Table 3. Sample stability under several conditions

Sample stability (mean ± SD, %, $n = 6$)	25°C				4°C		−80°C		−80°C[a]	
	1 h	2 h	3 h	6 h	12 h	24 h	24 h	72 h	24 h	72 h
20 ng/mL										
NNIC	100.9 ± 4.2	99.7 ± 5.0	100.4 ± 6.1	100.7 ± 4.2	100.3 ± 7.1	95.4 ± 3.6	99.0 ± 10.2	101.7 ± 7.0	97.5 ± 7.5	109.9 ± 14.6
NIC	91.9 ± 9.1	92.5 ± 10.6	95.9 ± 5.5	102.6 ± 11.9	95.7 ± 6.4	96.9 ± 8.3	98.4 ± 9.4	92.6 ± 12.5	115.1 ± 12.2	101.1 ± 6.0
NCOT	99.7 ± 1.6	101.1 ± 1.2	101.0 ± 1.4	103.6 ± 1.8	98.4 ± 1.2	98.9 ± 1.6	97.5 ± 4.7	109.5 ± 4.5	101.8 ± 1.8	106.1 ± 7.6
COT	98.3 ± 1.2	98.7 ± 2.1	104.8 ± 2.4	102.1 ± 2.4	96.9 ± 2.0	102.2 ± 2.0	109.4 ± 6.0	102.3 ± 6.4	104.8 ± 2.3	100.1 ± 2.4
HCOT	98.6 ± 1.7	98.6 ± 1.5	98.4 ± 1.3	101.0 ± 2.6	97.6 ± 1.3	98.9 ± 1.3	99.9 ± 2.6	95.7 ± 2.1	98.6 ± 1.0	98.0 ± 4.4
100 ng/mL										
NNIC	100.8 ± 2.2	106.7 ± 2.9	103.7 ± 3.7	102.8 ± 2.9	102.8 ± 3.3	106.9 ± 3.2	104.8 ± 3.4	106.6 ± 2.9	109.4 ± 3.5	113.7 ± 9.3
NIC	107.1 ± 10.0	108.9 ± 4.9	105.4 ± 7.4	108.4 ± 12.4	102.5 ± 5.9	104.2 ± 6.5	114.7 ± 17.7	109.8 ± 3.5	111.3 ± 7.0	108.2 ± 5.3
NCOT	104.5 ± 3.5	104.0 ± 1.3	109.2 ± 7.2	102.6 ± 5.0	99.3 ± 3.3	102.0 ± 2.9	102.1 ± 6.8	101.9 ± 3.0	107.2 ± 5.9	99.8 ± 2.3
COT	99.7 ± 1.5	109.3 ± 2.8	110.8 ± 2.0	102.3 ± 2.4	100.3 ± 1.2	109.0 ± 2.0	109.2 ± 4.8	101.7 ± 3.0	112.5 ± 2.6	107.2 ± 5.5
HCOT	102.4 ± 1.8	103.6 ± 1.3	104.2 ± 1.8	103.2 ± 1.3	98.5 ± 1.7	101.5 ± 1.6	102.4 ± 0.9	103.9 ± 3.1	108.3 ± 7.2	106.7 ± 5.7

[a] Freeze–thaw stability of the extract (three cycles).

Accuracy was expressed as the ratio of theoretical concentrations to average measured ones.

Serum sample stability was examined as well: stability of the unprocessed sample (24 and 72 h, −80°C), autosampler stability (6, 12, and 24 h, 4°C), processed sample preparation (1, 2, 3, and 4 h, 25°C), and freeze–thaw stability of the extract (three cycles).

Results and Discussion

Optimization of HILIC-MS/MS Conditions

The analytes that were dissolved in methanol at a concentration of 100 ng/mL were infused through an integrated syringe pump into the ESI probe at a rate of 10 µL/min to tune the mass spectrometer and optimize the acquisition parameters. Comparison of the results obtained in the positive and negative ion modes showed that a high response was obtained in the positive ESI mode. The optimized MRM conditions are given in Table 1.

NIC and its metabolites are highly polar compounds and are thus difficult to determine by reverse-phase liquid chromatography. Several approaches to retaining the desired compound on the stationary phase or separating other compounds are available. HILIC is characterized by the presence of a high initial organic modifier concentration to favor hydrophilic interactions between the solute and the hydrophilic stationary phase (Alpert, 1990). HILIC is an alternative to normal-phase chromatography as it uses a silica column and conventional reverse-phase mobile phase. Thus, the retention times of highly polar compounds are increased as the hydrophilicity of the solute is increased. HILIC can be easily used with a mass spectrometer since it is a variant of normal-phase chromatography. In HILIC, the hydrophilic stationary phase is used in combination with a mostly organic mobile phase, and elution is usually performed by increasing water concentration. HILIC-MS was recently employed to separate and quantify highly polar compounds in biological samples (Iwasaki *et al.*, 2006).

We examined whether the presence of formic acid in the mobile phase would increase both sensitivity and resolution, to obtain sharp peaks. Formic acid improved separation and sensitivity and the mobile phase that gave the most intense peak contained 0.001% formic acid (data not shown). When the mobile phase decreased in concentration and pH value, all compounds increased in peak intensity. However, the lower concentration of formic acid affected the retention time and peak shape of NIC, which was also retained by the HILIC column. For this reason, we concluded that the final concentration of mobile phase was set to 0.05% formic acid. MRM chromatograms are shown in Fig. 2. All analytes were well detected and separated. Moreover, the other compounds did not affect the identification or quantitation even when a high concentration of sample was injected.

Solid-phase Extraction Recovery

Optimization of the SPE procedure was carried out to meet three objectives. First, the overall conditions selected would provide the highest and most consistent absolute recovery of the target analyte. Second, the conditions for washing the SPE cartridges containing bound analyte would remove unwanted matrix components to the greatest extent possible without eluting the target analyte or IS. Third, elution conditions would subsequently recover the analyte efficiently while minimizing the elution of less polar matrix components (*Yu et al.*, 2008). Recovery was calculated from the peak area ratios (in percent) of extraction samples

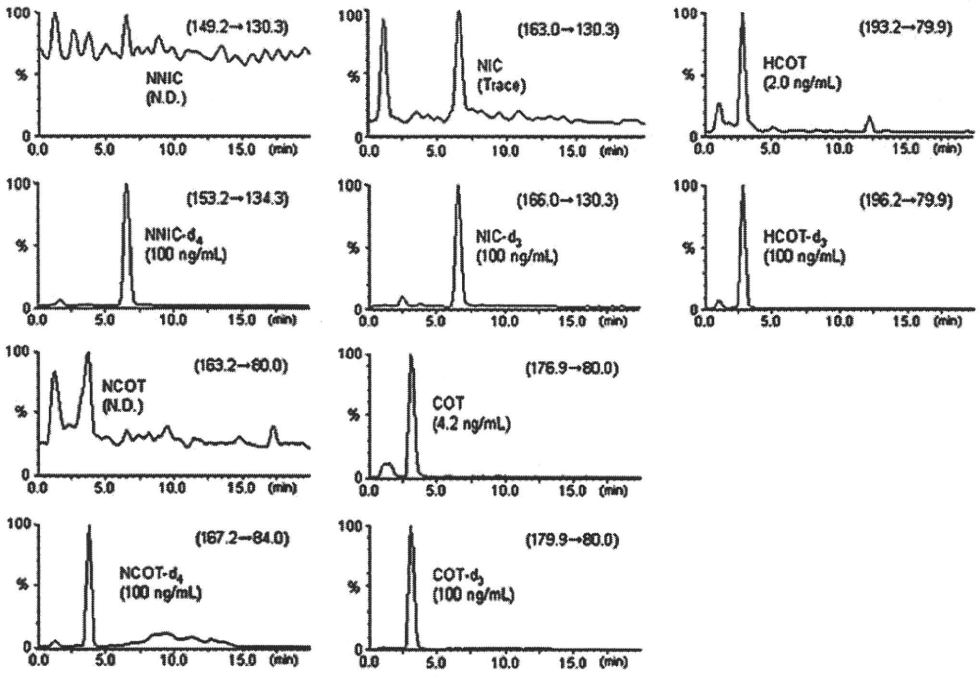

Figure 3. MRM chromatograms of nicotine and its metabolites in maternal serum sample (no. 18).

to control samples at each concentration and the results are shown in Table 2. The recoveries of NIC and its major metabolites, such as COT and HCOT, from human serum samples ranged from 86.2 and 107.7%.

To evaluate the matrix effect, peak areas of extracted pooled serum samples spiked with standards at QC concentrations after the extraction procedure were compared with those of QC samples (Matuszewski *et al.*, 2003; Chambers *et al.*, 2007). Table 2 shows the results of the matrix effect. The results indicated that NIC and its metabolites were influenced by the matrix effect through ion suppression. Although the magnitude of the matrix effect was not determined, the accuracy and precision of the method at this level were found to be within the limits required by the FDA. However, they indicated that matrix effects should be investigated to ensure that precision and sensitivity are not compromised.

Validation

The LODs of the HILIC-MS/MS method were determined by analyzing the response after injecting methanol containing NIC and its metabolites. The LOQs for NIC, COT and HCOT were 1 ng/mL. The correlation coefficient (r) was more than 0.99 and deviation of the calculated concentrations was acceptable within ±15% from nominal concentrations (Table 3). Moreover, in each analytical run, the highest calibrator containing all analytes (500 ng/mL) was injected immediately before a negative sample, allowing the quantification of potential carryover. There was no evidence of carryover at the method's LOD.

Precision and accuracy were assessed at LOQ, 10, 50, and 100 ng/mL. Table 4 contains intra- and inter-day assay precision and accuracy data for each concentration. Precision was determined by multiple analyses of the same sample at different days, and is expressed as RSD. Precision was evaluated by calculating intra- and inter-day assay at each QC concentration. Intra-day assay was less than 10% for all the analytes ($n = 6$). The differences from the target were less than 10% and considered clinically insignificant. Inter-day assay ($n = 6$) was less than 10% for each compound.

Accuracy, also called bias, expresses the similarity of the experimental mean value to the accepted reference value. It indicates systematic errors and is expressed as percentage deviation from the accepted reference value. Accuracy was acceptable in each case (threshold of ±15%) as the relative bias was lower than 14.2%. The RSDs for the intra-day assay as well as for the inter-day assay were within ±15%, which is acceptable according to the FDA guidelines for bioanalytical method validation (FDA, 2001).

The pooled serum sample was spiked with the analyte at 20 or 100 ng/mL and six samples were analyzed immediately following sample preparation. The remaining pooled serum sample was divided into two subsets (stable samples and control samples). Stable samples were stored at 25, 4 and −80°C, respectively (Table 5). Losses of less than 10% were observed on storage for 3 h at 25°C (room temperature storage) and for 24 h at 4°C (autosampler storage). There was no significant degradation and stability was maintained under routine laboratory conditions. Stability studies were conducted to ensure that the analytes were stable during the preparation, processing and analysis.

