Approach to distribution and accumulation of dibutyl phthalate in rats by immunoassay

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ABSTRACT

Dibutyl phthalate (DBP) is mainly taken up by the general population from food intake. To estimate intake of phthalates, determining distribution and accumulation of DBP in biological materials was a critical need. In this work, we set up two novel approaches with a monoclonal antibody specific to DBP to determine the distribution and accumulation of DBP in vivo. The contents of DBP in liver, kidney, stomach and testes were detected by immunofluorescence assays and indirect competitive ELISA. This data give directly evidence that indicates the distribution and accumulation of DBP in vivo. Double-label immunofluorescence assay provides with a visual approach to determination of the distribution and accumulation of DBP. It indicated that DBP accumulated in subcutaneous tissue such as sweat gland, hair follicle. Both of immunofluorescence assay and ELISA can be used to detect the content of DBP in biological materials. Our assays showed that DBP accumulated in viscera being rich in fat, such as liver, kidney and could overcome physiological barriers to penetrate testes. The date suggested that the accumulations of DBP exposed through dermal route were less than that of oral route and most of DBP was metabolized in 2 or 3 days.

1. Introduction

In recent years, plasticizer contamination triggers food security fear in many countries and regions. Phthalates are a group of synthetic chemicals with widespread use in the industrial production of numerous consumer products. They are used to soften plastics found in numerous flexible plastics products and personal care products such as food packaging and food-contact materials, cosmetics, toys, bags, drugs and building materials. During the last thirty years, numerous studies have been published concerning the presence of phthalate compounds in food and packaging materials (Cao, 2010; Fierens et al., 2012).

Phthalate esters are suspected to be involved in male reproductive health problems and have also been associated with several other health problems including obesity, diabetes, allergy, and asthma (Borch et al., 2006; Bornehag and Nanberg, 2010; Engel et al., 2010; Hauser et al., 2007; Matsuda et al., 2010; Ormond et al., 2009; Stahlhut et al., 2007; Stroheker et al., 2006). Among all phthalate esters, dibutyl phthalate (DBP) is second only to di-(2-ethylhexyl) phthalate in terms of such effects (Huang et al., 1999; Yie, 1993).

DBP is a phthalate diester that is used mainly as a plasticizer for nitrocellulose, polyvinyl acetate, and polyvinyl chloride; a lubricant; an antifoaming agent; and a skin emollient (Koo and Lee, 2004). DBP, the main kind of plasticizers used in China at present, is one of the main sources of indoor semi-volatile organic compounds (SVOCs) (Wang et al., 2010). DBP is a developmental toxicant in rats and mice (Fisher et al., 2003; Foster et al., 2000; Lamb IV et al., 1987). It is known to affect male fertility, cause testicular atrophy in young rats, and produce embryomortality and teratogenicity, in rodents (Gray et al., 1999, 2000; Kavlock et al., 2002; Moore et al., 2001; Mylchreest et al., 1998, 1999, 2000). Besides its role as endocrine disruptor, DBP has been shown to alter the expression of a number of genes. Expressions of 391 genes have been found significantly altered as a result of the exposure to

phthalates including DBP among approximately 30,000 genes queried (Euling et al., 2011; Liu et al., 2005). DBP is metabolized to monobutyl phthalate (MBP) in the stomach and small intestine and enters the bloodstream. The pharmacokinetics of DBP have been investigated in rats in experimental studies (Clewell et al., 2009). MBP and its primary metabolite, monobutyl phthalate – glucuronide (MBP-G), were measured in maternal and fetal after DBP doses where developmental effects have been found. MBP has been reported to be the active species responsible for adverse effects from DBP exposure (Ema et al., 1996; Kremer et al., 2005). It has been reported that both DBP and MBP suppress steroidogenesis by fetal-type Leydig cells in rats, but similar effects in vitro cannot be studied in primate (Hallmark et al., 2007).

DBP enters the environment in several different ways. These include: during production, distribution, and incorporation into polyvinylchloride resin; disposal in industrial and municipal landfills; waste incineration; and leaching from consumer products during use or after disposal. Because of its low vapor pressure, concentrations of DBP in outdoor air are low; they range from 100 to 102 ng/m^3 (Luks-Betlej et al., 2001). Although outdoor concentrations of DBP are low, indoor concentrations of DBP are relatively high (Weschler and Nazaroff, 2008). It is among the most abundant "manmade" SVOC commonly measured in indoor air and in settled dust. DBP is very stable and can enter the aquatic environment. There is a potential risk for ecotoxicological effects of DBP on aquatic species. Concentrations above 0.04 mg/L are shown to cause adverse effects on aquatic invertebrates and concentrations above 0.2 mg/L can have lethal effects. Although DBP can be biodegraded as a short chains phthalate, the degradation process is time-consuming in anaerobic environments. It has some potential to bioaccumulate in some organisms.

Because of its large and widespread use DBP is taken up by the general population from various sources (Clark et al., 2003; Wormuth et al., 2006). For humans, food intake is the most important exposure pathway for phthalates, followed by ingestion of dust and inhalation of indoor (Dickson-Spillmann et al., 2009; Janjua et al., 2008; Petersen and Jensen, 2010; Schlumpf et al., 2010; Weschler and Nazaroff, 2012; Wormuth et al., 2006). Intake of phthalates via food might cause different effects comparing with the routes by ingestion of dust and inhalation of indoor air. Studies to evaluate the potential adverse effects of exposure to dibutyl phthalate through various routes were a critical need.

Phthalate metabolite concentrations measured in urine samples were used to estimate phthalate daily intake in previous researches (Ferguson et al., 2011).To evaluate the potential adverse effects of exposure to DBP, the concentration of DBP and MBP, the major metabolite of DBP, in the blood and urine was often determined by gas chromatography, liquid chromatography and mass spectroscopy (Jen and Liu, 2006; Serodio and Nogueira, 2006; Zhang et al., 2008). The mean concentrations of MBP in urine were measured in the United States according to the National Health and Nutrition Examination Survey (NHANES). While MBP, the principal metabolite of DBP, has been measured in blood and urine in numerous studies, there are no studies that have measured DBP, itself, in blood or urine. And the environmental data consist primarily of estimates.

DBP concentrations are often either at or below detection limits. It is expensive and difficult to determine the concentration of DBP in organism. Although, the chromatographic techniques provide a low level of detection for phthalates, they are time consuming and have high instrumentation costs. To evaluate the potential adverse effects of exposure to dibutyl phthalate, methods for dibutyl phthalate determination must be developed.

