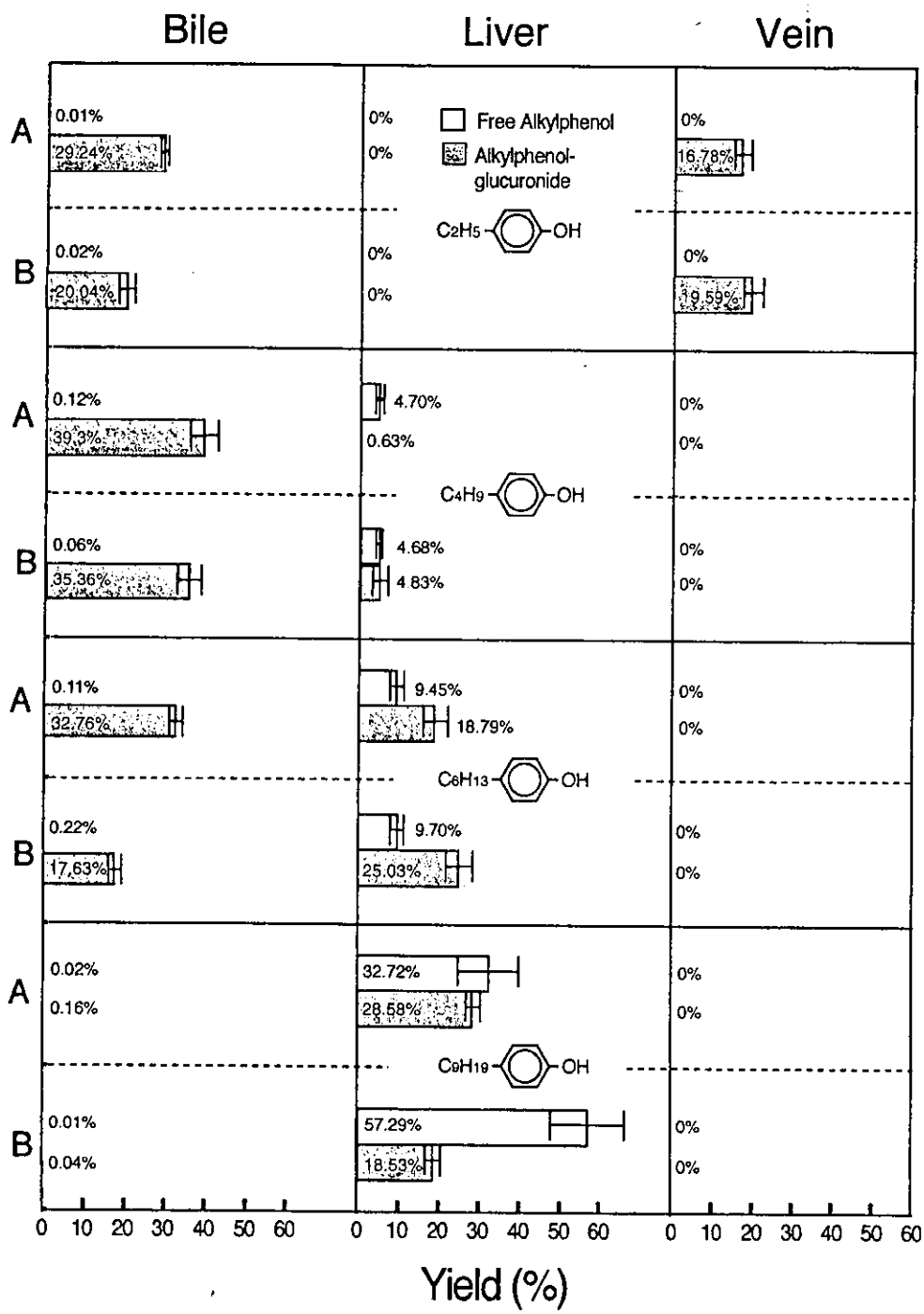

FIG. 4. *Alkylphenol metabolites in the bile, liver tissue, and vein after 1 h of perfusion in SD rats.*

Total amounts of alkylphenols and glucuronide conjugates, which were excreted into the bile and vein and remained in the liver during a 60-min period of liver perfusion, are shown. Numerals in the columns indicate the percentages of respective metabolites, free (□) or glucuronide conjugate (□). Data are represented in the upper box (A) when 0.025 mM alkylphneol was injected, in the lower box (B) when 0.050 mM nonylphenol was injected. About 30% of injected alkylphenols were absorbed to the inside of the silicon tubes during the perfusions. Recovery was calculated with the tube absorption of each alkylphenol. Parameters are shown as means ± S.E. (n = 4 animals or n = 3 animals in ethylphenol analysis).

both the bile and vein within 60 min (Fig. 3E). When 0.05 mM butylphenol and hexylphenol were perfused, these alkylphenols were also glucuronidated in the liver, but these glucuronides were not excreted completely from the liver within 60 min of perfusion (Fig. 3B). Trace amounts of nonylphenol-glucuronide were detected only in the bile (Fig. 3, C and D). The alkylphenols remaining in the livers after 1 h of perfusion were extracted as described under *Materials and Methods*. The amounts of alkylphenols in the liver and that excreted into bile and vein during 1 h of perfusion are shown in Fig. 4. Recovery of alkylphenols after the perfusions were about 50 to 60% (Fig. 4). One of the reasons for this is that it was supposed that all other alkylphenol metabolites such as hydroxylated alkylphenols and conjugated alkylphenols with sulfate or glutathione remaining in the liver tissue were not extracted with acetonitrile completely, and/or

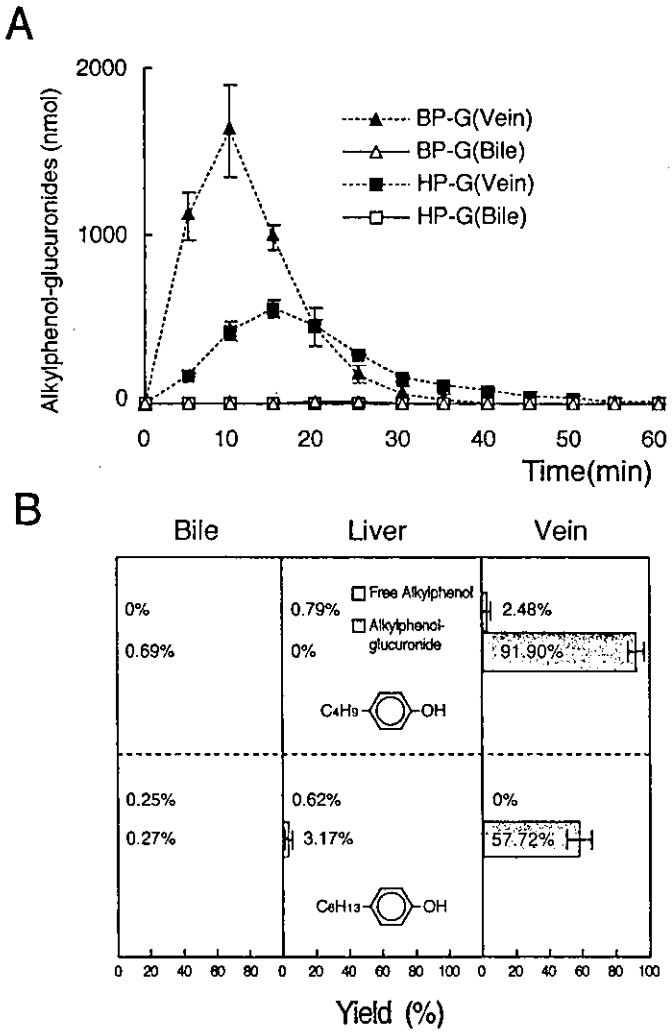

FIG. 5. *Excretion of alkylphenol metabolites during liver perfusion in EHBR.*

A, excretion of metabolites of butylphenol and hexylphenol during a 1-h period of liver perfusion in EHBR, which have a hereditary defect in MRP-2. B, total amounts of the alkylphenols and glucuronide conjugates excreted into the bile and vein, and the total amount of those that remained in the liver during the 60-min period of liver perfusion. Numerals in the columns indicate the percentages of the respective metabolites (free, □; or glucuronide conjugate, □). Parameters are shown as means ± S.E. (n = 4 animals).

these metabolites were not detected in this HPLC system. Unconjugated alkylphenols remained in the liver tissue, and small amounts of free alkylphenols were detected in the bile (Fig. 4). About 800 to 1000 nmol of injected nonylphenol could be conjugated as a glucuronide by the rat liver within 1 h of perfusion, but most of the glucuronide and free nonylphenol remained in the liver (Fig. 4). Other alkylphenols having shorter alkyl chains were excreted smoothly into the bile as glucuronides (Fig. 4). These results indicated that alkylphenols with shorter (including C6) alkyl chains were easily excreted into the bile as glucuronides and that only the glucuronide conjugate of nonylphenol, which has a longer C9 alkyl chain, was not easily excreted from the liver. The metabolites of butylphenol and hexylphenol in liver perfusion of EHBR, which are deficient in xenobiotic conjugates transporter MRP-2, are shown in Fig. 5. These alkylphenols were excreted into the vein as glucuronides, and only slight amounts of the metabolites were observed in the bile (Fig. 5A). Rapid excretion and clearance of butylphenol-glucuronide from the liver were observed (Fig. 5, A and B). The cumulative biliary excretion of the alkylphenol-glucuronides, which were produced in the liver, was severely impaired in EHBR compared with SD rats, indicating that alkylphenol-glucuronides were transported to the bile by MRP-2.

Discussion

The disposition of alkylphenols and their metabolites was investigated in SD rats and EHBR by a liver perfusion method. To determine the effects of nonylphenol on animals, it is essential to elucidate the metabolic fate of this compound. Various data on the metabolism and disposition of nonylphenol in fishes are available. The major metabolite (about half of the injected nonylphenol) in rainbow trout (*O. mykiss*) was excreted as a glucuronide conjugate of nonylphenol (Thibaut et al., 1998a,b), and other metabolites, including glucuronide conjugates of ring- or side chain-hydroxylated nonylphenol (Coldham et al., 1998), were detected. It has been reported that nonylphenol was extensively metabolized to the glucuronide conjugate in rats (Moffat et al., 2001; Doerge et al., 2002). Doerge et al. (2002) have reported that two glucuronide metabolites (NP-glucuronide and *p*-nonyl-catechol glucuronide) were detected in the rat liver and serum after oral administration. We detected NP-glucuronide as a major peak with slight shoulder peaks after the liver perfusion. Other metabolites may be contained in the shoulder peaks. In this study, rat hepatocytes showed a significant capability for glucuronidation of nonylphenol of about 800 to 1000 nmol/h by liver perfusion, but the transport of the nonylphenol-glucuronide across the canalicular membrane into the bile was delayed. These findings suggest that nonylphenol glucuronidation is mediated by UDP-glucuronosyltransferase isoform(s), such as UGT2B1, with high activity but that the resultant glucuronide having a long (C9) alkyl chain could not be transported by MRP-2.

