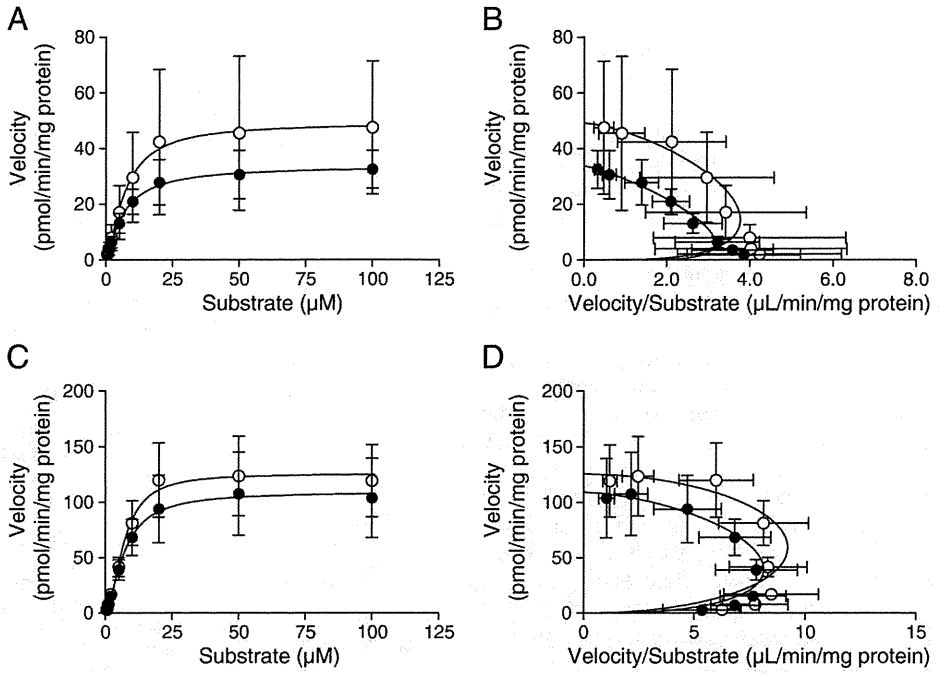

Fig. 5. Kinetics for SN-38 glucuronidation by liver microsomes and insect cell membranes expressing UGT1A1s of humans and cynomolgus monkeys. Substrate concentrations were 0.5–100 μM. Each point represents the mean ± SD of three separate experiments derived from independent preparations. (A) [*S*]–[*V*] plots for liver microsomes; (B) [*V*/*S*]–[*V*] plots for liver microsomes; (C) [*S*]–[*V*] plots for insect cell membranes expressing UGT1A1s; (D) [*V*/*S*]–[*V*] plots for insect cell membranes expressing UGT1A1s. ○, human liver microsomes or humUGT1A1; ●, cynomolgus monkey liver microsomes or monUGT1A1.

Although we do not have evidence of the effect of alamethicin on the kinetic profile of glucuronidation, the optimum concentration of 7-HFC glucuronidation for human liver microsomes differed among substrate concentrations in our preliminary study. Therefore, to evaluate uniformly the results of kinetic analysis, the glucuronidation activities toward 7-HFC, estradiol and SN-38 in human liver microsomes and recombinant UGT1A without alamethicin were determined in this study.

7-HFC glucuronidation in humans has been reported to be catalyzed by several UGT1A and UGT2B isoforms, except UGT1A4, although there is no information on the kinetics (Ghosal et al. 2004; Kaku et al. 2004). These reports showed that UGT1A6 and UGT1A9 have higher activities among UGT isoforms expressed in the liver, and the activity of UGT1A1 is 12–32% that of UGT1A6 and UGT1A9. In this study, the kinetics of 7-HFC glucuronidation by human liver microsomes was biphasic, and the K_m value of recombinant humUGT1A1 was remarkably low compared with those of high- and low-affinity phases, supporting the observation of the 7-HFC activity of each UGT isoform in previous reports (Ghosal et al. 2004; Kaku et al. 2004). In contrast, the kinetics of 7-HFC glucuronidation by cynomolgus monkey liver microsomes was monophasic, followed by the Michaelis–Menten model, and the expression profile of UGT isoforms in the liver was considered to be extensively different between humans and cynomolgus monkeys. In recombinant UGT1A1s, the glucuronidation of 7-HFC showed substrate inhibition kinetics in both humans and cynomolgus monkeys, and there was no significant difference in the levels of kinetic constants between humUGT1A1 and monUGT1A1. The kinetic profile of 7-HFC glucuronidation by liver microsomes and recombinant UGT1A1s of humans and cynomolgus monkeys was first suggested in this study.

Table 3
Kinetic parameters for SN-38 glucuronidation by liver microsomes and recombinant UGT1A1s of humans and cynomolgus monkeys.

	S_{50} (μM)	V_{max} (pmol/min/mg protein)	n	CL_{int} (μL/m i n/mg protein)
HLM	7.44 ± 0.83	49.4 ± 26.5	1.35 ± 0.14	4.21 ± 2.49
MLM	6.74 ± 0.38	33.8 ± 8.0	1.21 ± 0.04	3.29 ± 0.86
humUGT1A1	6.75 ± 0.50	126 ± 35	1.89 ± 0.08	12.5 ± 3.1
monUGT1A1	7.00 ± 1.59	110 ± 39	1.61 ± 0.28	9.87 ± 1.79

Each value represents the mean ± SD of three separate experiments derived from independent preparations. HLM, human liver microsomes; MLM, cynomolgus monkey liver microsomes.

Glucuronidation of E-3OH is a useful probe for UGT1A1 activity in humans (Senafi et al. 1994; Ritter 2000; Court 2005). The kinetics of E-3OH glucuronidation by human liver microsomes and recombinant UGT1A1s (humUGT1A1 and monUGT1A1) best fitted the Hill equation, indicating substrate activation, and agreed with the previous report of Fisher et al. (2000b); however, the kinetics of cynomolgus monkey liver microsomes appeared to follow a typical Michaelis–Menten model. Fisher et al. (2000b) have also suggested that E-3OH glucuronidation by human liver microsomes exhibited atypical kinetics. Although the cause of the species difference in the kinetics of E-3OH glucuronidation by liver microsomes is unclear at present, it may be due to the turnover level in each sample, as suggested for interindividual differences of E-3OH activities in human liver microsomes (Fischer et al. 2000b). K_m or S_{50} values of recombinant humUGT1A1 and monUGT1A1 were comparable to those of human and cynomolgus monkey liver microsomes, respectively, and no significant species difference in the kinetic parameter levels was observed. Thus, it was suggested that UGT1A1 is the predominant isoform responsible for E-3OH glucuronidation in both species.

We further determined SN-38 glucuronidation activity in liver microsomes and recombinant UGT1A1s of humans and cynomolgus monkeys. SN-38 is reportedly catalyzed mainly by UGT1A1 in humans (Hanioka et al. 2001; Gagné et al. 2002; Court 2005; Kiang et al. 2005). The kinetics of SN-38 glucuronidation as well as E-3OH glucuronidation was sigmoidal in all enzyme sources, and there were no significant differences in the kinetic levels of SN-38 glucuronidation by liver microsomes and recombinant UGT1A1s between humans and cynomolgus monkeys. The K_m values of human liver microsomes and

recombinant human UGT1A1 expressing insect or COS-1 cells have been reported to be 3.7–36 and 7.5–24 μM, respectively (Hanioka et al. 2001; Gagné et al. 2002; Jinno et al. 2003; Zhang et al. 2007). The S_{50} values of human liver microsomes (7.4 μM) and insect cell membranes expressing humUGT1A1 (6.8 μM) obtained in this study were comparable to those of K_m values in other studies. Thus, there were no remarkable species differences in the kinetics of 7-HFC, E-3OH and SN-38 glucuronidation by liver microsomes and recombinant UGT1A1 between humans and cynomolgus monkeys. The kinetic profile for glucuronidation was extensively different according to substrates and enzyme sources, although the reason for this phenomenon is unclear at present.

Comparison of the primary amino acid sequence of cynomolgus monkey UGT1A enzymes with the corresponding human enzymes revealed more than 90% identity. In addition, comparison of the conserved carboxyl-region demonstrates 97% sequence identity between humans and cynomolgus monkeys (Barbier and Bélanger 2003). These data generally illustrate the elevated homology of UGT1A isoforms and suggest similar *UGT1A* gene organization between humans and cynomolgus monkeys. Based on this information, Barbier and Bélanger (2003) have found that enzymatic activities toward estrogen (estradiol, estriol and estron) of UGT1As (UGT1A1 and UGT1A9) are similar between humans and cynomolgus monkeys, and reviewed the relevance of cynomolgus monkeys as an animal model for the study of steroid glucuronidation. The glucuronidation abilities toward 7-HFC and SN-38 as well as estradiol of monUGT1A1 were suggested to be also very similar to those of humUGT1A1 in this study; however, with respect to UGT1A6, we have demonstrated that the enzymatic properties of UGT1A6 were extensively different between humans and cynomolgus monkeys using serotonin and 4-methylumbelliferone glucuronidation as probes (Hanioka et al. 2006). The similarities and differences in the enzymatic properties of UGT1As between humans and cynomolgus monkeys seem to be isoform dependent.

In conclusion, we expressed human and cynomolgus monkey UGT1A1 enzymes in insect cells, and showed 7-HFC, E-3OH and SN-38 glucuronidation in recombinant UGT1A1s as well as in liver microsomes of humans and cynomolgus monkeys. The kinetics of 7-HFC and E-3OH glucuronidation by human liver microsomes were biphasic and sigmoidal, respectively, whereas the kinetics by cynomolgus monkey liver microsomes was monophasic followed by the Michaelis–Menten model. SN-38 glucuronidation by human and cynomolgus monkey liver microsomes exhibited autoactivation kinetics. The kinetics of 7-HFC, E-3OH and SN-38 glucuronidation by recombinant humUGT1A1 and monUGT1A1 fitted substrate inhibition (7-HFC glucuronidation) or the Hill equation (E-3OH and SN-38 glucuronidation), and each glucuronidation showed the same kinetic profile between humans and cynomolgus monkeys. There were no significant differences in the levels of kinetic parameters for 7-HFC, E-3OH and SN-38 glucuronidation between humans and cynomolgus monkeys in both liver microsomes and recombinant UGT1A1s. These findings suggest that human and cynomolgus monkey UGT1A1 enzymes have high homology in their amino acid sequences, and that their enzymatic properties are very similar. The information gained in this study should help with the in vitro–in vivo extrapolation of drug metabolism.