Application

To demonstrate the applicability of the method, prenatal tobacco-exposed maternal and cord sera were analyzed. One

Table 6. Quantification of maternal and cord serum

Compound	Non-smoker Sample No. 1–16	Smoker No. 17	No. 18	No. 19	No. 20
Maternal serum					
NNIC	ND	ND	ND	ND	ND
NIC	ND	ND	Trace	3.2	7.8
NCOT	ND	ND	ND	ND	5.4
COT	ND	ND	4.2	13.6	486.4
HCOT	ND	ND	2.0	ND	163.5
Cord serum					
NNIC	ND	ND	ND	ND	ND
NIC	ND	ND	ND	ND	ND
NCOT	ND	ND	ND	ND	2.6
COT	ND	ND	2.5	4.8	216.8
HCOT	ND	ND	1.3	ND	93.9

All values in ng/mL.

maternal serum sample contained 4.2 ng/mL COT and 2.0 ng/mL HCOT. The corresponding chromatograms are displayed in Fig. 3. All nonsmoker maternal and cord serum samples were negative for NIC and its metabolites (Table 6). In smokers, mean cotinine level in maternal serum was 126.1 ± 240.3 ng/mL and mean HCOT level was 41.4 ± 81.4 ng/mL. The concentrations of NIC metabolites in cord serum were generally two- to threefold lower than in serum from the mothers, but strong correlations were observed between maternal and cord serum.

Conclusions

A sensitive and specific HILIC-MS/MS method for the simultaneous detection and quantification of NIC and its metabolites in human maternal and cord sera is presented. The method has sufficient analytical sensitivity for the quantification of analytes using a very low volume of serum (100 µL). The method could be used to determine NIC and its metabolites in human maternal and cord sera in future studies of the effects of nicotine exposure on neonatal outcome.

Acknowledgements

This study was supported by grants from the Ministry of Health, Labour and Welfare, Japan.

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Bull Environ Contam Toxicol (2010) 85:92–96
DOI 10.1007/s00128-010-0051-8

Determination of Five Phthalate Monoesters in Human Urine Using Gas Chromatography-Mass Spectrometry

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Received: 29 January 2010 / Accepted: 14 June 2010 / Published online: 24 June 2010

Abstract We have developed a gas chromatography-mass spectrometry (GC–MS) method to determine five phthalate monoesters (monoethyl phthalate (MEP), mono-*n*-butyl phthalate (MBP), mono-(2-ethylhexyl) phthalate (MEHP), monoisononyl phthalate (MINP) and monobenzyl phthalate (MBz)) in human urine. Human urine samples were subjected to enzymatic deconjugation of the glucuronides followed by extraction with hexane. The extracted phthalate monoesters were methylated with diazomethane, purified on a Florisil column and then subjected to GC–MS analysis. The recoveries from urine spiked with five phthalate monoesters were 86.3%–119% with coefficients of variation of 0.6%–6.1%. We measured phthalate monoester levels in human urine by analyzing 36 samples from volunteers. MBP and MEP were detected in all samples, and their median concentrations were 60.0 and 10.7 ng/mL, respectively. MBzP and MEHP were found in 75% and 56% of samples, and their median concentrations were 10.9 and 5.75 ng/mL, respectively. MINPs were not detected in most samples (6% detectable). Women had significantly ($p < 0.05$) higher mean concentrations of MBP and MEP than men. The estimated daily exposure levels for the four parent phthalates excluding diisononyl phthalate ranged from 0.27 to 5.69 μg/kg/day (median).

Keywords Phthalate monoesters · Exposure · Urine · Gas chromatography-mass spectrometry

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Phthalates, diesters of phthalic acid, are widely used in many consumer products and as additives, solvents and plasticizers. Humans are ubiquitously exposed to phthalates (Blount et al. 2000a; Silva et al. 2004) and patients undergoing medical procedures such as transfusions, dialysis, cardiopulmonary bypass or aphaeresis are potentially more heavily exposed (Calafat et al. 2004; Green et al. 2005; Inoue et al. 2005). Certain phthalates such as dibutyl phthalate (DBP), butylbenzyl phthalate (BBzP), di-2-ethylhexyl phthalate (DEHP) and diisononyl phthalate (DINP) lead to testicular toxicity in rodents in an antiandrogenic manner (Parks et al. 2000). DBP, DEHP, BBzP, and their metabolites, mono-*n*-butyl phthalate (MBP), monobenzyl phthalate (MBzP), and mono-(2-ethylhexyl) phthalate (MEHP) are teratogenic in animals (Gray and Gangolli 1986; Mylchreest et al. 1998; Parks et al. 2000; Ema and Miyawaki 2001a, b). It has been reported that urinary concentrations of four phthalate metabolites, monoethyl phthalate (MEP), MBP, MBzP, monoisobutyl phthalate, were significantly associated with reduced anogenital distance in human male infants which was the first

demonstration of the subtle developmental effects in human (Swan et al. 2005).

More recent studies concerning general human exposure to phthalates have been carried out by measuring phthalate monoester metabolites mainly in urine (Koch et al. 2003; Hauser 2008), because phthalate diesters metabolize rapidly to their monoesters and are excreted in urine either as hydrolytic monoesters or the oxidative products (Silva et al. 2003). Furthermore, potential problems of diester contamination during sample collection, storage and analysis procedures may make it difficult to measure low baseline levels of phthalate diesters in serum and/or urine (Blount et al. 2000b). Several reports have described the assessment of phthalate exposure in the general population in the USA (Kohn et al. 2000) and Europe (Koch et al. 2003). However, reports relating to the Japanese are limited; Itoh et al. (2005) measured MBP and MEHP in urine samples from the general population.

For the quantitative detection of phthalate monoester metabolites in urine, a high-performance liquid chromatography-tandem mass spectrometric (LC–MS/MS) method has been developed (Silva et al. 2003). All the above-mentioned reports used LC–MS/MS method. To our knowledge, there are no reports of studies on phthalate monoester determination in urine using gas chromatography-mass spectrometry (GC–MS). In the present study we have developed a new method for the quantitative determination of five phthalate monoesters (MEP, MBP, MEHP, monoisononyl phthalate (MINP) and MBzP) in human urine using GC–MS. The method was used for analysis of human urine samples.

Materials and Methods

The study participants were 36 volunteers who were recruited from the staff of Aichi Prefectural Institute of Public Health, Nagoya, Japan. All of the volunteers reside in the Aichi Prefecture, Japan. Of the participants, 64% (23/36) were men and 36% (13/36) were women. 31% (11/36) were under 39 years of age, 25% (9/36) were between 40 and 49 years of age, and 44% (16/36) were over 50 years of age.

MEP, MBP, mono-2-ethylhexyl phthalate (MEHP), monoisononyl phthalate (MINP), MBzP, and their $^{13}C_4$-labeled internal standards were purchased from Cambridge Isotope Laboratories (MA, USA). Florisil® PR, β-glucuronidase solution from *Escherichia coli* and pesticide analysis grade sodium sulfate were purchased from Wako Pure Chemical Industries (Osaka, Japan). Bondesil-PSA (40 μm pore size) was purchased from Varian (CA, USA). Phthalic acid esters, analysis grade acetonitrile, hexane, acetone and sodium chloride were purchased from Kanto Chemical (Tokyo, Japan). The water used for the extraction was prepared by washing distilled water with hexane.

All of the experimental apparatus, including glassware and spatulas, were washed carefully with acetone and hexane, and then heated at 200°C for 2 h to remove any phthalates. They were washed with acetone and hexane just before use. Sodium chloride, sodium sulfate and Florisil® PR were heated at 200°C for 2 h. A reagent blank was analyzed before sample analysis in each batch.

A Florisil column was prepared by packing Florisil (1 g) and sodium sulfate (2 g) in turn into a glass syringe (15 mm × 110 mm). The column was washed with acetone (10 mL) and hexane (10 mL) before use.

Diazomethane was prepared by adding 0.5 g of *N*-methyl-*N'*-nitro-*N*- nitrosoguanidine to 3 mL of 20% sodium hydride solution; the diazomethane gas thus generated was dissolved in 10 mL of ice-cold methyl *t*-butyl ether.

Urine (2 mL) was placed into a centrifuge tube to which ammonium acetate (0.5 mL) was added and the sample was spiked with isotopically labeled internal standards (50 μL, 1 μg/mL). After β-glucuronidase (30 μL, 50 units/mL) was added, the sample was incubated at 37°C for 60 min. The sample solution was then acidified to pH 2 with 10% sulfuric acid. After adding hexane (5 mL) and sodium chloride (1 g), the sample solution was mixed for 3 min by vigorous shaking and then centrifuged at 3,000 rpm for 5 min. The hexane layer was collected and dried under a stream of nitrogen gas at 35°C. Diazomethane (0.5 mL) was added to the residue and the mixture was allowed to stand for 30 min at room temperature. The solution was dried under a stream of nitrogen gas at 35°C. The residue was dissolved in hexane (5 mL) and then loaded onto a Florisil column, which had been preconditioned with acetone (10 mL) and hexane (10 mL). After washing with hexane (3 mL), the phthalate monoester fraction was eluted with 5% acetone-hexane (10 mL). The eluate was concentrated to 1.0 mL under a stream of nitrogen gas at 35°C. An aliquot of each sample (2 μL) was injected into a GC–MS system.

GC–MS analysis was performed on an Agilent 6890 N GC/5973 N MSD instrument (Agilent Technologies, CA, USA). A 30-m HP-5MS SV column (J & W Scientific, CA, USA) of i.d. 0.25 mm and a film thickness of 0.5 μm was used. The initial oven temperature was 80°C. After holding at the initial temperature for 3 min, the temperature was increased to 240°C at a rate of 20°C/min, and then to 300°C at a rate of 10 °C/min, where it remained constant for 5 min. Helium was used as carrier gas at a flow-rate of 1.2 mL/min. The ion source temperature was 230°C and electron ionization was used as the ionization mode. The injection port was kept at 250°C. The ions used for selected ion monitoring (SIM) are summarized in Table 1; the ions observed as the base peak were used for quantification and

Table 1 Monitored ions for quantification and confirmation, and retention times for the measured methyl derivatives of phthalate monoesters

Analyte	Monitored ions (*m/z*)		Retention time (min)
	Quantification	Confirmation	
MEP	163	149, 176	9.04
MEP-$^{13}C_4$	167	153, 180	9.04
MBP	163	149, 181	10.18
MBP-$^{13}C_4$	167	153,185	10.18
MEHP	163	149, 181	12.09
MEHP-^{13}C4	167	153,185	12.09
MINP	163	149, 181	12.42
MINP-$^{13}C_4$	167	153,185	12.42
MBzP	163	91, 164	12.71
MBzP-^{13}C4	167	91, 168	12.71

Abbreviations: *MEP* monoethyl phthalate, *MBP* mono-*n*-butyl phthalate, *MEHP* mono-2-ethylhexyl phthalate, *MINP* monoisononyl phthalate, *MBzP* monobenzyl phthalate

the second and third most abundant ions were used for confirmation. The concentrations of five phthalate monoesters in urine were corrected with $^{13}C_4$-labeled internal standards. Reproducible calibration curves for the methyl derivatives of the five phthalate monoesters were obtained with correlation coefficients greater than 0.999 (known concentration versus analyte/internal standard) and they were linear over the range of 2–100 ng/mL.

