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ORIGINAL RESEARCH

Determination of bisphenol A in human breast milk by HPLC with column-switching and fluorescence detection

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ABSTRACT: A highly sensitive HPLC method was developed for the determination of xenoestrogenic compound, bisphenol A (BPA) in human breast milk samples. After a two-step liquid–liquid extraction, BPA was derivatized with fluorescent labeling reagent, 4-(4,5-diphenyl-1*H*-imidazol-2-yl)benzoyl chloride (DIB-Cl). The excess fluorescent reagent could be removed effectively using a column-switching system. The separation of DIB-BPA from endogenous materials in milk was carried out on two C_{18} columns and fluorescence intensity was monitored at 475 nm with the excitation of 350 nm. A good linearity ($r = 0.994$) was observed of BPA in the concentration range of 0.2–5.0 ng mL^{-1} in breast milk, and the detection limit was 0.11 ng mL^{-1} at a signal-to-noise ratio of 3. Intra- and inter-day precision (RSD, %) were less than 8.7 and 10.4, respectively. Twenty-three breast milk samples of healthy lactating women were analyzed for the BPA concentration; the mean value was 0.61 ± 0.20 ng mL^{-1}, with no correlation to the lipid content of milk samples. Copyright © 2004 John Wiley & Sons, Ltd.

KEYWORDS: bisphenol A; human breast milk; column-switching HPLC; fluorescence detection

INTRODUCTION

Bisphenol A (BPA) is an industrial chemical used widely as a monomer material of polycarbonate plastics, in the manufacture of the resin used to line food and beverage cans; as a component of plastic in dental fillings, and as a flame retardant. It has been reported that over one million tons of BPA are produced yearly world wide (Nagel *et al.*, 1999). According to the investigation by Japan Hygienic Olefin and Styrene Plastics Association in 1998, annual production of BPA in Japan is around 0.25 million tons. Of this amount, 4000 tons are used for food-related purposes. Humans exposure to BPA may arise through its leaching from related materials into foods or liquids (Kawamura *et al.*, 1998; Krishnan *et al.*, 1993) and in the saliva of patients treated with dental sealants (Noda *et al.*, 1999; Olea *et al.*, 1996). BPA exhibits weak estrogenic activity *in vitro* and *in vivo* (Gaido *et al.*, 1997; Steinmetz *et al.*, 1997, 1998; Bergeron *et al.*, 1999). It binds to human estrogen receptors (ER) and stimulates the transcriptional activity of both ER subtypes (Perez *et al.*, 1998; Kuiper *et al.*, 1997). Researchers have shown that BPA can exhibit xenoestrogenic effects *in vitro* at very low concentrations of 6 ppb (25 nM; Brotons *et al.*, 1995).

BPA is a lipophilic compound which has an octanol–water partition coefficient value (log *P*) around 3–4, suggesting that it has the potency to partition into fat and breast milk. In particular, contamination of human milk with BPA is important, because breast milk is the first food for newborns and thus they are possibly receiving BPA during their critical growth processes. This constitutes a severe threat to the health of infants and children. Since breast-feeding is the preferred nutrition, a better understanding of an infant's level of exposure to BPA is essential, particularly in Japan where information is sparse.

Until now, a number of analytical methods for the determination of BPA have been developed, including HPLC with UV detection (Nagel *et al.*, 1999; Careri *et al.*, 2001), electrochemical detector (ECD) (Inoue *et al.*, 2000; Sajiki, 2001), mass spectrometry (MS; Pedersen *et al.*, 1999), as well as gas chromatography (GC)-MS (Jeannot *et al.*, 2002; Zafra *et al.*, 2003). Enzyme-linked immunosorbent assay (ELISA) has also been applied to the analysis of BPA (De Meulenaer *et al.*, 2002). Generally, as sensitivities of HPLC-UV, HPLC-MS and GC-MS methods are not so high, sample enrichment is required. However, when limited volumes of samples such as biological fluids

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Abbreviation used: BPA, bisphenol A; DIB-Cl, 4-(4,5-diphenyl-1*H*-imidazol-2-yl) benzoyl chloride; ECD, electrochemical detector; SPE, solid-phase extraction; LLE, liquid-liquid extraction; LOD, limit of detection; RSD, relative standard deviation; M/S ratio, milk/serum ratio.