In this work, we set up two novel approaches to determination of distribution and accumulation of DBP in vivo with a monoclonal antibody specific to DBP (Wei et al., 2011).The described assay can provide useful estimates of excretion, distribution and metabolism of DBP through different routes.

2. Materials and methods

2.1. Animals

Six-week-old male Wistar rats arrived from Hubei Center for Disease Control and were housed in the Animal Care Facility of Central China Normal University, which is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care National. The animals were housed 2 or 3/cage on wood-chip bedding in an air-conditioned animal room at 22 ± 3 °C and 55% ± 10% humidity with a 12/12 h light–dark cycle and allowed free access to powdered diet and filtered tap water.

2.2. Treatment

DBP (Sigma–Aldrich) was dissolved in corn oil (99.5% pure, Shanghai Solvent Factory, Shanghai, China). Wistar rats (n = 5/group) were administered either dibutyl-phthalate (DBP – Sigma–Aldrich) or corn oil by epicutaneously exposure (cutaneous route) or gavage (oral route). For dermal exposure, rat abdomens were shaved using small animal clippers one day before exposure. Rats were epicutaneously smeared with DBP at 200, 400 mg/kg bw-d or corn oil. For oral route, Wistar rats (n = 5/group) were administered either dibutyl-phthalate (DBP – Sigma–Aldrich) at dose of 200, 400 mg/kg bw-d, by gavage (oral route), or corn oil (vehicle). Starting 24, 48, 72 h after the final treatment, the rats were euthanized. Skin, liver, kidney, stomach, testes from each rats were collected, snap frozen in liquid nitrogen, and stored individually at −80 °C.

2.3. Preparation for monoclonal antibody

Anti-DBP monoclonal antibody (MAb) was prepared according to Wei et al. (2011). Dibutyl 4-aminophthalate (DBAP), an aminated hapten with structural feature of DBP, was linked to BSA or OVA through diazotization to generate artificial antigen DBAP-BSA or DBAP-OVA. All the hapten-protein conjugates were characterized by UV–vis spectrometry. Then BALB/c mice were immunized with these artificial antigens. Mice were tail-bled 7–10 days after the third and the fourth injection. Indirect noncompetitive ELISA (inELISA) and Indirect competitive ELISA (icELISA) with 1.0 μg/mL heterologous coating protein conjugates were used to determine the titer and sensitivity of antibody in the serum. The mouse that generated polyclonal antibody with a combination of the best sensitivity and the highest titer received a final intraperitoneal injection of 200 μg of immunogen in 200 μL of PBS, 3 days before cell fusion. Cell fusion, hybridoma culture and selection were carried out essentially as described by Wei et al. (2011). Then female BALB/c mice were injected intraperitoneally with 0.5 mL of paraffin oil 7–10 days before receiving an intraperitoneal injection of 2×10^6 hybridoma cells suspended in PBS. Ascites fluid developed 2–3 weeks after the injection of the cells and was collected every other day for 6 days (James, 1995). The ascites fluid was centrifuged at 450 g for 5 min to remove cell pellet. The supernatant was diluted with equal volume of PBS and appropriate amount of silica dioxide (15 mg/mL) was added to the supernatant and incubated at room temperature for 30 min with rotation. The IgG from the pretreated ascites fluid was purified by caprylic acid/ammonium sulfate precipitation twice and dialysis and then stored at −70 °C. The concentration and purity of purified MAb were determined by taking absorbance at 280 nm and SDS–polyacrylamide gel electrophoresis, respectively.

2.4. Determining for the titer of anti-DBP MAb

Checkerboard titration, in which purified MAb was titrated against varying amounts of the coating hapten-conjugate was used to measure the reactivity of antibodies and to select an appropriate coating concentrations and antibody dilutions for icELISA (Crowther, 2001). The checkerboard assays were performed in inELISA. Microtiter plates were coated with 100 μL/well of hapten-protein conjugates (0, 0.25, 0.5, and 1 μg/mL) in 0.05 mol/L carbonate buffer (pH 9.6) and incubated overnight at 4 °C. The plates were washed three times with PBST (0.01 mol/L PBS containing 0.05% Tween-20, pH7.4, 200 μL/well) and were blocked with 3% (w/v) skim milk powder in PBS (250 μL/well) for 1 h at 37 °C. After another washing step, 100 μL/well of purified anti-DBP MAb (1:200, 1:400, 1:800, 1:1600, 1:3200, 1:6400) diluted in PBS containing 0.1% BSA was added. After incubation for 1.5 h at 25 °C, the plates were washed Subsequently, the plates were incubated for 1 h at 37 °C with 8000-fold diluted HRP anti-mIgG in PBS containing 0.1% BSA (100 μL/well). After another washing step, 100 μL/well of freshly prepared O-phenylenediamine (OPD) solution (0.4 mg/mL OPD and 0.012% H_2O_2 in 0.05 mol/L citrate–phosphate, pH 5.0) was added. The reaction was stopped with 2 mol/L sulfuric acid (50 μL/well) after an incubation of 15 min at room temperature keeping away from light. The absorbance was recorded at a primary wavelength of 492 nm with a reference wavelength of 630 nm.

2.5. Determining for the specificity of MAb

To determine the specificity of MAb, the ability of the MAb to recognize several structurally related compounds (analogues) was tested by performing hapten coated icELISA (Wei et al., 2011) and determining their respective IC_{50} (the concentration of analyte giving 50% inhibition) values. The cross-reactivity (CR) values were calculated according to the following equation CR (%) = IC_{50} DBP/IC_{50} analogue × 100. The hapten coated icELISA was modified to determine whether MBP affects the binding of DBP to the MAb. The increasing concentration of MBP (19.5–10,000 μg/mL) was added to the 12.5 μg/L of DBP, then twice as the titer of the anti-DBP Mab in 50 μL of assay buffer was added to the wells in the "competition" step of icELISA. The other procedures were the same as hapten coated icELISA under optimal condition.

2.6. Frozen sections

Each excised sample was immediately frozen in liquid nitrogen. The specimens were embedded in tissue freeze media optimum cutting temperature (OCT) compound (Electron Microscopy Sciences, Hatfield, PA) and frozen at −80 °C freezer. Several frozen sections (5–10 μm thickness) were cut from each sample. Each section was dried in 37 °C for 1 h. It was fixed in 4% Paraformaldehyde 30 min and then washed 3 times in PBS.

