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Quantitative detection of bisphenol A and bisphenol A diglycidyl ether metabolites in human plasma by liquid chromatography–electrospray mass spectrometry

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Abstract

Due to the ubiquity of epoxy resin compounds and their potential role in increasing the risk for reproductive dysfunction and cancer, the need for an assessment of human exposure is urgent. Therefore, we developed a method for measuring bisphenol A (BPA) and bisphenol A diglycidyl ether (BADGE) metabolites in human blood samples using high-performance liquid chromatography–electrospray ionization mass spectrometry (LC–MS). Human blood samples were processed using enzymatic deconjugation of the glucuronides followed by a novel sample preparation procedure using a solid-phase-cartridge column. This selective analytical method permits rapid detection of the metabolites, free BPA and a hydrolysis product of BADGE (BADGE-4OH) with detection limits in the low nanogram per milliliter range (0.1 ng ml^{-1} of BPA and 0.5 ng ml^{-1} of BADGE-4OH). The sample extraction was achieved by Oasis HLB column on gradient elution. The recoveries of BPA and BADGE-4OH added to human plasma samples were above 70.0% with a standard deviation of less than 5.0%. This selective, sensitive and accurate method will assist in elucidating potential associations between human exposure to epoxy-based compounds and adverse health effects.

Keywords: Bisphenol A; Bisphenol A diglycidyl ether

1. Introduction

Bisphenol A diglycidyl ether (BADGE) is an epoxy resin monomer obtained by a condensation reaction between epichlorohydrin and bisphenol A (BPA) (Fig. 1). Epoxy-based solution coatings are

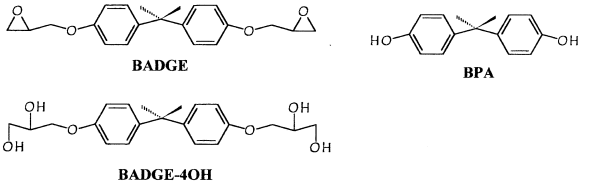

Fig. 1. Structure of BADGE, BPA and hydrolysis product of BADGE. BADGE, bisphenol A diglycidyl ether (M.W. 340); BADGE-4OH, bisphenol A bis(2,3-dihydroxypropyl) ether (M.W. 376); and BPA, bisphenol A (M.W. 228).

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used in many applications as additives for a variety of plastic materials, plastic coatings in food-packing and dental materials. Much research has been carried out to determine the analysis of BADGE and BPA in foods such as water, wine samples and canned fish in oil [1–5]. In addition, determination studies of BADGE and BPA in dental materials have been reported [6,7]. Therefore, it is highly possible that these compounds leach into food or saliva, and that humans are exposed to epoxy-compounds.

Recently, studies of the estrogenic effect of BPA [8–10], food extracts obtained from lacquered cans containing BADGE and BPA [11] and composite resins used in dentistry have been published [7]. Additionally, BADGE, its hydrolysis products and the chlorohydrin of BADGE were examined for their genotoxicity by an in vitro assay [12]. However, this is becoming a serious issue, only very few methods have been reported for determination of epoxy-base compounds in healthy exposed human.

Humans are potentially exposed to epoxy resin compounds, BPA, BADGE, hydrolysis products and the chlorohydrin of BADGE contained in foods, water, or dental materials. Measurement of an internal dose, or bio-marker of exposure, is an aspect of assessing exposure. As BPA is glucuronidated in liver microsomes for the metabolism of BPA [13], free BPA occurs only at low levels in blood of healthy human bloods [14,15]. BPA in humans and animals is metabolized to a glucuronide of BPA which appears to bioaccumulate. In the same way as BADGE, Climie et al. suggested that a fairly rapid metabolism occurred and the major metabolic transformation of BADGE was the hydrolytic ring-opening of the two epoxy rings to form diols [16,17]. BADGE was unstable in aqueous simulants, rapidly affording hydrolysis product corresponding to the opening of epoxy rings [18,19] (Fig. 1). These reports indicate that BADGE will not be detected in human blood or urine samples. An analytical approach is therefore required to measure the BADGE metabolite responsible for human exposure.

We present a novel method for the accurate, precise and sensitive measurement of the glucuronide of BPA and the hydrolysis product of BADGE (BADGE-4OH) in human blood. The use of β-glucuronidase enzyme to hydrolyze glucuronide metabolites allows the quantification of both the free and glucuronidated form of each BPA and BADGE-4OH. To satisfy the requirements of accurate and rapid sample preparation, a novel sample pretreatment procedure is developed for determining trace concentrations of these compounds in plasma using solid-phase based cartridge column on gradient elution.

