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Detection of Bisphenol-A in Dental Materials by Gas Chromatography-Mass Spectrometry

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The xenoestrogenic substance bisphenol-A is widely used as a synthetic precursor of resin monomers, such as bisphenol-A diglycidyl methacrylate. Reports describing the release of bisphenol-A from polymerized resin into saliva have aroused considerable concern regarding exposure to xenoestrogen by dental treatment. The purpose of the present study was to demonstrate a reliable methodology of detecting the trace amounts of bisphenol-A in dental materials. Bisphenol-A was separable from bisphenol-A diglycidyl methacrylate, which is often employed as the principal dimethacrylate monomer, by selective extraction with a Sep-Pak C18 cartridge. Using this extraction method in combination with a gas-chromatography mass-spectrometry, we have obtained evidence that all unpolymerized materials used in this study were contaminated with bisphenol-A. Quantitative analysis using a deuterium-labeled compound as an internal standard revealed bisphenol-A contents in commercial dental materials ranging from<1 μg/g material to about 20μg/g material. The polymerized dental materials released up to 91.4 ng bisphenol-A /g material into phosphate buffered saline during 24-h incubation. These results indicate that bisphenol-A can be released from dental materials, however the leachable amount would be less than 1/1000 of the reported dose (2μg/kg body weight/day) required for xenoestrogenisity *in vivo*.

Key words : bisphenol-A, xenoestrogen, gas chromatography-mass spectrometry

INTRODUCTION

Recent reports describing the presence of bisphenol-A (BPA, Fig. 1) in dental materials[1)] have aroused considerable concern regarding exposure to xenoestrogen by dental treatement[2)]. Feldman *et al.*[3)] first reported that BPA acts as an estrogenic substance and can be released from polycarbonate flasks during autoclaving. As BPA is the starting material for manufacture of bisphenol-A diglycidyl methacrylate (bis-GMA, Fig. 1), which is often employed as the principal dimethacrylate monomer in dental materials, Olea *et al.*[1)] attempted to determine the xenoestrogen in the resin composites and fissure sealants frequently used in restorative and/or pediatric dentistry. They showed in vitro xenoestrogenisity of the bis-GMA based sealant and identified BPA by high performance liquid chromatography (HPLC) and gas chromatography-mass spectrometry (GC-MS). Furthermore, they demonstrated that BPA up to 931

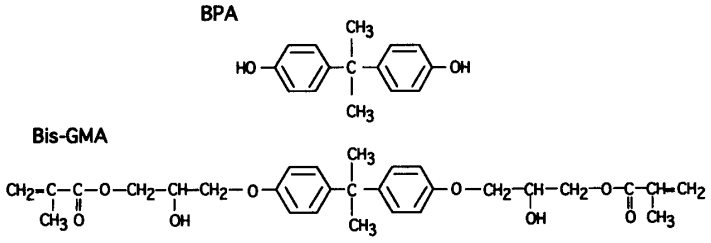

Fig. 1 Chemical structures of bisphenol-A (BPA) and bisphenol-A diglycidyl methacrylate (bis-GMA).

μg was released in saliva during the first hour after application of 50 mg the sealant. In contrast, other investigators argued that none of the tested sealants were shown to release detectable amounts of BPA[4,5]. These demonstrations were conducted by similar strategies for identification and quantification of BPA, suggesting that the unreliable methodologies used to detect the trace amounts of the xenoestrogenic compound in dental materials caused diverse results.

Since Bowen *et al.*[6] introduced a resin composite composed of bis-GMA, triethyleneglycol dimethacrylate (TEGDMA), 2-hydroxyethyl methacrylate (2-HEMA) and silicon dioxide as an inorganic filler, the material has been utilized extensively and became indispensable in most fields of dentistry. Furthermore, fissure sealants which frequently contain bis-GMA as the principal dimethacrylate monomer are increasingly used in preventive dentistry. Although these bis-GMA based materials are widely used in daily treatments, the xenoestrogenisity of dental materials is still obscure due to the conflicting observations described above. The purpose of the present study was to establish a reliable procedure for detecting BPA and to verify the content of xenoestrogen in commercial dental materials. The present study shows that leachable BPA from light-cured dental materials can be detected by GC-MS. The amounts, however, were far less than those observed by Olea *at al.*[1].

MATERIALS AND METHODS

Materials

Dentin bonding agent Clearfil Photo Bond (lot #287) and the fissure sealants Teeth Mate A (lot #0O06) and Teeth Mate F-1 (lot #0C09) were obtained from Kuraray Co., Ltd. (Okayama, Japan). Another fissure sealant Concise (lot #19980317) was purchased from 3M (St. Paul, MN). The composite resins Palfique Estelite U (A3) (lot #162), Silux Plus (U) (lot #7EA), and Clearfil Photo SC (lot #0O40) were obtained from Tokuyama Corp. (Yamaguchi, Japan), 3M, and Kuraray, respectively. Authentic Bis-GMA (lot #1029H) and TEGDMA (lot #0223U) were obtained from Shin-Nakamura Chemical Co., Ltd. (Wakayama, Japan). Authentic BPA ($C_{15}H_{16}O_2$=228.29) and 2-HEMA were purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan) and Merck (Schuchardt, Germany), respectively. Deuterium

labeled BPA (D-BPA, $C_{15}D_{16}O_2$=244.38) was obtained from Aldrich Chemical Co. Inc. (Milwaukee, WI). Sep-Pak Plus cartridges (short body) were obtained from Waters Corp. (Milford, MA). *N,O*-bis (trimethylsilyl) trifluoroacetamide (BSTFA) with 1 % trimethylchlorosilane (TMCS) was purchased from Pierce (Rockford, IL).