We calculated the estimated daily intake of phthalates by using the following equation (Kohn et al. 2000; Koch et al. 2003; Itoh et al. 2005): intake (μg/kg/day) = ME × CE/(f × 1,000) × (MW_d/MW_m). ME is the creatinine-adjusted concentration of the phthalate monoester (μg/g creatinine), CE is the personal daily creatinine excretion (mg/kg/day), which was calculated using the following equation (Kawasaki et al. 1991): CE (male) = − 12.63 × age + 15.12 × body weight (kg) + 7.39 × height (cm) − 79.90, or CE (female) = −4.72 × age + 8.58 × body weight (kg) + 5.09 × height (cm) − 74.95. The values of the ratios of urinary excretion to total elimination (f) were 0.024 for DEHP, 0.69 for DEP and DBP, and 0.73 for BBzP (Koch et al. 2003). MW_d and MW_m are the molecular weights of the parent diesters and their monoesters, respectively.

The Mann–Whitney U-test was used for statistical analyses. Differences were considered to be significant at $p < 0.05$.

Results and Discussion

For extraction of phthalate monoesters in biological samples, different solid-phase extraction (SPE) and solvent extraction methods are used. Among a variety of commercially available solid-phase extraction cartridges, we examined three types of cartridge; Abselut NEXUS (VARIAN), GL-Pak Glass SPE PLS-3 (GL Science, Tokyo, Japan) and Oasis® HLB Glass (Waters, MA, USA). However, the background levels of MEHP and MBP for all cartridges were high at about 60 and 8 ng/mL, respectively, even though the cartridges were prewashed with acetone and hexane repeatedly. We then examined hexane and acetonitrile for extraction of phthalate monoesters. Although both solvents extracted phthalate monoesters in urine effectively, acetonitrile also extracted co-existing substances in urine. Hexane was consequently selected as the extraction solvent.

For purification of methyl derivatives of phthalate monoesters methylated with diazomethane, we examined the Florisil single layer column and the Florisil and Bondesil-PSA dual layer column. A Florisil single layer column was selected because both columns showed the same clean-up effect. For elution from a Florisil column, we examined a mixture of solvents; acetonitrile/hexane and acetone/hexane. Acetone/hexane was selected because this solvent mixture gave the most satisfactory recoveries, while acetonitrile/hexane gave poor recoveries of methylated MEP (<10%). The optimized procedures including the extraction followed by Florisil column purification are described in the Materials and Methods section. Typical SIM chromatograms of a mixture of methyl derivatives of five standard phthalate monoesters and a urine sample are shown in Fig. 1.

The recoveries from urine spiked with 250 ng/mL of each of the five phthalate monoesters were examined by calculating the ratio of the amount of analytes recovered

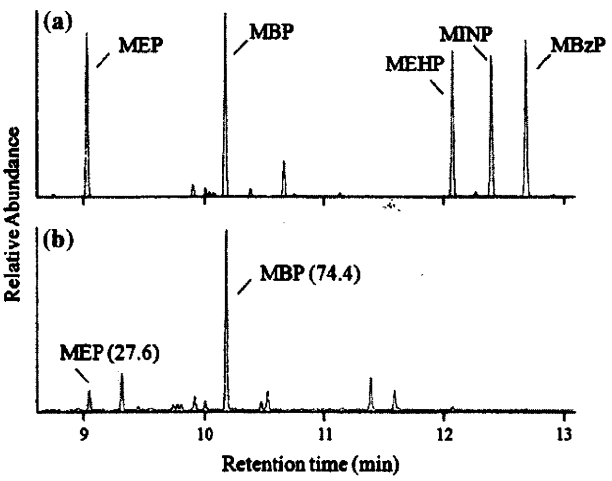

Fig. 1 Typical SIM chromatograms of **a** a mixture of methyl derivatives of five standard phthalate monoesters and **b** a urine sample. *Numbers in parentheses* indicate the found concentrations (ng/mL)

Table 2 Recoveries and LOQ of phthalate monoesters

Analyte (ng/mL)	Recovery (%)	CV[a] (%)	LOQ[b]
MEP	86.3	2.2	1
MBP	119	6.1	1
MEHP	100	0.6	5
MINP	98.0	0.8	1
MBzP	89.1	2.1	5

Results are means of three replicate determinations

[a] *CV* coefficients of variation

[b] *LOQ* limit of quantification

after Florisil column purification to the amounts originally added. Overall recoveries and coefficients of variation were found to be satisfactory; these values were 86.3%–119% and 0.6%–6.1%, respectively (Table 2). To determine the background levels of the five phthalate monoesters originating from sample preparation, a blank test was carried out using hexane-washed water instead of urine. The blanks (n = 3) contained < 1.0 ng/mL for MBP, 1.6 (±0.09) ng/mL for MEP, 3.0 (±0.5) ng/mL for MEHP, <1.0 ng/mL for MINP and <5.0 ng/mL for MBzP. The limit of quantification (LOQ) for each of the five phthalate monoesters was calculated as $10S_0$, where S_0 is the value of the standard deviation obtained by analyzing quintuplicate sets of the blank analysis, or as $10S_1$, where S_1 is the value of the standard deviation obtained by analyzing quintuplicate sets of the lowest level of the standard sample. The LOQ for each of the five phthalate monoesters is summarized in Table 2. With careful control of contamination, as described in the Materials and Methods section, sample analysis was achieved with a low-level background, which allowed us to evaluate precisely the concentration of phthalate monoesters.

This method was then applied to the analysis of urine samples obtained from 36 volunteers (Table 3). MBP and MEP were detected in all samples, and their median concentrations were 60.0 and 10.7 ng/mL, respectively. MBzP and MEHP were found in 75% and 56% of samples, and their median concentrations were 10.9 and 5.75 ng/mL, respectively. MINP was not detected in most samples (detectable in 6%). The concentrations of MBP and MEP showed larger inter-individual variation: 9.09–194 ng/mL for MBP and 1.36–1,350 ng/mL for MEP, whereas those of MBzP, MEHP and MINP showed less inter-individual variation; <LOQ-40.6 ng/mL for MBzP, <LOQ-29.6 ng/mL for MEHP and <LOQ-3.00 ng/mL for MINP. We speculate that these results may reflect the differences in the individuals' use of products containing the parent phthalate diesters. DBP and diethyl phthalate (DEP) are used mostly in consumer products (detergents, soaps, cosmetics, shampoo, and perfumes), while BBzP, DEHP and DINP are used primarily for industrial purposes as plasticizers (Silva et al. 2004; Mortensen et al. 2005). Women had significantly ($p < 0.05$) higher mean concentrations of MBP and MEP (114 and 110 μg/g creatinine) than did men (58.7 and 12.6 μg/g creatinine). The fact that women had higher concentrations of MBP and MEP than men was most likely attributable to women's increased use of personal care products, such as hair care products, cosmetics, and perfumes as indicated by Silva et al. (2004). Urinary levels of phthalate monoesters in approximately 2,540 samples collected from participants in the National Health and Nutrition Examination Survey (NHANES), 1999–2000 in the USA have been reported (Silva et al. 2004). The median concentration of MBP and MEHP was 2.7- and 2.5-fold lower in the NHANES 1999–2000 population (21.9 and 3.08 μg/g creatinine) than in the present study (58.7 and 7.73 μg/g creatinine), whereas that of MEP in the NHANES 1999–2000 population (141 μg/g creatinine) was almost tenfold higher than that in the present study (13.7 μg/g creatinine). The median concentrations of MBzP were almost the same in both studies (13.3 μg/g creatinine for NHANES 1999–2000 population and 11.4 μg/g creatinine for the present study). Despite the limited numbers of samples in the present study, these results may represent the differences in the use of phthalate diesters between the USA and Japan.

Human exposure to phthalate diesters has been estimated by calculating the daily intake using the concentrations of the phthalate monoesters in urine (Kohn et al. 2000; Koch et al. 2003; Itoh et al. 2005). Estimates of the intake of four phthalate diesters excluding MINP are shown

Table 3 Concentrations of phthalate monoesters in urine

Analyte	No. of Positives	Concentration			
		Measured(ng/mL)		Creatinine-adjusted (μg/g creatinine)	
		Median	Range	Median	Range
MBP	36 (100)[a]	60.0	9.09–194	58.7	22.8–554
MEP	36 (100)	10.7	1.36–1,350	13.7	3.06–944
MBzP	27 (75)	10.9	<LOQ–40.6	11.4	<LOQ–39.4
MEHP	20 (56)	5.75	<LOQ-29.6	7.76	<LOQ-56.2
MINP	2 (6)	<LOQ	<LOQ-3.00	<LOQ	<LOQ-6.38

[a] Numbers in parentheses indicate percentages

Table 4 Comparison of the intake estimates for four phthalate diesters

Phthalate diester	Median intake value (μg/kg/day)				TDI (μg/kg/day)
	This study (n = 36)	Kohn et al. (n = 289)	Koch et al. (n = 85)	Itoh et al. (n = 36)	
DEHP	5.69	0.71 (3.14)[a]	10.3	1.8 (5.48)	40–140
DBP	1.47	1.5	5.22	1.3	100
DEP	0.29	12	2.32	–	5,000
BBzP	0.27	0.88	0.60	–	200

[a] Numbers in parentheses indicate the recalculated intake value by using the fractionary urinary excretion factor of 0.024 reported by Koch et al. (2003)

in Table 4 together with other previous assessments. The median values in the present study were highest for DEHP intake and decreased in the order DEHP, DBP, DEP, and BBzP. The median values of DEHP intake were higher in the present study (5.69 μg/kg/day) than those reported by Kohn et al. (2000) (0.71 μg/kg/day) and Itoh et al. (2005) (1.8 μg/kg/day), and were lower than those reported by Koch et al. (2003) (10.3 μg/kg/day). However, previous assessments used different fractional excretion values, suggesting that the calculated daily intake values have inherent uncertainties. When DEHP intake values are recalculated using the fractional urinary excretion factor (0.024) reported by Koch et al. (2003), these values range from 3.14 to 10.3 μg/kg/day (Table 4) which are comparatively similar values. The establishment of a method for calculating the intake estimates remains to be elucidated.

In summary, we have developed a gas chromatography–mass spectrometry method to determine the concentration of five phthalate monoesters in human urine. Using this method we were able to measure precisely the level of phthalate monoesters in human urine obtained from 36 volunteers. This method will help to evaluate human exposure to phthalates.

Acknowledgments This study was supported by a grant from the Ministry of Health, Labor, and Welfare, Japan.

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Bull Environ Contam Toxicol (2010) 84:212–216
DOI 10.1007/s00128-009-9919-x

Determination of Phthalates in Diet and Bedding for Experimental Animals Using Gas Chromatography-Mass Spectrometry

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Received: 23 June 2009 / Accepted: 18 November 2009 / Published online: 1 December 2009

Abstract We have developed a gas chromatography–mass spectrometry method to measure five phthalates (dibutyl phthalate, butylbenzyl phthalate, di-2-ethylhexyl phthalate, diisooctyl phthalate, and diisononyl phthalate) in diets and beddings for experimental animals. The recoveries from diets and beddings spiked with five phthalates were 98.8%–148% with coefficients of variation of 0.4%–7.8% for diets and 94.7%–146% with coefficients of variation of 1.0%–5.0% for beddings. We analyzed commercial animal diets and beddings, and found that the levels of phthalates varied from sample to sample; the concentrations of five phthalates were 141–1,410 ng/g for diets and 20.5–7,560 ng/g for beddings.