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are available, the lack of sensitivity becomes a serious problem in the use of these detection methods. Inoue *et al.* (2000) reported a highly sensitive HPLC-ECD method for the determination of BPA in human serum with the detection limit of 0.05 ng mL^{-1}. However, an ECD detector requires stricter maintenance to keep its working ability compared with conventional UV or fluorescence detectors.

In our previous study, an HPLC-fluorescence detection for the determination of BPA in several biological fluids (maternal and umbilical cord sera, ascitic fluid) was developed (Kuroda *et al.*, 2003). In this study, in order to assess breast-fed newborns' exposure to BPA, an alternative column-switching HPLC-fluorescence detection method was developed for the determination of BPA in human breast milk. To our knowledge, until now, there has been no information concerning BPA level in breast milk of lactating women, and the potential exposure to BPA of their newborn babies. Monitoring BPA in human breast milk would provide the information needed to assess the exposure to BPA of adults and their offspring.

EXPERIMENTAL

Reagents and chemicals. BPA [2,2-(4-hydroxyphenyl)-propane], triethylamine (TEA), 25% ammonia solution, acetic acid, sodium acetate, hexane and chloroform were of reagent grade purchased from Wako Pure Chemical Industries Ltd (Osaka, Japan). Acetonitrile and methanol were of HPLC grade (Wako). DIB-Cl was synthesized in our laboratory as reported previously (Nakashima *et al.*, 1995), which is now available from Tokyo Kasei Kogyo Co. Ltd (Tokyo, Japan). Water was deionized, distilled and passed through a water purification system (Puric-Z, Organo Co., Tokyo).

HPLC system. The HPLC system consisted of two pumps (LC-10AS, Shimadzu, Kyoto, Japan), a shimadzu RF-10A_{XL} fluorescence detector, a 7125 injector with a 5 μL loop (Rheodyne, Cotati, CA, USA), a SPV-N-6 column-switching valve with a 200 μL loop (GL Sciences, Tokyo, Japan) and a Rikadenki R-01 recorder (Tokyo, Japan; Fig. 1). The mobile phases were acetonitrile–H_2O–methanol (72:13:15, v/v/v) for precolumn (mobile phase 1) and acetonitrile–0.1 M acetate buffer (pH 5.5)–methanol (55:12:33, v/v/v) for the separation column (mobile phase 2), which were delivered at flow rates of 0.10 and 0.30 mL min^{-1}, respectively. The temperature for both columns was maintained at 35°C in a column oven (Omron E5C3, Tokyo, Japan). Samples were injected onto precolumn (Wakosil-5C18RS, 150 × 1.0 mm i.d., Wako) and eluted with mobile phase 1 to pass through the loop of column switching valve. Ten and half minutes after injection, the valve was switched so that the analyte was loaded onto the separation column (Vydac Protein and Peptide C_{18}, 150 × 4.6 mm i.d., Vydac, Hesperia, CA, USA) and eluted with mobile phase 2 to the fluorescence detector. This valve position was kept until the analysis was finished. Fluorescence intensity was monitored at 475 nm with the excitation of 350 nm.

Preparation of BPA standard solution. BPA stock solution at a concentration of 100 μg mL^{-1} was prepared in acetonitrile. This stock solution was subsequently used in the preparation of working standards by further dilution with acetonitrile. The stock solution was stable for at least 3 months when stored at 4°C. Milk samples spiked with BPA were prepared by evaporating 10 μL of each working standard and adding 100 μL of milk to result in the concentration range 0.2–5.0 ng mL^{-1}.

Preparation of BPA-free water. BPA-free water was prepared by passing the deionized water through an ENVI-18 disk (diameter 47 mm, Supelco, Bellefonte, PA, USA) five times. The disk was preconditioned by washing with 500 mL of methanol to remove traces of BPA on disk resins prior