2.7. Immunofluorescence assays

Each specimen was embedded, sectioned and fixed. Washed with PBS, the frozen sections were blocked with 5% milk in PBS at room temperature for 1 h and then incubated at 4 °C overnight with the purified anti-DBP antibody (1:100 dilution with 1% milk in PBS). After washes in PBS containing 0.1% Tween-20, all samples were incubated with a secondary antibody (fluorescein isothiocyanate-conjugated, affinity-purified goat-rabbit IgG) at 1:100 dilution in PBS containing 2% Goat Serum for 1 h at room temperature under dark. All sections were counterstained with 3,8-Diamino-5-[3-(diethylmethylammonio)propyl]-6-phenylphenanthridinium diiodide (PI). Sections were mounted with Vectashield (Brunschwig chemie, Amsterdam, The Netherlands) and coverslipped. Observations and photography were obtained with an Axioskop 40 microscope equipped with epifluorescence optics and a cool-CCD camera (Zeiss) or a confocal microscope (Leica, Heerbrugg, Switzerland).

2.8. Conjugate coated indirect competitive ELISA

An icELISA was used to assess the sensitivity of the MAb to free DBP and the crossreactivities of structurally related compounds to the antibody. N,N-dimethylformamide (DMF) was used to prepare standard solution and assay buffer (0.01 mol/L PBS containing 0.1% BSA, 10% DMF and 1.6% NaCl). For competition, 50 μL of standards or diluted samples in assay buffer were placed in the wells, and 50 μL of the anti-DBP MAb diluted with assay buffer was added. After incubated for 1.5 h at 25 °C, the plate was washed. The following procedure was followed as described for the inELISA. With the icELISA format, analytes that do not react with the antibodies would produce the maximum absorbance; conversely, analytes that do react with the antibody would decrease in percentage of absorbance. Standards and samples were run in triplicate wells, and the mean absorbance values were processed. Standard curves were obtained by plotting absorbance against the analyte concentration and fitted with a logistic three-parameter equation $y = (A−Ai)/(A−A0) * 100$, where A is the absorbance value with no analyte present, Ai is the absorbance value with analyte present, $A0$ is the absorbance value of blank.

2.9. Statistical methods

The contents of DBP were evaluated by immunofluorescence assays and measurement of fluorescence intensity using softer *Image-Pro Plus*. All analyses were performed using softer origin75.

3. Results

3.1. Preparation for monoclonal antibody

A monoclonal antibody specific to DBP was produced from a stable hybridoma cell line resulting from the fusion of a mouse myeloma with B cells obtained from a mouse immunized with purified, artificial antigen DBAP-OVA. Indirect noncompetitive ELISA (inELISA) with 0.25, 0.5, 1 μg/mL DBAP-OVA as a coated antigen were used to determine the titer and sensitivity of anti-DBP MAb. The results of the titers of anti-DBP MAb are shown in Table 1. The optimal coating concentration of DBAP-OVA and dilution of MAb determined by checkerboard titration were 0.5 μg/mL and 1:1600 (1 μg/mL), respectively.

3.2. The specificity of MAb

We evaluated MAb recognition to some chemicals structurally resembling portions of DBP structure (Table 2). The most sensitive

Table 2
Cross-reactivity of some compounds structurally related to DBP by hapten coated icELISA.

Analyte	IC_{50} (ng/ml)[a]	CR%
DBP	17.7 ± 3.2	100
DBAP	64.7 ± 14.1	27.4
MBP	–	7.3% inhibition at 10 mg/ml
DMP	–	7.98% inhibition at 10 mg/ml
DEP	–	5.08% inhibition at 10 mg/ml
DEHP	–	1.12% inhibition at 10 mg/ml
DOP	–	1.15% inhibition at 10 mg/ml

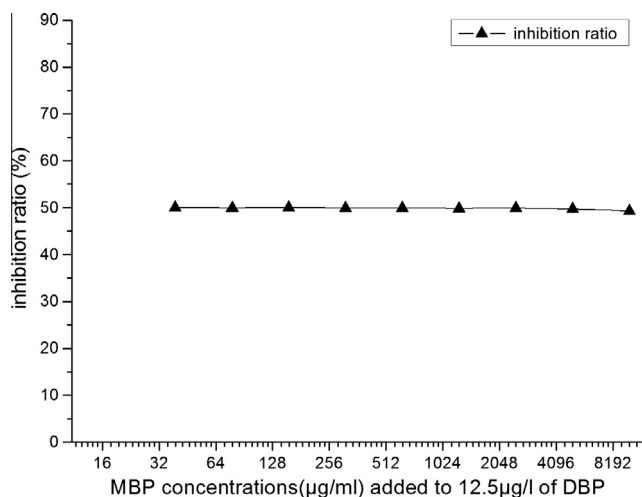[a] Each data represents the mean of ± SD of three replicates (three plates on the same day).

Fig. 1. Determining whether MBP affects DBP binding to the anti-DBP MAb.

Table 1
Titers of anti-DBP MAb.

Coating concentration (μg/mL)	Times of dilution/absorbance at 492 nm					
	200	400	800	1600	3200	6400
1	2.391	2.057	1.752	1.408	1.110	0.803
0.5	2.034	1.847	1.519	1.187	0.903	0.658
0.25	1.643	1.372	1.107	0.846	0.576	0.413