The substrate specificity of MRP-2 has been studied by comparing the transportation activity across the bile canalicular membrane in normal rats with that in transport-deficient rats (Paulusma et al., 1996) or in EHBR, which have a hereditary defect in MRP-2 (Fernandez-Checa et al., 1992; Takenaka et al., 1995; Yamazaki et al., 1996). It has been demonstrated that glutathione conjugates, glucuronides of xenobiotics, sulfates of several bile acids, and some organic anions are substrates for MRP-2, but the mechanism of the transport and the substrate specificity of the transporter have not been clarified yet. In this study, it was found that transportation activity of MRP-2 decreased with increase in the number of alkyl chains of alkylphenols, suggesting that after MRP-2 recognizes or binds to the glucuronic acid anion, the long alkyl chain of alkylphenol-glucuronide disturbs the transportation across the membrane. The transporter, which mediates the transportation of glucuronides into the vein from liver cells, can transport only an ethylphenol-glucuronide having the shortest alkyl chain, indicating that metabolic distribution and excretion of alkylphenol is caused by the length of the alkyl chain. It has been shown that MRP-2 has transportation activity of glucuronide conjugates of various xenobiotics having large molecular mass (Yamazaki et al., 1996), suggesting that inability of MRP-2 to transport nonylphenol-glucuronide is due to the shape or hydrophobicity of the long (C9) alkyl chain.

Finally, nonylphenol has been shown to reduce the level of CYP1A1 expression in murine Hepa-1c1c7 cells (Jeong et al., 2001) and to inhibit in vitro CYP1A1 activity in rat liver microsomes (Lee et al., 1996) and activities of human cytochrome P450s, including steroidogeneic CYP17 activities (Niwa et al., 2002). Oral administration of nonylphenol decreased hepatic testosterone hydroxylation and CYP2C expression level (Laurenzana et al., 2002). Estradiol binding to the estradiol receptor (ER) was significantly inhibited by nonylphenol (Danzo, 1997). These results suggest the possible inhibition of cytochrome P450s and ER functions by nonylphenol, which delays excretion from the liver.

One of the toxicological implications for the delay of excretion of nonylphenol and nonylphenol-glucuronide is estimated to the binding to and inhibition of ER (Tabira et al., 1999); another is the inhibition of MRP-2 which excretes various glucuronides such as estradiol glucuronide (Ito et al., 2001) and bilirubin glucuronide (Kusuhara et al., 1998). The delay of nonylphenol excretion is possible because of the adverse effects of nonylphenol on rat reproductive systems (Laws et al., 2000).

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BISPHENOL A GLUCURONIDATION AND ABSORPTION IN RAT INTESTINE

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ABSTRACT:

Bisphenol A, an environmental estrogen, can be leached from plastic tableware and from the coating of food and drink cans, orally exposing human beings to the compound. The present study focuses on the absorption and metabolism of bisphenol A in the rat intestine, as elucidated experimentally by segmented everted intestine. One hour after the application of 2 μmol of bisphenol A to the mucosal fluid, the absorption of bisphenol A was slightly greater in the colon (48.6%) than in the proximal jejunum (37.5%). In the serosal side, unconjugated bisphenol A appeared in small amounts, increasing distally (maximal 1.6 nmol, colon). Large amounts of the bisphenol A glucuronide were then transported into the serosal side, also increasing distally (proximal, 80.4 nmol; distal, 478.4 nmol). The greatest amount of the glucuronide (~573 nmol) was excreted into the mucosal side of the small intestine, whereas in the colon, mucosal excretion was minimal (67.2 nmol). On high-dose application of bisphenol A to the mucosal fluid, the transported unconjugated bisphenol A increased markedly throughout the intestine and colon. These results suggest that bisphenol A in the intestinal lumen is glucuronidated almost exclusively during its passage through the intestinal wall.

A growing number of industrial chemicals has been reported to act as endocrine disrupters in mammals and other animals (Hoyer, 2001). One of the prominent environmental hormones, bisphenol A[3] (2,2-bis[4-hydroxyphenyl]propane), has demonstrated estrogenic activity having adverse effects on the reproductive system (Kim et al., 2001; Chen et al., 2002). Bisphenol A is widely used in the manufacture of epoxy, polycarbonate, and polyester-styrene resins (National Toxicology Program, 1982), and traces of the compound can be easily leached from food containers and tableware made of such plastics and can be taken up by human beings through eating and drinking (Brotons et al., 1995; vom Saal et al., 1998). In vitro, bisphenol A has stimulated cell proliferation as well as the induction of progesterone receptors on MCF-7 human breast cancer cells (Krishnan et al., 1993). In the reproductive tract of female rats, a single high dose of the compound (37.5–150 mg/kg) was reported to induce cell differentiation, and c-fos proto-oncogene expression (Steinmetz et al., 1998). Exposing pregnant CF-1 mice for 7 days to bisphenol A (2.4 mg/kg) evoked early puberty of the female offspring, significantly hastening vaginal opening and onset of estrous (Howdeshell et al., 1999).

Bisphenol A introduced orally into the body must pass through the intestine and liver before arriving at the reproductive organs, where irreversible damage may be inflicted. To elucidate the mechanism governing the detrimental effects on the target organs, it is essential to clarify metabolism and disposition of the compound during its journey through the gastrointestinal tract. Previously, we found that bisphenol A in rat liver is extensively glucuronidated by UGT2B1, an isoform of UDP-glucuronosyltransferase (Yokota et al., 1999). Additional study showed that the compound is glucuronidated in rat perfused liver and is excreted exclusively into the bile (Inoue et al., 2001). In the intestine, however, which provides the foremost barrier against ingested toxicants, behavior of the compound has not been delineated.

In experiments applying 1-naphthol to rat-everted intestine, glucuronidation activity toward the phenolic compound was evident, and the resultant glucuronide was expelled from the enteric mucosal cells into the lumen (Inoue et al., 1999). These observations, together with our hepatic findings to date, led us to conduct the present work using everted intestine to determine the fate of bisphenol A that enters the intestine of the rat.

Materials and Methods

Chemicals. Bisphenol A was purchased from Kanto Chemical Co. (Tokyo, Japan); high-performance liquid chromatography (HPLC) grade acetonitrile from Labscan Ltd. (Dublin, Ireland); β-glucuronidase (type B-1; from bovine liver) from Sigma-Aldrich (St. Louis, MO). Bisphenol A glucuronide purified from the bile after rat liver perfusion with 7.5 μmole bisphenol A (Inoue et al., 2001) was quantified by HPLC by using the difference between β-glucuronidase-treated sample and untreated sample, and was used as a standard.

Animals. Male Sprague-Dawley rats (8-weeks old; 300–340 g) were used in all experiments. The rats were housed under standard conditions and given food and water ad libitum. The animals were handled according to the Laboratory Animal Control Guidelines of Rakuno Gakuen University, which is based on the Guide for the Care and Use of Laboratory Animals of the U.S. National Institutes of Health.

Preparation of Everted Intestine. Krebs Ringer's bicarbonate buffer (135.0 mM Na^+, 5.0 mM K^+, 2.5 mM Ca^{2+}, 1.2 mM Mg^{2+}, 122.4 mM Cl^-, 25.0 mM HCO^{3-}, 10.0 mM glucose) was used in all experiments. The buffer

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[3] Abbreviations used are: bisphenol A, 2,2-bis[4-hydroxyphenyl]propane; HPLC, high performance liquid chromatography; MRP, multidrug resistance associated protein.

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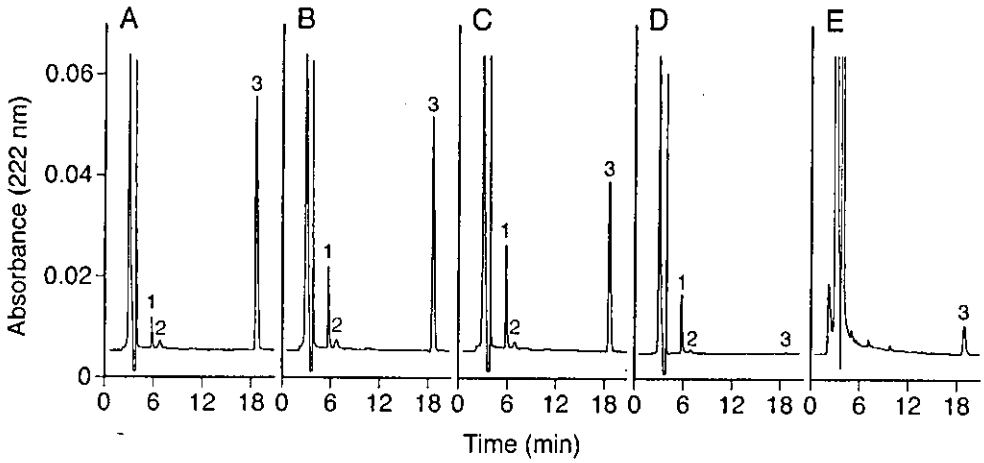

FIG. 1. *HPLC chromatograms of mucosal and serosal buffer solutions derived from rat everted intestine treated with bisphenol A (50 μM).*

Samples here are from the ileum, segment IV (see Fig. 1). Mucosal buffer solution sampled at 20-min incubation (A), 40 min (B), and 60 min (C); serosal buffer solution at 60-min incubation (D); serosal buffer solution treated with β-glucuronidase (E). Peak 1, bisphenol-A glucuronide increased with incubation time; peak 2, unknown peak; and peak 3, unconjugated bisphenol A.

solution was aerated by 95% O_2 + 5% CO_2 and the pH was adjusted to 7.4. The rats were euthanized by decapitation, and the jejunum, ileum, and colon were flushed with cold Krebs Ringer's buffer. The bowels of the animals were excised and prepared according to a modification of the segmentation and eversion method described previously (Inoue et al., 1999). Briefly, with the exception of the duodenum, the excised small intestine was lavaged and divided into four sections of equal length as quickly as possible. The distal portion of each section was excised and trimmed to 10 cm and designated I, II, III, and IV in distal order, with segment I being from the jejunum and segment IV from the distal ileum. In the same manner, the colon was excised, washed, and trimmed to a final segment of 10 cm taken from the distal end.