2. Experimental

2.1. Materials

All solvents for LC–MS were HPLC grade, while the other solvents used for sample preparation were pesticide grade purchased from Wako Pure Chemical Industries Ltd. (Tokyo, Japan). Bisphenol A (BPA) standard was purchased from Kanto Chemical Industries Ltd. (Tokyo, Japan). Bisphenol A bis(2,3-dihydroxypropyl) ether (BADGE-4OH) standard were obtained from Fluka Chemie AG (Buchs, Switzerland). The ultra-pure water was purified with a Milli-Q water purification with EDS polisher system (Millipore, Bedford, MA, USA). Concentrated solutions (1.0 mg ml^{-1}) of BPA and BADGE-4OH were prepared in acetonitrile, and prepared at 0.1~1000 ng ml^{-1} as required by addition of acetonitrile–water (1:1).

2.2. Samples

We evaluated the suitability of the developed method for determining BPA and BADGE metabolite levels in healthy human blood specimens. Human plasma samples were stored at −80°C until analysis.

2.3. Instrumentation and conditions

The liquid chromatography–electrospray mass spectrometry (LC–MS) was performed using an Agilent 1100 MSD-SL system (Agilent Technologies, Palo Alto, USA). Samples volumes of 5.0 μl were injected. The column oven was controlled at 40°C. A CAPCELL PAK MG C_{18} (2.0ϕ×250 mm) reversed-phase column (Shiseido Co., Tokyo, Japan) was used. LC separation was carried out using a mobile phase from 0.01% acetic acid in water

(Solvent A) and acetonitrile (solution B). The gradient mode was as follows; 0–12 min at 40% solution B, then 12–14 min a liner increase from 40 to 100% solution B, hold at 100% solution B. The flow-rate was 300 μl min^{-1}. The working conditions for electrospray ionization MS were as follows; the drying nitrogen gas temperature was set at 350°C and the gas was introduced into the capillary region at a flow-rate 12 l min^{-1}; the capillary was held at a potential of 3500 V relative to the counter electrode for the negative-ion mode. The fragmentor voltages were fixed at 140 V for BPA and 100 V for BADGE-4OH during one chromatographic run. When working in the selected ion monitoring (SIM) mode, the *m/z* 227 and 435 ions, which were assigned as the $[M-H]^-$ of BPA and $[M+CH_3COO]^-$ of BADGE-4OH respectively, were monitored.

Calibration equations were obtained for BPA and BADGE-4OH using a series of standard solutions over the concentration range 1.0–100 ng ml^{-1}. The repeatability was evaluated by replicate analyses ($n=12$) of the standard solution at a concentration of 10 ng ml^{-1}. The calibration graphs for these standards of the SIM peaks were linear throughout the range tested.

2.4. Sample preparation

One milliliter of human plasma or serum sample was buffered with ammonium acetate (200 μl, 1.0 *M*, pH 6.8). After *Escherichia coli* β-glucuronidase (15 μl, 89 U ml^{-1}; Fluka Chemie AG, Buchs, Switzerland) was added, the samples were sealed in a glass tube and gently mixed. The quantitative glucuronidase hydrolysis required to release free BPA resulted in incubation at 37°C for 3 h. After enzymatic deconjugation, the samples were fraught with water to a total volume of 1.5 ml. Samples were subsequently filtered with an Acrodisc LC 25 mm syringe filter (0.45 mm PVDF membrane).

The samples were prepared by using solid-phase based cartridge column (Oasis HLB cartridge column 2.1×20 mm, Waters Co., Milford, MA, USA). The clean up and extraction of blood samples was achieved with cartridge column by using elution in water–acetonitrile. The sample preparation system consisted of a pump, auto-sampler for HPLC and the SPE column. The sample solution was injected in 500 μl by using an auto-sampler. Solvent phase A consisted of pure-water and solvent phase B consisted of acetonitrile for pesticide analytical grade. The following solvent programming was used: isocratic 100% solvent A, then increasing linearly to 100% solvent B after 10 min, maintaining this eluent for 10 min and then going back to the initial conditions for 5 min at a flow-rate of 0.5 ml min^{-1}. The optimal fractions for BADGE-4OH and BPA were collected, then the solutions were evaporated to dryness under a stream of nitrogen at 40°C. The samples were reconstituted in 200 μl of methanol. The obtained samples were measured by LC–MS.