[a] Absorbance at 492 nm under different concentrations of coating antigen (DBAP-OVA) and times of dilution of MAb.
[b] The last dilution of MAb that gave an absorbance at 492 nm of >1.0 was judged as the titer.
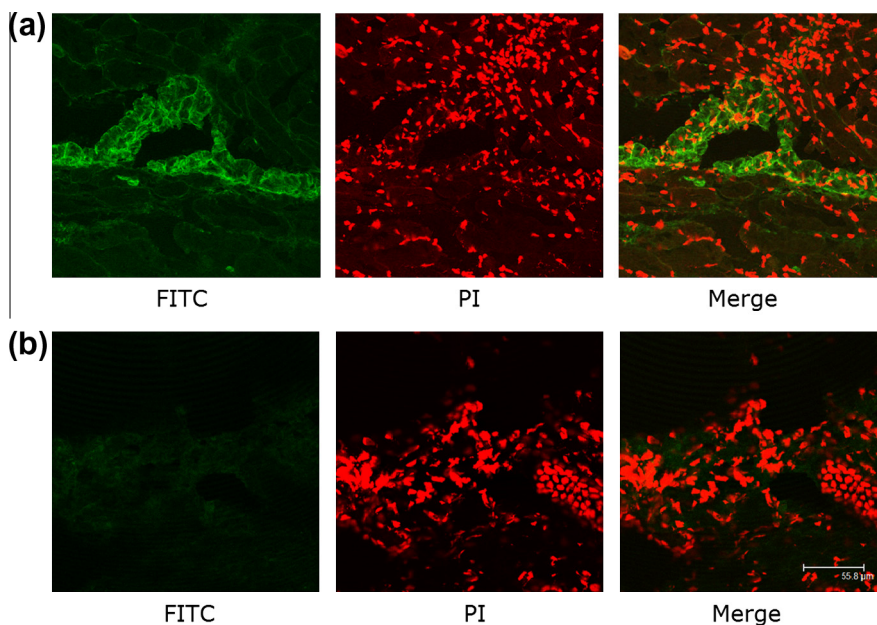
Fig. 2. Adherence and internalization of DBP in dermas and subcutaneous tissue cells. (a) Group of 400 mg DBP and (b) negative control group. Ten rats were epicutaneously smeared with DBP at 400 mg/kg bw-d or corn oil for 3 days. The treated skin was collected, embedded, sectioned and fixed. Each section was fixed and processed for double-immunofluorescence labeling. The DBP immersed in the cell was detected with the monoclonal antibody of DBP. PI-stained nuclei are shown in red. In merged images, DBP are shown green and nuclei are shown in red. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
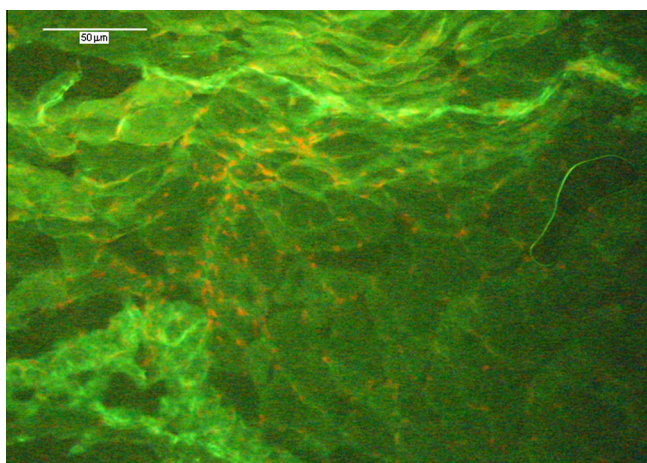

Fig. 3. DBP accumulations in sweat gland.

As MBP is the major metabolite of DBP, to determine whether MBP affects DBP binding to the MAb, The increasing concentration of MBP from 19.5 μg/mL to 10 mg/mL was added to 12.5 μg/L of DBP (the concentration of DBP giving 50% inhibition at twice as the titer of the MAb) at the system of icELISA. It was found that there is no substantial affection on inhibition ratio of DBP (Fig. 1).

3.3. Immunofluorescence assays DBP exposure by dermal route

Ten rats were epicutaneously smeared with DBP at 200, 400 mg/kg bw-d or corn oil for 3 days. No dead rats were found in any of the groups. Immunofluorescent showed that the monoclonal antibody of DBP reacted with frozen tissue sections of the rats treated with DBP, but not with the rats treated with corn oil counterparts. Confocal laser scanning fluorescence microscopy indicates that DBP were immersed in the cell actin cytoskeleton of cutaneous cells and/or dispersed in the cytoplasm and the perinuclear region (Fig. 2).

DBP accumulated in subcutaneous tissue such as sweat gland, hair follicle, this also was shown by fluorescence microscopy (Figs. 3 and 4).

3.4. Immunofluorescence assays DBP exposure by oral route

Administered DBP at dose of 400 mg/kg bw-d for 5 days by gavage, Wistar rats were euthanized. Some organs were collected and
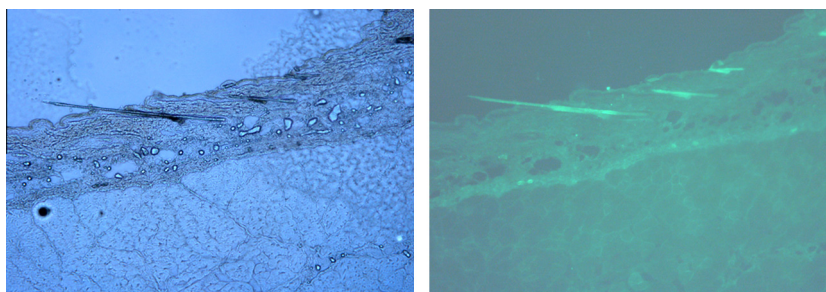
assay was obtained for DBP, and its CR was considered as 100%. For all of the other compounds tested, the CRs achieved were very low except that for DBAP, the aminated hapten with structural feature of DBP (27.4%).
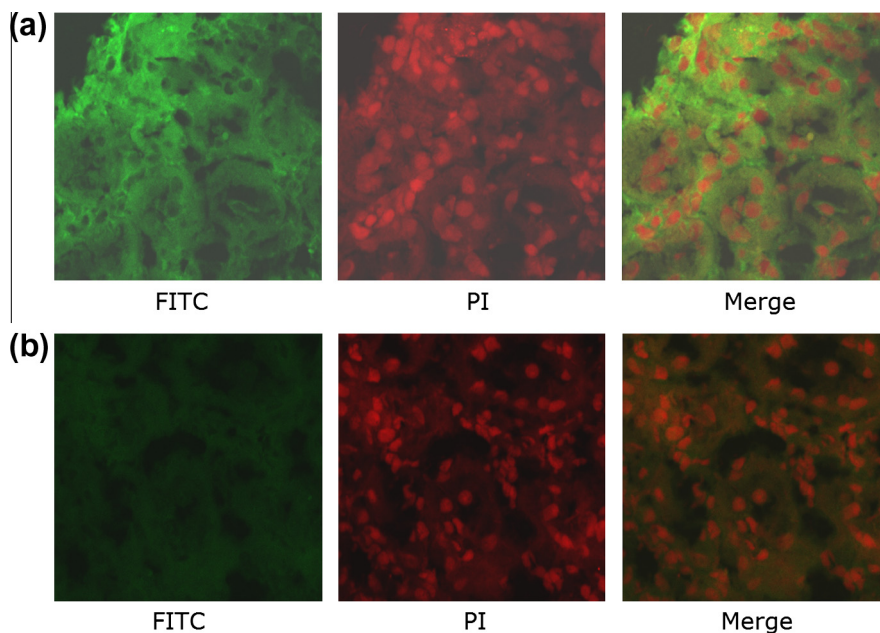

Fig. 4. DBP accumulations in hair follicle.