Conflict of interest statement

The authors have no duality of interest to declare.

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TOXICOKINETICS AND METABOLISM

Functional characterization of human cytochrome P450 2E1 allelic variants: in vitro metabolism of benzene and toluene by recombinant enzymes expressed in yeast cells

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Abstract Benzene and toluene are common organic solvents currently in worldwide industrial usage, which are metabolized mainly by hepatic cytochrome P450 2E1 (CYP2E1) in humans. Genetic polymorphism of *CYP2E1* in 5′-flanking and coding regions has been found previously in Caucasian and Chinese populations. In this study, the effects of *CYP2E1* alleles causing amino acid substitutions (*CYP2E1*2*, *CYP2E1*3* and *CYP2E1*4*; wild-type, *CYP2E1.1A*) on benzene hydroxylation and toluene methylhydroxylation were studied using recombinant CYP2E1 enzymes of wild-type (CYP2E1.1) and variants (CYP2E1.2 having Arg76His, CYP2E1.3 having Val389Ile and CYP2E1.4 having Val179Ile) expressed in yeast cells. The K_m, V_{max} and CL_{int} values of CYP2E1.1 were 10.1 mM, 9.38 pmol/min/pmol CYP and 0.99 nL/min/pmol CYP for benzene hydroxylation, and 3.97 mM, 19.9 pmol/min/pmol CYP and 5.26 nL/min/pmol CYP for toluene methylhydroxylation, respectively. The K_m, V_{max} and CL_{int} values for benzene and toluene metabolism of CYP2E1.2, CYP2E1.3 and CYP2E1.4 were comparable to those of wild-type CYP2E1. These findings may mean that the polymorphic alleles of *CYP2E1* causing amino acid substitutions are not directly associated with the metabolic activation of benzene and toluene. The information gained in this study should help to identify the variations in the toxicity of environmental pollutants.

Keywords Benzene · Toluene · Metabolism · Cytochrome P450 2E1 (CYP2E1) · Genetic polymorphism

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Introduction

Benzene and toluene are organic solvents widely used in a variety of industries and can also be encountered as household products or sniffing solvents. These compounds have been suggested to cause hematotoxicity, genotoxicity or carcinogenicity in humans (Fishbein 1988; Lauwerys and Buchet 1988; McMichael 1988; Snyder et al. 1993). It is considered that the urinary metabolites of benzene (glucuronide/sulfate conjugates of hydroxylated compounds (phenol, hydroquinone and catechol), *trans*, *trans*-muconic acid and *S*-phenylmercapturic acid) and toluene (hippuric acid and glucuronide/sulfate conjugates of *o*-cresol for toluene) are very important indices for the biological monitoring of individuals exposed to these compounds (Fishbein 1984, 1985).

Benzene has been shown to be metabolized to benzene oxide by hepatic cytochrome P450 (CYP) enzymes (mainly CYP2E1) in mammals (Koop et al. 1989; Guengerich et al. 1991; Nakajima et al. 1990, 1993). Benzene oxide is non-enzymatically rearranged to phenol, which can undergo another CYP oxidation to give hydroquinone (Kalf 1987; Koop et al. 1989; Snyder and Hedli 1996). The toxicity of benzene has been suggested to arise from free radicals formed when metabolites such as hydroquinone are further oxidized to form semiquinones (Greenlee et al. 1981; Smith et al. 1989; Snyder et al. 1993). Toluene metabolism in mammals has also been reported to catalyze CYP

enzymes to benzyl alcohol and *o*- and *p*-cresol (Nakajima et al. 1993; Hanioka et al. 1995; Tassaneeyakul et al. 1996; Kim et al. 1997). In rats and humans, the aliphatic hydroxylation forming benzyl alcohol as the major metabolic pathway is catalyzed by mainly CYP2E1, whereas the aromatic hydroxylation forming *o*- and *p*-cresol as the minor metabolic pathways is catalyzed by not only CYP2E1 but also by other CYP isoforms, such as CYP1A2 and CYP2Bs (Nakajima et al. 1991, 1992, 1993, 1997; Kim et al. 1997).

Members of the CYP superfamily catalyze the oxidative metabolism of exogenous compounds, such as drugs and environmental pollutants, as well as endogenous substances, such as steroids and fatty acids (Gonzalez 1990; Nelson et al. 1996; Rendic and Di Carlo 1997). CYP2E1 is expressed mainly in the liver and metabolizes compounds of relatively low molecular weight (Gonzalez 1990; Ingelman-Sundberg et al. 1993; Nelson et al. 1996). It has been suggested that there are large inter-individual variations in the protein level and catalytic activity of CYP2E1 in human liver microsomes (Yoo et al. 1988; Shimada et al. 1994). Previous in vivo studies of Caucasian populations using chlorzoxazone as a probe drug reported a several-fold variation in clearance with a normal distribution of activities and suggested that such variation was caused in part by the genetic polymorphism of *CYP2E1* (Kim and O'Shea 1995; Lucas et al. 1995).

Twelve allelic variants have been identified using restriction fragment polymorphism analysis or DNA sequencing to date (http://www.cypalleles.ki.se/cyp2e1.htm). In the coding region, three allelic variants termed *CYP2E1*2* (227G > A, Arg76His), *CYP2E1*3* (1165G > A, Val389Ile) and *CYP2E1*4* (535G > A, Val179Ile) with amino acid substitutions have been found in Caucasian and Chinese populations at frequencies of 1.3–2.6% (Hu et al. 1997; Fairbrother et al. 1998). One variant, *CYP2E1*2*, has been reported to affect the metabolic ability toward chlorzoxazone and 4-nitrophenol of the CYP2E1 enzyme (Hu et al. 1997; Fairbrother et al. 1998; Hanioka et al. 2003); however, there has been no report about the effect of genetic polymorphism of *CYP2E1* on the metabolism of environmental pollutions.

Since CYP2E1 metabolizes volatile organic compounds, such as benzene and toluene, the genetic polymorphism of *CYP2E1* has been regarded as an important environmental risk factor similar to those of *N*-acetyltransferase and glutathione *S*-transferase (Brockmöller et al. 1996; Thier et al. 2003). The purpose of this study was to clarify whether the amino acid substitutions caused by *CYP2E1*2* and *CYP2E1*3* affect the metabolism of benzene and toluene. To achieve this, CYP2E1 enzymes of wild-type (CYP2E1.1) and variants (CYP2E1.2, CYP2E1.3 and CYP2E1.4) were heterologously expressed in yeast cells, and the in vitro metabolism of benzene to phenol and toluene to benzyl alcohol was examined.

Materials and methods

Materials

pYES2/CT yeast expression vector was purchased from Invitrogen (Carlsbad, CA, USA); yeast nitrogen base was from BD Diagnostics (Franklin Lakes, NJ, USA); Zymolyase 100T was from Seikagaku Corporation (Tokyo, Japan); benzene, phenol, toluene, benzyl alcohol, *p*-methylbenzyl alcohol and 4-nitrophenol were from Nacalai Tesque (Kyoto, Japan); cytochrome c from horse heart and chlorzoxazone and 6-hydroxychlorzoxazone were from Sigma–Aldrich (St. Louis, MO, USA); $NADP^+$, glucose 6-phosphate and glucose 6-phosphate dehydrogenase were from Oriental Yeast (Tokyo, Japan); rabbit anti-human CYP2E1 antibody was from Novus Biologicals (Littleton, CO, USA); peroxidase-conjugated goat anti-rabbit immunoglobulin was from Zymed Laboratories (South San Francisco, CA, USA); and enhanced chemiluminescence-plus reagents were from GE Healthcare Bio-Sciences (Little Chalfont, UK). All other chemicals and reagents were of the highest quality commercially available.

Expression of CYP2E1 enzymes

CYP2E1*1A cDNA cloned into pGEM-T vector (pGEM-T/CYP2E1*1A) (Hanioka et al. 2007), wild-type CYP2E1 plasmid, was used as a template to generate point mutations in the target sites. The cDNAs of CYP2E1*2, CYP2E1*3 and CYP2E1*4 were constructed with a QuikChange site-directed mutagenesis kit according to the manufacturer's instructions using the primers listed in Table 1. All CYP2E1 plasmids were sequenced to confirm successful mutagenesis. The wild-type and variant CYP2E1 cDNAs were subsequently subcloned into the pYES2/CT yeast expression vector. The pYES2/CT vectors containing CYP2E1 cDNAs were used to transform INV*Sc*1/OR (Hanioka et al. 2007) by the lithium acetate procedure (Schiestl and Gietz 1989). The yeast transformants were cultivated, and the microsomal fractions were prepared as described previously by (Hanioka et al. 2007). The microsomes were stored at –80°C until used.

Assay for CYP2E1 holo- and apoproteins

Yeast cell microsomes were diluted to a protein concentration of 5.0 mg/mL with 100 mM potassium phosphate buffer (pH 7.4) containing 20% (v/v) glycerol and 0.4% (w/v) Emulgen 911, and total functional CYP contents

Table 1 Primers used for site-directed mutagenesis

Mutation	Primer name	Sequence	Location
227G>A	CYP2E1*2-FP	5′-GTGGGCTCGCAGC**A**CATGGTGGTGATG-3′	214–240
	CYP2E1*2-RP	5′-CATCACCACCATG**T**GCTGCGAGCCCAC-3′	
1165G>A	CYP2E1*3-FP	5′-CCAAGGGCACAGTC**A**TAGTGCCAACTCTGG-3′	1,151–1,180
	CYP2E1*3-RP	5′-CCAGAGTTGGCACTA**T**GACTGTGCCCTTGG-3′	
535G>A	CYP2E1*4-FP	5′-CGCGCCCTGCAAC**A**TCATAGCCGACATC-3′	522–549
	CYP2E1*4-RP	5′-GATGTCGGCTATGA**T**GTTGCAGGGCGCG-3′	

Bold and underlined letters indicate the mutation sites introduced by PCR-based mutagenesis

were spectrophotometrically measured as reduced carbon monoxide (CO) spectra according to the method of Omura and Sato (1964) using 91 $mM^{-1}cm^{-1}$ as an absorption coefficient for the 450–490 wavelength couple. Total CYP2E1 protein levels of holo- and apoforms in yeast cell microsomes were determined by Western blot analysis. Microsomal proteins (5.0 μg) were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Laemmli 1970) and electrotransferred to a polyvinylidene fluoride sheet as described by Towbin et al. (1979). The sheet was incubated with rabbit anti-human CYP2E1 antibody (diluted at 1:10,000) as the primary antibody and then with peroxidase-conjugated goat anti-rabbit immunoglobulin (diluted at 1:5,000) as the secondary antibody. Immunoreactive proteins were visualized with chemifluorescence (enhanced chemiluminescence-plus reagents), and the band densities were relatively determined with ImageJ v1.34 (National Institutes of Health, MD, USA).