The five trimmed segments were turned inside out and fixed on a polyethylene tube in the mucosal buffer solution (40 ml). Serosal buffer solution (40 ml) was pumped through the everted bowels (tube pump MP-32N; EYELA, Tokyo, Japan) at 5 ml per min via polyethylene tubes. Bisphenol A was added to the mucosal buffer solution in concentrations of 10, 50, and 100 μM, and reaction products were collected independently from the serosal and mucosal sides at 0, 20, 40, and 60 min after each addition of the compound.

HPLC Analysis of Reaction Products. The mucosal and serosal samples were filtered by a disposable disk filter (HLC-DISK3) from Kanto Chemical Co. and stored at −80°C until analysis. The samples were analyzed by an HPLC system (Tosoh, Tokyo, Japan) based on the method described previously (Yokota et al., 1999). Briefly, the samples were eluted with a solution of acetonitrile/H_2O/acetic acid (37:63:0.1 v/v/v) on a constant flow rate at 1 ml/min. The eluted samples were analyzed with UV 222-nm detection using TSK-gel ODS-80Ts-reversed phase column (4.6 × 250-mm; Tosoh Tokyo, Japan). The results were recorded with C-R6A integrator from Shimadzu (Tokyo, Japan).

β-Glucuronidase Reaction. After being filtered, the various mucosal and serosal buffer solutions were allowed to react with the β-glucuronidase (Inoue et al., 2001), and the products were analyzed by HPLC to verify whether the metabolite was the glucuronide. Hardly any sulfatase activity of the β-glucuronidase was detected by HPLC under the same conditions as those for β-naphthyl sulfate, indicating that the metabolite was in fact the glucuronide.

Statistical Analysis. All data were presented as the mean ± S.E., and the means were compared by use of analysis of variance, with the p value of 0.05 as the level of significance.

Results

High-Performance Liquid Chromatography of Buffer Solution. Elution profiles obtained by HPLC are shown in Fig. 1. In mucosal buffer solution from the intestinal segments treated with 50 μM bisphenol A, three peaks (numbered as 1, 2, and 3) were observed. With increasing incubation time, the peak eluted at ~5.6 min (peak 1) increased gradually, and the last peak (peak 3) decreased (Fig. 1, A-C). In serosal buffer solution, a modest peak (peak 3) of the substrate appeared at ~19 min (Fig. 1D). In the solution containing β-glucuronidase, which cleaves the glucuronide, unconjugated bisphenol A appeared (Fig. 1E; peak 3), signaling that the first peak (peak 1) represented the bisphenol-A glucuronide. An unidentified peak eluted at ~6.8 min (peak 2) did not change dynamically with incubation time.

Bisphenol A Absorption and Transport. On each addition of bisphenol A (10, 50, or 100 μM) to the mucosal side, bisphenol A concentration in the mucosal fluid decreased with incubation. As shown in Table 1, the rate of bisphenol A disappearance from the mucosal compartment was notable on a high dose of bisphenol A (100 μM). Although the distal intestine showed the greatest extent of bisphenol A disappearance, the data were not significant among the five segments of the intestine ($p > 0.052$).

Bisphenol A was absorbed from the mucosal side and transported to the serosal side, as evidenced by the appearance of small amounts of unconjugated bisphenol A. The appearance of serosal bisphenol A increased distally (Fig. 2) and accelerated markedly with the high-dose application (100 μM).

Bisphenol A Glucuronidation. Bisphenol-A glucuronide expelled into the mucosal side as well as that transported into the serosal side increased with the incubation period (Fig. 3). In contrast to the excretion of the glucuronide to the mucosal side, an ~10-min time lag was observed in the transport of glucuronide into the serosal side, suggesting that the intestinal wall may have interfered with the diffusion of the glucuronide in the everted intestine experimental system. Although both the excretion and transport of the glucuronide increased in relation to the administrative dose of bisphenol A, the amounts appeared to reach plateau with a high dose (100 μM) of the compound.

In the small intestine, the greatest amount of the glucuronide was secreted into the mucosal side, whereas in the colon, the mucosal secretion of the glucuronide was diminished (Fig. 4). Glucuronide secretion to the serosal side was mininum in the proximal small intestine and increased with progression distally to the colon.

Fate of Bisphenol A at 60-min Postapplication. In the small intestine at 60-min postapplication of low-dose bisphenol A (10 μM), the absorbed substrate was almost completely glucuronidated, and the

TABLE 1

Bisphenol A disappearance from the mucosal compartment of rat everted intestinal segments within 60-min incubation

Bisphenol A was added to the mucosal buffer solution of each segment in concentrations of 10 μM, 50 μM, and 100 μM. The amount of bisphenol A disappearance (mean ± S.E.) was obtained by subtracting the final amounts of bisphenol A in the mucosal buffer solution after 60-min incubation. I, II, III, and IV indicate the intestinal sites in distal order from the ligament of Trietz.

Dose	Bisphenol A Disappearance (nmol/10-cm bowel/60 min)				
	I	II	III	IV	Colon
μM					
10	175 ± 22	158 ± 14	163 ± 14	198 ± 18	244 ± 7
50	751 ± 92	996 ± 98	862 ± 111	1086 ± 105	1163 ± 98
100	1325 ± 324	1581 ± 282	1556 ± 143	1592 ± 220	2016 ± 114

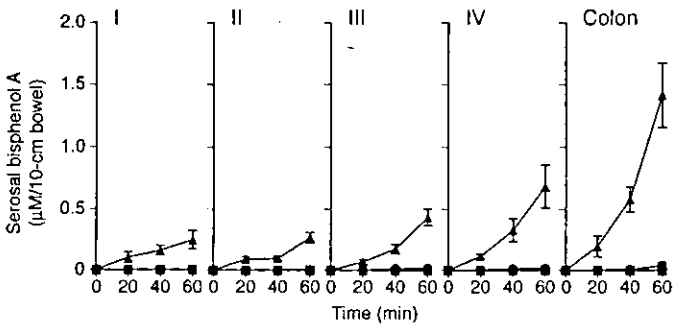

FIG. 2. *Absorption of unconjugated bisphenol A in the individual serosal fluid of rat everted intestine and colon.*

The jejunum, ileum, and colon were segmented into lengths of 10 cm each, and bisphenol A was added to the mucosal buffer solution of each segment in concentrations of 10 μM (-■-), 50 μM (-●-), and 100 μM (-▲-). Segments I, II, III, and IV are from the jejunum and ileum in distal order.

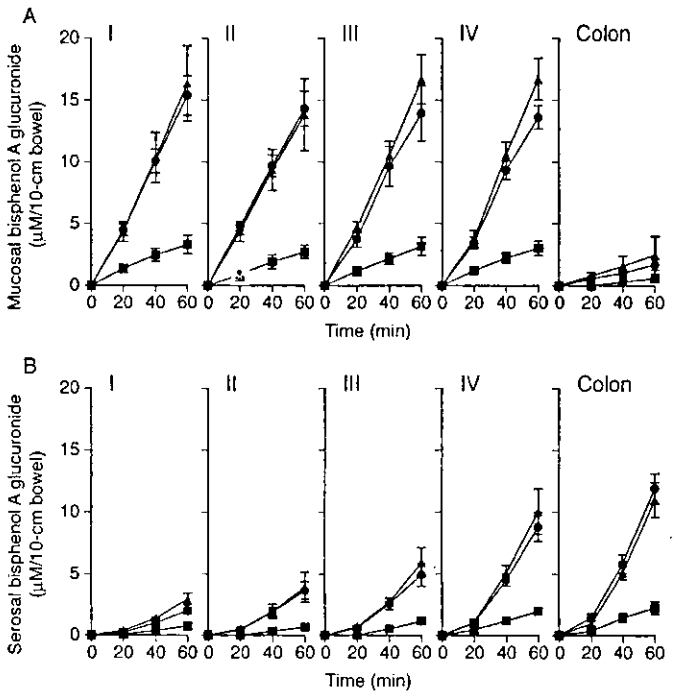

FIG. 3. *Glucuronidation of bisphenol A in rat everted intestine.*

A, the resulting glucuronide excreted to the mucosal side; B, the resulting glucuronide transported to the serosal side. Symbols are the same as those of Fig. 2.

resulting glucuronide was expelled into the mucosal and serosal buffer solutions (Fig. 5). In contrast, the amount of bisphenol A that eluded detection increased on a high dose of bisphenol A (100 μM). The amount remaining unaccounted for (i.e., of unknown fate) was especially prominent in the colon.

In proportion to the dose, in the present experimental system employing everted intestine, 50 μM bisphenol A effected optimal glucuronidation of the compound (Fig. 5). At 60-min postapplication of 50 μM bisphenol A to the mucosal buffer solution, ~37% of the compound was absorbed into intestinal segments I to IV, of which ~83% was glucuronidated. About 74.7% of the glucuronide was excreted to the mucosal side, and ~25.3% was transported to the serosal side.

Discussion

Results of this study affirm that, in the intestine of Sprague-Dawley rats exposed to bisphenol A, 1) most of the compound absorbed by the intestine is glucuronidated within the intestinal wall; 2) the resulting glucuronide is eliminated preferentially into the mucosal side in the small intestine and into the serosal side in the colon; and 3) on a high-dose exposure to bisphenol A, the relative absorption of unconjugated bisphenol A increases dramatically.

Bisphenol A Glucuronidation during Absorption. As suggested by the present results, the proximal intestine is seen as playing a highly protective role against ingested bisphenol A. Bisphenol A in the lumen of the rat intestine was highly glucuronidated during its passage through the intestine, with most of the compound excreted to the mucosal side as glucuronide, which is low in estrogenic activity (Matthews et al., 2001). This was particularly evident for the proximal jejunum, where mucosal excretion of the glucuronide greatly exceeded serosal excretion. Thus, it appears that the proximal jejunum defends the body against potential adverse effects of orally introduced bisphenol A by limiting entry of the free compound into the blood stream and by curtailing exposure to the middle and distal parts of the intestine. In line with these results, low exposure has been reported in association with oral intake of bisphenol A (Pottenger et al., 2000). Comparing the concentration-time profiles for bisphenol A in the blood of F344 rats exposed intraperitoneally and those exposed orally to the compound, Pottenger et al. (2000) found that oral administration results in a low exposure to unconjugated bisphenol A. In the light of our present findings, the diminution of exposure to unconjugated bisphenol A on oral administration may be ascribed to high glucuronidation of the compound in the proximal intestine, which is the foremost barrier to damage from oral administration.