Fig. 5. Immunofluorescence labeling DBP on kidney. (a) Group of exposure to DBP and (b) negative control group. Wistar rats (n = 5/group) were administered either dibutyl-phthalate (DBP – Sigma–Aldrich) at dose of 200, 400 mg/kg bw-d, by gavage (oral route), or corn oil (vehicle). All animals were euthanized and samples were collected at 24, 48, 72 h following 5 days exposure to DBP. Each section was fixed and processed for double-immunofluorescence labeling. The DBP immersed in the cell was detected with the monoclonal antibody of DBP. PI-stained nuclei are shown in red. In merged images, DBP are shown green and nuclei are shown in red. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 6. Immunofluorescence labeling DBP on liver. (a) Group of exposure to DBP and (b) negative control group. Wistar rats (n = 5/group) were administered either dibutyl-phthalate (DBP – Sigma–Aldrich) at dose of 200, 400 mg/kg bw-d, by gavage (oral route), or corn oil (vehicle). All animals were euthanized and samples were collected at 24, 48, 72 h following 5 days exposure to DBP. Each section was fixed and processed for double-immunofluorescence labeling. The DBP immersed in the cell was detected with the monoclonal antibody of DBP. PI-stained nuclei are shown in red. In merged images, DBP are shown green and nuclei are shown in red. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
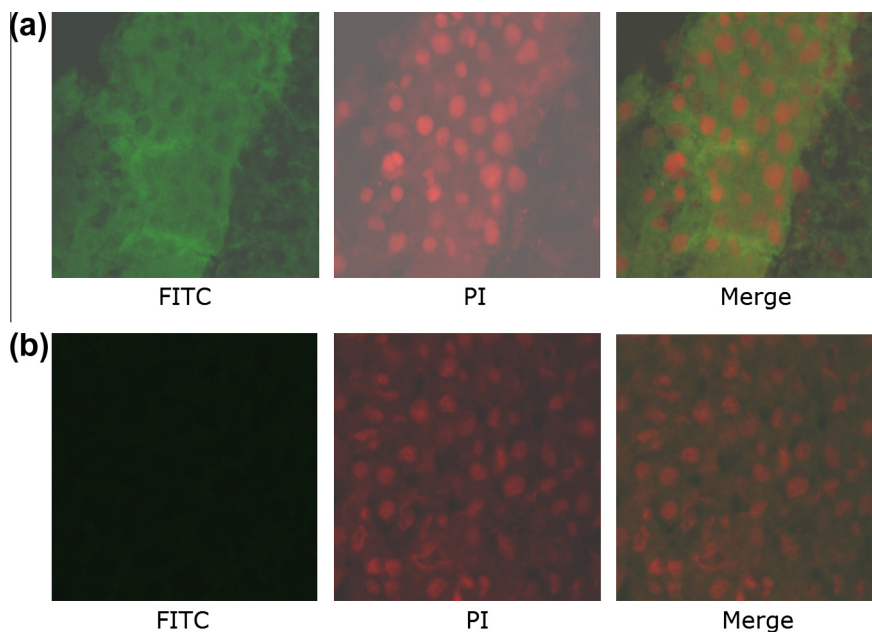
cut into frozen sections and tested by double-immunofluorescence labeling. The distribution and accumulations of DBP were detected with the monoclonal antibody of DBP in the cells of liver, kidney, stomach and testes from each rat (Figs. 5–8). Especially, the accumulations of DBP in the liver and kidney were more obvious than those of other organs from their stronger fluorescence intensity.