Keywords Phthalates · Animal diets · Beddings · Gas chromatography-mass spectrometry

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Phthalates are used as additives, as solvents, and as plasticizers in many consumer products. The widespread manufacture, use, and disposal of phthalates have caused ubiquitous environmental pollution and humans are regularly exposed to phthalates (Blount et al. 2000; Silva et al. 2004; Kato et al. 2005). Certain phthalates, such as dibutyl phthalate (DBP), butylbenzyl phthalate (BBzP), di-2-ethylhexyl phthalate (DEHP) and diisononyl phthalate (DINP), have been shown to disrupt development of the reproductive tract in male rodents in an antiandrogenic manner (Parks et al. 2000). Concern has been raised about phthalates in relation to effects on the reproductive tract in adult males and the development of male offspring in humans (Duty et al. 2003; Swan et al. 2005).

When animal toxicology of phthalates is studied, it is necessary to take into account the exposure from the diet and the experimental environment. Since the complete exclusion of phthalate may well be impossible, the precise concentration of phthalates in the diet, bedding and water used for feeding, the air in the experimental animal room, etc. could be important information needed to attain reliability of animal experiments. To our knowledge, no study of phthalate contamination of diets or experimental environments has been reported.

We have developed a gas chromatography-mass spectrometry (GC-MS) method to determine five commonly used phthalates (DBP, DEHP, BBzP, DINP and diisooctyl phthalate (DIOP)) in animal diets and beddings. The method was used for the analysis of commercial diets and beddings for experimental animals. Additionally, in order to investigate the other causes of rodent exposure to phthalates in feeding conditions, we analyzed water supplied to the animals, and samples of air taken from the animal room.

Materials and Methods

DBP, DEHP, BBzP, DIOP, DINP, DBP-3,4,5,6-d_4, DEHP-3,4,5,6-d_4, BBzP-3,4,5,6-d_4, and DOP-3,4,5,6-d_4 were purchased from Kanto Chemical (Tokyo, Japan). DNP-3,4,5,6-d_4 was purchased from Hayashi Pure Chemicals (Osaka, Japan). Phthalic acid esters and analytical grade acetonitrile, hexane, acetone and sodium chloride were purchased from Kanto Chemical. Pesticide analysis grade sodium sulfate was purchased from Wako Pure Chemical Industries (Osaka, Japan). Bondesil-PSA (40 μm pore size) was purchased from Varian (CA, USA). Florisil® PR was purchased from Wako Pure Chemical Industries. The water used for extraction was prepared by washing distilled water with hexane.

Rodent diets were obtained from CLEA Japan Inc. (Tokyo, Japan), Oriental Yeast Co. Ltd (Tokyo, Japan), and Nihon Nosan Kogyo Co. Ltd (Tokyo, Japan). Animal beddings were obtained from Charles River Laboratories Japan Inc. (Yokohama, Japan), Japan SLC, Inc. (Hamamatsu, Japan), CLEA Japan Inc., and Oriental Yeast Co. Ltd.

Since some of phthalates analyzed in this study are very abundant and contamination is practically unavoidable (Takatori et al. 2004), the contamination preventive measures were given as follows. No plastic apparatus was used in these experiments. The reagents and solvents were used just after opening or were not left for a long time after opening. All of the experimental apparatus, including glassware and spatulas, were washed carefully with acetone and hexane, and then heated at 200°C for 2 h to remove any phthalates. They were washed with acetone and hexane just before use. Sodium chloride, sodium sulfate and Florisil® PR were heated at 200°C for 2 h. Before starting the experiment, we took various precautionary measures, such as shortening fingernails, washing hands well with soap, covering the laboratory bench with aluminum foil, etc. A blank analysis was carried out before sample analysis in each batch. The evaporator was cleaned before use by evaporating 10 mL of acetone.

A Florisil and Bondesil-PSA column was prepared by packing Florisil (1 g), Bondesil-PSA (0.5 g) and sodium sulfate (2 g) in turn into a glass syringe (15 mm × 110 mm). The column was washed with acetone (10 mL) and hexane (10 mL) before use.

Stock solutions of native standards (DBP, DEHP, BBzP, DIOP, DINP) and isotope-labeled internal standards (DBP-3,4,5,6-d_4, DEHP-3,4,5,6-d_4, BBzP-3,4,5,6-d_4, DOP-3,4,5,6-d_4, DNP-3,4,5,6-d_4) were prepared in acetonitrile and stored at −20°C in Teflon-capped glass bottles until use. They were mixed at the desired ratio and serially diluted for calibration curves. The peak area ratio of analyte to isotope-labeled internal standard was used for quantification. Reproducible calibration curves for five phthalates were obtained with correlation coefficients greater than 0.999 (known concentration vs analyte/internal standard). They were linear over the range of 10–1,000 ng/mL for DBP, BBzP and DEHP and 50–1,000 ng/mL for DIOP and DINP.

The present method for analysis of phthalates in diets is a modification of that described by Tsumura et al. (2001). A sample of the diet (5 g) was weighed into a centrifuge tube, followed by water (5 mL) and acetonitrile (20 mL), and spiked with isotopically labeled internal standards (4 μg/mL, 25 μL). The sample was homogenized for 1 min using a homogenizer (Physcotron, Microtec Co. ltd), and then centrifuged at 3,000 rpm for 5 min. The acetonitrile layer was collected, and the residual homogenate was extracted again with 75% acetonitrile in water (20 mL). The acetonitrile layers were combined, sodium chloride (1.5 g) was added, and the mixture was shaken vigorously for 5 min. The acetonitrile layer was collected, hexane saturated with acetonitrile (4 mL) was added, and the mixture was shaken vigorously for 5 min. The acetonitrile layer was evaporated to dryness under reduced pressure at 35°C. The residue was dissolved in water (2 mL) and hexane (5 mL) and the mixture was shaken vigorously for 30 s. The solution was centrifuged at 3,000 rpm for 5 min, and the hexane layer was removed and saved. Hexane (3 mL) was added to the water layer, and extracted as described above. The hexane layers were combined and loaded onto a Florisil and Bondesil-PSA dual layer column, which was preconditioned with acetone (10 mL) and hexane (10 mL). After washing the column with hexane (3 mL), phthalates were eluted with 5% acetone in hexane (10 mL). The eluate was evaporated to dryness under reduced pressure at 35°C, and then dissolved in hexane (1 mL). An aliquot of each sample (2 μL) was injected into a GC-MS system.

The present method for analysis of phthalates in bedding is a modification of that described by Tsumura et al. (2001). A sample of bedding for experimental animals (5 g) was weighed into a centrifuge tube followed by acetone (40 mL), and spiked with isotopically labeled internal standards (4 μg/mL, 25 μL). The sample was left for 1 h and then shaken vigorously for 10 min. The solution was evaporated to dryness under reduced pressure at 35°C. The residue was treated as described for diets.

Analysis of phthalates in water was carried out as described (Glick 1998). Water (30 mL) from a tap in the experimental animal room was weighed into a centrifuge tube followed by hexane (10 mL), spiked with isotopically labeled internal standards (4 μg/mL, 25 μL), then shaken vigorously for 10 min. The hexane layer was evaporated to dryness under reduced pressure at 35°C. The residue was dissolved in hexane (1 mL) and an aliquot of each sample (2 μL) was injected into a GC-MS system.

Analysis of phthalates in air was carried out according to the method reported by the Ministry of Health, Labour and Welfare of Japan (2000). Sampling was done using an SP208-10L (GL Science, Tokyo, Japan) pump attached to an AERO cartridge SDB 400 (GL Science) with a sampling rate of 5 L/min for 24 h. After the addition of isotopically labeled internal standards (4 μg/mL, 25 μL), the collected phthalates were extracted with acetone (2 mL) by ultrasonication for 10 min. After centrifugation at 3,000 rpm for 5 min, the supernatant was collected. An aliquot of each sample (2 μL) was injected into a GC-MS system.

GC-MS analysis was performed on an Agilent 6890 N GC/5973 N MSD instrument (Agilent Technologies, CA, USA). A 30 m HP-5MS SV column (J & W Scientific, CA, USA) with 0.25 mm i.d. and 0.5 μm film thickness was used. The initial oven temperature was 80°C. After holding at the initial temperature for 2 min, the temperature was increased to 240°C at a rate of 40°C/min, and then to 300°C at a rate of 10°C/min, where it remained constant for 5 min. Helium was used as carrier gas at a flow-rate of 1.2 mL/min). The ion source temperature was 230°C and electron ionization was used as the ionization mode. The injection port was kept at 250°C. The ions used for selected ion monitoring are summarized in Table 1. DIOP was determined as the two highest peaks, and DINP as the five main peaks in the chromatogram as described by Tsumura et al. (2001).

Table 1 Retention times, quantification and confirmation ions for the measured phthalates: dibutyl phthalate, DBP; butyl benzyl phthalate, BBzP; di-2-ethylhexyl phthalate, DEHP; diisooctyl phthalate, DIOP; diisononyl

Analyte	Retention time (min)	Quantification ion	Confirmation ion
DBP	8.0	149	205, 223
DBP-d_4	8.0	153	209, 227
BBzP	10.2	149	91, 206
BBzP-d_4	10.2	153	91, 210
DEHP	11.3	149	167, 279
DEHP-d_4	11.3	153	171, 283
DIOP	11.5–12.1	149	279
DOP-d_4	12.5	153	283
DINP	12.8–13.9	149	293
DNP-d_4	14.2	153	297

Results and Discussion

The recoveries from diets and beddings spiked with 100 ng/g of DBP, BBzP and DEHP and 500 ng/g of DIOP and DINP were examined. Overall recoveries of the three repeated measurements are summarized in Table 2; these values were 98.8%–148% with coefficients of variation (CV) of 0.4%–7.8% for diets and 94.7%–146% with CV of 1.0%–5.0% for beddings. Recoveries of BBzP, DIOP and DINP were satisfactory (98.8%–113% for diets; 94.7%–119% for beddings); however, those of DBP and DEHP were relatively high (123%–148%).

The elimination of phthalate contamination is key for precise measurement. Blanks originating from sample analysis were therefore examined using phthalates-free water instead of diet and bedding. The blanks from diet sample analysis were 5.0 (±1.9) ng/g for DEHP, <3.0 ng/g for DBP and BBzP, and <20 ng/g for DIOP and DINP (n = 5), and those from bedding sample analysis were 6.6 (±2.5) ng/g for DEHP, 3.1 (±0.3) ng/g for DBP, <3.0 ng/g for BBzP, and <20 ng/g for DIOP and DINP (n = 5). The limit of quantification (LOQ) for each of five phthalates was calculated as $10S_0$, where S_0 is the value of the standard deviation obtained by analyzing quintuplicate sets of the blank analysis, or as $10S_1$, where S_1 is the value of the standard deviation obtained by analyzing quintuplicate sets of the lowest level of standard sample. The LOQ for each of five phthalates is summarized in Table 2. With our careful control of contamination, sample analysis was achieved with a low-level background, which allowed us to evaluate the amount of phthalates precisely. Typical chromatograms of a standard mixture and of diet and bedding samples are shown in Fig. 1.