Previously, we showed that bisphenol A glucuronidation in the liver is mediated by UGT2B1, an isoform of UDP-glucuronosyltransferase, and that the isoform is not expressed in rat intestine (Yokota et al., 1999). Generally, the UGT2B family glucuronidates steroid hormones (Turgeon et al., 2001). To our knowledge, the steroid UDP-glucuronosyltranferases are still unclear in rat intestine. In human intestine, however, UGT2B-family isoforms have been reported by Radominska-Pandya et al. (1998). This leads us to conjecture that one

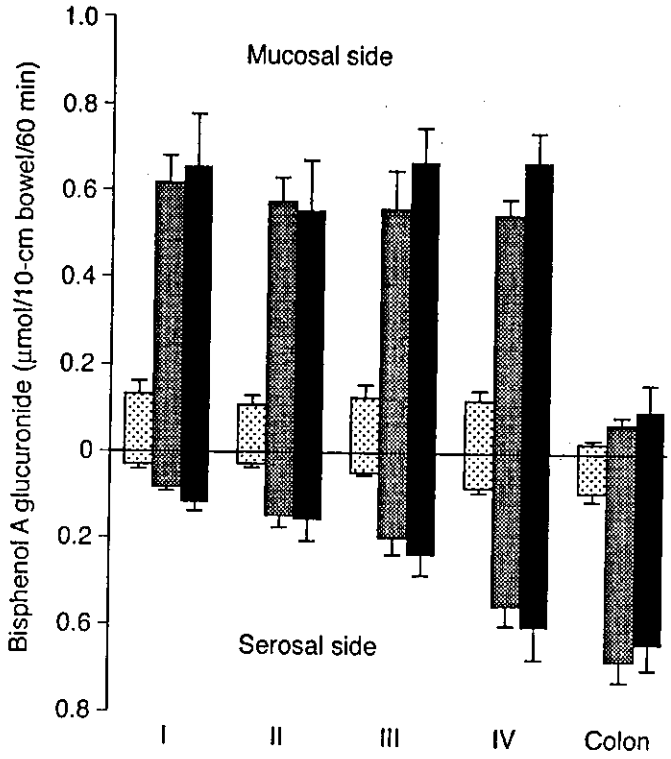

FIG. 4. *Bisphenol-A glucuronidation and excretion to the mucosal side and transport to the serosal side within 60-min incubation in relation to the dose applied. Bisphenol A was added to the mucosal buffer solution of each segment in concentrations of 10 μM (□), 50 μM (▨), and 100 μM (■).*

I, II, III, and IV indicate the intestinal sites in distal order from the ligament of Trietz.

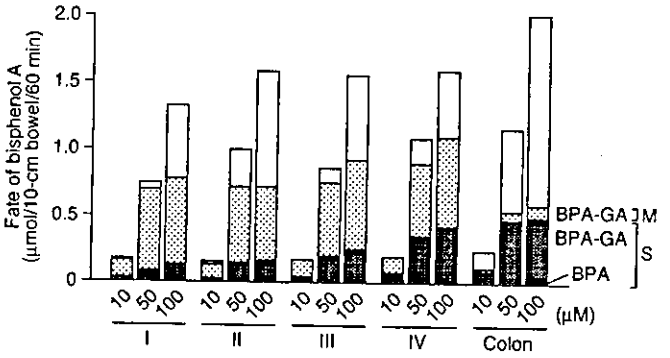

FIG. 5. *Absorption and glucuronidation of bisphenol A at 60-min incubation in rat everted intestine in relation to the dose applied.*

Concentrations (10, 50, and 100 μM) of bisphenol A added into the mucosal buffer solution are depicted horizontally. Total decrease of mucosal bisphenol A during 60-min incubation is depicted in the sum total of the fractional column in each intestinal segment. Bisphenol A glucuronide secreted to the mucosal side (□), bisphenol A glucuronide transported to the serosal side (▨), bisphenol A transported to serosal side (■) bisphenol A of unknown fate (□). BPA, bisphenol A; BPA-GA, bisphenol A glucuronide; M, secreted into mucosal side; S, transported into serosal side.

or more steroid UDP-glucuronosyltransferase isoenzymes of the UGT2B family other than UGT2B1 are responsible for catalyzing bisphenol A within the rat intestinal wall. Further studies are needed to identify the suspected isoenzyme that may catalyze glucronidation of bisphenol A in rat intestine.

Excretion of the Resulting Glucuronide. Whereas in the intestine the bisphenol A glucuronide was excreted into the mucosal side, the direction of elimination was reversed in the colon, where transport was into the serosal side. Recently, ATP-dependent transporters have been described as mediating the transport of the glucuronide across the cell membrane (Oude Elferink et al., 1995). In rat liver a member of the ATP-binding cassette family, namely, multidrug resistance associated protein (MRP), is reported to be capable of mediating transmembrane excretion of a wide range of amphiphathic compounds, including bilirubin-, estrogen- and xenobiotic-glucuronide (Yamazaki et al., 1996). In the rat intestine, MRP2, localized in the apical domain of the enterocyte, is distributed in the proximal intestine (Mottino et al., 2001) and MRP3, localized in the basolateral domain, is distributed mainly in the ileum and colon (Rost et al., 2002). Intriguingly, the apical and basolateral directions of bisphenol A glucuronide excretion in our study parallels the distribution patterns of MRP2 and MRP3, respectively. Thus the supposition may be made that the elimination direction of bisphenol A glucuronide is governed by the distribution of an organic anion transporter system such as MRP.

Appearance of Serosal Bisphenol A in the Colon. As the lumen was the site into which large amounts of the bisphenol A glucuronide were eliminated in our study, presumably the excreted glucuronide would flow with the luminal contents into the distal intestine. In the colon, most likely the glucuronide would be deconjugated by lumen bacterial β-glucuronidase, an enzyme known to generate toxic and carcinogenic substances (Reddy et al., 1992). Deconjugation by lumen bacterial β-glucuronidase is known to be involved in the reactivation of an antitumor chemical derived from Irinotecan (Kaneda and Yokokura, 1990). Furthermore, a deglucuronidated Irinotecan derivative (SN-38 glucuronide) is reabsorbed in the distal intestine, where it damages the mucosa (Takasuna et al., 1996). In the light of these reports, the notable absorption and transport of unconjugated bisphenol A to the serosal side in the rat colon in our study suggests that the deconjugated bisphenol A is eventually reabsorbed by the colon. This proposition concurs with Upmeier et al. (2000), who recently, in a toxicokinetic study of bisphenol A in female DA/Han rats, have shown the possibility of enterohepatic recirculation and protracted absorption of bisphenol A from the intestinal tract. These findings bear out that, for ingested bisphenol A, the metabolism and disposition of the compound in the intestinal tract play a pivotal role in mediating the degree of toxic damage by the compound.

Generally, the paramount issue in the study of adverse effects of bisphenol A has to do with oral exposure to the chemical in low doses (Feldman, 1997). Rubin et al. (2001) has described adverse effects in rat offspring after maternal administration. Conversely, other studies have shown no adverse effects (Cagen et al., 1999; Ema et al., 2001). Thus, the toxicity of low doses of bisphenol A remains controversial. We believe that the sensitivity of an animal to ingested bisphenol A reflects the condition inside the intestine (e.g., the luminal contents and the types of bacterial flora). Further studies are required to clarify the correlation between catalytic reactivation of bisphenol A glucuronide by luminal flora and adverse effects caused by bisphenol A.

Conclusion

Because the intestine absorbs environmental estrogens introduced orally into the body, it is important to trace the fate of such compounds before inflow into the bloodstream. Evidence is accruing that most bisphenol A that is ingested is glucuronidated during its absorption by the small intestine and by its passage through the liver (Inoue et al., 2001). The present study has established that bisphenol A is excreted into the intestinal lumen as a glucuronide, bearing out that the gastrointestinal tract is a strategic pathway against invasion of bisphenol A at target organs such as the gonads and the brain. Further

work is warranted to determine the fate of the enteroluminal glucuronide in its complete pathway before excretion.

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Original

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Development of Analytical Method for Determining Trace Amounts of BPA in Urine Samples and Estimation of Exposure to BPA

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Abstract

We have developed a reliable analytical method for determining trace amounts of bisphenol A (BPA), a suspected endocrine disruptor, in urine samples by use of GC/MS so that daily exposure of human bodies to BPA can be estimated. We administered BPA-d_{16} (100 μg) to volunteers in order to conduct an excretion experiment and found that the BPA was absorbed quickly through the digestive tract and excreted mainly as a glucuronide conjugate into urine, in an amount of almost 100 % in 24 hours. The results suggest that determining the BPA content of urine samples enables estimation of the exposure to BPA.

The results of our analysis of urine from adults show that the average total BPA concentration was 0.82 ng/mℓ (0.14~5.47 ng/mℓ, n=91) and that the average free BPA concentration was 0.08 ng/mℓ (0.01~0.27 ng/mℓ, n=11). Also, from a determination of whole-day urine samples, the exposure to BPA was estimated to be 1.68 μg/day (0.48~4.5 μg/day, n=22) on the average.

Key words: BPA, endocrine disruption, GC/MS, exposure

INTRODUCTION

Bisphenol A [2,2-bis(4-hydroxyphenyl)propane; BPA], a suspected endocrine disruptor, has been widely used in large amounts as a raw material for polycarbonate, epoxy resin, phenol resin, polyester, antioxidant, and a stabilizer for vinyl chloride. This has led to a great concern about human exposure to BPA and potential effects on the human body. BPA does not exhibit high acute toxicity; LD_{50} in rats is 3250 mg/kg (oral)[1]. Some reports have stated that BPA is an endocrine disruptor: BPA shows a weak estrogenic activity in an E-SCREEN test on the cellular level[2], and promotes the multiplication of human MSF-7 breast cancer cells[3]. In animal tests, upon exposure to BPA female mice show a decrease in the number of litters delivered and the number of offspring per litter, as well as a decrease in birth weight, and male mice show a decrease in the number of offspring, an increase in the weight of seminal vesicle, and a deterioration in the motility of sperm cells[4].

Conventionally, exposures to a substance via differ-

ent pathways are estimated from the concentrations of the substance in food, air, water, and the like, as well as the amounts of intake, and the estimated amounts are added together in order to estimate the amount of exposure to a chemical substance. However, the conventional method has a drawback in that a large amount of work is required. If the exposure to BPA can be estimated from the amount of excreted BPA in urine, the amount of work and cost will be substantially reduced.

