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Short Communication

Direct competitive immunosorbent assay for detection of MEHP in human urine

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HIGHLIGHTS

- A specific monoclonal antibody against MEHP with high affinity of 4.8×10^8 was prepared.
- A direct competitive immunosorbent assay on detecting MEHP was reported with high specificity and sensitivity.
- MEHP was tested by the established dcELISA showing higher MEHP concentration than that reported before.
- The established ELISA method could be used to monitor MEHP in human urine for assessing human exposure to DEHP.

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ABSTRACT

Di-(2-ethylhexyl) phthalate (DEHP) is the most commonly used plasticizer for flexible polyvinyl chloride (PVC), which is also known as one of the environmental endocrine disruptors with the reproductive, developmental and embryonic toxicity after entering human body. Mono-2-ethylhexyl phthalate (MEHP) is one of the most complicate metabolites from DEHP in vivo and responsible for many toxic effects of DEHP. In order to evaluate human exposure to DEHP, a direct competitive enzyme-linked immunosorbent (dcELISA) based on monoclonal antibody (mAb) was developed to detect MEHP. A hybridoma cell line 4B9 secreting mAb against MEHP was prepared, and the horseradish peroxidase (HRP) labeled antigen as a probe in the dcELISA was made. After optimization of ELISA reaction conditions, the standard curve with a linear range from 0.56 to 1000 ng mL^{-1} and a detection limit of 0.39 ng mL^{-1} was established. The cross-reactivities of anti-MEHP mAb to other ten phthalate esters were less than 5% except for mono-methylphthalate (MME). The average recoveries of MEHP from distilled water and negative human urine were both between 87.4% and 94.72% with coefficient of variation (CV) less than 5%. Here, the ELISA method on detecting MEHP was successfully established and applied to real urine sample analyses and the results were confirmed by HPLC. Furthermore, it was indicated that the immunoassay was reliable and suitable for monitoring MEHP.

1. Introduction

Phthalate acid esters (PAEs), these kinds of the important environmental hormones, are mainly used as additives in flexible polyvinyl chloride (PVC) products. In recent years, with the mass production of plastic products leading to the wide uses of the PAEs, PAEs have become one of the most common pollutants in the environment. Although only a few phthalates are produced at the industrial scale, it was estimated by the World Health Organization (WHO) that the annual production of phthalates can approach to millions of tons. One of the most important PAEs is Di-(2-ethylhexyl) phthalate (DEHP) which accounts for about 50% of the world production of phthalates (Chao and Cheng, 2007). DEHP is a primary component in polyvinyl chloride plastics used in numerous household products, children's toys, construction materials, personal care products (such as body lotion and deodorant), furniture upholstery and medical devices (Food and Drug Administration, FDA, 2001; Cirillo et al., 2013). As DEHP is not chemically bound to the polymer matrix, it can be released into the external environment during the processes of manufacturing and using products (Bagó et al., 2004; Khan and Jung, 2008). Therefore, human beings have many opportunities to be exposed to DEHP regarded as a potential hazard. People approaching to DEHP are assumed to be

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mainly through food, water, and air inhalation (Koch et al., 2003b), especially patients may be easier to expose to DEHP than the general population (Faouzi et al., 1999) when receiving transfusions (Peck et al., 1979; Hanawa et al., 2000; Takatori et al., 2008; Satoshi et al., 2012) or dialysis (Sharman et al., 1994). At present, DEHP has been paid great attention among the world because of its potential harm on public health, especially after the event of plasticizer happened in Taiwan last year (Susana et al., 2011), more concern was given to DEHP in China.

After entering the human body, DEHP was metabolized into monoesters and the corresponding glucuronides by the liver and kidney. via animal toxicological experiments, DEHP and its primary metabolite mono-(2-ethylhexyl) phthalate (MEHP) represent reproductive and developmental toxicity (Lee et al., 2004; Maire et al., 2005; Bonilla and Mazo, 2010; Kang et al., 2011), and have the danger of malformation, cancer and mutation. Moreover, MEHP appears to be in possession of greater potential toxicity (Anas et al., 2003; Dalman et al., 2008) and has antiandrogenic activity 10 times greater than DEHP (Frederiksen et al., 2007; Martinez-Arguelles et al., 2013). A recent study suggested that DEHP via MEHP suppresses estradiol production in the ovary, leading to anovulation (Lovekamp-Swan and Davis, 2003). Thereby, it is significant to evaluate the exposure of DEHP in the environment and understand the relationship between the content of DEHP in human body and certain disease by detecting MEHP in urine.