The levels of fluorescence intensity in those of organs were converted to integrated option density (IOD SUM) using softer Image-Pro Plus (Fig. 9). The The IOD SUM value computed by this softer indicate the relative amount of immunoreactant. According to the IOD SUM of these images, the relative contents of DBP in those of organs were compared.
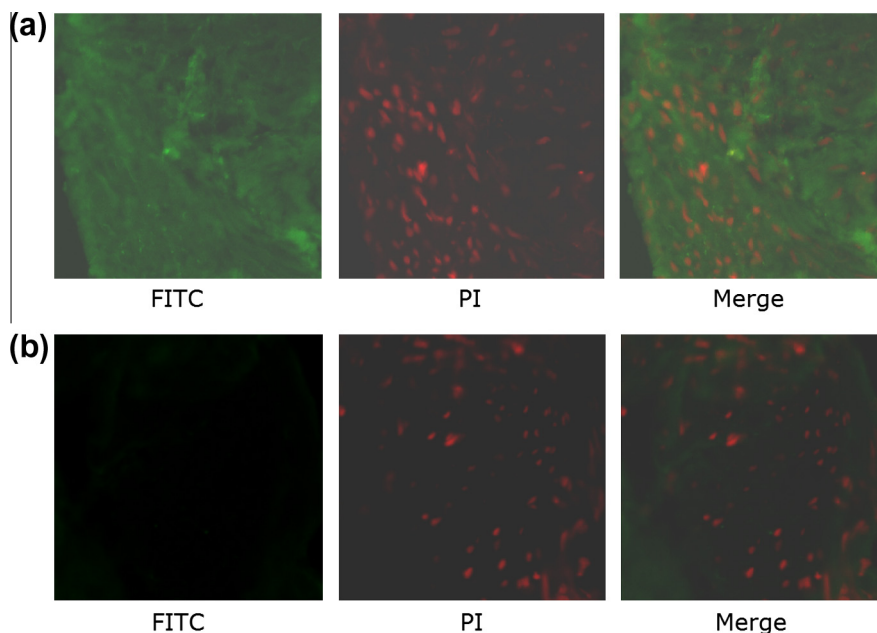
Fig. 7. Immunofluorescence labeling DBP on stomach. (a) Group of exposure to DBP and (b) negative control group. Wistar rats (n = 5/group) were administered either dibutyl-phthalate (DBP – Sigma–Aldrich) at dose of 200, 400 mg/kg bw-d, by gavage (oral route), or corn oil (vehicle). All animals were euthanized and samples were collected at 24, 48, 72 h following 5 days exposure to DBP. Each section was fixed and processed for double-immunofluorescence labeling. The DBP immersed in the cell was detected with the monoclonal antibody of DBP. PI-stained nuclei are shown in red.In merged images, DBP are shown green and nuclei are shown in red. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 8. Immunofluorescence labeling DBP on testes. (a) Group of exposure to DBP and (b) negative control group. Wistar rats (n = 5/group) were administered either dibutyl-phthalate (DBP – Sigma–Aldrich) at dose of 200, 400 mg/kg bw-d, by gavage (oral route), or corn oil (vehicle). All animals were euthanized and samples were collected at 24, 48, 72 h following 5 days exposure to DBP. Each section was fixed and processed for double-immunofluorescence labeling. The DBP immersed in the cell was detected with the monoclonal antibody of DBP. PI-stained nuclei are shown in red. In merged images, DBP are shown green and nuclei are shown in red. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
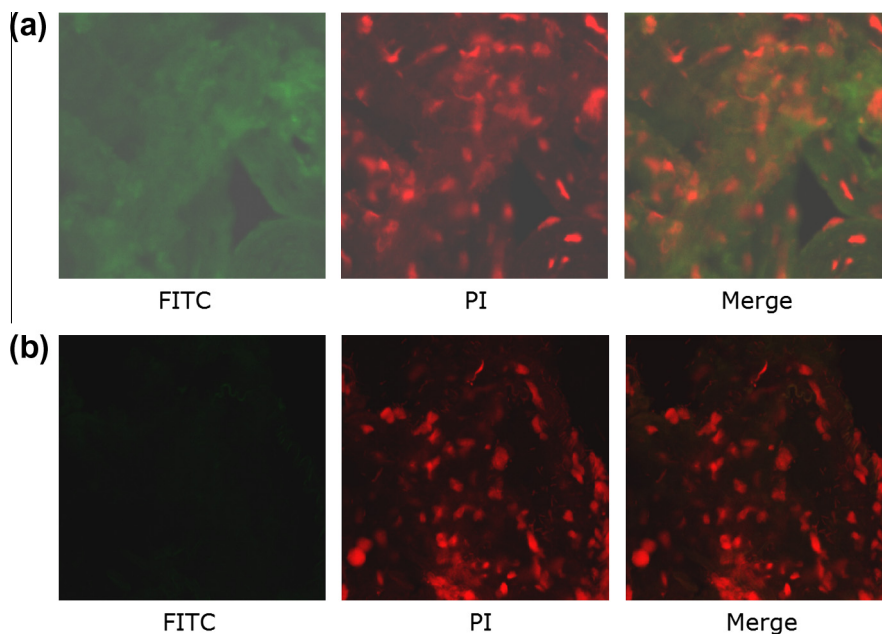
3.5. Immunofluorescence assays metabolize of DBP

Starting 24, 48, 72 h after the 5 days exposure to DBP, the rats were euthanized. To study phthalate metabolize, the accumulations of DBP were evaluated by immunofluorescence assays and measurement of fluorescence intensity using softer *Image-Pro Plus*.

According to the IOD SUM value, the fluorescence intensity of samples collected at 48 or 72 h following the 5 days exposure to DBP was significantly decreased in the rats given DBP at dose of 400 mg/kg bw-d (Fig. 10). This significant difference also was observed in the sections of liver (Fig. 11). This change indicates that DBP was metabolized to MBP or other metabolite in 2 or 3 days.
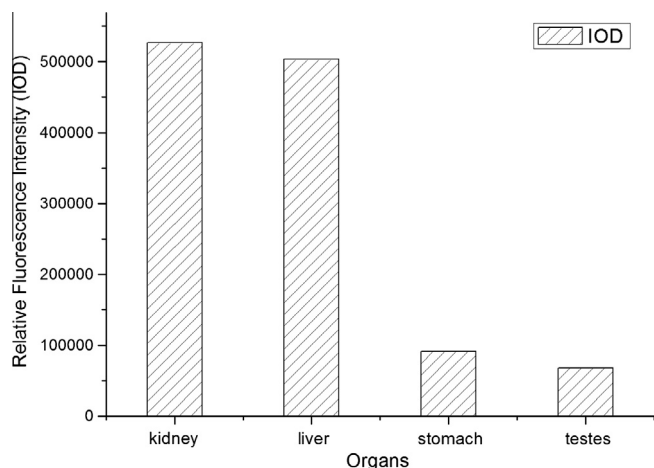
Fig. 9. comparing the levels of fluorescence intensity in the sections of liver, kidney, stomach and testes using softer *Image-Pro Plus*.
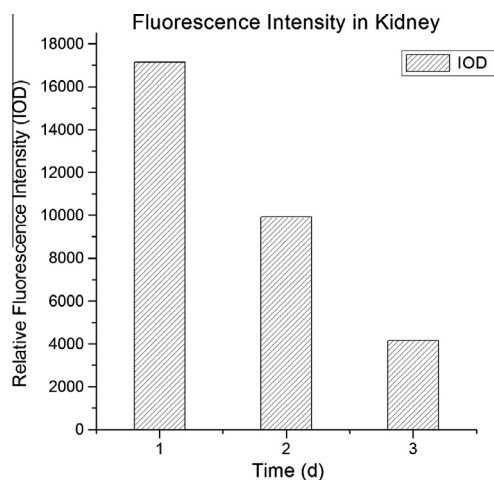

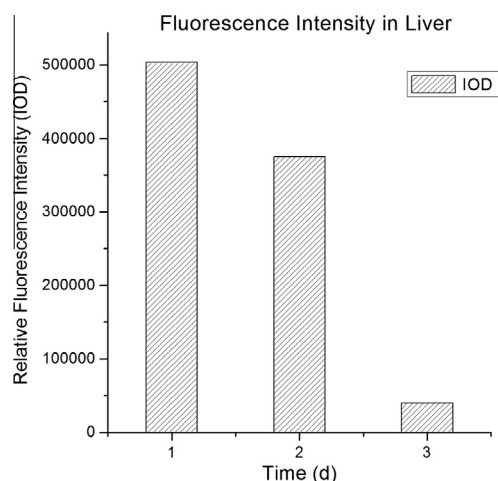**Fig. 10.** Comparing the levels of fluorescence intensity of kidney in different time using softer *Image-Pro Plus*.