3. Results and discussion

3.1. Detection of BPA and BADGE-4OH

In the mass spectral investigation by ESI–MS through flow injection analysis of BPA and BADGE-4OH standard solutions (10 μg ml^{-1}), the *m/z* 227 and 435 ions, which were assigned as the $[M-H]^-$ ion of BPA and the $[M+CH_3COO]^-$ ion of BADGE-4OH, respectively, were observed as the main peaks (Table 1).

The most important parameters affecting LC–MS for determination of compounds are the fragmentor voltage and the mobile phase on concentration of acetic acid in water. In order to establish the optimum fragmentor voltages for the detection of BPA and BADGE-4OH, the signals of the *m/z* 227 for BPA and *m/z* 435 for BADGE-4OH vs. fragmentor voltage were investigated. Standard solutions of BPA and BADGE-4OH were infused with various compositions of the mobile phase (water–acetic acid–acetonitrile) into the ESI interface while the fragmenter voltage was varied. The main signals of *m/z* showed a maximum in 0.01% acetic acid at 100 V for BADGE-4OH and 140 V for BPA, respectively (Fig. 2). In addition, for the determination of quality the fragmentation was produced by altering the skimmers collision-induced dissociation for a fragmentor voltage. The fragment ions were efficiently generated (Fig. 3). Moreover, the condition of ESI–MS shown in Table 1 was established.

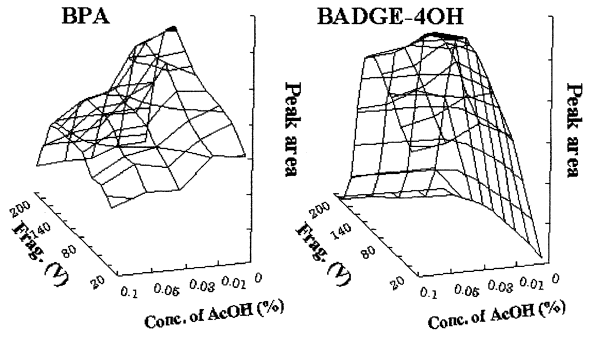

Fig. 2. Co-optimization of mobile phase solution (acetic acid in water base) and fragmentor voltage for BPA (m/z 227) and BADGE-4OH (m/z 435). The concentration range was 0–0.1% acetic acid in water. The fragmentor voltage range was 20–200 V.

3.2. Chromatographic condition

The detection limits (DLs) were calculated according to IUPAC with $3S_b = A_s - A_b$ (A_s is the average of the sample signal, A_b is the average of the blank signal and S_b is the standard deviation of the blank signal). So, the DL of BADGE-4OH was 0.5 ng ml^{-1} and the DL of BPA was 0.1 ng ml^{-1}. The chromatographic determination characteristics of these standards are presented in Table 2. It was possible to separate and to determine these compounds in a single run of 15 min by using LC–MS with SIM. The method yields a highly precise determination of standards and might be applied to the detection of trace amounts of BADGE-4OH and BPA in blood samples.

Table 1
Condition of detecting BADGE-401I and BPA by ESI-MS

Analyte	Monitored ion (m/z)	Fragment ion (m/z)	Optimal fragmentor voltage (V)
BPA	227	211	140
BADGE-4OH	435	301	100