Reported methods for determining BPA include GC/MS[5-8], LC-MS[9-12], HPLC[13,14], and ELISA[15]. However, most of the instruments used for collection, storage, and clean-up of samples are composed of high polymer materials. Thus they may contaminate samples, resulting in an overestimation when trace amounts of BPA in biological samples are to be determined. Accordingly, urgent demand exists for a highly reliable and sensitive analytical method for determining BPA that is free from the risk of contamination.

In our method, the BPA in urine is subjected to an enzymolysis and then to a solid phase extraction by use of a C_{18} cartridge. The extract is trimethylsilylated (TMS), and the TMS derivative obtained is purified with a florisil cartridge, and then determined using GC/MS-SIM. We aim to estimate the human daily exposure to BPA by determining the concentration and amount of excreted BPA in urine samples by employing our method.

EXPERIMENTAL SECTION

Reagents

BPA was a product of Wako Pure Chemicals. ^{13}C-BPA was purchased from Cambridge Isotope. Purified water was obtained by filtering super pure water through an activated charcoal cartridge. (β-Glucuronidase was a product of Wako Pure Chemicals intended for biochemical use. C_{18} cartridge used was a Supelclean ENVI-18 (0.5 g) from Supelco, and the florisil cartridge was a Supelclean ENVI-florisil (0.5 g) from Supelco. BSTFA (*N,O*-bis[trimethylsilyl]trifluoroacetamide) was purchased from GL Sciences, Inc. Methanol, ethyl acetate, *n*-hexane, and acetone were products of Kanto Kagaku intended for analysis of agricultural chemical residues. Phosphoric acid was a product of Wako Pure Chemicals of special grade.

Apparatus

The apparatus used were an ultrasonic washer (Shibata SU-3TH), a vacuum pump (Yamato WP-25), a solid phase extraction equipment (Spelco VISIPREP DL), a super pure water production equipment (Millipore Milli-Q-ST), and a GC/MS (JEOL GC-mate).

Collection of urine samples

All the urine samples were collected after the procedure of informed consent.

Exposure experiment: A subject orally consumed 100 mℓ of a drink containing 100 μg of BPA-d_{16}, and urine samples were collected at predetermined intervals for 26.5 hours after intake (n=1). Twelve male and thirteen female volunteers each orally consumed 100 mℓ of a drink containing 50 μg of BPA-d_{16}, and urine samples were collected for 5 hours after intake.

Urine samples were collected from 46 male and 23 female volunteers at least twice, and samples from the same subject were combined.

Whole-day urine samples were collected from 11 male and 11 female volunteers.

Method

Total BPA: 100 mℓ of a urine sample was placed in a conical flask equipped with a ground stopper, and 100 μℓ of β-glucuronidase solution and 0.1 μg of ^{13}C-BPA were added to the flask. The mixture was subjected to an enzymolysis at 37 ℃ for 90 minutes. To the resultant mixture, 1 mℓ of 7.5 M phosphoric acid was added in order to adjust pH to 3 or lower. The mixture thus obtained was loaded onto a C_{18} cartridge that had been activated with 5 mℓ of methanol and 10 mℓ of purified water, to extract BPA. After the cartridge was washed with 10 mℓ of 10 % methanol, 3 mℓ of methanol was added to elute BPA. The eluate was collected in a 100 mℓ eggplant-shaped flask. To the eluate, 20 mℓ of ethyl acetate was added, and concentrated to dryness by use of a rotary evaporator. To the flask, 200 μℓ of BSTFA and 2 mℓ of acetone were added, and the mixture was allowed to stand overnight to be trimethylsilylated, and the stripped of acetone by use of a rotary evaporator. To the resultant mixture, 2 mℓ of *n*-hexane was added and dissolved by use of an ultrasonic washer. The resultant mixture was loaded onto a florisil cartridge that had been prewashed with 5 mℓ of *n*-hexane, and the eluate was collected in a test tube. Subsequently, the flask was

washed twice with 2 mℓ of *n*-hexane each time, and the mixtures obtained were loaded onto the cartridge. The eluate was added to the previously obtained eluate in a test tube. The resultant eluate was concentrated to 1 mℓ by blowing nitrogen gas, and then subjected to an analysis by GC/MS-SIM.

Free BPA : To 200 mℓ of a urine sample, which had not been subjected to enzymolysis, 2 mℓ of 7.5 M phosphoric acid and 0.05 μg of ^{13}C-BPA were added. The subsequent procedure was the same as that described above for total BPA.

Calibration curve: BPA was placed in test tubes stepwise in an amount of 10~200 ng, and 100 ng of ^{13}C-BPA was added as a surrogate, and then 200 μℓ of BSTFA was added. The volume of the resultant mixture was increased to 1 mℓ by adding acetone. The mixture obtained was allowed to stand overnight and then subjected to a GC/MS-SIM. A calibration curve was constructed on the basis of area ratios relative to ^{13}C-BPA.

GC/MS conditions

GC separation was carried out with an HP-5890 series Ⅱ. GC conditions were as follows: column DB-5MS, inner diameter 0.32 mm, length 30 m, film thickness 0.25 μm; column temperatures 70 ℃ (2 min) —20 ℃ /min—150 ℃—10 ℃ /min—300 ℃ (5 min), inlet port temperature 250 ℃; carrier gas He, flow velocity 1 mℓ/min; injection method splitless, purge off 1 min.

MS analysis was carried out with an JEOL GC-mate. The SIM conditions were as follows: ion source temperature 230 ℃; ionization voltage 70V; monitor ions (m/z), BPA (357, 372), BPA-d_{16} (368), and ^{13}C-BPA (369).

RESULTS AND DISCUSSION

Enzymolysis of conjugates

Insoluble compounds taken into the body are converted into highly water-soluble glucuronide conjugates during the metabolic process before excretion. Phenol compounds are believed to be excreted mainly as a glucuronide conjugate. Thus, we studied the amount of β-glucuronidase and the incubation time required for decomposition of the glucuronide conjugate of BPA. The results show that the decomposition was almost completed by addition of 50 μℓ of β-glucuronidase to 100 mℓ of urine followed by incubation at 37 ℃ for 60 minutes. In our analysis using real samples, we chose to add 100 μℓ of β-glucuronidase and perform enzymolysis at 37 ℃ for 90 minutes, in order to provide a margin of safety.

Extraction, washing, and elution using the C_{18} cartridge

Phenol compounds are generally subjected to solid phase extraction at a pH of 3 or lower. The C_{18} cartridge used in our analysis allows extraction under this condition. In our analysis, after the cartridge was washed with 10 % methanol, an addition of 3 mℓ of methanol allowed elution of BPA. No elution of BPA from the cartridge was observed.

Trimethylsilylation of BPA

Since BPA is absorbed in a GC column when subjected to direct analysis at low concentrations, determination of BPA at extremely low concentrations is difficult and requires derivatization. Commonly used forms of derivatization include pentafluorobenzylization (PFB) and trimethylsilylation (TMS). For our analysis, since we use urine samples which can be collected in a large amount, we chose TMS. Since urine samples are considered to contain a variety of substances that act on BSTFA, 200 μℓ of BSTFA was added to each sample, and the resultant mixture was allowed to stand overnight. Fig. 1 shows the mass spectrum of BPA-TMS, and Fig. 2 shows the mass spectrum of ^{13}C-BPA-TMS.

Clean-up using the florisil cartridge

Since urine samples contain a variety of metabolites, trimethylsilylation of extracts was found to produce a tar-like substance and require a clean-up treatment for determination. We studied a clean-up using a florisil cartridge and found that *n*-hexane caused trimethylsilylated compounds of BPA to elute easily and that clean-up was achieved by merely allowing a reagent dissolved in *n*-hexane to pass through the cartridge.

Excretion of BPA

In order to study the kinetics of excretion of BPA, 100 mℓ of a drink containing 100 μg of BPA-d_{16} was administered, and urine samples were collected at predetermined intervals for 26.5 hours after the intake. The results are shown in Fig. 3. The concentration of BPA in urine reached 90 ng/mℓ after 30 minutes, then

decreased to 26 ng/mℓ 60 minutes after intake, and 5 hours after intake, the concentration further decreased to the proximity of the concentration before the intake. BPA was found to be absorbed quickly through the digestive tract and excreted mainly as a glucuronide conjugate into urine in almost 100 % in 24 hours.

BPA concentrations in urine

The average BPA concentration in urine samples was 0.81 ng/mℓ (0.14~5.47 ng/mℓ), and three subjects had a concentration higher than 2 ng/mℓ. The subject who had the highest concentration, 5.47 ng/mℓ, had an extremely small amount of urine. Fig. 4 shows an SIM chromatogram of urine sample and Fig. 5 shows a histogram which has a bell shape with the mean almost at the center. The analysis of whole-day urine samples (Table 1) shows that the average concentration of BPA was 0.81 ng/mℓ (0.24~2.03 ng/mℓ),

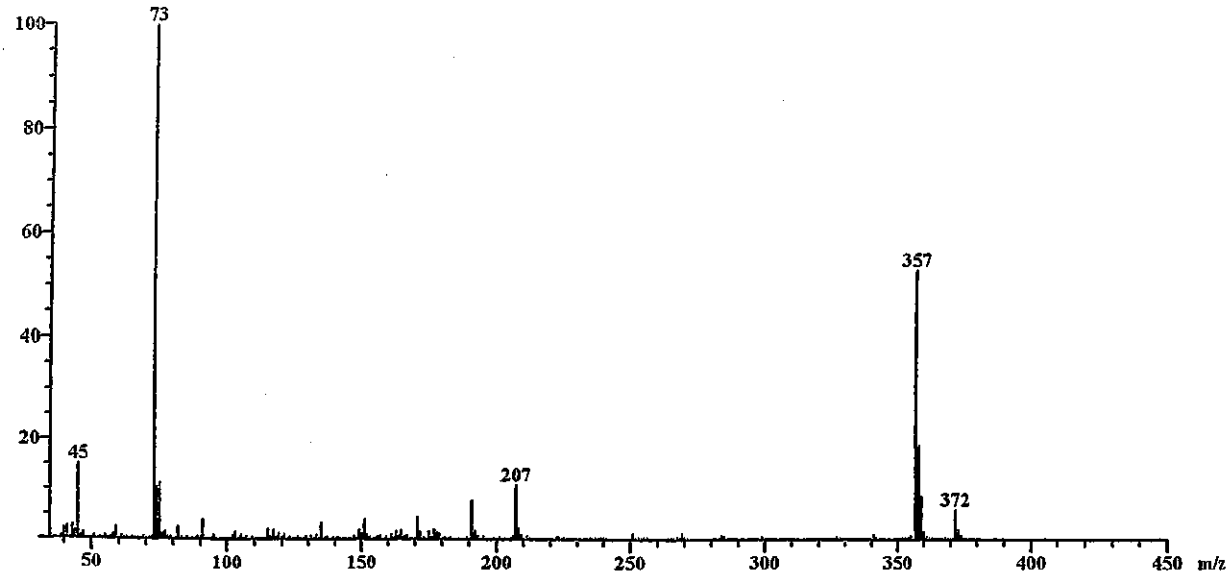

Fig. 1 EI mass spectrum of the BPA derivative. Measurement conditions: column DB-5MS, inner diameter 0.32 ㎛, length 30 m, film thickness 0.25 ㎛; column temperature 70℃ (2min)-20℃/min-150℃-10℃/min-300℃(5min); carrier gas He at a flow rate of 1 mℓ/min; ion source temperature 230℃: ionization voltage 70V

