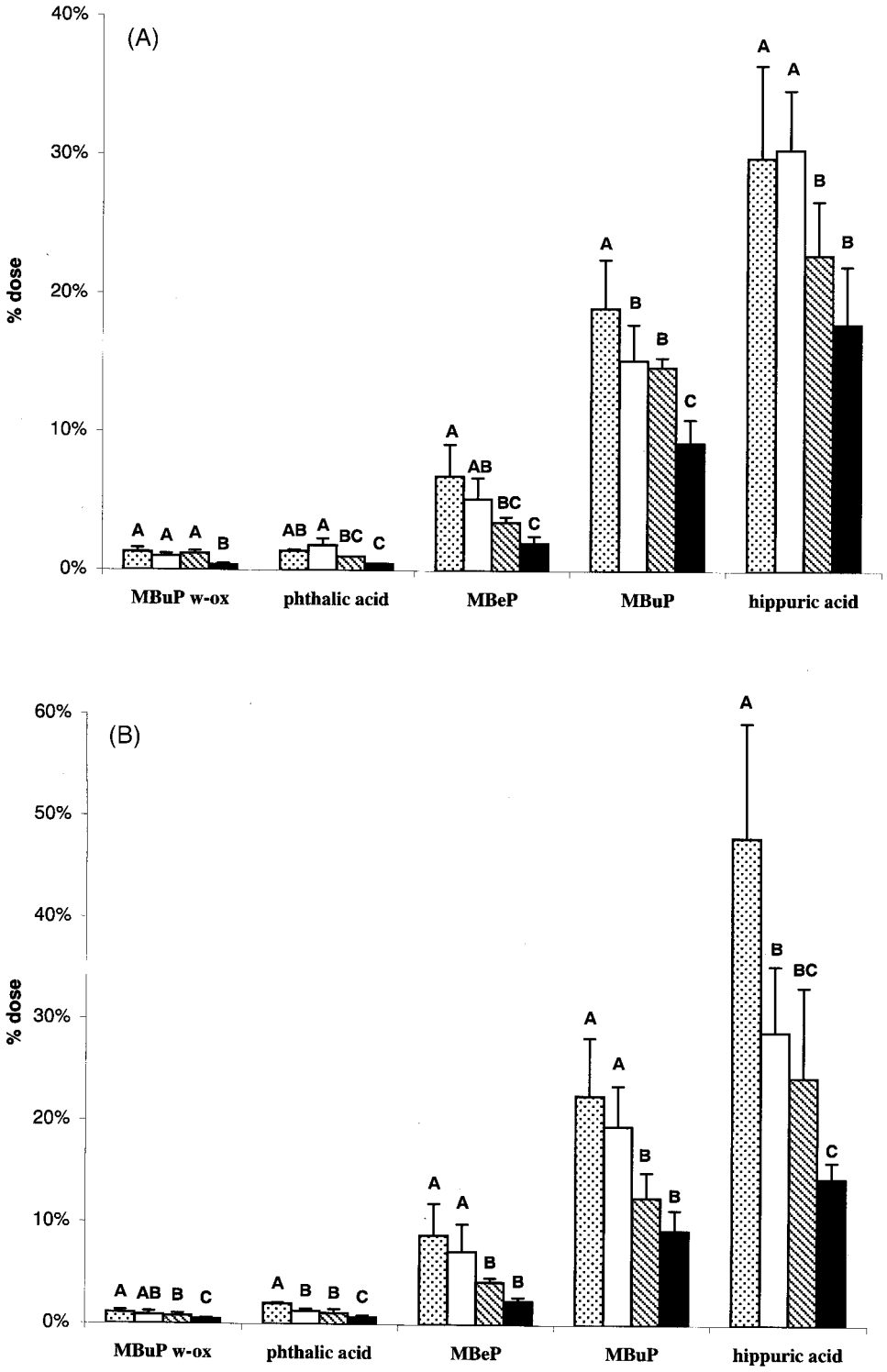

Fig. 6. Excretion of BBP metabolites, hippuric acid, mono-*n*-butyl phthalate, mono-*n*-benzyl phthalate, mono-*n*-butyl phthalate ω-oxidized and phthalic acid , after multiple oral dosing at levels of 150 (⚃), 475 (□), 780 (▨) and 1500 mg/kg body weight/day (■). (A) 24 hr after the first gavage (day 1); (B) 24 hr after the second gavage (day 2). Data are presented as means $\pm$ SD (n = 10) of two independent experiments. Dose effect is determined by comparison of means for each metabolite. Means with the same letter are not significantly different ($P < 0.05$).