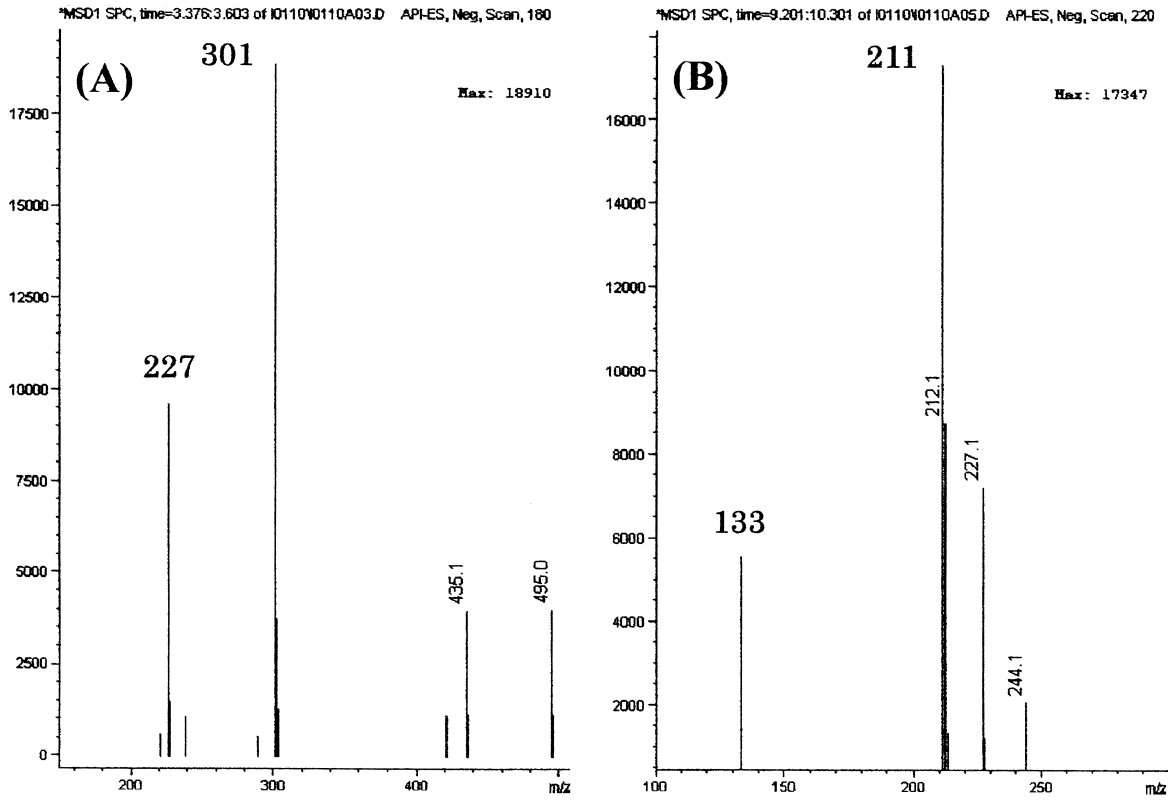

Fig. 3. LC–ESI–MS spectra of BADGE-4OH at fragmentors of 180 V (A) and BPA at fragmentors of 220 V (B).

Table 2
Chromatographic validation of detecting BADGE-4OH and BPA

Analyte	Retention time (min)	RSD_{RT} [a] (%)	RSD_{PA} [b] (%)	DL (ng ml^{-1})	Linear range (ng ml^{-1})	*r*
BADGE-4OH	3.7	0.26	0.77	0.5	1.0–100	0.999
BPA	9.4	0.13	0.30	0.1	1.0–100	0.999

[a] RSD_{RT} (%): The relative standard deviation within the series of replicate analyses ($n=12$) of the retention time.
[b] RSD_{PA} (%): The relative standard deviation within the series of replicate analyses ($n=12$) of the peak area.

3.3. Sample preparation

The novel pretreatment was carried out by using an Oasis HLB cartridge column. In addition, pure-water and acetonitrile for pesticide analytical grade were used for pretreatment of trace levels of BPA and BADGE-4OH in blood samples. The effluent was fractionated, the fractions were collected, and measured by LC–MS.

Based on the result of the determination of BPA and BADGE-4OH in each fraction, we decided to use those fractions in which the compounds have been most selectively isolated form plasma samples (Fig. 4). These fractions were evaporated to dryness, then adjusted in methanol and examined for their recoveries and standard deviations (SD's). The recoveries of BADGE-4OH and BPA were obtained using 1.0 ml of plasma spiked with a concentrated standard. These recoveries were 97.4% of BADGE-4OH and 74.5% of BPA, and the SD's were 4.6% of BADGE-4OH and 3.5% of BPA ($n=3$). Fig. 5 shows BADGE-4OH and BPA chromatograms form the spiked plasma samples used for determination of the recovery. The method is successfully applied to plasma samples pretreated. Thus, we decided to use this technique for a simple and selective pretreatment and quantitative determination of BADGE-4OH and BPA in blood samples.