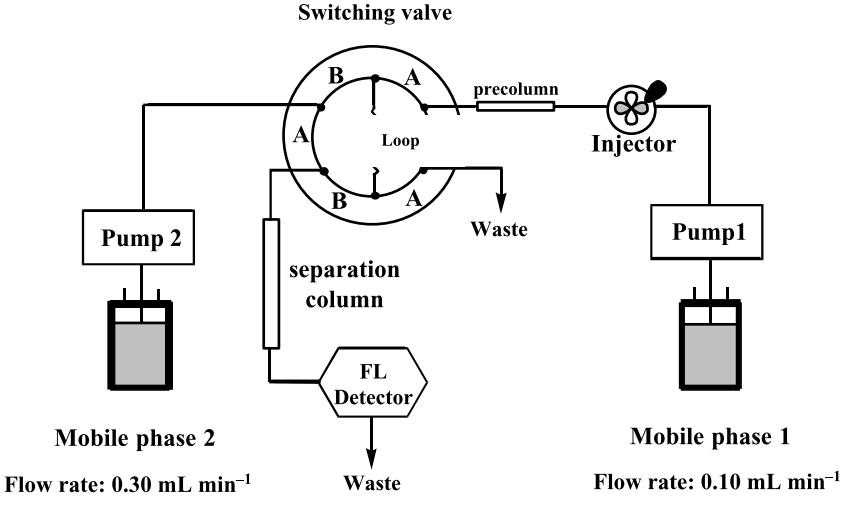

Figure 1. Schematic diagram of the HPLC-column switching system. Position of A and B in column-switch valve shows elute directions of HPLC system before and after column switching, respectively.

to use. This operation was performed on a clean bench in order to prevent environmental contamination.

Breast milk samples. Breast milk samples were collected from twenty-three healthy lactating women including primiparas and multiparas. Glass tubes were used throughout the sampling and storage to avoid contamination of BPA. The samples were kept frozen at –20°C until analysis. All donors were given informed consents for the monitoring of BPA levels.

Solid-phase extraction (SPE) for milk fractionation. ODS cartridges (Sep-pak plus C_{18}, 360 mg/3 mL, Waters, Milford, MA, USA) were preconditioned with 50 mL of methanol, followed by 2 mL of BPA-free water. Then 100 μL of milk sample were loaded onto the cartridge, and washed with 3 mL of BPA-free water. Finally, cartridges were eluted with a step gradient of acetonitrile aqueous solutions for fractionation. Each fraction was then evaporated to dryness under nitrogen gas stream.

Two-step liquid–liquid extraction (LLE) for milk sample clean-up. Milk samples were thawed and homogenized by sonication for 60 min. A 100 μL portion of the homogenate was transferred into a glass test tube, and to this were added 200 μL of BPA free water and mixed well. After adding 1.0 mL of hexane, the mixture was vortex-mixed for 1 min and then centrifuged at 1300***g*** for 10 min. The aqueous layer (280 μL) was collected (care should be taken to avoid transferring the hexane layer) and 100 μL of 0.2 M hydrochloric acid was added for acidification. Thereafter, 1.0 mL of chloroform was added and vortex-mixed for 1 min. After centrifugation at 1300***g*** for 10 min, 900 μL of the organic layer were collected and evaporated to dryness under nitrogen gas stream.

Derivatization reaction. To the residue of the eluates and the residue of the extract, 100 μL of 5 mM DIB-Cl suspension in acetonitrile and 5 μL of 1.5 M TEA were added, and reacted for 20 min at 35°C in a hot dry bath (E5C3, Omron, Tokyo, Japan). The reaction was stopped by adding 10 μL of 12.5% ammonia solution. After 10 min, 10 μL of 5% acetic acid were added for neutralization. The resultant reaction mixture was passed through a filter (0.45 μm, HLC-DISK 3, Kanto Chemical Co. Inc., Tokyo) and a 5 μL portion of it was injected onto the HPLC system.

Assay validation. Standard calibration curves were constructed by spiking breast milk with a known amount of BPA in the concentration range 0.2–5.0 ng mL^{-1}. The milk standards were also used to determine the intra- and inter-day precision and accuracy ($n = 5$) of the method.

Triglyceride assay. In order to compare the BPA concentration in breast milk to lipid level, triglyceride was assayed by an enzymatic method using a commercially available kit (Triglyceride E-test, Wako, Tokyo, Japan). The procedure was following the instruction of the kit. Milk samples were diluted 10-fold with water. A working curve of triglyceride was prepared, from which the triglyceride concentration of milk sample was calculated.