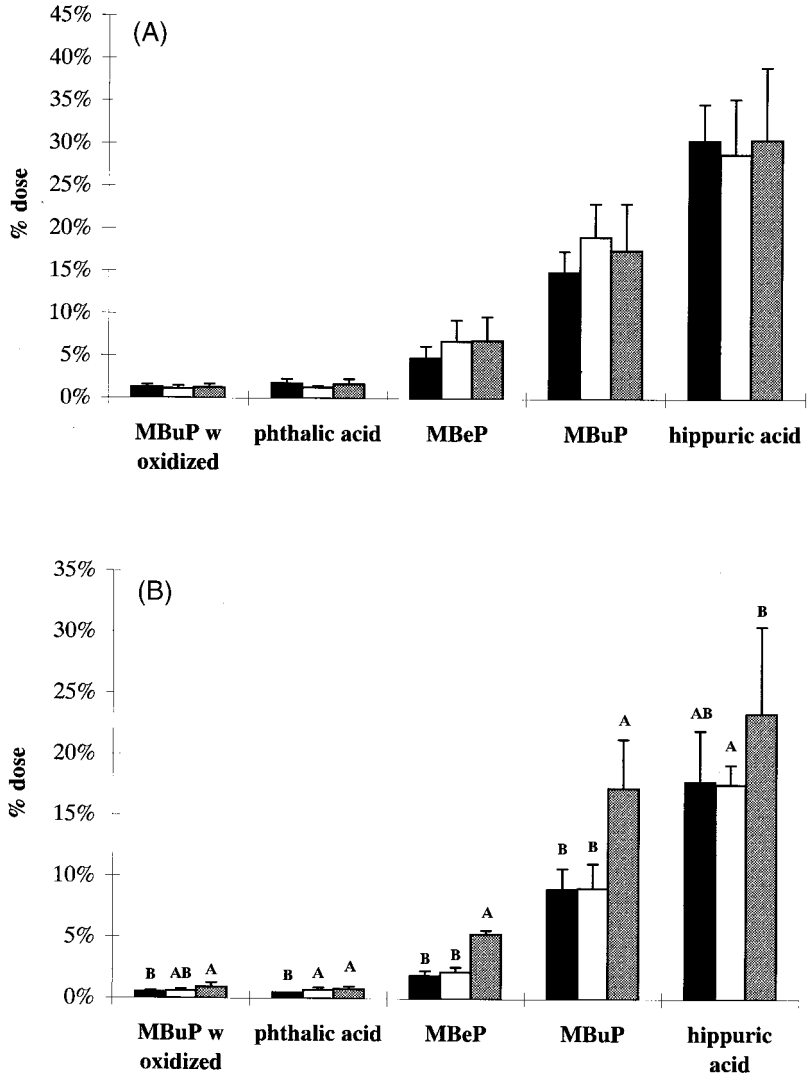

Fig. 7. Quantification of urinary metabolites of BBP after multiple dosing. BBP was administered to rats [(A) 475 mg/kg body weight/day and (B) 1500 mg/kg body weight/day] for 3 consecutive days and urines collected 24 hr after each dose (■, day 1; □, day 2; ▩, day 3). Data are presented as means ± SD (n = 10) of two independent experiments. Time effect is determined by comparison of means for each metabolite. Means with the same letter are not significantly different ($P < 0.05$).

Eigenberg *et al.* (1986) were not able to identify hippuric acid in male rat urines.

We did not identify any glucuronide in the female rat urines as mentioned by Eigenberg *et al.* (1986). Indeed, 2–21% of the dose was identified as glucuronide of the monophthalate derivatives in the male F344 rat urine. Sex differences can occur at various levels of the overall conjugation process (Mulder, 1986).

Butanol, the alkyl chain issued from BBP hydrolysis, is never recovered in rat urines but is probably metabolized earlier into the corresponding acid and further by the mitochondria.

Like MEHP (Albro *et al.*, 1973; Lhuguenot *et al.*, 1988), MBuP appears to undergo ω-oxidation (1–2%) in the female rat urines. In the rat, ω-oxidation products could lead to compounds which are further metabolized by peroxisomal β-oxidation (Mannaerts and Van Veldoheven, 1993), but we did not identify any trace of β-oxidized metabolite. The lateral chain might be too short to be further oxidized (Lake *et al.*, 1987). ω-oxidation of MBuP have been identified in different species (rat, guinea pig, hamster) during DBP metabolic studies since MBuP is a common metabolite of both BBP and DBP (Tanaka *et al.*, 1978). 3-Hydroxybutyl hydrogen phthalate, a ω-1 oxidized metabolite, has also been detected in the urines of male rats and hamster when DBP was orally administered to these species. Phthalic acid has been identified in the female rat urine (2% of the dose). Phthalic acid is a common molecule of phthalate diesters and is present in rat urines when DEHP (Albro *et al.*, 1982) or DBP (Tanaka *et al.*, 1978) is orally ingested. Phthalic

acid might be also recovered in faeces when oral phthalic acid doses administered to rats were increased (Williams and Blanchfield, 1974).

Ema *et al.* (1993, 1994, 1995a) have shown the similarity in the dependence of gestational days of treatment on the manifestation of the teratogenicity and spectral malformations induced by BBP and DBP. Results obtained in this study with BBP in female rats together with previously demonstrated teratogenic activities of monoesters MBuP (Ema *et al.*, 1995b) and MBeP (Ema *et al.*, 1996a,b) suggest that the monoesters MBuP and/or MBeP, rather than the corresponding diester, are responsible for the teratogenic effects of BBP.

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