Assay for benzene hydroxylation and toluene methylhydroxylation

The activities of benzene hydroxylation and toluene methylhydroxylation in yeast cell microsomes expressing wild-type and variant CYP2E1s were determined by measuring the formation of phenol and benzyl alcohol, respectively, according to the methods reported previously by (Nakajima et al. 1991, 1993 and Hanioka et al. 1995) with some modifications. The incubation mixture contained benzene (0.5–50 mM) or toluene (0.2–20 mM) as a substrate, yeast cell microsomes expressing CYP2E1s (500 μg protein/mL) and an NADPH-generating system (1 mM $NADP^+$, 10 mM glucose 6-phosphate, 2 U/mL glucose 6-phosphate dehydrogenase and 5 mM $MgCl_2$) in 50 mM potassium phosphate buffer (pH 7.4) in a final volume of 500 μL. Both substrates were dissolved in methanol. The final concentration of methanol in the incubation mixture was 1% (v/v). The reaction was initiated by the addition of the NADPH-generating system after preincubation at 37°C for 1 min. After incubation at 37°C for 10 min, the reaction was terminated by adding 100 μL of 15% zinc sulfate, followed by 100 μL of saturated barium hydroxide solution. The mixtures were spiked with 2.0 nmol of *p*-methylbenzyl alcohol as an internal standard and gently vortexed for 10 s. The supernatant was filtered with a polytetrafluoroethylene membrane filter (0.45 μm pore size, Millipore, Billerica, MA, USA), and a 50 μL portion of the filtrate was subjected to high-performance liquid chromatography (HPLC) with an Inertsil ODS-80A column (4.6 mm i.d. × 150 mm; GL Sciences, Tokyo, Japan). The column was maintained at 40°C. The products (phenol for benzene hydroxylation and benzyl alcohol for toluene methylhydroxylation) were eluted isocratically with water–acetonitrile (75:25, v/v) at a flow rate of 1.0 mL/min. UV detection was performed at 200 nm. Under these conditions, the retention times of phenol, benzyl alcohol and *p*-methylbenzyl alcohol were 6.4, 5.1 and 9.3 min, respectively. The limits of detection for phenol and benzyl alcohol were 50 and 100 pmol/mL with a signal-to-noise ratio of 3, respectively. Intra-day ($n = 5$) and inter-day ($n = 5$) precision did not exceed 10% in any of the assays.

Other methods

Protein concentrations of microsomes from yeast cells expressing wild-type and variant CYP2E1s were determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard. NADPH-cytochrome P450 reductase (OR) activities were measured as described previously, with a substrate concentration of 50 μM (Phillips and Langdon 1962). Chlorzoxazone 6-hydroxylation activities were determined by HPLC as described previously by (Hanioka et al. 2007). The substrate and protein concentrations of yeast cell microsomes were 50–5,000 μM and 500 μg protein/mL, respectively.

Data analysis

Kinetic parameters such as K_m and V_{max} for benzene hydroxylation, toluene methylhydroxylation and chlorzoxazone 6-hydroxylation were estimated by analyzing Michaelis–Menten plots using Prism v5.01 software

(GraphPad Software, San Diego, CA). Intrinsic clearance (CL_{int}) values were determined as the ratio of V_{max}/K_m. All values are expressed as the mean ± SD of three donors/animals or three separate experiments derived from independent preparations. Statistical comparisons were performed by one-way ANOVA with Dunnett's *post hoc* test using Prism v5.01 software. Differences were considered statistically significant when the p value was <0.05.

Results

Expression of wild-type and variant CYP2E1s in yeast cells

The expression levels of CYP2E1 proteins in microsomal fractions obtained from yeast cells transfected with wild-type and variant CYP2E1 cDNAs were examined by reduced CO difference spectral and Western blot analyses. The reduced CO difference spectra of yeast cell microsomes expressing CYP2E1.1, CYP2E1.2, CYP2E1.3 and CYP2E1.4 proteins showed a Soret peak at around 450 nm (Fig. 1). The expressed CYP level of CYP2E1.1 was 61.7 pmol/mg of microsomal protein. The levels of CYP2E1.3 and CYP2E1.4 were comparable to that of CYP2E1.1, whereas the level of CYP2E1.2 was 42.9% that of CYP2E1.1 (Table 2). The expression levels of wild-type and variant CYP2E1 proteins in yeast cell microsomes were also assessed by Western blot analysis which recognized both holo- and apoforms. All constructs, except the negative control (mock), yielded immunodetectable CYP2E1 protein (Fig. 2). The staining band intensities of variant CYP2E1s were 65.8–87.4% that of CYP2E1.1, and this difference was not significant (Table 2). OR activities in yeast cell microsomes expressing wild-type and variant CYP2E1s were further determined. OR activity of CYP2E1.1 was 0.17 μmol/min/mg protein, and there were no significant differences in the OR activities of wild-type and variant CYP2E1s.

Table 2 CYP contents and OR activities in microsomes from yeast cells expressing wild-type and variant CYP2E1s

Variant	CYP		
	Reduced CO[a] (pmol/mg protein)	WB[b] (% of CYP2E1.1)	OR (μmol/min/mg protein)
CYP2E1.1	61.7 ± 21.1	100 ± 23	0.17 ± 0.04
CYP2E1.2	26.5 ± 5.75	65.8 ± 12.9	0.14 ± 0.02
CYP2E1.3	61.4 ± 17.4	85.3 ± 10.8	0.16 ± 0.03
CYP2E1.4	83.7 ± 10.9	87.4 ± 17.6	0.16 ± 0.02

Each value represents the mean ± SD of three separate experiments derived from independent preparations

[a] Reduced CO difference spectral analysis

[b] Western blot analysis

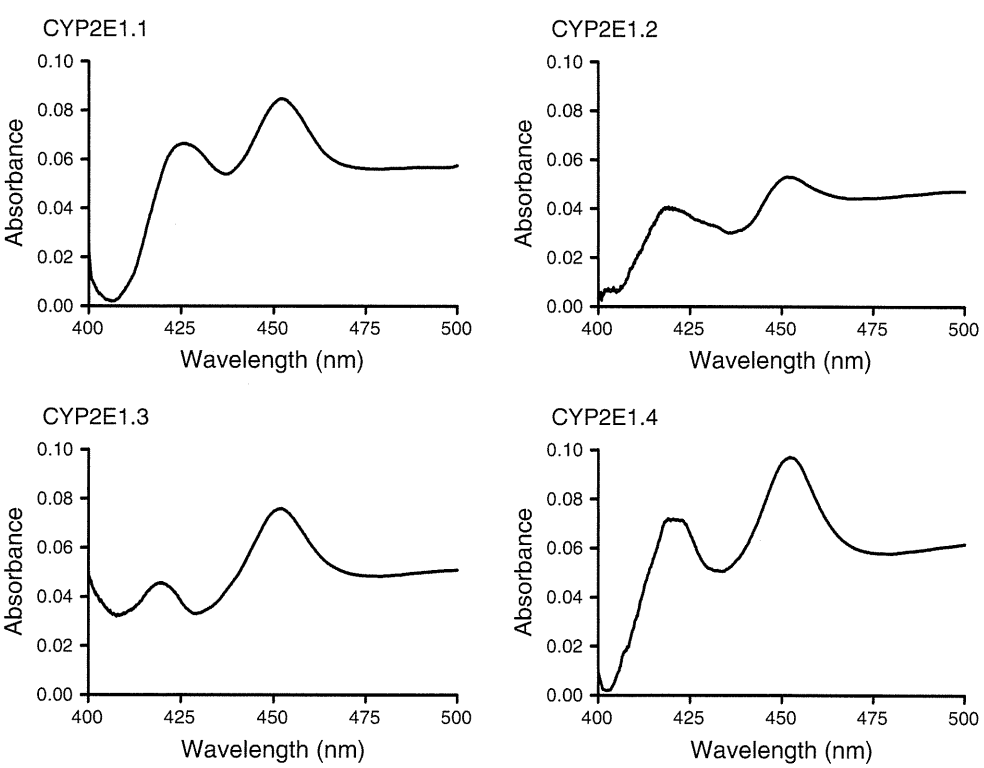

Fig. 1 Reduced CO difference spectra of yeast cell microsomes expressing wild-type and variant CYP2E1s. Pooled microsomes from three independent preparations are expressed. The microsomal protein concentrations used were 5.0 mg/mL

Benzene hydroxylation and toluene methylhydroxylation activities of wild-type and variant CYP2E1s expressed in yeast cells

Benzene hydroxylation and toluene methylhydroxylation activities in yeast cell microsomes expressing wild-type and variant CYP2E1s were determined, and kinetic analyses were then performed. No activity in yeast cell microsomes of the negative control was detected with any substrate (data not shown). The [*S*]-[*V*] plots and kinetic parameters are shown in Fig. 3 and Table 3 for benzene hydroxylation, and Fig. 4 and Table 4 for toluene methylhydroxylation, respectively. Benzene hydroxylation and toluene methylhydroxylation in yeast cell microsomes expressing wild-type and variant CYP2E1s showed single-site Michaelis–Menten kinetics. The K_m, V_{max} and CL_{int} values of CYP2E1.1 were 10.1 mM, 9.38 pmol/min/pmol CYP and 0.99 nL/min/pmol CYP for benzene hydroxylation, and 3.97 mM, 19.9 pmol/min/pmol CYP and 5.26 nL/min/pmol CYP for toluene methylhydroxylation, respectively. There were no significant differences in the K_m, V_{max} and CL_{int} values between wild-type CYP2E1 and each variant of CYP2E1 in any oxidative metabolism.