Table 2 Recoveries and LOQ of phthalates

Analyte	Diet			Bedding		
	Recovery (%)	CV[a] (%)	LOQ[b] (ng/g)	Recovery (%)	CV (%)	LOQ (ng/g)
DBP	123	7.8	10	132	5.0	5
BBzP	98.8	0.4	10	102	1.0	10
DEHP	148	1.8	20	146	4.2	25
DIOP	109	0.5	50	119	4.3	50
DINP	113	1.3	50	94.7	4.3	50

Results are means of three replicate determinations

[a] *CV* coefficients of variation

[b] *LOQ* limit of quantification

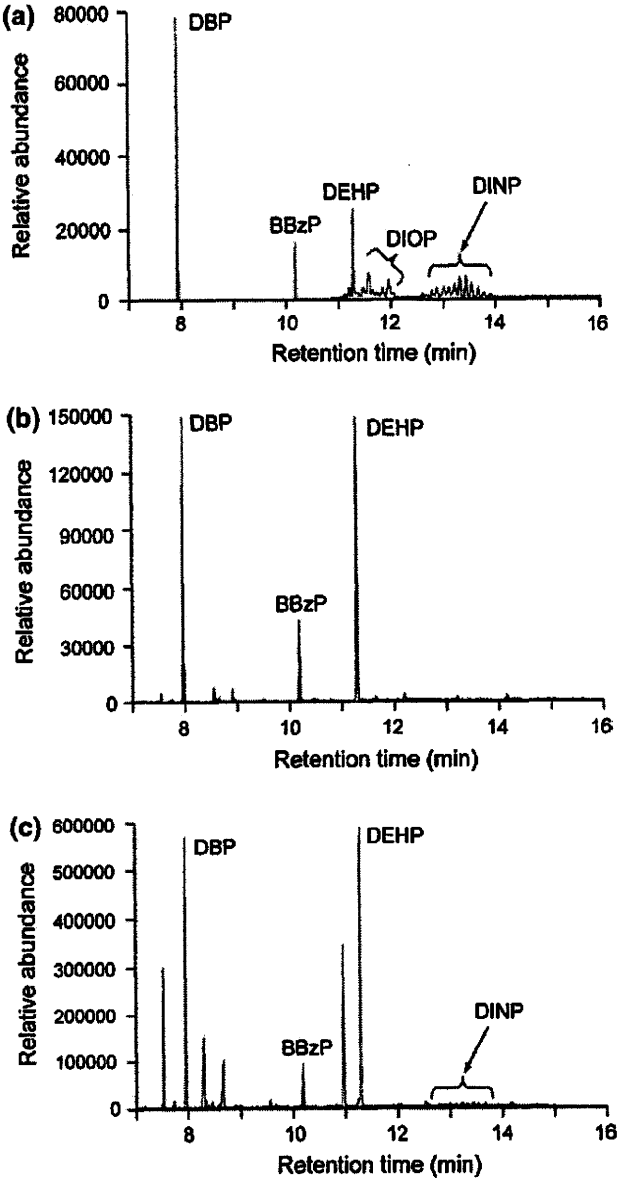

Fig. 1 Typical selected ion monitoring chromatograms of **a** a standard mixture of five phthalates, **b** diet and **c** bedding

We evaluated the suitability of the method for detecting the levels of phthalate in 12 commercial animal diets and 13 bedding materials (Table 3). All samples were analyzed just after opening the package. Two of the most frequently detected phthalates were DBP, which was found in all diets and beddings, and DEHP, which was found in all diets and in 77% of the beddings. BBzP was not detected in most samples (15%–33% detectable), and DINP was detected in only one sample of bedding. DIOP was not detected in any diet or bedding. The levels of phthalates varied from sample to sample; the concentrations of five phthalates were 141–1,410 ng/g for diets and 20.5–7,560 ng/g for beddings.

Table 3 Concentrations of phthalates in commercial animal diets and beddings (ng/g)

Sample no.	DEHP	DBP	BBzP	DIOP	DINP	Sum
Diet						
D1	143	179	<LOQ	<LOQ	<LOQ	322
D2	160	46.3	<LOQ	<LOQ	<LOQ	206
D3	116	25.1	<LOQ	<LOQ	<LOQ	141
D4	511	146	157	<LOQ	<LOQ	814
D5	156	41.4	<LOQ	<LOQ	<LOQ	197
D6	146	134	<LOQ	<LOQ	<LOQ	280
D7	118	36.1	<LOQ	<LOQ	<LOQ	154
D8	205	80.4	<LOQ	<LOQ	<LOQ	285
D9	281	205	22.2	<LOQ	<LOQ	508
D10	422	403	18.4	<LOQ	<LOQ	843
D11	257	344	<LOQ	<LOQ	<LOQ	601
D12	431	944	33.4	<LOQ	<LOQ	1,410
Bedding						
B1	449	1,380	<LOQ	<LOQ	<LOQ	1,830
B2	<LOQ	757	<LOQ	<LOQ	<LOQ	781
B3	280	19.6	<LOQ	<LOQ	<LOQ	300
B4	498	765	<LOQ	<LOQ	<LOQ	1,260
B5	<LOQ	6.0	<LOQ	<LOQ	<LOQ	20.5
B6	187	128	<LOQ	<LOQ	<LOQ	315
B7	262	130	440	<LOQ	<LOQ	832
B8	420	66.4	<LOQ	<LOQ	<LOQ	486
B9	132	538	<LOQ	<LOQ	<LOQ	670
B10	5,070	1,390	900	<LOQ	198	7,560
B11	547	381	<LOQ	<LOQ	<LOQ	928
B12	443	55.5	<LOQ	<LOQ	<LOQ	499
B13	<LOQ	30.5	<LOQ	<LOQ	<LOQ	46.5

Results are means of duplicate determinations

The results of our study demonstrated that the levels of phthalates varied from sample to sample. The highest value for the concentration of five phthalates in diets (1,410 ng/g) was 10 times higher than the lowest value. If a rat weighing 150 g eats 12 g of this diet daily (Poon et al. 1997), 16,920 ng of phthalates would be ingested, which corresponds to an oral administration of 0.113 mg/kg per day. The levels of phthalates in the diets analyzed in this study are about 20–2,000 times lower than those fed in reported developmental and reproductive toxicity experiments (Poon et al. 1997; Tyl et al. 1988; Arcadi et al. 1998). However, it is necessary to take into account the progress of the contamination during storage after opening the packaging of diets.

In terms of the contamination level in beddings, one sample (no. B10), made of recycled paper, showed a remarkable level of contamination at a concentration of

Table 4 Concentrations of five phthalates in water supplied to the animals, and samples of air taken from the animal room

Sample	DEHP	DBP	BBzP	DIOP	DINP	Sum
Water (ng/mL)						
Water1	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Water2	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Water3	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Air (ng/m^3)						
Air1	31.4	357	<LOQ	<LOQ	<LOQ	388
Air2	27.6	320	<LOQ	<LOQ	<LOQ	348
Air3	31.9	261	<LOQ	<LOQ	<LOQ	293

Results are means of duplicate determinations

five phthalates of 7,560 ng/g. Although we cannot estimate the exposure to phthalates from beddings through oral intake by licking and dermal absorption, it would be better not to use heavily contaminated bedding to avoid the potential exposure to high levels of phthalates.

In order to investigate the other causes of rodent exposure to phthalates under various feeding conditions, we analyzed water supplied to the animals, and samples of air taken from the animal room (Table 4). None of the five phthalates was detected in the water (lower limits of quantification: DBP, BBzP and DEHP, 0.5 ng/mL; DIOP and DINP, 2 ng/mL). DBP and DEHP were detected in all samples of air with concentration ranges of 261–357 ng/m^3 and 27.6–31.9 ng/m^3, respectively. Other three phthalates were not detected (lower limits of quantification: BBzP, 5 ng/m^3; DIOP and DINP, 20 ng/m^3). The contamination level in air taken from the animal room was 293–388 ng/m^3 for five phthalates. The average exposure from air amounts to 101 ng/day when we assume an average daily inhalation of 0.29 m^3 air per rat (The ICH Steering Committee 1997). This level of exposure by inhalation would be about 125 times lower than the intake from diets. These results indicate that the major source of exposure to phthalates may be the diet, although the possibility cannot be denied completely that direct incorporation of these phthalates from the lung without hydrolysis occurs.

In summary, we have developed a GC-MS method to determine five phthalates in diets and beddings used for experimental animals. We analyzed commercial animal diets and beddings, and found that both of them were polluted by phthalates, especially DBP and DEHP, suggesting that dietary exposure to phthalates routinely occurs. The total exclusion of phthalates from the experimental environment is probably impossible. Therefore, the contamination levels in the diet and bedding should be measured. Additionally, it would be wise to monitor the contamination levels of water and the air in the animal room, although the exposure from water and air was low.

Acknowledgments This study was supported by a grant from the Ministry of Health, Labor, and Welfare, Japan.

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Journal of Reproduction and Development, Vol. 56, No. 1, 2010

—Full Paper—

Resistance to 5-aza-2′-deoxycytidine in Genic Regions Compared to Non-genic Repetitive Sequences

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Abstract. The DNA methyltransferase (Dnmt) inhibitor and demethylating agent 5-aza-2′-deoxycytidine (5azadC) has been used to induce cellular differentiation and gene activation. It has been approved for treating several kinds of malignancies due to its ability to reactivate silenced tumor suppressor genes. Considering the potential effect of 5azadC on non-targeted genomic regions in normal cells, we investigated its effect on repetitive sequences and selected gene loci, *Oct-4*, *Sall3*, *Per1*, *Clu*, *Dpep1* and *Igf2r*, including tissue-dependent and differentially methylated regions, by treating mouse NIH/3T3 fibroblast cells with concentrations of 5azadC ranging from 0.001 to 5 μM. Demethylation of minor satellite repeats and endogenous viruses was concentration dependent, and the demethylation was strong at 1 and 5 μM. In genic regions, the methylation level decreased only at 0.1 μM, but was minimally altered at concentrations lower or higher, regardless of the abundance of CpG sites. Thus, repeats are strongly demethylated, but genic regions are only demethylated at effective doses. Genes were activated by 5azadC treatment and were accompanied by a unique combination of histone modifications in genic regions, including an increased level of H3K9me3 and a decreased level of AcH3. Increase of H3K9me3 in genic regions was not observed in Dnmt knock out cells. We identified differential effects of 5azadC on repetitive sequences and genic regions and revealed the importance of choosing appropriate 5azadC doses to achieve targeted gene recovery.

Key words: 5-aza-2′-deoxycytidine, Decitabine, DNA methylation, Epigenetics, Histone modification

(J. Reprod. Dev. 56: 86–93, 2010)

DNA methylation is one of the epigenetic events associated with gene regulation and function. Hypermethylation of promoter regions of tumor suppressor genes causes silencing of the genes that lead to cancer [1–3]. Thus, reversing the methylation status of gene promoters to their prevalent methylation states has become a treatment option for certain cancer types. To date, there are many types of demethylating agents that have been shown to inhibit promoter methylation and reactivate silenced genes [4–6]. Some of these have been approved or are in clinical studies to be developed as cancer drugs [7].