Fig. 11. Comparing the levels of fluorescence intensity of liver in different time using softer *Image-Pro Plus*.
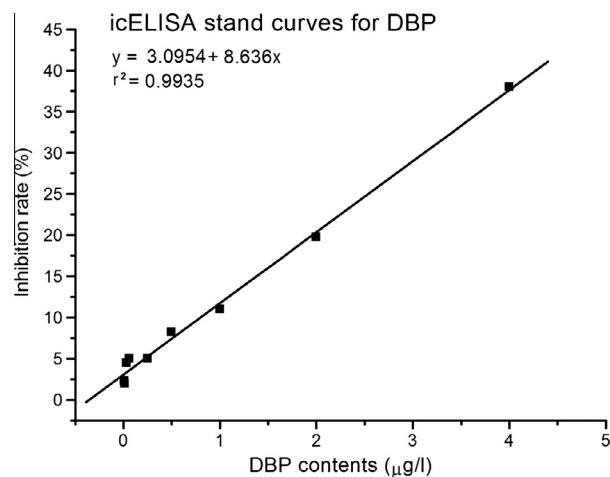

Fig. 12. Representative icELISA standard curves for DBP. Each point represents the mean of ± SD of three replicates (one plate on three different days). The standard curve was calculated according to the 3-parameter equation $y = (A-Ai)/(A-A0) * 100$, where A is the absorbance value with no analyte present, Ai is the absorbance value with analyte present, $A0$ is the absorbance value of blank.

3.6. Indirect competitive ELISA determining accumulations of DBP

The optimal coating concentration of DBAP-OVA and dilution of MAb determined by Indirect noncompetitive ELISA (inELISA) were 0.5 µg/mL and 1:1600 (1 µg/mL), respectively. An icELISA was used to determine DBP contents of analytes. Standard curves were obtained by plotting inhibition ratio against the standards (DBP) concentration (Fig. 12).

After 5 days exposure to DBP through dermal route or oral route, samples of blood, urine, liver and kidney from the exposed rats were collected at 24, 48, 72 h and DBP contents of them were determined by Indirect noncompetitive ELISA, respectively (Fig. 13). According to the inhibition ratio and regression equation of standard curve, concentrations of DBP in the tested tissues at 24, 48, 72 h were calculated. The DBP contents of samples collected at 48 h were significantly decreased than those collected at 24 h, and those collected at 72 h were less than those collected at 72 h. These data confirm the results of immunofluorescence assays and indicate that DBP was metabolized in 2 or 3 days. Exposure to the same dose of DBP, the accumulations of DBP in rats exposed through dermal route was less than that of oral route. To calculate the percentages of daily-administered dose, DBP concentrations in tested tissues at different time were converted from µg/L to mg/kg according to their densities then divided by the 400 mg/kg bw-d of administered dose (Table 3). Only about 0.1–0.25% of the daily administered dose by oral route or dermal route was found in blood, kidney, liver and urine within 24 h, about 0.03–0.1% and 0.004–0.02% of the dose was found at 48 h and 72 h.

4. Discussions

Phthalates are a group of chemicals with widespread use in the industrial production of numerous consumer products. They are suspected to be involved in male reproductive health problems and have also been associated with several other health problems including obesity and asthma. To estimate the daily intake of phthalates, the main concern has been the levels of phthalate metabolites in the blood and urinary excretion (Becker et al., 2009), whereas no data is available concerning the levels of DBP in other biological materials, and the environmental data consist primarily of estimates.
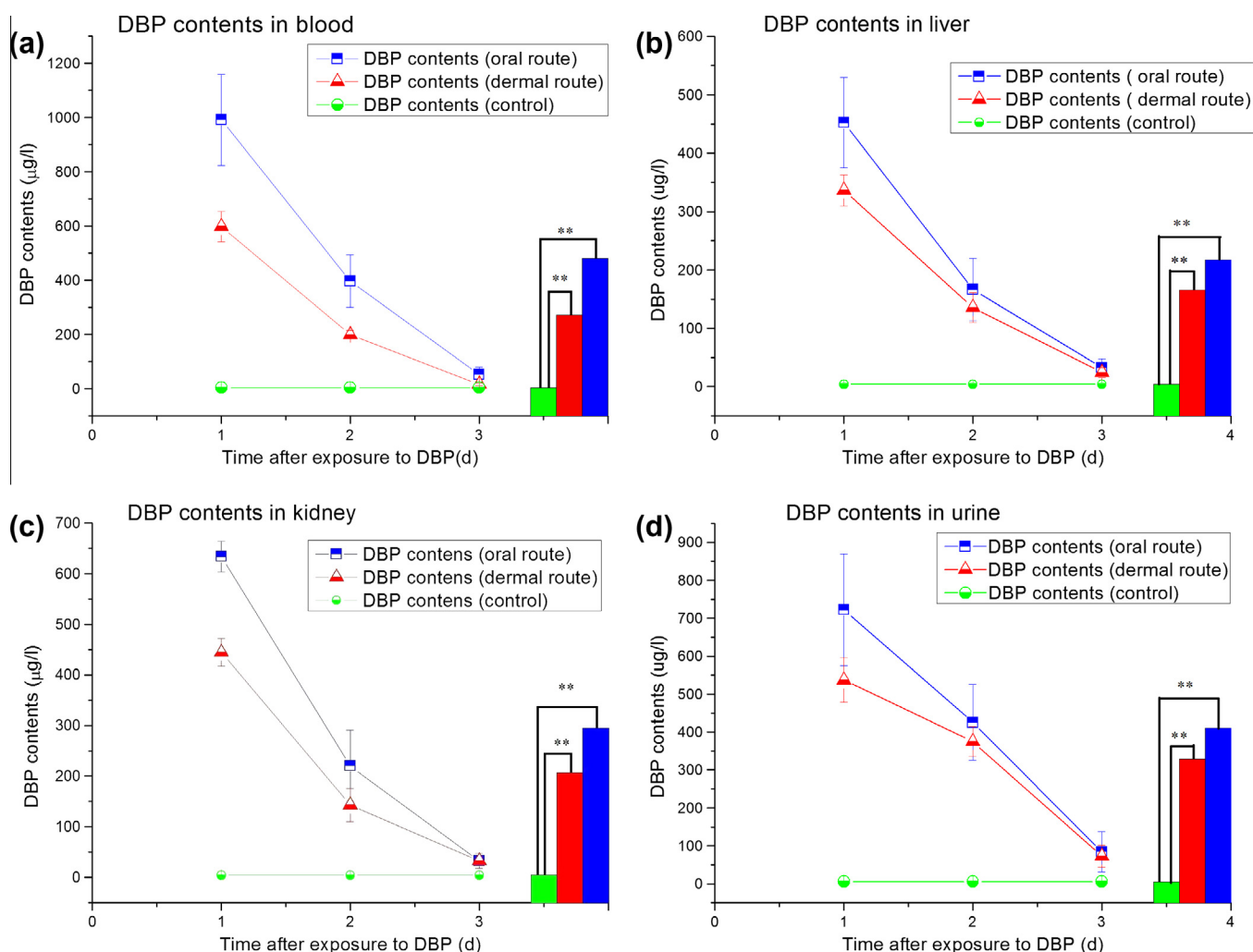
Fig. 13. DBP contents (a) DBP contents in blood (b) DBP contents in liver (c) DBP contents in kidney and (d) DBP contents in urine.