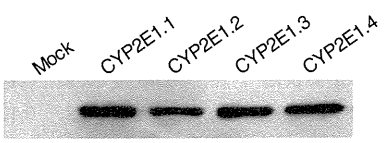

Fig. 2 Immunoblotting of yeast cell microsomes expressing wild-type and variant CYP2E1s. Pooled microsomes from three independent preparations are expressed. The microsomal protein levels applied were 5.0 μg/lane

Chlorzoxazone 6-hydroxylation activities of wild-type and variant CYP2E1s expressed in yeast cells

Chlorzoxazone 6-hydroxylation activities as a typical CYP2E1 probe in yeast cell microsomes expressing wild-type and variant CYP2E1s were also determined. The [*S*]-[*V*] plots and the kinetic parameters are shown in Fig. 5 and Table 5, respectively. Chlorzoxazone 6-hydroxylation

Table 3 Kinetic parameters for benzene hydroxylation by microsomes from yeast cells expressing wild-type and variant CYP2E1s

Variant	K_m (mM)	V_{max} (pmol/min/pmol CYP)	CL_{int} (nL/min/pmol CYP)
CYP2E1.1	10.1 ± 2.1	9.38 ± 2.15	0.99 ± 0.47
CYP2E1.2	10.2 ± 2.2	9.13 ± 2.47	0.95 ± 0.31
CYP2E1.3	13.6 ± 2.6	6.97 ± 2.80	0.56 ± 0.34
CYP2E1.4	13.6 ± 2.5	5.37 ± 1.70	0.40 ± 0.09

Each value represents the mean ± SD of three separate experiments derived from independent preparations

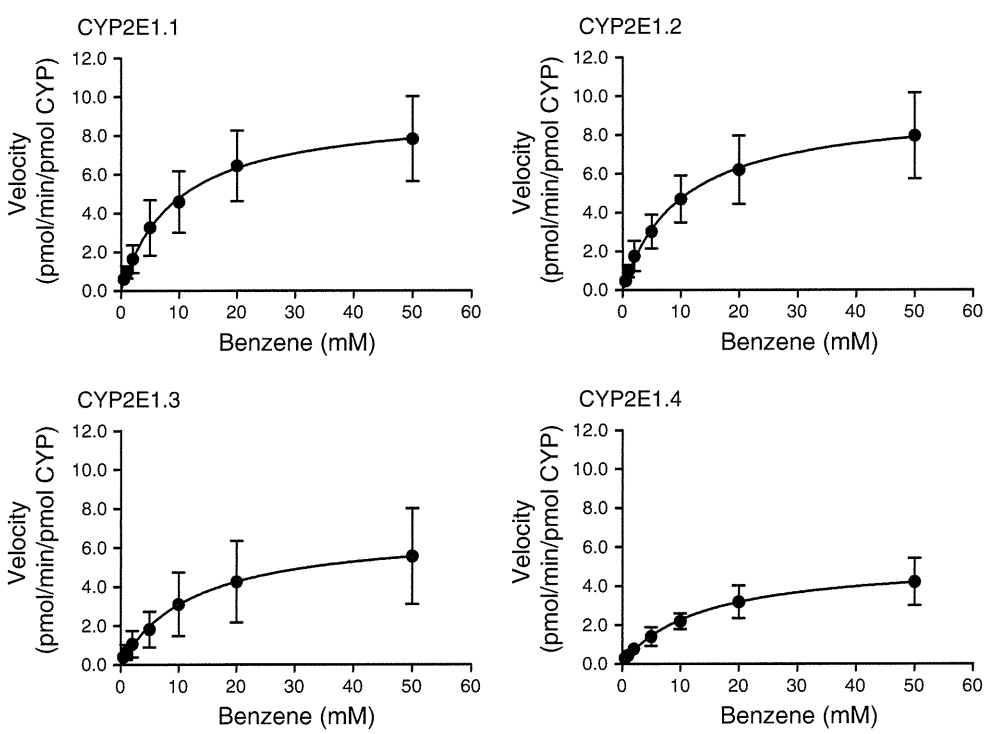

Fig. 3 [*S*]-[*V*] plots for benzene hydroxylation by yeast cell microsomes expressing wild-type and variant CYP2E1s. Each point represents the mean of three separate experiments derived from independent preparations. The substrate concentrations used were 0.5–50 mM

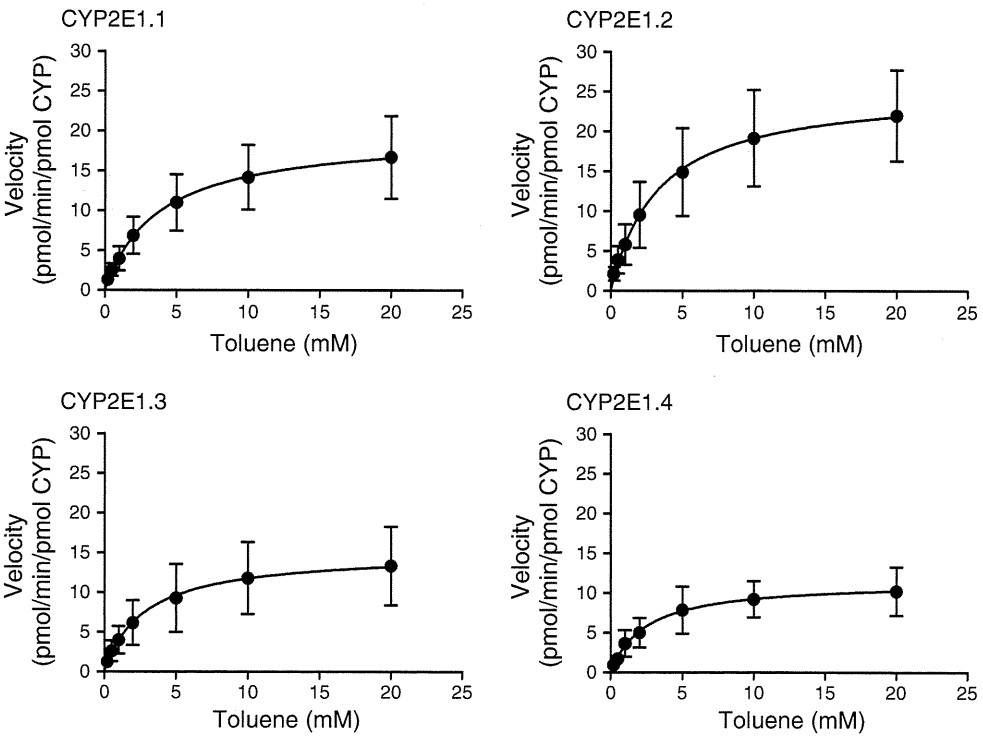

Fig. 4 [*S*]-[*V*] plots of toluene methylhydroxylation by yeast cell microsomes expressing wild-type and variant CYP2E1s. Each point represents the mean of three separate experiments derived from independent preparations. The substrate concentrations used were 0.2–20 mM

Table 4 Kinetic parameters for toluene methylhydroxylation by microsomes from yeast cells expressing wild-type and variant CYP2E1s

Variant	K_m (mM)	V_{max} (pmol/min/pmol CYP)	CL_{int} (nL/min/pmol CYP)
CYP2E1.1	3.97 ± 1.02	19.9 ± 6.2	5.26 ± 2.12
CYP2E1.2	3.81 ± 1.41	25.9 ± 6.0	7.92 ± 4.42
CYP2E1.3	3.26 ± 1.39	15.3 ± 5.2	5.39 ± 2.92
CYP2E1.4	2.54 ± 0.43	11.6 ± 3.1	4.77 ± 2.06

Each value represents the mean ± SD of three separate experiments derived from independent preparations

by yeast cell microsomes expressing wild-type and variant CYP2E1s showed single-site Michaelis–Menten kinetics. The K_m, V_{max} and CL_{int} values of CYP2E1.1 were 1.57 mM, 9.78 pmol/min/pmol CYP and 6.12 nL/min/pmol, respectively. The K_m, V_{max} and CL_{int} values of CYP2E1.2, CYP2E1.3 and CYP2E1.4 were not significantly different from those of wild-type CYP2E1.

Discussion

Benzene and toluene are common organic solvents currently in worldwide industrial usage, and a large number of workers are exposed to these chemicals (World Health Organization 1996). Previous reports have shown that CYP2E1 is a key enzyme involved in the toxicity of benzene and toluene (Guengerich et al. 1991; Tassaneeyakul et al. 1996; Kim et al. 1997; Nakajima et al. 1997). The metabolic activity of CYP2E1 toward xenobiotics both in vivo and in vitro has been reported to be extensive, and genetic polymorphism in *CYP2E1* has been considered to be at least partly responsible (Kim and O'Shea 1995; Lucas et al. 1995; Song 1996). Since *CYP2E1* polymorphism has been suggested to be closely associated with the incidence of several cancers, it may be an important risk factor for susceptibility to environmental pollutants (Song 1996; Tanaka et al. 2000). In this study, benzene hydroxylation and toluene methylhydroxylation were investigated in yeast cell microsomes expressing wild-type and variant CYP2E1s.

The expression of wild-type and variant CYP2E1 protein in yeast cells was confirmed by reduced CO difference spectral and Western blot analyses. Although there were no statistically significant differences in CYP levels between CYP2E1.1 and each variant CYP2E1 in any analysis, the decreasing tendency of holo- and apoproteins CYP levels of CYP2E1.2 was observed. Hu et al. (Hu et al. 1997) have reported that CYP2E1*2 cDNA reduces the expression level of protein to about 40% of CYP2E1*1 cDNA but not mRNA in the expression system using COS-1 cells. We have also previously found that the CYP level of CYP2E1.2 expressed in COS-1 cells was about 30% of CYP2E1.1, and that the transcription and translation rates for each expression plasmid of variant CYP2E1 were very similar to those for wild-type CYP2E1 (Hanioka et al.