The cytosine analog 5-aza-2′-deoxycytidine (5azadC), also known as decitabine, has been widely used as a DNA methyltransferase (Dnmt) inhibitor to reverse aberrant hypermethylation [8, 9]. It has been approved for hematological malignancies, showing favorable results with low dose treatment [10, 11]. Known to have dual modes of action, 5azadC at low doses induces gene hypomethylation, whereas high doses of 5azadC induce cytotoxicity and cause severe side effects in patients [12, 13].

Nearly 40% of the mouse genome is composed of repetitive sequences including different classes of interspersed repeats, such as LINEs, SINEs, LTR elements and satellites, that are mainly found in heterochromatin regions [14]. Most repeats are densely methylated, and methylation in repeats reflects the global methylation level [15, 16]. Loss of methylation in repeats causes genomic instability [17, 18]. Conversely, genes comprise only a small portion of the genome. Tissue-dependent and differentially methylated regions (T-DMRs) are unique sequences in genic regions that are methylated depending on tissue or cell types. T-DMRs have been widely observed, including in undifferentiated embryonic stem cells, normal tissues and even in cloned mice [19–21]. Both repetitive regions and T-DMRs serve as important markers for methylation analysis, as repeats could be used to estimate global methylation, whereas T-DMRs could serve as references for cell- or tissue-specific methylation.

Previous reports show that Dnmts exhibit functional cooperation on genomic regions [22, 23]. We reported previously that Dnmt1, Dnmt3a and Dnmt3b share targets in the same CpG islands with T-DMRs, and each Dnmt has target preferences depending on the genomic regions [24]. Dnmt3a and Dnmt3b prefer T-DMRs of genic regions, whereas Dnmt1 prefers repetitive sequences.

The demethylating effect of 5azadC is exerted by binding to Dnmts [4]. Since Dnmts have multiple targets, there is the potential of having a genome-wide demethylating effect when using 5azadC. Demethylation of non-targeted genomic regions might occur, not only in cancer cells but also in normal cells. In addition, there are diverse interactions between DNA methylation and histone modifications in euchromatic and heterochromatic regions [25, 26]. The epigenetic status of T-DMRs is regulated by the interplay between DNA methyltransferases, histone modification enzymes, nuclear proteins and other epigenetic factors that cooperate to form cell- and tissue-specific DNA methylation profiles [27,

Received: March 17, 2009
Accepted: October 3, 2009
Published online in J-STAGE: December 9, 2009

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28]. It may be possible to induce hypomethylation-independent activation of gene expression and downstream responses. To know whether 5azadC induces an invariable effect on different genomic regions, we investigated the effect of 5azadC on non-genic repetitive sequences and some genic regions including T-DMRs in fibroblast cells.

Materials and Methods

Reagents, cell culture and genome extraction

All reagents were purchased from Wako Pure Chemicals (Osaka, Japan) unless stated otherwise.

NIH/3T3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (JRH, Lenexa, KS, USA) and 50 unit/ml penicillin / 50 μg/ml streptomycin (Invitrogen) at 37 C in 5% CO_2 in air. Prior to treatment with 5-aza-2'-deoxycytidine (5azadC; Sigma-Aldrich, St. Louis, MO, USA; diluted with sterile water to the concentrations required), cells were plated at 1×10^5 cells/150 mm dish and cultured for 24 h. Cells were treated with 5azadC to final concentrations ranging from 0.001 to 5μM. Sterile water was substituted for 5azadC in the untreated control. The medium was changed every 24 h, and cells were collected after 3 days for DNA extraction.

Wild type ES cells (J1) and mutant ES cells deficient in *Dnmt*1 (*Dnmt*1$^{-/-}$; c/c), *Dnmt*3a and *Dnmt*3b (*Dnmt*3a$^{-/-}$3b$^{-/-}$; 7aabb) were cultured on gelatin coated dishes with ES medium containing 1000 U/ml leukemia inhibitory factor (Chemicon, Temecula, CA, USA) as previously described [24]. J1, c/c and 7aabb cells were harvested at passage numbers 32, 17 and 17, respectively.

Cells were incubated in lysis buffer (150 mM EDTA, 10 mM Tris-HCl, pH 8.0, and 1% SDS) containing 10 mg/ml proteinase K (Merck, Darmstadt, Germany) at 55 C for 20 min. Following phenol/chloroform/isoamyl alcohol extraction twice, genomic DNA was precipitated with ethanol and dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0).

Cell proliferation assay

NIH/3T3 cells were seeded into 96-well plates at 1×10^3 cells per well, 24 h before 5azadC was added. Cells were treated with 5azadC at final concentrations of 0 (as the control), 0.001, 0.005, 0.01, 0.05, 0.1, 0.5, 1.0, 5.0 and 10.0 μM for 3 or 4 days at 37 C in 0.5% CO_2 in air with medium changes every 24 h. Four hours before plate reading, 10 μl of Cell Proliferation Reagent WST-1 (Roche, Penzberg, Germany) was added. The absorbance of each sample was measured against a background control using an ELISA reader at an absorption wavelength of 450 nm.

Analysis of the methylation status of repetitive sequences by Southern blotting

Genomic DNA (5 μg) was digested with the restriction enzyme *Msp*I (Takara, Kyoto, Japan) or *Hap*II (Takara) and electrophoresed on a 0.8% agarose gel. Following hydrolyzation with 0.25 N HCl and denaturation with 1.5 M NaCl/0.5 N NaOH, the DNA was transferred onto a nylon membrane. The membrane was probed with pMO for endogenous C-type retrovirus (MoMuLV; Genbank accession NC_001501) and pMR150 for minor satellite repeats (X14469 and X07949). Probes were labeled with the Gene Images random prime labeling module (Amersham Pharmacia, Little Chalfont, Buckinghamshire, UK). Hybridization and detection were performed using the Gene Images CDP-star detection module (Amersham Pharmacia) according to the manufacturer's instructions.

Bisulfite restriction mapping and sequencing

Genomic DNA, digested with *Eco*RI, was denatured by incubating with 0.3 M NaOH at 37 C for 15 min. Sodium metabisulfite (pH 5.0) and hydroquinone were added to a final concentration of 2 M and 0.5 mM, respectively, and the mixture was incubated at 55 C for 18 h in the dark. Bisulfite modified DNA was purified with the Wizard DNA Clean-Up System (Promega, Madison, WI, US), and the bisulfite reaction was terminated with NaOH at a final concentration of 0.3 M at 37 C for 15 min. The sample was neutralized by adding NH_4OAc, pH 7.0 (3 M, final concentration), and was precipitated with ethanol. Purified DNA was dissolved in sterile water and amplified using Immolase (Bioline, Tokyo, Japan) with the primer sets as follows: 5'-TAAGGGTAGGTATATAGGTGTGGT-3', F, and 5'-TCTACCCCCTTTAAAAATCACTTTAA-3', R, for ODE; 5'-TGGGTTGAAATATTGGGTTTATTT-3', F, and 5'-CTAAAACCAAATATCCAACCATA-3', R, for OPR; 5'-GGGAAGGGGATTTTGTTATTGTAGT-3', F, and 5'-CATAAACCCAACAACAAACCCATCT-3', R, for *Per1*; 5'-GTTAGGGTTTTTTTAG-GGTATTAGT-3', F, and 5'-CCCTAATCTACCCAACATATACAAA-3', R, for *Sall3*. The PCR conditions were as follows: 95 C for 10 min, followed by 40 cycles of denaturation at 94 C for 30 sec, annealing at 55 C for 30 sec and extension at 72 C for 1 min, with a final extension at 72 C for 10 min.

Oct-4 distal enhancer, *Oct-4* proximal enhancer and promoter PCR products were digested with *Taq*I (Takara) at 65 C, and *Per*1 and *Sall*3 PCR products were digested with *Hpy*CH4IV (NEB, Ipswich, MA, USA) at 37 C for 3 h. Restricted fragments were assessed by agarose gel electrophoresis. Images were recorded and semi-quantified using the ImageJ software provided by the National Institutes of Health (http://rsbweb.nih.gov/ij/). The relative DNA methylation level of each genic region was calculated by the formula: DNA methylation status (%)=$100 \times I^C / (I^{UC} + I^C)$, where I^C and I^{UC} represent the intensities of the digested and undigested bands, respectively.

For bisulfite sequencing, PCR products were cloned into pGEM T-Easy vector (Promega, Madison, WI, USA), and 10 clones were sequenced for each sample. The primer sets used were 5'-TGGGCTGAAATACTGGGTTCACCC-3', F, and 5'-CTGAAGCCAGGTGTCCAGCCATG-3', R, for *Oct-4*; 5'- GGTTGGGGAATTGGTTGTT-3', F, and 5'-CAACCTACTCCTAAATCCTCCA-3', R, for *Dpep1*; 5'- TAGTGAGTGGGGATGTAGTATTATGG-3', F, and 5'-AACCCCTAAACAACTTCAAAATTTT-3', R, for *Clu*; and 5'-GTTTAGAATATTGGTGAGTAGTGGG-3', F, and 5'-CCTTAAAATAAAAATAAACATCTTAAA-3', R, for *Igf2r*, with the following PCR conditions: 95 C for 10 min, followed by 40 cycles of denaturation at 94 C for 30 sec, annealing at 55 C for 30 sec and extension at 72 C for 1 min, with a final extension at 72 C for 10 min.

RNA extraction and RT-PCR

Total RNA was extracted with TRIzol reagent (Invitrogen) according to the manufacturer's instructions. First strand cDNA was synthesized with SuperScript™ III First-Strand Synthesis System for RT-PCR (Invitrogen), and RT-PCR was performed using Taq DNA Polymerase (Promega) with primers as follows: 5'-CAGGAGTGTGTGAGGGAG-3', F, and 5'-GGTGTCACTGTCCGACTTGC-3', R, for *Dnmt1*; 5'-ACCCATGCCAAGACTCACCTTC-3', F, and 5'-TCCACCTTCTGAGACTCTCCAG-3', R, for *Dnmt3a*; 5'-TCAGACACGAAGGATGCTCC-3', F, and 5'-ACAGGGTACTCCTGCACATG-3', R, for *Dnmt3b*; 5'-TTCTACAATGAGCTGCGTGTGG-3', F, and 5'-ATGGCTGGGGTGTTGAAGGT-3', R, for *β-actin*; 5'-GGCGTTCGCTTTGGAAAGGTGTTC-3', F, and 5'-CTCGAACCACATCCTTCTCT-3', R, for *Oct-4*; 5'- CCAGTCGAAGATGCTCAACA-3', F, and 5'- TGTGATGGGGTCAGAGTCAA-3', R, for *Clu*; 5'- ATGCGGTATCTGACCCTCAC-3', F, and 5'-ATCTGCAAAGCGTCCTTCAT-3', R, for *Dpep1* and 5'-CAACGTCTGTGGAAATGTGG-3', F, and 5'- CAGCCCATAGTGGTGTTGAA-3', R, for *Igf2r*. The PCR conditions were as follows: 95 C for 1 min, followed by 30 cycles of denaturation at 94 C for 30 sec, annealing at 60 C for 30 sec and extension at 72 C for 1 min, with a final extension at 72 C for 5 min.