Several methods on detecting DEHP in environment have been reported (Luster et al., 1978; Albro et al., 1984). Furthermore, in the United States, DEHP exposure has been estimated by measuring the concentration of specific urinary metabolites (such as MEHP) (Silva et al., 2004b; Adibi et al., 2008) which helps to avoid the contamination from the ubiquitous parent compounds, thus allowing for a more accurate assessment of DEHP exposure to the general population (Blount et al., 2000a). Moreover, MEHP possessing biologically relevant activity is a valid biomarker when evaluating effect of DEHP on human health. The conventional methods to detect MEHP include gas chromatography, high-performance liquid chromatography (HPLC), and mass spectrometry (Liang et al., 2008). Despite the fact that accurate and reliable results can be obtained in these techniques, they are also time-consuming and expensive, and require skilled personnel and complicated sample pre-treatment. Moreover, they are unable to analyze a large number of samples simultaneously. The immunoassay based on specific antibodies is a rapid, reliable, economic, simple, and sensitive method for identifying and quantifying the target compounds in samples (Krasnova et al., 2001; Ahn et al., 2004; Ngo and Narinesingh, 2005; Wang et al., 2006). Up to date, there are no reports about the use of immunoassay techniques to measure phthalate metabolites. In this study, a direct competitive enzyme-linked immunosorbent (dcELISA) method based on the specific monoclonal antibody and enzyme-tagged antigen was established to detect MEHP in human urine for assessing human exposure to DEHP.

2. Materials and methods

2.1. Reagents, solutions, and cell lines

Mono-2-ethylhexyl phthalate (MEHP, purity 99.0 atom %D) and other ten cross-reactants including Mono-butylphthalate (MBP), Mono-benzyl phthalate (MBzP), Mono-methylphthalate (MME), Diethyl-hexylphthalate (DEHP), Di-n-octyl phthalate (DNOP), Dimethyl phthalate (DMP), Diisooctyl phthalate (DIOP), Diethyl phthalate (DEP), Dibutyl phthalate (DBP) and Butyl benzyl phthalate (BBP) were obtained from the Dr. Ehrenstorfer GmbH, Augsburg, Germany. Activated horseradish peroxidase kit was bought from Pierce Company. Bovine serum albumin (BSA), ovalbumin (OVA), complete and incomplete freunds adjuvant (CFA and IFA), polythyleneglycol-1000 (PEG), RPMI1640, fetal bovine serum (FBS), HT (hypoxanthine/thymidine), HAT (hypoxanthine/aminopterin/thymidine), OPD (O-Phenylenediamine) and horseradish peroxidase-conjugated goat anti-mice IgG (HRP-IgG) were all purchased from Sigma.

The regularly used solutions included phosphate buffered saline (PBS, 10 mM sodium phosphate buffer with pH 7.4 containing 140 mM NaCl), washing buffer (PBST, PBS containing 0.05% Tween 20), coating buffer (50 mM carbonate buffer with pH 9.5), blocking buffer (1% NH_4Cl in PBS), substrate reagent (0.4 mg mL^{-1} OPD and 0.15% (v/v) of 30% H_2O_2 in phosphate citrate buffer with pH 5.0).

Myeloma Cells SP2/0 were reserved by Key Laboratory of Zoonosis Research, Ministry of Education (China). The ELISA was carried out in 96-well polystyrene microtiter plates (Costar).

2.2. Experimental animals

Female Balb/C mice, 8–10 weeks old for immunization and 10–12 weeks old for ascites producing, were raised in cages with food and water available at any time, and a natural daylight, at the ambient temperature of 20 °C ± 2 °C, and relative humidity of 50 ± 5% (Dearman et al., 2008). All the animal experiments were performed with abidance by the provisions of EU animal management practices (1986.11.24).

2.3. Preparation and confirmation of protein–MEHP conjugate

The conjugates of protein–MEHP were prepared using a modified method (Nuria et al., 2007). Briefly, 1 mg of MEHP, 0.4 mg of N-hydroxysuccinimide and 0.8 mg of N,N-Dicyclohexyl carbodiimide (DCC) were mixed in 100 μL of DMF. The mixture was then incubated for 2 h at room temperature in an orbicular shaker (100 rpm). Forty microliter aliquot of the reaction mixture was added to the carrier protein BSA (0.96 mg in 50 μL of 0.01 M PBS) and OVA (0.68 mg in 50 μL of 0.01 M PBS), respectively. The reaction was continued for another 2 h at room temperature. The conjugates were dialyzed against 0.01 M PBS (pH 7.4) at 4 °C for 24 h with four changes of dialysate to remove the residual reagents, and then they were dispensed and stored at −20 °C. The reagent-treated carrier proteins (free of MEHP) were also prepared in the same way as control. The conjugates were analyzed by a modified method (Zhou et al., 2009a) of non-denaturalized polyacrylamide gel electrophoresis (PAGE).

2.4. Preparation of mAb against MEHP

2.4.1. Animal immunization

One hundred microgramme of MEHP-BSA (immunogen) in 100 μL of PBS (0.01 M, pH 7.4) with an equal volume of CFA or IFA was emulsified according to the method (Zhou et al., 2009b). The mice were immunized by intraperitoneal injection with 100 μL of the CFA emulsion. The mice were boosted with an equal volume of the IFA emulsion in the same manner every 2 weeks. The sera collected at the seventh day after the third immunization were tested for serum titers of binding to MEHP–OVA, and the carrier proteins OVA or BSA. The mouse with 6×10^3 or higher of the serum titer binding to MEHP–OVA was selected to be spleen donors for hybridoma production and was dealt with intravenous injection (i.v.) boosts of 50 μg immunogens at the third day before the cell fusion.

2.4.2. Hybridoma screening and mAb characteristics

The spleen cells from the immunized mice were fused with myeloma cells (SP2/0) at a ratio of 5–10:1 under the reaction of polyethylene glycol-1000 (PEG) and dispensed in the 96-well cell