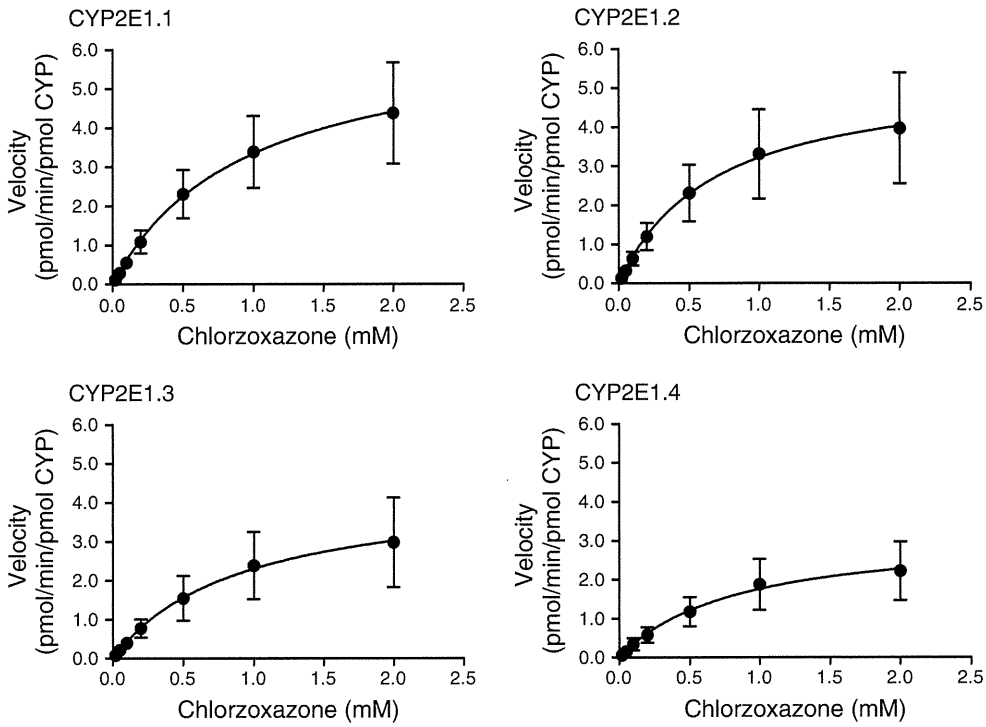

Fig. 5 [*S*]-[*V*] plots for chlorzoxazone 6-hydroxylation by yeast cell microsomes expressing wild-type and variant CYP2E1s. Each point represents the mean of three separate experiments derived from independent preparations. The substrate concentrations used were 0.05–5.0 mM

Table 5 Kinetic parameters for chlorzoxazone 6-hydroxylation by microsomes from yeast cells expressing wild-type and variant CYP2E1s

Variant	K_m (mM)	V_{max} (pmol/min/pmol CYP)	CL_{int} (nL/min/pmol CYP)
CYP2E1.1	0.92 ± 0.11	6.51 ± 2.04	6.92 ± 1.67
CYP2E1.2	0.66 ± 0.10	5.41 ± 2.09	7.99 ± 2.10
CYP2E1.3	0.89 ± 0.16	4.41 ± 1.80	4.87 ± 1.55
CYP2E1.4	0.84 ± 0.13	3.24 ± 1.11	3.88 ± 1.35

Each value represents the mean ± SD of three separate experiments derived from independent preparations

2003). Thus, the expression profile of CYP2E1 obtained in this study using the yeast cell expression system agreed with those of previous reports, and it is possible that the reduction in the CYP expression level of CYP2E1.2 is due to the altered stability or folding efficiency of the protein.

Nedelcheva et al. (1999) and Tassaneeyakul et al. (1996) have reported that the activities of benzene hydroxylation and toluene methylhydroxylation were highly correlated with CYP2E1-specific activities and the immunoreactive CYP2E1 level in human liver microsomes, suggesting the importance of CYP2E1 in benzene and toluene metabolism; therefore, we examined the effect of *CYP2E1*2*, *CYP2E1*3* and *CYP2E1*4* on benzene hydroxylation and toluene methylhydroxylation using the recombinant CYP2E1 enzymes expressed in yeast cells. Additionally, chlorzoxazone 6-hydroxylation activities were also determined to characterize the enzymatic function of variant CYP2E1 enzymes toward a typical CYP2E1 substrate. All variant CYP2E1s with amino acid substitutions were capable of catalyzing benzene and toluene oxidation as well as wild-type CYP2E1 at the substrate concentrations examined, and the kinetics of all CYP2E1 enzymes fitted the typical Michaelis–Menten model. Although the in vitro metabolism of benzene and toluene in humans has been studied using liver microsomes and recombinant CYP enzymes (Tassaneeyakul et al. 1996; Kim et al. 1997; Nakajima et al. 1997; Nedelcheva et al. 1999), the kinetic parameters for benzene hydroxylation and toluene methylhydroxylation by recombinant CYP2E1 enzymes were estimated for the first time in this study. The K_m values for benzene hydroxylation and toluene methylhydroxylation of CYP2E1.1 were millimolar levels, and the values were generally higher than those of other typical CYP2E1-dependent oxidation by recombinant CYP2E1 and liver microsomes (Peter et al. 1990; Tassaneeyakul et al. 1993; Fairbrother et al. 1998; Shimada et al. 1999; Hanioka et al. 2003, 2007). The K_m, V_{max} and CL_{int} values for benzene hydroxylation and toluene methylhydroxylation of CYP2E1.2, CYP2E1.3 and CYP2E1.4 were comparable to those of wild-type CYP2E1, and the kinetic parameters of chlorzoxazone 6-hydroxylation as a typical probe for CYP2E1 were not also affected by the corresponding amino acid substitutions of variant CYP2E1s. These findings suggest that Arg76His, Val389Ile and Val179Ile substitutions in the CYP2E1 enzyme hardly affect benzene and toluene metabolism.

X-ray crystal structures of several mammalian CYP enzymes (rabbit CYP2B4 and CYP2C5, and human CYP2C8, CYP2C9, CYP2D6 and CYP3A4) have been reported to date (Williams et al. 2000, 2003, 2004; Schoch et al. 2004; Scott et al. 2004; Rowland et al. 2006). Homology modeling of human CYP2E1 has been generated using CYP2C5 crystal structures as a template, and six substrate recognition sites (SRSs) and a putative active site for CYP2E1 have been identified (Lewis 2002, 2003; Lewis et al. 2003). Phe205 and Phe298 of CYP2E1 have been suggested to be able to form π–π stacking with the benzoxazole ring of chlorzoxazone, whereas Asp202 and The303 of CYP2E1 enter into hydrogen bonding with the chlorzoxazone hydroxyl and chloro groups, respectively. These amino acid residues are in SRS-2 and SRS-4 and have been regarded to assist in positioning the substrate cooperatively such that the 6-hydrogen is directly over the haem iron at the distance of 5.7 Å. The amino acids of interest in this study, Arg76, Val389 and val179, are not found in any SRSs; therefore, we consider that the profile of the metabolism of benzene and toluene by wild-type and variant CYP2E1s is similar.

In conclusion, we expressed three variant CYP2E1s as well as wild-type CYP2E1 in yeast cells, and the kinetics of benzene hydroxylation and toluene methylhydroxylation were determined. The K_m, V_{max} and CL_{int} values of CYP2E1.2, CYP2E1.3 and CYP2E1.4 were comparable to those of wild-type CYP2E1 in any oxidative metabolism, and the kinetic parameters of chlorzoxazone 6-hydroxylation as a typical probe for CYP2E1 were not also affected by the corresponding amino acid substitutions of variant CYP2E1s. These findings may mean that the polymorphic alleles of *CYP2E1* causing amino acid substitutions are not directly associated with the metabolic activation of benzene and toluene. The information gained in this study should help to identify the variations in the toxicity of environmental pollutants.

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Analysis of phthalic acid diesters, monoester, and other plasticizers in polyvinyl chloride household products in Japan

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The aim of this study was to determine the concentrations of six phthalic acid diesters (PAEs) [di(2-ethylhexyl) phthalate (DEHP), di-*n*-butyl phthalate (DBP), butyl benzyl phthalate (BBP), diisononyl phthalate (DINP), di-*n*-octyl phthalate (DNOP), and diisodecyl phthalate (DIDP)], two non-phthalic plasticizers [di(2-ethylhexyl) adipate (DEHA), 2,2,4-trimethyl-1,3-pentanediol diisobutylate (TMPDIB)], and mono 2-ethylhexyl phthalate(MEHP) in polyvinyl chloride (PVC) household products that children often places in their mouths and/or contact with their skin (41 products, 47 samples) in Japan. The detection frequencies of the studied compounds were as follows: DEHP (79 %), DINP-2 (13 %), DINP-1 (11 %), DBP (8.5 %), DEHA (8.5 %), DIDP (4.3 %), and DNOP (2.1 %). Concentrations of these compounds ranged from 0.021 % to 48 %. BBP and TMPDIB were not detected in the all samples. Most samples contained DEHP and DINP at high concentrations over 0.1 %. High concentrations of PAEs were detected in PVC household products that appear appealing to children and can possibly be licked and chewed by them. Di(2-ethylhexyl) terephtalete, diisononyl 1,2-cyclohexanedicarboxylic acid, acetyl tributyl citrate, and di(2-ethylhexyl) 4-cyclohexene-1,2-dicarboxylate used as substitute plasticizers were also detected in several samples. MEHP was present in 70 % of the samples, with concentrations ranging from trace amounts to 140 μg/g. The ratios of MEHP against DEHP were 6.2×10^{-4} to 1.6×10^{-1} %. MEHP in the household products investigated in this study was most probably an impurity in DEHP. The high concentrations of PAEs detected in products that children often place in their mouth reveal the importance of replacing plasticizers in common household products, and not just children's toys, with safer alternatives.

Keywords: Phthalic acid diester and monoester, plasticizer, polyvinyl chloride, household products.

Introduction

Phthalic acid diesters (PAEs) are widely used as plasticizers in various products, particularly those made from polyvinyl chloride (PVC). In 2009, Japan produced 197,930 tons of PAEs annually,[1] including 125,281 tons of di(2-ethylhexyl) phthalate (DEHP), 59,822 tons of diisononyl phthalate (DINP), 4,041 tons of diisodecyl phthalate (DIDP), and 1,216 tons of di-*n*-butyl phthalate (DBP).

Since PAEs easily migrate from plastic products and their content in plastic material is high, many toxicological investigations of PAEs have been carried out in order to determine whether they have an adverse effect on human health. Reproductive and developmental toxicities of DEHP, DBP, and butyl benzyl phthalate (BBP) have been reported in rats and mice.[2–4] Although testicular toxicity of DINP has not been observed, its hepatic toxicity has been reported in rats.[5] When male and female marmosets were treated with DEHP by oral gavage from weaning to sexual maturity, no histological changes were observed in them,[6] indicating that toxicological sensitivity is different between primates and rodents for some unknown reason.