Table 3
DBP concentrations in tissues at different time as percentages of the daily-administered dose.

Time and route		Percentage in blood (%)	Percentage in liver (%)	Percentage in kidney (%)	Percentage in urine (%)
1 Days	Oral route	0.248 ± 0.042	0.113 ± 0.019	0.158 ± 0.007	0.180 ± 0.037
	Dermal route	0.149 ± 0.014	0.084 ± 0.006	0.111 ± 0.006	0.134 ± 0.015
2 Days	Oral route	0.099 ± 0.024	0.042 ± 0.013	0.055 ± 0.017	0.106 ± 0.025
	Dermal route	0.050 ± 0.004	0.034 ± 0.006	0.036 ± 0.008	0.093 ± 0.009
3 Days	Oral route	0.013 ± 0.007	0.008 ± 0.004	0.008 ± 0.004	0.021 ± 0.013
	Dermal route	0.004 ± 0.001	0.006 ± 0.002	0.008 ± 0.004	0.018 ± 0.007

Data are expressed as mean ± S.D ($n = 5$).

In this study, the contents of DBP in liver, kidney, stomach and testes were detected with the monoclonal antibody by immunofluorescence assays and indirect competitive ELISA. This data give directly evidence that indicates the distribution and accumulation of DBP in vivo.

The concentration of DBP and MBP, are often either at or below detection limits. It is expensive and difficult to determine the concentration of DBP in organism. Both of ^{14}C isotopic tracer method and chromatographic techniques to determine the concentration of DBP in previous studies are time consuming and have high instrumentation costs or high requirements for equipment. Although, the chromatographic techniques provide a low level of detection for phthalates, these methods require extensive purification, experienced technicians and expensive equipment and reagents. In contrast, immunoassay is a fast, simple, and economic

analytical method. Because of its strong selectivity and sensitivity, efforts for sample cleanup can be reduced to a minimum, which makes the immunoassays highly convenient tools for high throughput studies for a large number of samples in a short period of time (Plaza et al., 2000). Nevertheless only a few attempts have been made to develop sensitive and specific immunoassays for phthalates. Zhang et al. has reported a competitive fluorescence immunoassay for determination of DBP in water and food packaging samples based on polyclonal antibody (Zhang et al., 2006). Yanaihara et al. developed a direct competitive enzyme-linked immunosorbent assay (ELISA) for phthalates also based on polyclonal antibody (Yanaihara et al., 2002). Nevertheless, polyclonal antibody is restricted by immunized animals and cannot be produced unlimitedly. Furthermore, the character of polyclonal antibody from different immunized animals is different, which made

it difficult to standardize the measurement. Thus, more attempts are needed to obtain immunochemical methodologies capable of detecting this phthalate.

In this work, we set up two novel approaches, immunofluorescence assays and indirect competitive ELISA to determination of the distribution and accumulation of DBP in vivo. Double-label immunofluorescence assays, the approach pursued in this study, provide with a visual approach to determination of the distribution and accumulation of dibutyl phthalate (DBP) in rats. It showed that DBP accumulations in subcutaneous tissue such as sweat gland, hair follicle. Confocal laser scanning fluorescence microscopy indicates that DBP were immersed in the cell actin cytoskeleton of cutaneous cells and/or dispersed in the cytoplasm and the perinuclear region. Both of immunofluorescence assays and ELISA can be used to detect the content of DBP in biological materials. The results of them confirmed each other. Our assays show that DBP accumulations in viscera being rich in fat, such as liver, kidney. Perhaps due to the high solubility in adipose tissue, the content of DBP in these organs is higher than others. Although there are several physiological barriers that DBP penetrates testes have to overcome, the fluorescence signal indicating accumulations of DBP in testes can be observed by immunofluorescence assays. This suggested that DBP can act directly on the testes.

Dibutyl phthalate is rapidly absorbed and excreted after oral administration as was demonstrated in studies in laboratory animals (European Commission, 2004). The data of our assays also confirmed this. Only 0.1–0.25% of the dose was found in blood, kidney, liver and urine within 24 h. At 72 h after administration only trace amounts (<0.02%) were detected in tissues. Because the major part of DBP is hydrolysed to MBP and unchanged DBP was small amounts and hard to detect, the concentrations of MBP but not DBP itself were used to estimate absorption and excretion. However, the absorption, distribution and accumulation of DBP in rats were revealed by the directly data of DBP level in this work. Comparing with DBP concentrations of samples collected at different time following exposure to DBP, it was found that the DBP level of samples at 48 h after administration was 40–60% of that at 24 h, while the DBP level at 72 h was only 10% of that at 24 h.

The results of ELISA show that the accumulations of DBP in rats exposed through dermal route were less than that of oral route, though exposures to the same dose of DBP. This means that humans are exposed to phthalates through ingestion is more principal way, comparing with dermal contact.

5. Conclusions

In this study, we have developed a fast, simple, and economical analytical method to detect DBP in vivo. The distribution and accumulation of DBP in rats were determined by two novel approaches with a monoclonal antibody specific to DBP in vivo. Double-label immunofluorescence assay provides with a visual approach to detect the distribution and content of dibutyl phthalate in biological materials. Both of immunofluorescence assay and ELISA can be used to detect the content of DBP in biological materials. The results showed that DBP accumulated in subcutaneous tissue (sweat gland, hair follicle) and viscera being rich in fat (liver, kidney) and DBP could overcome several physiological barriers to penetrate testes. The results suggested that the accumulations of DBP in rats exposed through dermal route were less than that of oral route and indicated that most of DBP was metabolized in 2 or 3 days.

6. Conflict of Interest

The authors declare that there are no conflicts of interest.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fct.2013.01.045.

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