Chromatin immunoprecipitation (ChIP) assay

ChIP assays were performed as described previously [20] using a Chromatin Immunoprecipitation (ChIP) Assay Kit (Cat. No. 17-295; Upstate Biotechnology, Lake Placid, NY, USA) with anti-acetylated histone H3 and H4 antibodies (Cat. No. 06-599 and 06-598; Upstate Biotechnology), anti-trimethylated H3K4 and H3K9 (Cat. No. ab8580 and ab8898; Abcam, Cambridge, UK) and anti-dimethylated H3K4, H3K9 and H3K27 (Cat. No. 07-030, 07-212 and 07-452; Upstate Biotechnology). Normal rabbit IgG (Cat. No. 12-370; Upstate Biotechnology) was used as a negative control to verify immunoprecipitation specificity. PCR was performed using primers as follows: 5'- GTGAGGTGTCCGGTGACCCAAGGCAG-3', F, and 5'- CGGCTCACCTAGGGACGGTTTCACC-3', R, for *Oct-4*; 5'- TGCTCTGGAGACACAGGAAA-3', F, and 5'-CTGGGGAAGAAAGCCAAGAT-3', R, for *Clu* ChIP 1; 5'-ATTGCAGTGATGCCAGATGA-3', F, and 5'- ACGCACAGCAGGAGAATCTT-3', R, for *Clu* ChIP 2; 5'-CTCCTCTTGTGGCTCCCTAA-3', F, and 5'-GGCTCCACAGAGTGCCAAG-3', R, for *Dpep1*. PCR was performed under the following conditions: 95 C for 10 min, followed by 30 cycles of denaturation at 94 C for 30 sec, annealing at 55 C for 30 sec and extension at 72 C for 1 min, with a final extension at 72 C for 10 min. The amount of each PCR product on an ethidium bromide-stained gel image was evaluated using the ImageJ software.

Results

Demethylating effect of 5azadC on repetitive sequences

We examined cell survival under different concentrations of 5azadC by WST-1 assay. After 72 h, the viable cell number was largely reduced at concentrations higher than 0.5 μM and was severely affected at higher concentrations (Fig. 1A). A similar effect was observed in the 96-h culture. Only minimal effects on viability were observed in cells treated with less than 0.1 μM 5azadC.

To determine the effect of 5azadC on the methylation of repetitive sequences, cells were treated with 0.001 μM to 5 μM 5azadC, and the methylation status of repetitive sequences was analyzed using methylation-sensitive restriction enzymes. Southern hybridization was performed using two probes of differentially localized repetitive sequences, minor satellite repeats located in the centromeric regions and endogenous C-type viruses interspersed across the mouse genome [14, 24, 29]. Minor satellite repeats were demethylated extensively starting at the 0.1 μM concentration (Fig. 1B), and 1 μM was sufficient to induce the maximum demethylating effect. Similarly, endogenous viruses showed aggressive loss of methylation at the 0.1 μM to 5 μM treatment levels, but slight demethylation could be observed at a concentration as low as 0.001 μM. Thus, the repetitive sequences were strongly demethylated by 5azadC. The results confirmed a previous report that 5azadC is effective in inducing demethylation dose-dependently from 0.1 μM to 5 μM [30].

Effect of 5azadC on genic regions

We next examined the effect of 5azadC on T-DMRs of genic regions. *Oct-4* (*Pou5f1*) has T-DMRs in the CpG-rich promoter/proximal enhancer region and distal enhancer region (Fig. 1C; 20). The DNA methylation status of the T-DMRs was analyzed using bisulfite restriction mapping focusing on *Taq*I restriction sites. At 0.001 and 0.01 μM 5azadC, the methylation levels of the investigated regions changed little compared to the untreated ones. A significant loss of methylation in both regions was observed at 0.1 μM, indicating that this concentration was able to induce demethylation in both genic regions and the repetitive sequences. In the 1 and 5 μM treated samples, however, both T-DMRs had nearly the same methylation levels as in the untreated control, in contrast to the extensive demethylation observed in the repetitive sequences at these concentrations.

Sall3 has a T-DMR located at an edge of a CpG island, which is methylated only in the trophoblast cell lineage [19, 21]. The T-DMR is aberrantly methylated in the placental genome of cloned mice [21]. *Per1*, which is involved in generating circadian rhythm, has a few CpGs in the upstream promoter region. Similar to *Oct-4*, 0.001 and 0.01 μM 5azadC had minimal effects on these loci. The methylation levels decreased significantly following 0.1 μM 5azadC treatment, but were only slightly decreased at 1 μM, and remained unchanged at 5 μM.

Bisulfite sequencing was performed on several gene loci containing CpG-rich promoters, including *Oct-4*. Hypomethylation of the *Clusterin (Clu)* promoter is associated with high gene expression in the rat testis and epididymis [31]. *Dpep1*, a renal *Dipeptidase* gene, has been reported to be a tumor marker candidate in malignancies [32]. All investigated loci had hypermethylated promoter regions in NIH/3T3 cells (Fig. 2A). Bisulfite sequencing results for the *Oct-4* promoter region validated the restriction mapping results showing that 0.1 μM was more effective in inducing demethylation in genic regions compared with 1 μM. Similar dose-dependent demethylation patterns were also

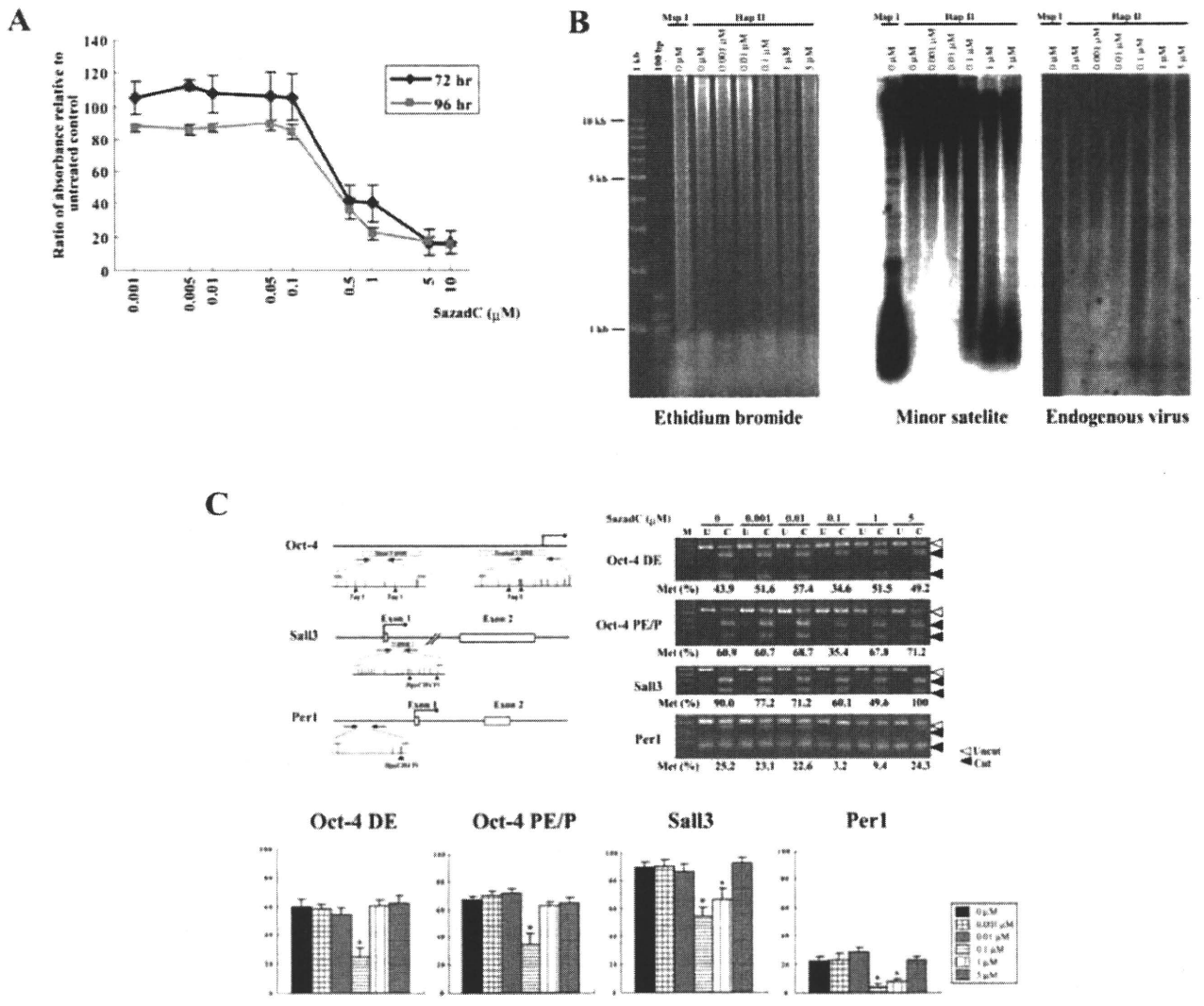

Fig. 1. Different demethylating effects of 5azadC on repetitive sequences and genic regions. A: A WST-1 cell proliferation assay was performed on cells treated with 5azadC at the indicated concentrations for 72 and 96 h. Cell number was estimated by the absorbance, and then represented as the ratio relative to the untreated control, the absorbance of which was arbitrarily set to 100. The values represent means ± S.E. of 3 independent cultures. B: Analysis of the methylation status of repetitive sequences by Southern hybridization. Genomic DNA (5 μg), digested with *Hap*II, was hybridized to probes for minor satellite repeats and endogenous viruses. As a control for complete digestion, DNA from the untreated control was digested with *Msp*I. C: Methylation analysis of T-DMRs by bisulfite restriction mapping. A schematic diagram of each investigated locus is shown in the top left panel. T-DMRs are represented by bars, and CG sites are represented by vertical lines. The locations of the amplified regions relative to each respective transcription start site are as follow: *Oct-4* distal enhancer (DE), -3086 to -2646; *Oct-4* proximal enhancer/promoter (PE/P), -420 to +31; *Sall3*, +1253 to +1665; and *Per1*, -1087 to -929. Each locus contains 2 *Taq*I or *Hpy*CH4IV restriction sites, respectively (triangles). PCR products were digested with restriction enzymes and electrophoresed on agarose gel (top right panel). The relative DNA methylation level was calculated based on the relative intensities of cut to uncut bands, indicated below each cut lane. M, marker; U, uncut; C, cut with restriction enzyme. The bottom panel indicates the methylation level of each gene locus at each concentration; the values are presented as means ± S.E. of 3 independent PCRs of 2 cultures. * $P<0.05$ (Student's *t*-test).

observed in the promoter regions of *Clu* and *Dpep1*.

We also analyzed the DMR2 region of the *Igf2r* imprinted gene, which is differentially methylated depending on its parental origin [33]. Due to allele-specific methylation, half of the clones were methylated in the untreated control. As observed in other genes, the DMR2 region appeared demethylated at 0.1 μM, but was not affected by 1 μM 5azadC.