In vitro percutaneous absorption rates of DEHP and DBP are slower for humans than rats;[7,8] further, patch tests of DEHP, DINP, and DIDP revealed no skin sensitization potential.[9] However, contact dermatitis from the DBP and benzalkoniunm chloride in Timodine® has been reported.[10] Furthermore, both DBP and DEHP displayed adjuvant effects during contact and atopic dermatitis.[11]

In 1999, the European Union (EU) temporarily restricted the use of DEHP, DBP, BBP, DINP, DIDP, and di-*n*-octyl phthalate (DNOP) in toys and childcare goods intended to be placed in the mouth of children under 3 years old.[12] In 2005, this decision was amended to limit DEHP, DBP, and BBP to less than 0.1 % in all plastic toys and childcare products and DNOP, DINP, and DIDP to less than 0.1 % in products that children can place in their mouth.[13] In 2003, DEHP was restricted by the Food Sanitation Law of Japan to below 0.1 % in the plastic containers

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that come into with food and in toys for preschool children (under 6 years old) and DINP was restricted in products that children can place in their mouth.[14]

Amending this regulation to be similar to the current EU regulation is currently under consideration in Japan.[15–17] Although it is considered that the exposure of children to PAEs in toys and childcare products has been reduced, oral PAE exposure can occur from licking and chewing plastic household products that are not included in the list of regulated products. Exposure may also occur from hand-to-mouth contact with households products, similar to what has been found for brominated flame retardants (BFRs) and perfluoroalkyl compounds present in household products .[18,19] However, relatively little is known about the concentrations of PAEs in household products[20,21] compared to that in toys for preschool children.[22–24]

In this study, therefore, we attempted to elucidate the concentrations of the six PAEs regulated in the EU in household products made with PVC that children can place in their mouth and can come in contact with their skin. Furthermore, we determined the concentrations of di(2-ethylhexyl) adipate (DEHA) and 2,2,4-trimethyl-1,3-pentanediol diisobutylate (TMPDIB), both of which are compounds used as replacements for PAEs.[21] Phthalic acid monoesters are major toxic metabolites of PAEs.[3,4] Recently, the presence of mono 2-ethylhexyl phthalate (MEHP), one such phthalic acid monoester, in house dust was reported.[25] However, since only a few studies have investigated MEHP in PVC products,[26] we determined the concentrations of MEHP in household products in this study.

Materials and methods

Samples

Household products made from PVC were purchased from several retail and online stores in Japan from September 2009 to February 2010. A total of 41 products (47 samples) were analyzed. They were divided into classes on the basis of usage and separated by color and flexibility as much as possible (Table 1).

Materials

Environmental grade DBP, DEHP, BBP, and DEHA were obtained from Kanto Chemical Co., Inc.(Tokyo, Japan). DNOP, DIDP, and TMPDIB were obtained from Fluka (Buchs, Switzerland), Dr. Ehrenstorfer GmbH (Augsburg, Germany), and Tokyo Chemical Ind., Co., Ltd. (Tokyo, Japan), respectively. Deuterated PAEs (DBP, DEHP, BBP, and DNOP), and anthracene-d_{10} were obtained from Kanto Chemical Co., Inc.. MEHP and MEHP-d_4 were obtained from Hayashi Pure Chemical Ind., Ltd. (Osaka, Japan), and the isomers DINP-1 (CAS.68515-48-0) and DINP-2 (CAS. 28553-12-0) were obtained from Kanto Chemical Co., Inc., and Wako Pure Chemical Ind., Ltd. (Osaka, Japan), respectively. DINP-1 and 2 contained different kinds of isomers.[5] Pesticide residue grade acetone, hexane, ethyl acetate, and diethyl ether were obtained from Wako Pure Chemical Ind., Ltd., Kanto Chemical Co., Inc., and Sigma-Aldrich (St. Louis, MO, USA). HPLC grade acetonitrile was obtained from Kanto Chemical Co., Inc. Phthalate analysis grade sodium chloride and anhydrous sodium sulfate, analytical grade sodium hydroxide, and heavy metal analysis grade hydrochloric acid were obtained from Wako Pure Chemical Ind., Ltd. Deionized water was produced by Milli-Q Synthesis A10 (Millipore, Tokyo, Japan). All utensils made of glass, metal, or Teflon were heated at 250°C for more than 12 h to prevent contamination.

Sample processing

PAEs, DEHA, and TMPDIB were processed and analyzed as previously described[27] with minor modifications. The sample was cut and 0.1 g of the sample was placed into a glass tube, with 3 mL of acetone/hexane = 3/7 (v/v) and left overnight at 37°C. The volume of the sample solution was adjusted to 10 mL and diluted to the appropriate volume (100–5000-fold). Finally, 50 μL of an acetone solution containing 2 μg mL^{-1} of the internal standard (DNOP-d_4, 0.2 μg mL^{-1}) was added to 1 mL of the diluted sample solution, and this solution was analyzed by gas chromatography/mass spectrometry (GC/MS). The concentrations of DINP and DIDP were determined using the sum of total peak area of their isomers similar to previous studies.[22,24,28]

A blank test was performed concurrently to rule out the possibility of contamination with PAEs during sample processing. PAEs [DBP (0.05 %), BBP (0.05 %), DEHP (0.05 %), DNOP (0.05 %), DINP (0.2 %), and DIDP (0.2 %)], DEHA (0.05 %), and TMPDIB (0.05 %) were added to the sample that did not contain the compounds studied, and GC/MS analysis was performed with the 100-fold diluted sample to obtain the recovery, method detection limit (MDL), and method quantification limit (MQL). MDL[29] and MQL[30] were calculated as follows:

$$\mathrm{MDL} = 3.3 \times \rho/\mathrm{ar} \quad (1)$$

$$\mathrm{MQL} = 10\rho \quad (2)$$

where ρ is the standard deviation obtained from the results of a low-concentration analysis; a is the slope of the standard curve; and r is the relative sensitivity. The obtained recoveries, MDL, and MQL are listed in Table 2.

MEHP was analyzed as described[26] with minor modifications. A 50 μL of acetone solution containing 2 μg mL^{-1} of the surrogate standard MEHP-d_4 was added to 1 mL of the extracted sample solution. The solution was dried using a gentle N_2 stream, and the residue was dissolved in 1 mL

Table 1. List of the PVC household products studied.

Usage	*Name of products*		*Country*	*Sample Name*
Stationery	card case		China	A1
	card case		China	A2
	eraser		Vietnam	A3
	card case		China	A4
	card case		China	A5
	cutting mat		Taiwan	A6
	eraser		Japan	A7
	eraser		Japan	A8
	eraser		Japan	A9
	desk mat		Japan	A10
	clip		China	A11
Accessories and others	shower cap[a]		Vietnam	B1
	emblem of rain coat		China	B2
	non-slip hook of baby carriage		China	B3
Outdoor and sports goods	ball		China	C1
	ring float	mouthpiece	China	C2_1
		body (clear)		C2_2
		body (blue)		C2_3
	beach ball	mouthpiece	China	C3_1
		body		C3_2
	shuttlecock	head	China	C4_1
		case		C4_2
	ball		unknown	C5
	ball		Taiwan	C6
	yoga mat		China	C7
Small articles	case		China	D1
	card holder		China	D2
	pass case	synthetic leather	China	D3_1
		clear part		D3_2
	key cover		China	D4
	mobile phone strap[b]	tag	Korea	D5_1
		body		D5_2
	mobile phone strap[b]		unknown	D6
	mobile phone strap[b]		Japan	D7
	mobile phone strap[b]		China	D8
	key holder		Japan	D9
Furniture aids	edge cover		China	E1
	sucking disc		China	E2
	floor mat		Japan	E3
	floor mat		Japan	E4
	edge cover (ball type)		China	E5
	casement lock		China	E6
	edge cover		China	E7
Furniture	children sofa		China	F1
	pipe chair		China	F2
	cushion		China	F3
	children chair		Vietnam	F4

[a]According to FT-IR analysis, PVC might not be used or might not be the dominant material for shower cap (B1), although the label on the products reads "PVC and others."
[b]Liquid crystal display (LCD) cleaner type.

of acetonitrile. Then, 5 % sodium chloride solution (3 mL) and 0.01 mol L^{-1} sodium hydroxide solution (0.5 mL) were added in the sample solution. Next, 2 mL of hexane was added to the sample solution and the mixture was shaken by hand for 5 min to remove excess PAEs. After shaking, the sample solution was centrifuged for 2 min (3000 rpm) and the hexane phase was disposed. This extraction procedure was repeated 4 times. Then, the pH of the sample solution was adjusted to pH 3 with 0.01 mol L^{-1} hydrochloric acid. Next, 2 mL of ethyl acetate was added in the sample

Table 2. GC retention times, quantifying and quabfying ions, recoveries (%) and its coefficients of variation (C,V,%) (n=3) and MDL[a] and MQL[b] of the compounds studied.

	M.W.	*Retention time (min)*	*Quantifying ion (m/z)*	*Qualifying ion (m/z)*	*Recovery*	*C.V.*	*MDL*	*MQL*
Phthalate diesters[c]								
DBP	278.3	11.68	149	223	100	0.74	0.00021	0.0038
BBP	312.4	13.53	149	206	101	1.9	0.00049	0.0099
DEHP	390.6	14.24	149	167	109	1.8	0.00061	0.0098
DNOP	390.6	15.04	279	149	86	2.0	0.0047	0.0086
DINP-1	418.6	14.8–15.8	293	149	102	1.3	0.0064	0.026
DINP-2	418.6	15–16	293	149	100	2.9	0.0075	0.059
DIDP	446.7	15.2–16.8	307	149	106	2.4	0.011	0.052
Others[d]								
TMPDIB	286.4	9.63	71	243	98	2.3	0.0017	0.011
DEHA	370.6	13.63	129	147	88	2.0	0.0062	0.0087
Internal standard								
DBP-d_4	282.3	11.66	153					
BBP-d_4	316.4	13.52	153					
DEHP-d_4	394.6	14.21	153					
DNOP-d_4	394.6	15.01	153					
Anthracene-d_{10}	188.3	11.02	188					
Mono ethylhexyl phthalate[e]								
MEHP	278.3	12.02	163	149,181	76	4.0	0.029	1.5
MEHP-d_4	282.3	12.01	167					

[a]MDL: (3.3 × standard deviation)/(slope of calibration curve × relative sensitivity), Unit: PAEs, DEHA and TMPDIB (%), MEHP ($\mu g\ g^{-1}$).
[b]MQL: 10 × standard deviation, Unit: PAEs, DEHA and TMPDIB (%), MEHP ($\mu g\ g^{-1}$).
[c]DBP, BBP, DEHP and DNOP were quantified using corresponding deuterated compounds as internal standard, DINP and DIDP were quantified using DNOP-d_{10} as internal standard.
[d]TMPDIB and DEHA were quantified using anthracene-d_{10} as internal standard.
[e]MEHP was quantified using MEHP-d_4.

solution. The mixture was shaken by hand for 5 min and the ethyl acetate layer was separated from the aquatic phase by centrifugation for 2 min (3000 rpm).