RESULTS AND DISCUSSION

Chromatographic conditions

In our previous study, a highly sensitive HPLC-fluorescence detection for the determination of BPA in several biological fluids (maternal serum, umbilical cord serum, ascetic fluid, etc.) was developed (Kuroda *et al.*, 2003). The excess labeling reagent was removed using a column-switching technique. In this study, the effect of the column-switching time on the collection of DIB-BPA fraction was studied over 9.0–12.0 min, using a BPA spiked milk sample in the HPLC system. Maximum and constant peak height of DIB-BPA was observed in the range 10.5–11.0 min; 10.5 min was chosen as optimum since complete separation of DIB-BPA from endogenous materials could be achieved.

Breast milk sample clean-up

LLE using chloroform was first applied to milk sample pretreatment, followed by derivatization with DIB-Cl as described above. However, in the chromatogram, no peak corresponding to DIB-BPA was observed for breast milk spiked with BPA (5.0 ng mL^{-1}). The same result was obtained when BPA was added to the residue of chloroform extracts evaporated. These results suggested that the derivatization reaction between BPA and DIB-Cl could not be completed because of the existence of interferences in the extracts of milk sample.

To clarify this problem, fractionation of milk sample by SPE was performed as follows: after loading the blank milk sample onto the cartridge, it was washed as described above, then eluted with acetonitrile–H_2O (1:1, v/v) to collect 1.0 mL each for fractions 1–4, and finally eluted with 3.0 mL of acetonitrile for fraction 5. Fractions 1–5 were then evaporated, spiked with 5.0 ng mL^{-1} of BPA standard and derivatized. The reactivity of BPA with DIB-Cl was calculated as the ratio of peak height of DIB-BPA in the presence of milk contents to that of standard. As shown in Fig. 2, reactivities of BPA in fractions 1–4 were higher than 80%, while no reaction occurred in fraction 5. Thus, it could be considered that a one-step LLE is not adequate for milk sample clean-up, as lipophilic interferences extracted into the chloroform layer interrupt the derivatization of BPA with DIB-Cl. An SPE clean-up for milk samples was then examined. The fractionation experiment was repeated again using milk sample spiked with 5.0 ng mL^{-1} of BPA. As shown in Fig. 2, recoveries of BPA in fractions 1–5 were 2.0, 68.0, 11.0, 0.3 and 0.0%. Almost all of the BPA was eluted in fraction 2. Considering that evaporation of the aqueous solution would be substantially time-consuming, a two-step LLE was tried to remove the interference materials with

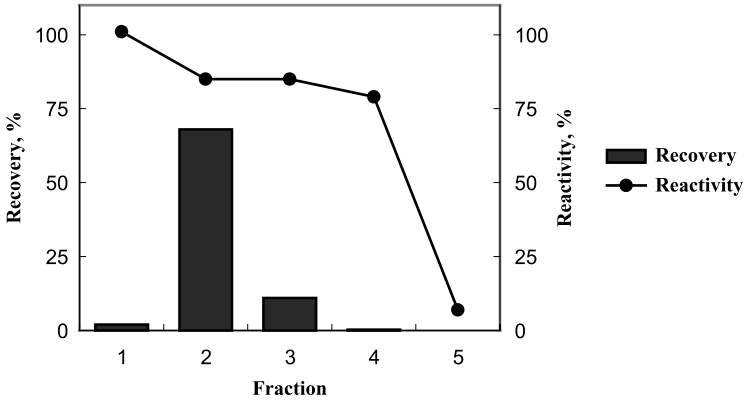

Figure 2. Recovery and fluorescence derivatization reactivity of fractionated milk sample spiked with BPA (5.0 ng mL^{-1}). Data are expressed as mean values of duplications.

Table 1. Intra- and inter-day precisions and accuracy of BPA in breast milk

	Intra-day assay (n = 5)		Inter-day assay (n = 5)	
Concentration (ng mL^{-1})	Precision (RSD, %)	Accuracy (%)	Precision (RSD, %)	Accuracy (%)
0.5	8.7	96.4	10.4	102.0
1.0	6.3	100.2	6.2	105.2
3.0	0.9	100.2	4.7	98.9

n-hexane followed by the extraction of BPA with chloroform from the breast milk sample.