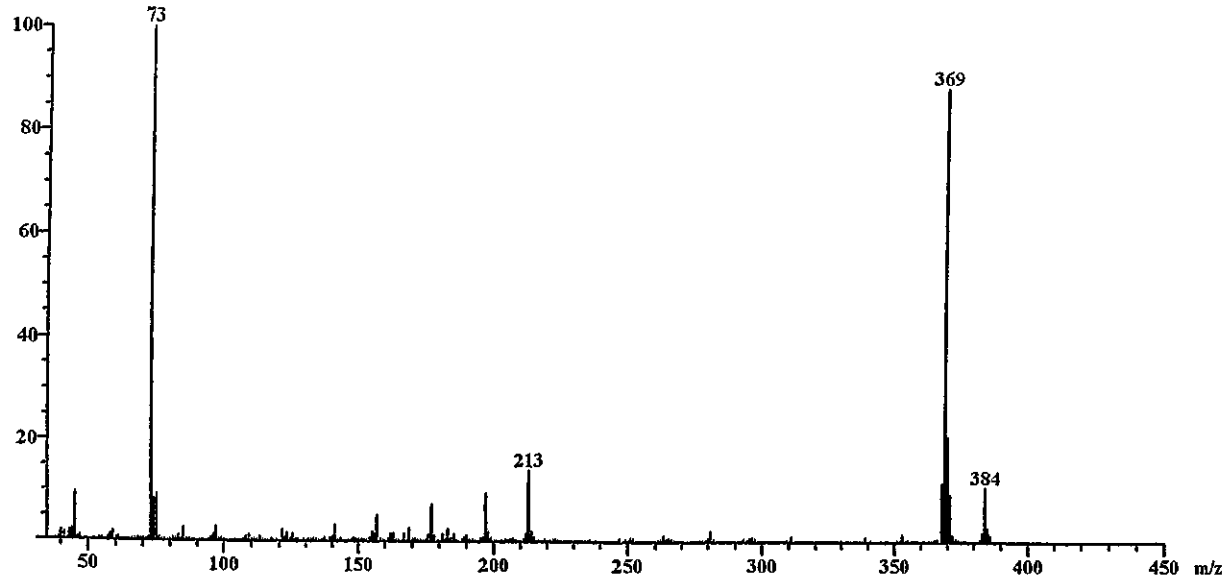

Fig. 2 EI mass spectrum of the ^{13}C-BPA derivative. Measurement conditions were the same as Fig. 1

the average amount of urine was 2055 mℓ (1030～3900 mℓ), and that the average amount of excreted BPA was 1.68 μg (0.48～4.5 μg/day). The amount of excreted BPA was found to be significantly less than the amount we had expected. This may be due to a drop in exposure to BPA achieved by an improvement in inner coatings of cans and the like. The excreted BPA is considered to be derived from food, but the type of food it is derived from remains unknown.

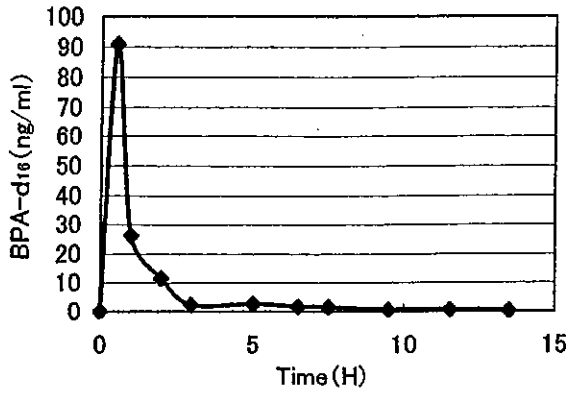

Fig. 3 Relationship between the concentration of the total BPA-d_{16} in urine and the elapsed time after 100 mℓ of a drink containing 100 μg of BPA-d_{16} was consumed

The conjugates of BPA and free BPA are considered to have different endocrine disrupting effects. Thus, determination of free BPA in urine was required. A drink containing 50 μg of BPA-d_{16} was given to volunteers, and urine samples were collected for 5 hours after intake. The results are shown in Fig. 6. The average total BPA concentration was 57.2 ng/mℓ (26.5～80 ng/mℓ), and the average free BPA concentration was 1.13 ng/mℓ (0.13～5.8 ng/mℓ). The average amount of excreted BPA was 38 μg (17.6～48.6 (μg), which means that 76 % of the given BPA was excreted after 5 hours. The average ratio of free BPA was 2.0 % (0.34～8.1 %). The subjects who showed higher ratios of free BPA may have been affected by their body conditions; in particular, liver function on the day of the experiment. Normal urine samples were also analyzed. The average total BPA concentration was 0.56 ng/mℓ (0.19～1.38 ng/mℓ), and the average free BPA concentration was 0.08 ng/mℓ (0.01～0.27 ng/mℓ). The average ratio of free BPA was 12 % (2.6

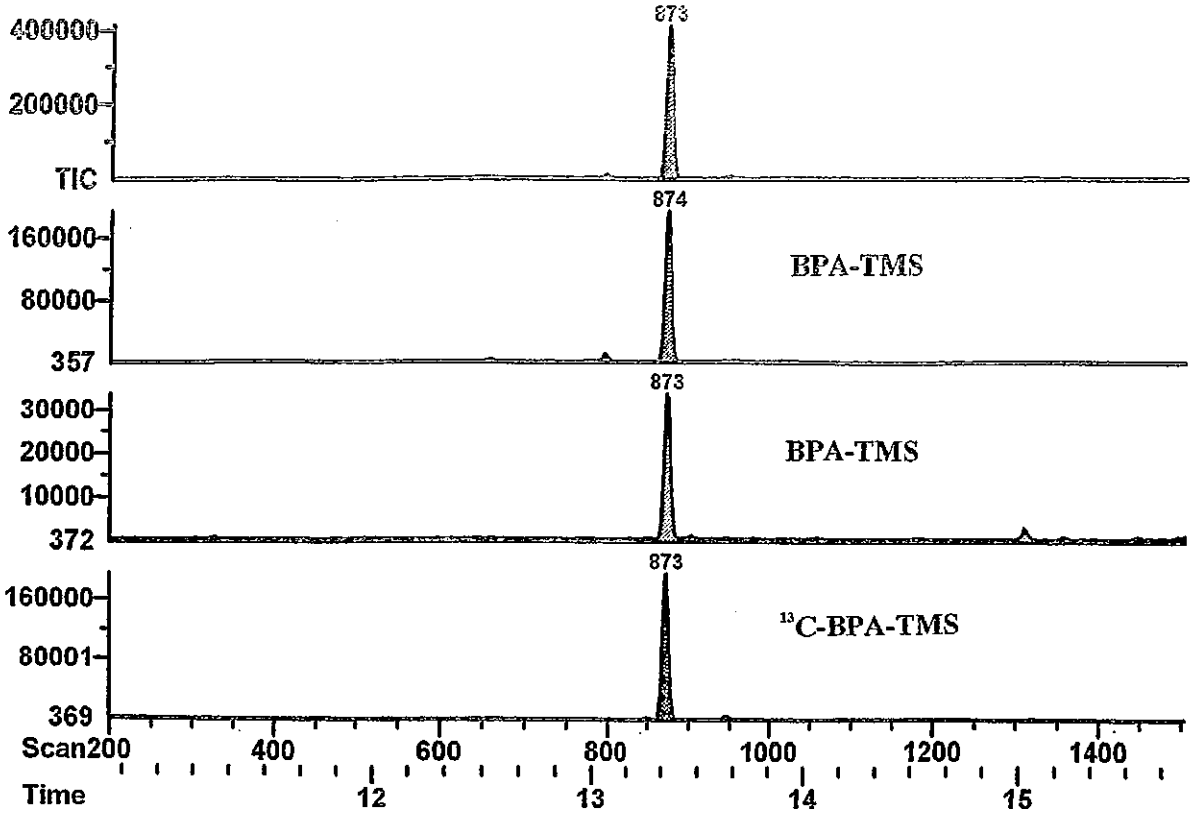

Fig. 4 SIM chromatogram of a real urine sample. Measurement conditions were the same as given in 2.5 GC/MS conditions

~29 %).

CONCLUSION

1. We developed a reliable analytical method for determining trace amounts of BPA in urine.
2. We conducted an excretion experiment by administering 100 μg of BPA-d_{16} to volunteers and found that BPA was absorbed quickly through the digestive tract and excreted mainly as a glucuronide conjugate into urine, in almost 100 % in 24 hours. Thus, the determination of the BPA in whole-day urine samples is considered to enable the estimation of the exposure to

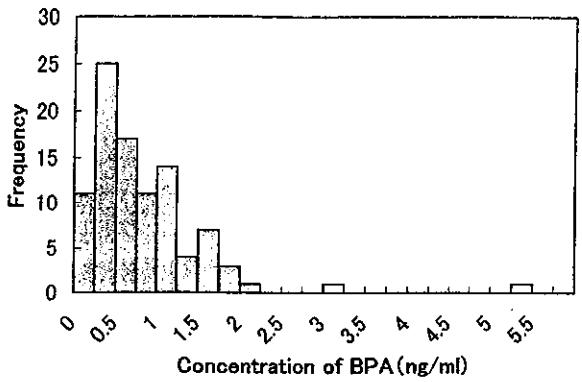

Fig. 5 Histogram of the BPA in urine samples (n =91)

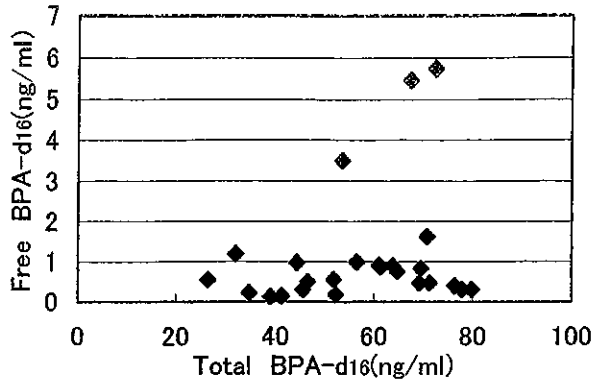

Fig. 6 Relationship between the concentrations of the total BPA-d_{16} and the free BPA-d_{16} in urine after 100 mℓ of a drink containing 50 μg of BPA-d_{16} was consumed

Table 1 Results of whole day urine samples

No.	Volume(ml)	BPA(ng/ml)	BPA(μg/day)
1	1567	0.36	0.56
2	1832	1.20	2.20
3	1846	0.57	1.05
4	1030	1.17	1.21
5	1910	0.64	1.22
6	2271	0.27	0.61
7	2125	0.58	1.23
8	2586	1.28	3.31
9	1296	0.37	0.48
10	2293	0.24	0.55
11	2137	0.82	1.75
12	1146	0.80	0.92
13	2664	0.43	1.15
14	1903	2.03	3.86
15	2800	1.57	4.40
16	2120	1.20	2.54
17	1950	0.93	1.81
18	1843	0.45	0.83
19	1460	0.62	0.91
20	1490	0.40	0.60
21	3060	1.48	4.53
22	3900	0.34	1.33
Ave.	2055±665	0.81±0.49	1.68±1.26

BPA.