This extraction procedure was performed twice and the ethyl acetate layer was combined and dehydrated with anhydrous sodium sulfate. The dehydrated solution was evaporated and dried under gentle N_2 stream. The residue was dissolved in 1 mL of acetone, and MEHP was methylated with diazomethane-diethyl ether.[31] After the methylation procedure, the sample solution was dried under gentle N_2 stream and the residue was dissolved in 1 mL of acetone. Finally, 50 μL of acetone solution containing 2 $\mu g\ mL^{-1}$ of anthracene-d_{10} used for confirmation of recovery of the surrogate standard was added, and this solution was then analyzed by GC/MS. MEHP was quantified by MEHP-d_4, and a blank test was performed in the same manner as that described above. MEHP was added to the sample (5 $\mu g\ g^{-1}$), and the recovery, MDL, and MQL were obtained in the same manner to that described above. These values are shown in Table 2.

GC/MS analysis

All samples in this study were analyzed using a Focus GC with a DSQII MS (Thermo Fisher Scientific, Waltham, MA, USA). A DB-5MS fused silica capillary column (length: 30 m, internal diameter: 0.25 mm, film thickness: 0.25 μm, Agilent, Santa Clara, CA, USA) was used for analysis in this study. The carrier gas used was He with a flow rate of 1.0 mL min^{-1}. The temperatures of the injector, transfer line, and ion source were 230, 280, and 250°C, respectively. The sample was injected in the splitless mode and the injected volume was 1 μL. The GC oven temperature was initially maintained at 60°C for 2 min and the temperature increased to 310°C at a rate of 20°C min^{-1}. The oven temperature was then maintained at 310°C for 10 min. The MS was operated in the electron ionization (EI) mode at 70 eV and the analysis was performed using the selected ion monitoring (SIM) mode. The retention times and the quantifying and qualifying ions are listed in Table 2.

Results and discussion

Concentrations of PAEs, DEHA and TMPDIB in the PVC household products

The concentrations of PAEs, DEHA and TMPDIB in the PVC household products are listed in Table 3. The concentration range, detection number, and detection frequency of PAEs, DEHA, and TMPDIB are listed in Table 4. The following PAEs were detected at high frequency: DEHP was present in 79 % of samples (concentration range: 0.021–47 %), DINP-2 in 13 % (0.075–48 %), DINP-1 in 11 %

Table 3. Concentrations of PAEs(%), TMPDIB(%), DEHA(%), and MEHP($\mu g\ g^{-1}$) in the PVC household products.

Usage	*Sample Name*	*DBP*	*BBP*	*DEHP*	*DNOP*	*DINP-1*	*DINP-2*	*DIDP*	*TMPDIB*	*DEHA*	*MEHP*
Stationery	A1	–[a]	–	0.021	–	–	–	–	–	–	–
	A2	–	–	17	–	–	–	–	–	–	6.4
	A3	–	–	41	–	–	–	–	–	–	20
	A4	–	–	–	–	–	–	–	–	–	–
	A5	–	–	19	–	–	–	–	–	–	6.6
	A6	–	–	17	0.26	–	1.8	1.4	–	0.32	75
	A7	18	–	30	–	–	–	–	–	–	8.9
	A8	7.4	–	35	–	–	–	–	–	–	51
	A9	17	–	29	–	–	–	–	–	–	7.3
	A10	–	–	21	–	–	–	–	–	–	tr[b]
	A11	–	–	11	–	–	–	–	–	–	5.2
Accessories and others	B1	–	–	–	–	–	–	–	–	–	–
	B2	–	–	0.88	–	–	27	–	–	–	–
	B3	–	–	19	–	–	–	–	–	–	11
Outdoor and Sports goods	C1	–	–	–	–	0.58	–	–	–	–	–
	C2_1	–	–	0.097	–	–	0.075	–	–	–	–
	C2_2	–	–	–	–	–	–	–	–	–	–
	C2_3	–	–	–	–	–	–	–	–	–	–
	C3_1	–	–	–	–	–	–	–	–	–	–
	C3_2	–	–	–	–	–	–	–	–	0.052	–
	C4_1	–	–	32	–	–	–	–	–	–	37
	C4_2	–	–	0.076	–	–	–	–	–	–	–
	C5	–	–	–	–	–	–	–	–	0.035	–
	C6	–	–	37	–	–	–	–	–	–	21
	C7	0.52	–	18	–	–	–	–	–	–	23
Small articles	D1	–	–	13	–	–	–	–	–	–	7.0
	D2	–	–	24	–	–	–	–	–	–	13
	D3_1	–	–	16	–	8.7	–	–	–	–	3.7
	D3_2	–	–	15	–	–	–	–	–	–	3.5
	D4	–	–	–	–	–	–	–	–	–	–
	D5_1	–	–	10	–	35	–	–	–	–	12
	D5_2	–	–	18	–	–	–	–	–	–	9.3
	D6	–	–	26	–	–	–	–	–	–	1.6
	D7	–	–	7.0	–	–	1.8	5.8	–	0.41	110
	D8	–	–	8.7	–	8.5	–	–	–	–	6.1
	D9	–	–	–	–	–	–	–	–	–	–
Furniture's aids	E1	–	–	23	–	–	–	–	–	–	13
	E2	–	–	1.5	–	–	48	–	–	–	tr
	E3	–	–	36	–	–	–	–	–	–	63
	E4	–	–	27	–	–	–	–	–	–	63
	E5	–	–	30	–	–	–	–	–	–	30
	E6	–	–	12	–	12	–	–	–	–	14
	E7			47							30
Furniture	F1	–	–	21	–	–	–	–	–	–	140
	F2	–	–	16	–	–	1.5	–	–	–	2.1
	F3	–	–	25	–	–	–	–	–	–	24
	F4	–	–	20	–	–	–	–	–	–	20

[a]Not detected.
[b]Between MDL and MQL.

(0.58–35 %), DBP in 8.5 % (0.52–18 %), DIDP in 4.3 % (1.4–5.8 %), DNOP in 2.1 % (0.26 %). The total detection frequency of DINP (DINP-1 + DINP-2) was 23 %. DEHA was detected in 4 samples (0.035–0.41 %). Recently, Abe et al. reported that the detection frequency of PAEs in PVC toy products was 32 %,[32] which is lower than that reported in a previous study[24] conducted in Japan in 2001. Furthermore, they also found a high detection frequency of TMPDIB (48 %) in PVC toy products in Japan.[32] However, the detection frequency of PAEs in household products considered in this study was still higher than that in PVC toy products.

Table 4. Concentration, detection number, and detection frequency (%).

Range (%)	*DBP*	*BBP*	*DEHP*	*DNOP*	*DINP-1*	*DINP-2*	*DIDP*	*TMPDIB*	*DEHA*
<0.1	0	0	3	0	0	1	0	0	2
0.1 ~ 1<	1	0	1	1	1	0	1	0	2
1 ~ 10<	1	0	4	0	2	3	1	0	0
10 ~ 20 <	2	0	13	0	1	0	0	0	0
20 ~ 30 <	0	0	10	0	0	1	0	0	0
30≦	0	0	6	0	1	1	0	0	0
Detection number	4	0	37	1	5	6	2	0	4
Detection frequency	8.5	0	79	2.1	11	13	4.3	0	8.5

In addition, BBP and TMPDIB were not detected in the all samples in this study. Some samples, e.g., A6, contained several kinds of PAEs. Most samples contained DEHP and DINP in high concentrations (over 0.1 %). In some previous studies, detection frequencies of DINP in toy products in Japan[22–24] and other countries[33] were higher than the corresponding detection frequencies of DEHP. Furthermore, similar detection frequencies of DINP and DEHP in PVC toys and childcare articles were reported in the market survey carried out in Germany, Austria, and Switzerland.[34] However, detection frequencies of DEHP in household products were higher than those of DINP,[20] which is in agreement with the results of our study.

Two of the products studied, the float ring (C2) and the beach ball (C3), displayed the safety toy mark and safe use of plasticizer mark, indicating that their contents conform to the regulation of PAEs by the Japan Toy Association and the Japan Inflatable Vinyl Products Manufactures Association, respectively. However, the mouth-piece of the float ring (C2_1) contained 0.097 % of DEHP and 0.075 % of DINP-2. The presence of these PAEs might be due to impurities of other plasticizers or contamination during the manufacturing process.[24,33] Four eraser samples were studied (A3, A7–9), and all of them contained high levels of DEHP and A7–9 also contained high levels of DBP. DEHP and DBP are often used together in household products;[20,21] the total concentration of PAEs (DEHP + DBP) in the eraser samples was over 40 %. Erasers that do not contain PVC or phthalates have recently become available, but our results indicate that a large proportion of erasers currently sold still contain high levels of PAEs. DINP-1 and 2 showed similar detection frequencies in the PVC containing household products.

Since small amounts of DEHP can be present as an impurity in DINP,[33] the DEHP detected in the emblem sample (B2) could be an impurity of DEHP. DINP and DEHP can be use as a mixture to improve resistance to low temperatures.[1] Therefore, DINP might have been used intentionally with DEHP for the pipe chair (F2) that can be used outdoors. Even though DIDP production is greater than DBP production in Japan[1] and it has been found in PVC toys in other countries,[33,34] its levels were only recently investigated in toys from Japan.[32] In our study, DIDP was detected in the cutting mat (A6) and mobile phone strap (D7). Recently, Abe et al. investigated the concentration of DIDP in the PVC toys circulated in Japan.[32] In this study as well, the presence of DIDP in household products was observed.

Exposure to PAEs from PVC household products

Our study has confirmed that high amounts of PAEs, particularly DEHP, are presented in PVC household products in Japan. In Japan, the tolerable daily intake (TDI) of DEHP was established as being 40–140 μg kgbw^{-1} day^{-1} on the basis of its testicular and reproductive toxicity in rats[2] and the TDI of DINP was established as being 150 μg kgbw^{-1} day^{-1} on the basis of its hepatic toxicity in rats.[5] Sugita et al. conducted an oral release study of DINP to estimate the amount of daily oral exposure of babies to PAEs derived from soft PVC baby toys.[35]

For a migration test, they used PVC products containing 38–58 % of DINP, and concluded that there were no differences in migration amounts between PVC products containing different concentrations of DINP and that the migration tendency of DEHP was similar to that of DINP. In addition, they found the migration amounts of DINP and DEHP to be greater than those of TDI in certain cases. The erasers and the mobile phone straps (LCD cleaner type) investigated in this study are small; therefore, children could easily lick, chew, and ingest these products. As a result, it is important to reduce the probability of contact of children with such products containing high PAE concentrations.