The optimal condition for two-step LLE was tested by adjusting the pH value of milk sample in a range of 4–13 [acetate buffer (pH 4–6), BPA-free water (pH 7), borate buffer (pH 9–13)] before extracting with *n*-hexane. The highest peak height of DIB-BPA was obtained at pH 7, therefore, BPA-free water was used for the following experiment. Under this condition, only less than 3% of BPA partitioned to the hexane layer.

Method validation

A calibration curve of BPA in breast milk sample was linear over the concentration range 0.2–5.0 ng mL^{-1} with a correlation coefficient of 0.994. A limit of detection (LOD) of 0.11 ng mL^{-1} was obtained at a signal-to-noise ratio of 3.

The extraction recovery of BPA from milk was determined by comparing the peak height of BPA in milk after two-step liquid–liquid extraction to that of the corresponding BPA standard. Recovery of BPA from milk was found to be approximately 70%.

The precision and accuracy of the method were determined by intra- and inter-day assay variance. These results are shown in Table 1. RSD (%) were less than 8.7 and 10.4% for intra-day and inter-day assays, respectively; accuracy was in the range 96.4–100.2 and 98.9–105.2%, respectively. Chromatograms obtained with blank milk and milk spiked with BPA are shown in Fig. 3.

Determination of BPA in human breast milk samples

The proposed method was applied to the determination of BPA in human breast milk samples from 23 healthy lactating women. As shown in Table 2, the levels of BPA were in the range 0.28–0.97 ng mL^{-1} with the mean concentration 0.61 ± 0.20 ng mL^{-1}. Animal experiments have shown that BPA is eliminated from the body due to the effective metabolism pathway resulting in the formation of a biologically inactive metabolite, BPA-monoglucuronide, and thus seems not to accumulate extensively inside animal bodies (Snyder *et al.*, 2000; Pottenger *et al.*, 2000; Yoo *et al.*, 2000). This supports our results that trace amounts of BPA were detected in breast milk samples.

The triglyceride contents as well as pH value of each milk sample were also assayed in this study. Triglycerides constitute 98% of human milk lipids and are thus an appropriate measure of fat content. Triglyceride contents measured ranged from 0.74 to 48.98