These results provide evidence that 5azadC has a strong demethylating effect on repetitive sequences, but its effect on genic regions is limited to a certain effective dose. The expression levels

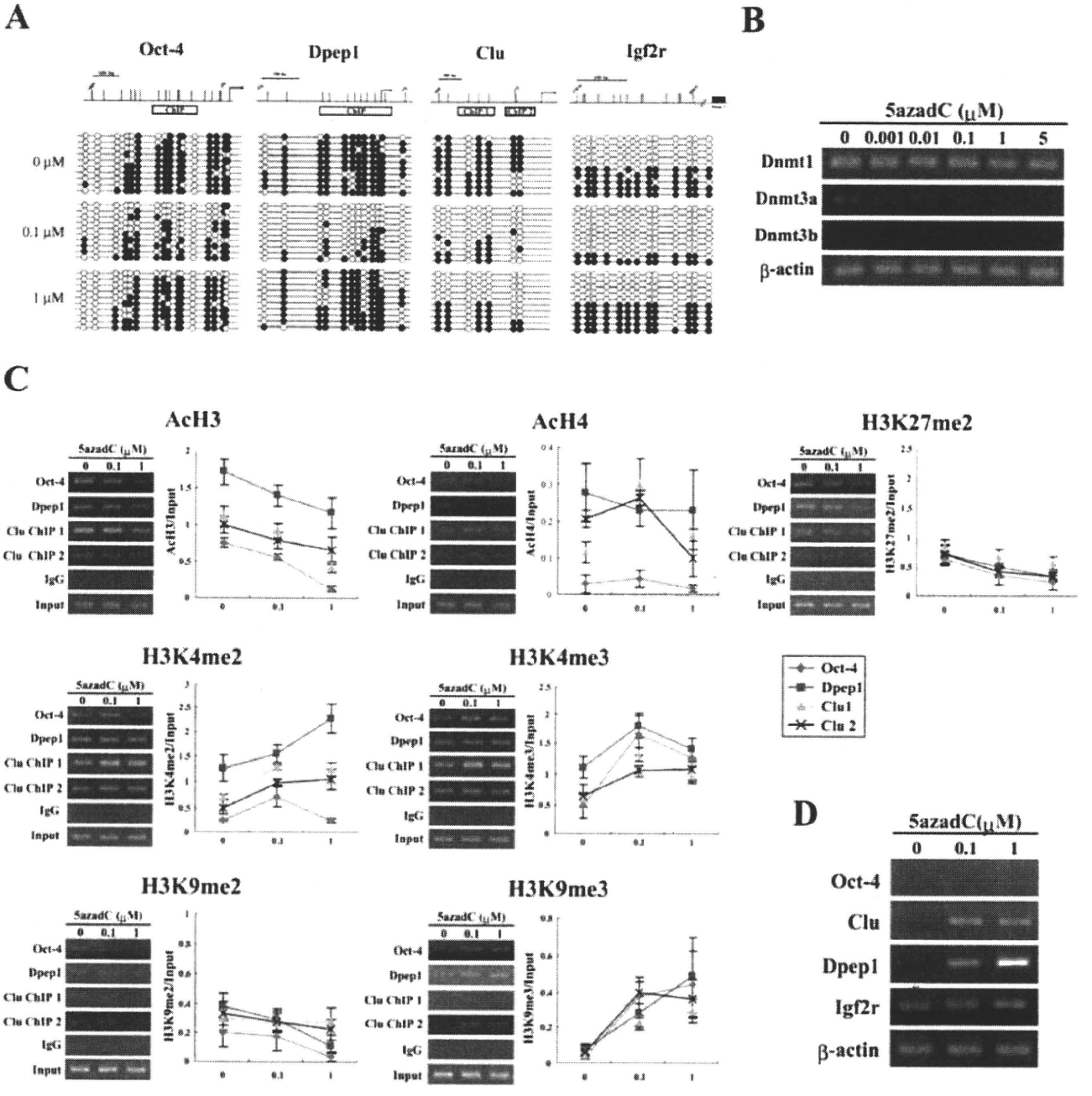

Fig. 2. Effect of 5azadC on DNA methylation, histone modification and gene expression. A: DNA methylation analysis of 4 gene loci, *Oct-4*, *Clu*, *Dpep1* and *Igf2r*, using bisulfite sequencing. The top panel shows the location of individual CpG sites (vertical lines) in genic regions amplified by PCR. The 0, 0.1 and 1 μM 5azadC-treated DNA was bisulfite converted and sequenced. The methylation status of each CpG site is represented by open (unmethylated) or closed (methylated) circles. B: Expression of *Dnmt1*, *Dnmt3a* and *Dnmt3b* at 0–5 μM 5azadC analyzed by RT-PCR. The expression level of *β-actin* was used as the internal control. C: Histone modification analysis of 0, 0.1 and 1 μM 5azadC-treated samples by ChIP. Analyzed regions are indicated by boxed "ChIP" in the schematic diagram in A. DNA immunoprecipitated with respective antibodies was amplified by PCR and electrophoresed (left panels). The relative intensities of PCR bands to those of the input DNA, presented as the means ± S.E. of 3 independent PCRs of 3 cultures, are shown in the right panels. D: RT-PCR results for the expression of each gene following 5azadC treatment.

of *Dnmt*1, *Dnmt*3a and *Dnmt*3b were not affected by 5azadC at concentrations from 0.001 to 5 μM (Fig. 2B), indicating that the partial retention of methylation observed at high concentrations of 5azadC was not due to increased Dnmt expression.

5azadC induced a unique combination of histone tail modifications

Since 0.1 and 1 μM 5azadC showed rather unexpected, different demethylation effects on genic regions, we investigated changes in histone modifications by performing a ChIP assay with antibodies against euchromatic and heterochromatic marks. The AcH3 level in genic regions continuously declined as the concentration of 5azadC increased (Fig. 2C), which is in contrast to the condition in decondensed chromatin [26]. Such changes were not observed in AcH4, which was less enriched in genic regions.

Both the H3K4me2 and H3K4me3 levels increased with 5azadC treatment, in almost all investigated regions, and high levels were correlated with increased gene expression (Fig. 2D). Elevation of H3K4me2 in *Dpep1* at 1 μM 5azadC corresponded to a remarkable transcriptional increase, and the relatively low abundance of H3K4me2 in the *Oct-4* promoter region at any level of 5azadC did not induce *Oct-4* expression. H3K4me3 was more enriched by 0.1 μM than by 1 μM 5azadC treatment.

Heterochromatin-associated H3K9me2 and H3K27me2 marks continuously decreased with 5azadC treatment. In contrast, elevation of H3K9me3 was observed, with higher enrichment at 1 μM compared with 0.1 μM 5azadC in most regions. Altogether, 5azadC treatment at different concentrations was accompanied by a distinct combination of changes in euchromatic and heterochromatic histone marks in genic regions.

Increase of H3K9me3 correlates with partially methylated regions

Previous studies show that H3K9 methylation directs DNA methylation [34, 35]. To date, our data have shown that 5azadC induces partial demethylation, not complete demethylation, in genic regions. To determine whether increased H3K9me3 is associated with DNA methylation, we investigated enrichment of H3K9 methylation in the *Dpep1* and *Clu* promoter regions in Dnmt-deficient cells. The promoter regions of *Dpep1* and *Clu* were heavily methylated in wild type ES cells and were demethylated in *Dnmt1* and *Dnmt 3a 3b* (Fig. 3A). The increase of H3K9me3 in the genic regions of Dnmt knockout cells was not as obvious as those in the 5azadC-treated NIH/3T3 cells (Fig. 3B). Therefore, increased H3K9me3 correlated with partially methylated regions, such as in 5azadC-treated cells. Similar to 5azadC-treated cells, H3K9me2 decreased in *Dnmt1* cells, but was increased in *Dnmt3a 3b* cells.

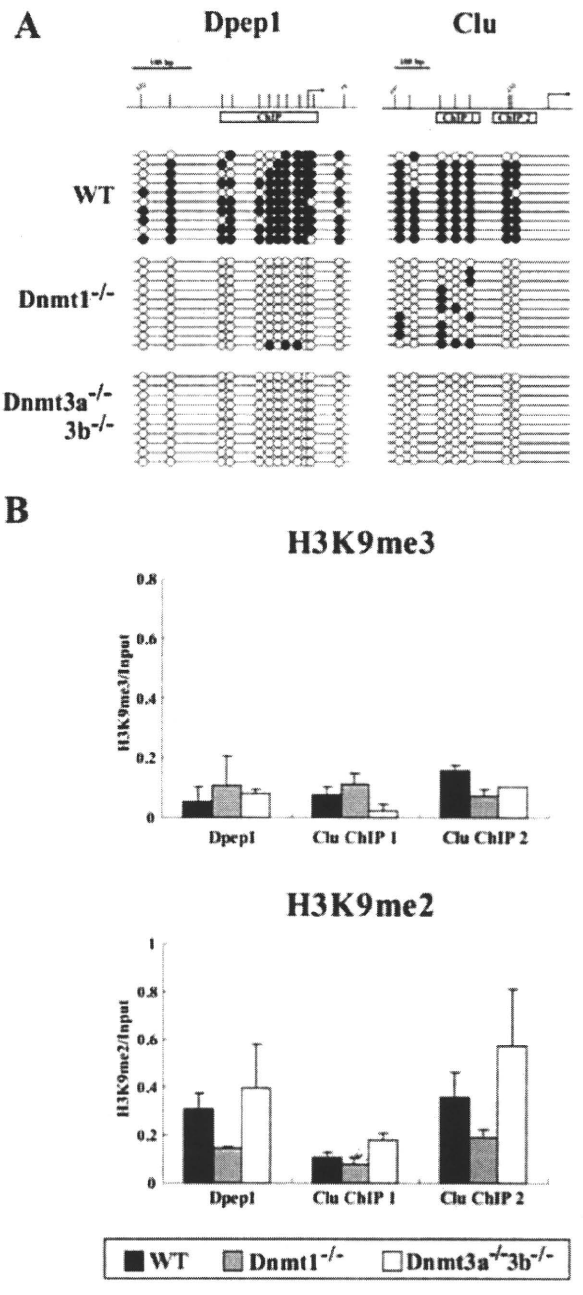

Fig. 3. DNA methylation and H3K9 methylation status of *Dpep1* and *Clu* in Dnmt knockout ES cells. A: DNA methylation analysis of *Dpep1* and *Clu* by bisulfite sequencing. B: H3K9 di- and trimethylation levels in the regions shown in panel A assessed by ChIP. The bar charts show the relative intensities of PCR bands to those of the input; the values are presented as means ± S.E. of 3 independent PCRs of 2 cultures.

Discussion

The current results demonstrate that 5azadC has differential effects on non-genic repetitive sequence and genic regions (Fig. 4). Importantly, non-genic repetitive sequences are susceptible to 5azadC, whereas genic regions are only demethylated by effective low doses.

Demethylation of repetitive sequences was concentration-dependent, whereas genic regions were only demethylated at effective concentrations, but not at higher concentrations. Thus,