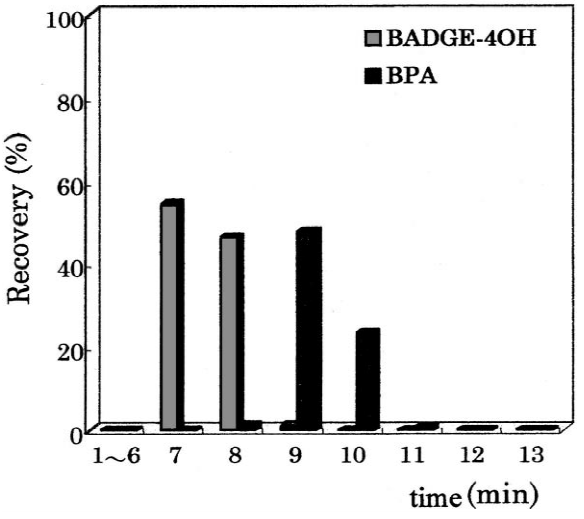

Fig. 4. Extractive fractions for determination of BADGE-4OH and BPA from 0 to 14 min. A plasma spiked with a standard solution (plasma concentration of 100 ng ml^{-1} of sample) was extracted by Oasis HLB cartridge column.

3.4. Application

Epoxy resins find widespread use as additives for plastic, adhesives and coatings in canned foods. The majority of the products are based on resins that use BADGE produced from BPA as starting material. The recent paper by Howdeshell et al. described that mouse fetuses exposed to BPA at a dose within the range typical of the environmental exposure level of humans alters the postnatal growth rate and brings on early puberty in these mice [20]. Therefore, it is highly possible that these compounds leach into the environment, foods and dental materials, and that humans are exposed to trace amount of these compounds. This is becoming a serious issue, but there are no analytical data to elucidate potential associations between human exposure to these compounds and adverse health effects. In this study, we targeted metabolites of BPA and BADGE. The determination of trace amounts of BPA is significant due to the contamination problems outlined previously [14,15]. Our study avoids potential problems of the previously reported method using enzymatic deconjugation. The proposed method was applied to healthy human blood samples to establish the levels of glucuronidation of BPA and BADGE-4OH in epoxy resin

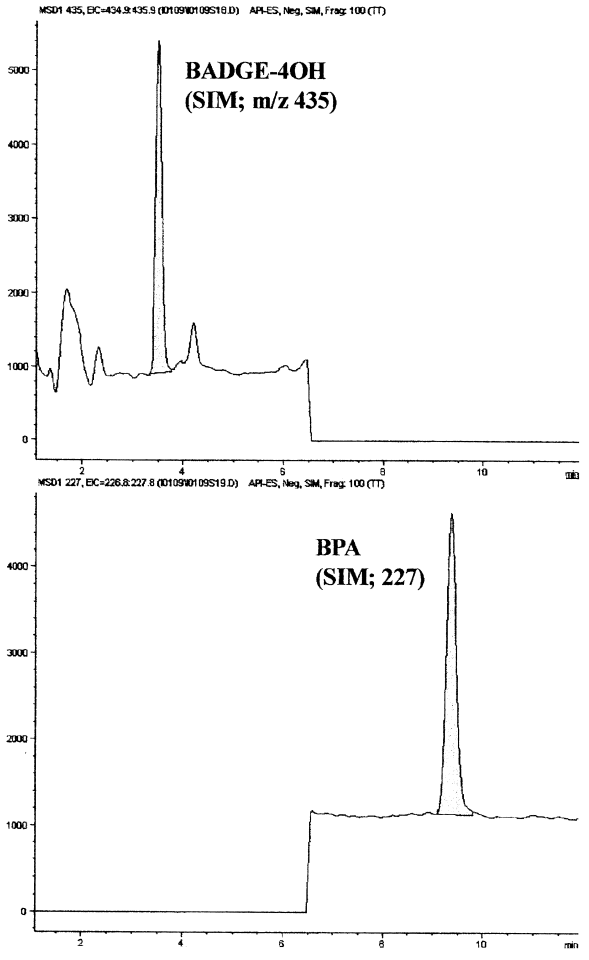

Fig. 5. Chromatograms in plasma spiked with a standard solution of BADGE-4OH and BPA. A 1.0 ml of plasma spiked with a solution of 100 ng/ml (plasma concentration of 10 ng ml^{-1} of sample) was extracted by an Oasis HLB cartridge column in 7–9 min for BADGE-4OH and 9–11 min for BPA.

exposure. Glucuronidation of BPA and BADGE-4OH concentrations in healthy human blood samples ($n=3$) showed only very low levels: ND-1.0 ng/ml of free BPA and ND-1.2 ng/ml of BADGE-4OH. ND was the limit of quantification in plasma. Concerning the detection of BPA and BADGE-4OH; the limit of quantification for measuring BPA and BADGE-4OH in plasma was each 0.6 ng/ml (signal of these compounds/background noise of control sample).

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