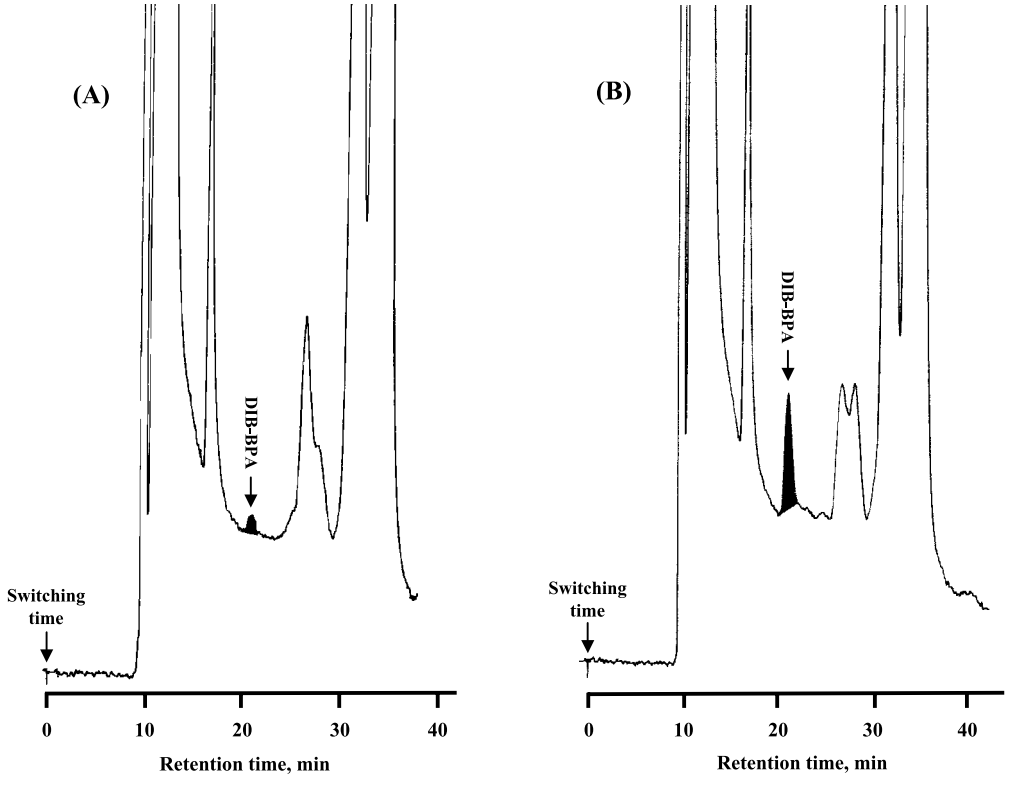

Figure 3. Typical chromatograms corresponding to (A) breast milk, (B) breast milk spiked with 2.0 ng mL^{-1} of BPA.

Table 2. BPA concentrations and the corresponding triglyceride content in human breast milk

Sample no.	BPA concentration (ng mL^{-1})	pH value	Triglyceride content (%)
01	0.69	6.6	1.70
02	0.58	6.6	2.42
03	0.69	6.4	4.50
04	0.46	—[a]	—[a]
05	0.86	6.4	2.94
06	0.58	6.4	2.32
07	0.58	6.6	2.16
08	0.69	6.4	2.92
09	0.47	6.6	1.48
10	0.28	6.8	1.30
11	0.63	6.6	0.74
12	0.76	6.6	15.61
13	0.44	6.8	4.82
14	0.31	6.8	12.76
15	0.97	6.6	48.98
16	0.97	6.4	25.89
17	0.58	6.8	19.28
18	0.75	6.6	15.21
19	0.80	6.6	8.29
20	0.74	6.6	20.09
21	0.41	6.8	36.18
22	0.44	6.8	43.94
23	0.28	6.8	41.22
Mean ± SD	0.61 ± 0.20	6.62 ± 0.15	14.31 ± 15.54
Median	0.61	6.60	6.56
Range	0.28–0.97	6.40–6.80	0.74–48.98

[a] Triglyceride content and pH value were not measured because of insufficient milk sample.

with a mean value of 14.31 ± 15.54%, and pH values ranged from 6.20 to 6.80 with a mean value of 6.62 ± 0.15 (Table 2). No correlation ($r = 0.02$) could be observed between BPA concentrations and triglyceride levels in milk samples. However, as several factors such as milk sampling before or after a meal, etc., were unclear in this study, further investigations are needed to clarify the influences of these factors on the relationship between BPA concentrations and lipid content of milk.

BPA concentrations in six milk specimens were further compared with those in corresponding maternal and umbilical cord blood sera from each individual volunteer, as reported in our previous study (Kuroda *et al.*, 2003). As a result, BPA levels in breast milk samples were relatively higher than those in maternal sera [0.46 ± 0.20 ng mL^{-1} ($n = 9$)] with the milk/serum ratio (M/S ratio) being 1.3, and were as same as those in umbilical cord sera [0.62 ± 0.13 ng mL^{-1} ($n = 9$)]. Yoo *et al.* (2001) reported consistent results that concentrations of BPA in milk exceeded those in serum, with the M/S ratio being 2.4–2.7 after intravenous administration of BPA to Sprague–Dawley rats. The M/S ratio obtained in this study was slightly lower, which might be due to the oral and daily exposure to BPA at low concentration levels, while in the experiment of Yoo *et al.*, BPA was administered by bolus injection to rats, which resulted in a large amount of unbound BPA existing in plasma.

Until now, no evidence has shown that environmental exposure to BPA has adverse effects on human health. Hence, further studies such as long-term follow-up studies on human exposure to BPA are needed to assess the adverse effects of it in early life.

CONCLUSION

A highly sensitive column-switching HPLC method with fluorescence detection was developed for the determination of BPA in human breast milk samples. Sample clean-up was carried out using a two-step liquid–liquid extraction. A limit of detection as low as 0.11 ng mL^{-1} of BPA in milk sample could be obtained at a signal-to-noise ratio of 3. By the proposed method, BPA in milk samples were determined to be in the range 0.28–0.97 ng mL^{-1}. No correlation was observed between BPA concentrations in milk sample versus triglyceride levels of milk samples collected under the described conditions.

Acknowledgments

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