In Japan, it has been reported that PVC products mounted at the edge of furniture to prevent children from injury (such as E1, E5, E6, and E7) can be chewed on and ingested by children.[36] Large amounts of DEHP and DINP were contained in these products investigated in this study (maximum concentration of 47 %). Thus, it is advisable to replace plasticizers used in such household products with the suitable alternatives.

Hand-to-mouth contact is a important route of exposure to BFRs because of the dermal exposure of BFRs through house dust or direct contact with products containing BFRs.[18] Bisphenol A (BPA) can be transferred from thermal printer paper to the finger skin.[37] Since the PAE concentrations of the products considered in this study

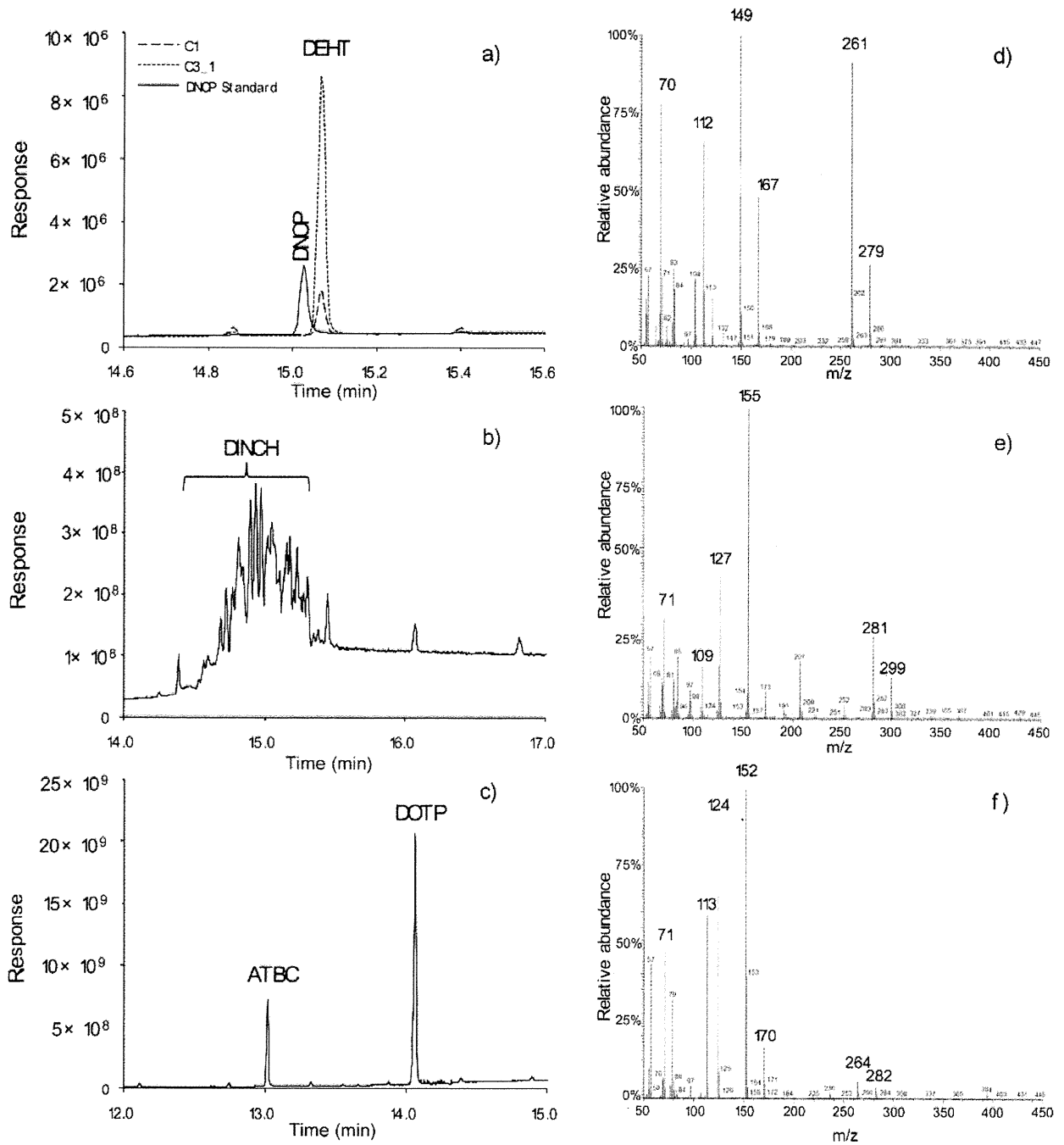

Fig. 1. Identification of unknown peaks in GC/MS chromatograms. a) Mass chromatogram of DNOP standard solution and samples C1 and C3_1 (selected ion monitoring mode: m/z = 149) and total ion chromatogram (scan mode: m/z = 50–450) of b) C2_2 and c) C5. Mass spectra of unknown peaks obtained by scan analysis (scan mode: m/z = 50–450) for d) DEHT (C1), e) DINCH (C2_2), and f) DOTP (C5).

are high, it is reasonable to assume that lager amounts of PAEs could be transferred to the skin surface from household products as compared to amounts of BFRs and BPA. Furthermore, PAEs may migrate easily from PVC products since they are not bound to plastic resin. Thus, a large amount of PAEs might be transferred to the skin surface from the chair, sofa, or mat samples investigated in this study. Although the dermal absorption of PAEs is not immediate,[7,8] determining of the level of dermal exposure is still important in order to estimate the amount of

PAEs ingested by hand-to-food or hand-to-mouth contact. Therefore, further study is necessary to estimate amounts of the PAEs transferred from household products to the skin surface.

Other plasticizers detected in PVC household products

Recently, Abe et al. detected and identified many kinds of non-phthalic plasticizers used in PVC toy products in Japan.[32] In this study, other compounds were also detected in the household products samples that contained no or small amounts of the PAEs studied. We also carried out identification in these unknown peaks. The retention time of the unknown peak detected in samples C1, C3_1, and D4 was very close to DNOP and had a characteristic fragment ion of phthalate ([m/z] = 149) (Fig. 1a). This peak was identified as based on EI-mass spectra reported in the previous study[38] and estimated di(2-ethylhexyl) terephthalate (DEHT) (Figs. 1a and d). The unknown compound detected in samples C2_1, C2_2, C2_3, and C3_2 was identified as diisononyl 1,2-cyclohexanedicarboxylic acid (DINCH) on the basis of EI-mass spectra[34,38] and the presence of isomers (Figs. 1b and e). Another unknown peak detected in samples C5 and D9 was identified as acetyl tributyl citrate (ATBC, Fig. 1c) using the NIST mass spectral library (NIST 05 ver2.0). ATBC is a non-phthalic plasticizer and has previously been detected in PVC toys and sports products[21,24,32] similar to DEHA and TMPDIB. Sample C5 also contained an additional unknown peak identified using NIST as di(2-ethylhexyl) 4-cyclohexene-1,2-dicarboxylate (DOTP, Figs. 1c and f). DOTP is used as plasticizer for PVC and lubber products. It is a cyclic fatty

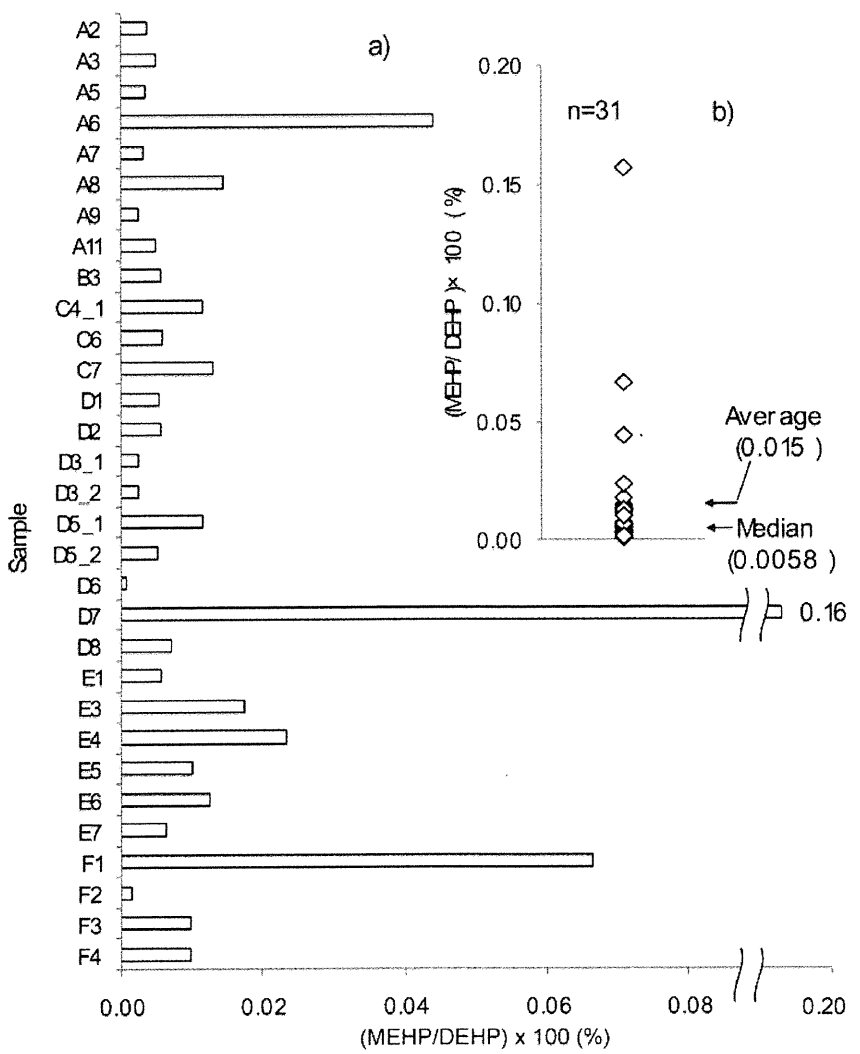

Fig. 2. MEHP in PVC household products. a) Ratios of MEHP against DEHP in the samples that contained MEHP at levels above the MQL, and b) their distribution.