3. The average total BPA concentration in urine samples was 0.82 ng/mℓ (0.14～5.47 ng/mℓ, n=91), and the average free BPA concentration was 0.08 ng/mℓ (0.01～0.27 ng/mℓ, n=11).

4. From the determination of whole-day urine samples, the exposure to BPA was estimated to be 1.68 μg/day (0.48～4.5 μg/day, n=22) on the average.

ACKNOWLEDGMENT

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Note

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GC及びGC/MSによる文具、化粧品、家庭用品等に含まれる可塑剤フタル酸及びアジピン酸エステル類の分析

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Analysis of Plasticizer Phthalate and Adipate Esters in Stationeries, Cosmetics, and Household Commodities by GC and GC/MS

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Abstract

Fourteen plasticizers (12 phthalates and 2 adipates) were analysed in commercially available stationeries, cosmetics, and household commodities by GC and GC/MS. Among 66 samples, dibutyl phthalate (DBP), di (2-ethylhexyl) phthalate (DEHP), diheptyl phthalate (DHpP), diisononyl phthalate (DINP), and di (2-ethylhexyl) adipate (DEHA) were detected in 32 samples (eraser: 20 samples, plastic bags for fiber-tipped pen: 4 samples, soft floor carpet: 5 samples, plastic tile: 1 sample, and plastic glove: 2 samples). Among them, 15 samples contained two different kinds of the plasticizers and 4 samples have three different kinds of the plasticizers. Erasers contained the plasticizers with high frequency of 95 % (20/21) and high concentrations ranging from 2.4 % to 35.2 %, and plastic bags showed frequency of 80 % (4/5) and the concentrations around 4.4-18.0 %.

Keywords：フタル酸エステル、アジピン酸エステル、文房具、化粧品
GC/MS; phthalate; adipate; stationery; cosmetic

I　緒言

フタル酸エステル及びアジピン酸エステル類はプラスチック製消しゴムの可塑剤として、あるいは化粧品、インク、接着剤、塗料等の成分保留剤として使用され[1-3]、我々の生活環境中に氾濫している[4,5]。これらの可塑剤の安全性に関する最近の研究ではげっ歯目類等の動物実験において、肝細胞内ペロオキシソーム増殖作用[6-8]、催奇形性[9-11]、アンドロゲン依存性の組織[12,13]への影響が報告されている。一方、これらの可塑剤は消化管、肺及び皮膚から速やかに吸収され[14]、特に側鎖の短いものは皮膚吸収性が高いとされていることから、我々はこれら可塑剤の暴露を日常的に受けているものと考えられる。したがって、これら文具、化粧品、家庭用品等からの可塑剤の暴露量を早急に把握する必要がある。しかし、現在までのところ、文具等をはじめとしたこれらのものに含まれる可塑剤の質的及び量的な調査は実施されていないので、早急な実施が必要と考えられる。そこで、文具、化粧品、家庭用品等中に含まれるフタル酸エステル及びアジピン酸エステル類を分析し、これら可塑剤の含有実態を調査することとした。

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II 実験

1. 試薬および標準溶液

n-ヘキサンは残留農薬測定用2000(和光純薬製)を、アセトンはフタル酸エステル測定用(関東化学製)を用いた。フタル酸ジエチル(DEP)、フタル酸ジプロピル(DPrP)、フタル酸ジイソブチル(DIBP)、フタル酸ジブチル(DBP)、フタル酸ジペンチル(DPeP)、フタル酸ブチルベンジル(BBP)、フタル酸ジヘキシル(DHxP)、フタル酸2-エチルヘキシル(DEHP)、フタル酸ジシクロヘキシル(DcHP)、フタル酸ジヘプチル(DHpP)、フタル酸ジオクチル(DOcP)、フタル酸ジイソノニル(DINP)、アジピン酸2-エチルヘキシル(DEHA)、アジピン酸ジイソノニル(DINA)については、すべて環境分析用(関東化学製)を用いた。フタル酸エステル類及びアジピン酸エステル類の標準溶液は、それぞれの濃度が1 μg/mLになるようにn-ヘキサンで調製し、それを適宜n-ヘキサンで希釈して用いた。

2. 試薬

名古屋市内の小売店で購入した文具49検体、化粧品9検体、家庭用品8検体を用いた。

3. 試験溶液の調製

クッションフロアはその表面をはぎ取ったのち5 mm角に細切し、消しゴム等その他の固形試料はそのまま5 mm角に細切し、いずれもその2.5 gをあらかじめアセトン及びn-ヘキサンで洗浄した50 mLメスフラスコに秤量した。n-ヘキサン40 mLを加え、一昼夜放置し、メスアップした。

インク、マニキュア等の液状試料は、あらかじめアセトン及びn-ヘキサンで洗浄した直径125 mmのガラス製シャーレに直径55 mmのNo.5Aのろ紙を置き、その上に0.5 gの試料を薄くのばした。その後、シャーレに20 mLのn-ヘキサンを加え、一昼夜放置し、あらかじめアセトン及びn-ヘキサンで洗浄した20 mLメスフラスコにn-ヘキサン溶液を移し、メスアップした。

4. 分析条件

4.1 GC条件

装置:GC-14B(FID検出器付、島津製作所製)
カラム:DB-5(0.53 mm×30 m、1.5 μm、J&W製)
カラム温度:200℃から280℃まで毎分5℃で昇温後、280℃で4分間保持した。
注入口及び検出器温度:280℃
キャリアガス:窒素、40 mL/min

4.2 GC/MS条件

装置:AUTO MASS SYSTEM II(日本電子製)カラム:HP-5MS(0.25 mm×30 m、0.25 μm、ヒューレットパッカード製)
カラム温度:150℃で1分間保持後、280℃まで毎分10℃で昇温し、280℃で5分間保持した。
イオン源温度:210℃ イオン化:EI イオン化電圧:70 eV検出方法:スキャン法(m/z 50-500) 試料注入方式:スプリットレス(1分間)

III 結果と考察

1. GCによる定量及びGC/MSによる同定

Table 1に示す14種の可塑剤(フタル酸エステル:12種類、アジピン酸エステル:2種類)の標準品を、実験の部に示すGC条件下で分析した。それらの保持時間はTable 1に、また検出頻度の高いフタル酸エステルとアジピン酸エステルのクロマトグラムをFig. 1Aに示した。検量線は50 μg/mLから2000 μg/mLの間で良好な直線性を示し、文具等の分析を行うために十分な定量性が得られたので、以下の研究では実験の部に示すGC条件を用いて可塑剤を定量することとした。

また、検出された可塑剤を同定するために実験の部に示すGC/MS条件下で14種の可塑剤の標準品を分析したところ、すべてのマススペクトルからモノエステル体に由来するイオン(DHpP:m/z 265、DBP:m/z 223、DEHP:m/z 279、DINP:m/z 293、DEHA:m/z 241)と、無水フタル酸に由来するイオン(DHpP、DBP、DEHP、DINP:m/z 149、DEHA:m/z 129)が観察され、良好に同定できることが判明した。したがって、以下の研究ではこのGC/MS条件を用いて可塑剤を同定することとした。

Table 1 Retention times of phthalates and adipates

Plasticizers	Retention time (min)
Diethyl phthalate (DEP)	1.27
Dipropyl phthalate (DPrP)	2.24
Diisobutyl phthalate (DIBP)	2.83
Dibutyl phthalate (DBP)	3.41
Dipentyl phthalate (DPeP)	5.27
Butyl benzyl phthalate (BBP)	7.41
Dihexyl phthalate (DHxP)	7.41
Diheptyl phthalate (DHpP, 4 peaks)	7.90-9.19
Di (2-ethylhexyl) adipate (DEHA)	8.00
Di (2-ethylhexyl) phthalate (DEHP)	9.88
Dicyclohexyl phthalate (DcHP)	9.68
Diisononyl adipate(DINA)	10.96
Diisononyl phthalate (DINP, 8 peaks)	12.00-14.42
Dioctyl phthalate (DOcP)	12.15

2. 文具、化粧品、家庭用品等の分析

市販の消しゴム等文具49検体、マニキュア等化粧品9検体、クッションフロア等家庭用品8検体について、実験の部に示す方法で、14種類の可塑剤(フタル酸エステル:12種類、アジピン酸エステル:2種類)を分析した。代表的な検体のクロマトグラムをFig. 1B～Eに、それらのマススペクトルをFig. 2に示した。GCクロマトグラム上のピークは、対応する可塑剤の保持時間に完全一致し、マススペクトルはそれぞれの可塑剤に由来するモノエステル体及び無水体のイオンが出現していたことから、それぞれのピークはFig. 1のクロ

マトグラム上に表記した可塑剤であると同定された。

定量結果はTable 2にまとめたが、66検体中32検体からフタル酸エステル及びアジピン酸エステルが検出された。その内訳は、消しゴムは21検体中20検体から、マーカーペンの透明ケース等5検体中4検体から、また、クッションフロア(5検体)、ビータイル(1検体)及び手袋(2検体)、からは全検体から検出された。全66検体中15検体(消しゴム：13検体、透明ケース：1検体、ビータイル：1検体)からは同時に2種類の、3検体(消しゴムのみ)からは3種類の可塑剤が検出された。

検出された可塑剤の種類としてはDEHPが最も高頻度に、66検体中30検体(消しゴム：18検体、透明ケース：4検体、クッションフロア：5検体、ビータイル：1検体、手袋：2検体)、かつ高濃度(3.6～39.5%；平均値：23.5%、中央値：25.1%)に検出された。次いで高頻度に検出されたのはDBP(14検体；消しゴムのみ)で、その濃度は5.1～19.1%(平均値：12.4%、中央値：12.5%)、DHpPが7検体(消しゴム：6検体、ビータイル：1検体)から2.0～35.2%(平均値：15.5%、中央値：11.7%)の濃度で検出された。また、DEHAとDINPが共に1検体(DEHA：透明ケース、DINP：消しゴム)から、それぞれ4.4%、6.9%の濃度で検出された。

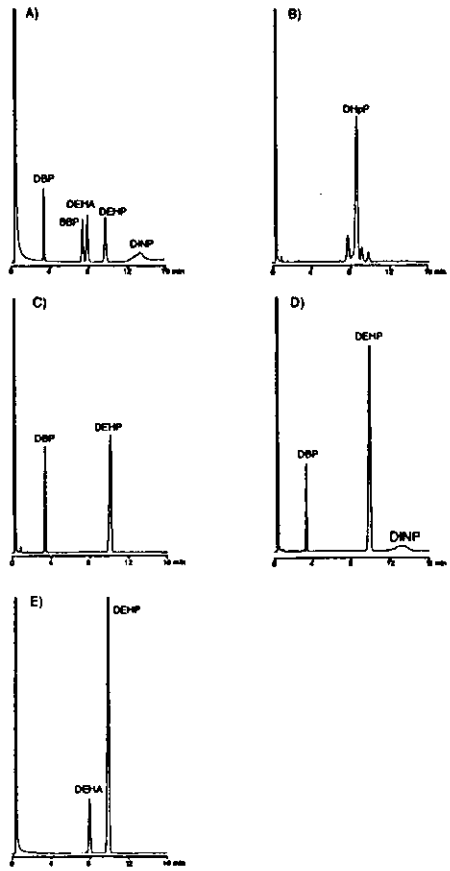

Fig. 1 Gas chromatograms of the extracts of the stationeries
A) Standards
B) Eraser 7
C) Eraser 10
D) Eraser 13
E) Fiber-tipped pen 1 (plastic bag)

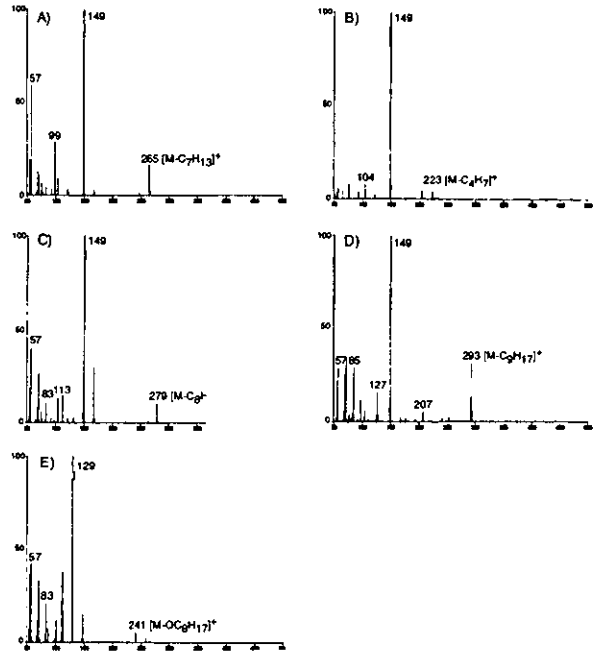

Fig. 2 Mass spectra of phthaltes and adipate in the stationeries
A) DHpP (eraser 7)
B) DBP (eraser 10)
C) DEHP (eraser 10)
D) DINP (eraser 13)
E) DEHA (fiber-tipped pen 1, plastic bag)

また、品目別の検出状況から、消しゴムには高頻度(95%、20/21)かつ高濃度(50%前後)にこれらの可塑剤が含有されていることが判明した。透明ケース類では80%の頻度(4/5)で15%前後の濃度の可塑剤が、手袋(2/2)及びクッションフロアでは全検体(5/5)から25から40%の濃度の可塑剤が検出された。

Ⅳ　結論

以上のように、我々の生活環境中に、多くの可塑剤を含んだ製品が氾濫していることが明らかとなり、これらの製品から可塑剤が、微量ながらも長期間にわたり生体内に取り込まれる可能性は否定できない。これらの可塑剤が及ぼす生体影響については、生体における変化が顕著に現れないため、その因果関係を直ちに立証することは困難であるが、さらに多岐にわたる暴露量評価と毒性評価及び疫学調査に基づく学際研究が必要と考えられる。

Ⅴ　謝辞

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Table 2 Concentration of phthalates and adipates in stationeries, cosmetics, and household commodities (%)

No	Sample	DBP	DEHA	DHpP	DEHP	DINP
1	Eraser 1	11.1	ND	ND	30.5	ND
2	Eraser 2	18.3	ND	ND	33.0	ND
3	Eraser 3	12.4	ND	ND	29.4	ND
4	Eraser 4	16.1	ND	ND	27.9	ND
5	Eraser 5	17.3	ND	ND	27.1	ND
6	Eraser 6	ND	ND	ND	ND	ND
7	Eraser 7	ND	ND	35.2	ND	ND
8	Eraser 8	4.1	ND	9.3	23.7	ND
9	Eraser 9	ND	ND	33.7	ND	ND
10	Eraser 10	15.8	ND	ND	29.0	ND
11	Eraser 11	ND	ND	2.4	30.7	ND
12	Eraser 12	17.9	ND	ND	33.1	ND
13	Eraser 13	7.0	ND	ND	27.4	6.9
14	Eraser 14	12.7	ND	ND	29.3	ND
15	Eraser 15	12.5	ND	ND	29.6	ND
16	Eraser 16	6.5	ND	5.9	23.9	ND
17	Eraser 17	5.1	ND	14.0	16.0	ND
18	Eraser 18	ND	ND	ND	19.1	ND
19	Eraser 19	ND	ND	ND	18.9	ND
20	Eraser 20	ND	ND	ND	18.9	ND
21	Eraser 21	19.1	ND	ND	23.7	ND
22	Clay 1	ND	ND	ND	ND	ND
23	Clay 2	ND	ND	ND	ND	ND
24	Clay 3	ND	ND	ND	ND	ND
25	Crayone 1	ND	ND	ND	ND	ND
26	Crayone 2	ND	ND	ND	ND	ND
27	Crayone pastel 1	ND	ND	ND	ND	ND
28	Crayone pastel 2	ND	ND	ND	ND	ND
29-1	Ball point pen (body)	ND	ND	ND	ND	ND
29-2	Ball point pen (ink)	ND	ND	ND	ND	ND
30	Ball point pen (ink)	ND	ND	ND	ND	ND
31-1	Fiber-tipped pen 1 (plastic bag)	ND	4.4	ND	12.4	ND
31-2	Fiber-tipped pen 1 (body)	ND	ND	ND	ND	ND
31-3	Fiber-tipped pen 1 (ink)	ND	ND	ND	ND	ND
32-1	Fiber-tipped pen 2 (plastic bag)	ND	ND	ND	ND	ND
32-2	Fiber-tipped pen 2 (body)	ND	ND	ND	ND	ND
32-3	Fiber-tipped pen 2 (ink)	ND	ND	ND	ND	ND
33-1	Fiber-tipped pen 3 (plastic bag)	ND	ND	ND	13.1	ND
33-2	Fiber-tipped pen 3 (body)	ND	ND	ND	ND	ND
33-3	Fiber-tipped pen 3 (ink)	ND	ND	ND	ND	ND
34-1	Fiber-tipped pen 4 (plastic bag)	ND	ND	ND	14.2	ND
34-2	Fiber-tipped pen 4 (body)	ND	ND	ND	ND	ND
34-3	Fiber-tipped pen 4 (ink)	ND	ND	ND	ND	ND
35	Fiber-tipped pen 5 (ink)	ND	ND	ND	ND	ND
36	Paints	ND	ND	ND	ND	ND
37	Colored pencil	ND	ND	ND	ND	ND
38	Pencil cap	ND	ND	ND	ND	ND
39-1	Pencil box (cover, outside)	ND	ND	ND	18.0	ND
39-2	Pencil box (cover, inside)	ND	ND	ND	16.1	ND
39-3	Pencil box (card holder)	ND	ND	ND	10.8	ND
39-4	Pencil box (pencil holder)	ND	ND	ND	ND	ND
39-5	Pencil box (body)	ND	ND	ND	ND	ND
40	Paste	ND	ND	ND	ND	ND
41	Paste	ND	ND	ND	ND	ND
42	Scissors (handle)	ND	ND	ND	ND	ND
43	Scotch tape	ND	ND	ND	ND	ND
44	Ruler	ND	ND	ND	ND	ND
45	Celluloid sheet 1	ND	ND	ND	ND	ND
46	Celluloid sheet 2	ND	ND	ND	ND	ND
47	Celluloid sheet 3	ND	ND	ND	ND	ND
48	Celluloid sheet 4	ND	ND	ND	ND	ND
49	Celluloid sheet 5	ND	ND	ND	ND	ND
50	Lipstick 1	ND	ND	ND	ND	ND
51	Lipstick 2	ND	ND	ND	ND	ND
52	Lipstick 3	ND	ND	ND	ND	ND
53	Nail color 1	ND	ND	ND	ND	ND
54	Nail color 2	ND	ND	ND	ND	ND
55	Nail color 3	ND	ND	ND	ND	ND
56	Nail color 4	ND	ND	ND	ND	ND
57	Powdery cosmetic	ND	ND	ND	ND	ND
58	Mascara	ND	ND	ND	ND	ND
59	Soft floor carpet 1	ND	ND	ND	27.0	ND
60	Soft floor carpet 2	ND	ND	ND	26.2	ND
61	Soft floor carpet 3	ND	ND	ND	23.8	ND
62	Soft floor carpet 4	ND	ND	ND	26.8	ND
63	Soft floor carpet 5	ND	ND	ND	27.0	ND
64	Plastic tile	ND	ND	2.0	3.6	ND
65	Plastic glove 1	ND	ND	ND	39.5	ND
66	Plastic glove 2	ND	ND	ND	22.8	ND

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