Polymerization of Dental Materials

Commercial unpolymerized materials were irradiated in a cylindrical glass tube (4 mm in diameter × 5 mm in hight) with a visible light source for 60 sec. The procedure has been reported to achieve an adequate degree of polymerization of bis-GMA based materials[7]. The polymerized samples were carefully removed from glass tube and incubated in 10 ml of phosphate buffered saline (PBS) for 24 h at 37℃.

Thin Layer Chromatography Analysis

A mixture of equal amounts of BPA and bis-GMA (1 mg) prepared with 40% methanol was introduced into the Sep-Pak C18 cartridge preconditioned with 100% methanol (10 ml) and water (10 ml). The cartridge was sequentially passed with 3 ml each of 40, 50, 60, 70, and 80% methanol. The eluate was evaporated to dryness under a nitrogen stream and the residue was dissolved in 100μ l of 100% methanol. A portion of the extract was spotted onto a TLC plate (silica gel 60 F254) and developed with ethyl acetate:hexane (1:2).

Extraction of Bisphenol A from Dental Materials

For detection of BPA in unpolymerized dental materials, a stock solution at a concentration of 10 mg/ml was prepared in a light protected glass tube with 100% methanol. After centrifugation (3000 rpm, 10 min) to remove insoluble filler, a portion (100μl) was transferred to 10 ml of 40% methanol and applied to a Sep-Pak C18 cartridge that had been preconditioned with 100% methanol (10 ml) followed by distilled water (10 ml). When polymerized materials were tested, PBS extracts were directly applied onto the preconditioned cartridge. To quantify the BPA content, 50 or 200 ng of D-BPA as an internal standard was added to the solution of dental materials before extraction. The cartridge was washed with 10 ml of 40% methanol and eluted with 5 ml of 70% methanol. The eluate was evaporated to dryness under a nitrogen stream at 40℃ and dissolved in 100μl of a sylilation reagent consisting of BSTFA containing 1 % TMCS. The reaction was carried out at 60℃ for 1 h. After derivatization, the excess reagent was evaporated under a nitrogen stream and the residue was dissolved in 50μl of ethyl acetate for GC-MS analysis.

Gas Chromatography-Mass Spectrometry Analysis

A portion (1μl) of silylated extracts of dental materials was introduced into a GC-MS system (Hewlett Packard 5890A-5970B, Palo Alto, CA), equipped with an apolar GC capillary column HP-1 (30 m × 0.25 mm ϕ, 0.25 μm film thickness, Hewlett Packard) and a splitless injector. The temperature programming was as follows; 100 ℃ (5 min), 100-300℃ (20℃/min), 300℃ (5 min). The temperature settings for the

injector and a transfer line were 200℃ and 300℃, respectively. Electron-impact mass spectra were obtained at 70 eV and are reported as m/z. Data was collected with either a scan mode or a selective ion monitoring (SIM) mode to identify or to quantify the compound, respectively. Ions at m/z 357 and 368 were selected for SIM analysis of BPA and its deuterium derivative, respectively.

RESULTS

Selective Extraction of BPA

A mixture consisting of equal amounts of bis-GMA and BPA was introduced into a Sep-Pak C18 cartridge and eluted with increasing concentrations of methanol. Components in the eluate were monitored by silica gel based TLC. Figure 2 illustrating a typical result of the solid phase extraction indicated that the cartridge retained all the applied components since no apparent band was observed in the wash-through (lane 1, 40% methanol) and 50%-methanol (lane 2) fractions. BPA that moved faster than bis-GMA on the TLC appeared in the 60%- and 70%-methanol fractions, though no apparent BPA band was observed in the 80%-methanol fraction (lane 5). In con-

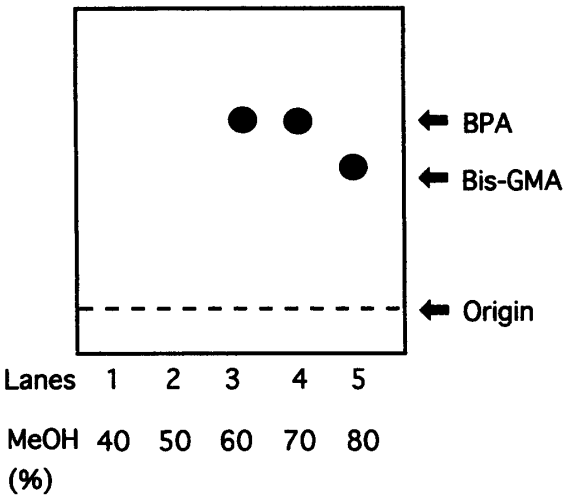

Fig. 2 Typical profile of thin layer chromatography (TLC) of BPA and bis-GMA.
A mixture of the equal amounts of BPA and bis-GMA was prepared with 40% methanol and introduced into the Sep-Pak C18 cartridge preconditioned with 100% methanol and water. The cartridge was sequentially passed with 3 ml each of 40~80% methanol as illustrated. A portion of the eluate was spotted onto the TLC plate (silica gel 60 F254) and analyzed under UV radiation.

trast, bis-GMA eluted with 80% methanol and no apparent bis-GMA band was observed in the 60% -and 70%-methanol fractions (lanes 3 and 4). These results indicate that BPA can be separable from the major component bis-GMA using the Sep-Pak cartridge. However, 2-HEMA and TEGDMA, which are the common monomers added to bis-GMA based dental resins, were eluted in the fractions where BPA appeared (data not shown).

Detection of BPA in bis-GMA

Since the ether bond of bis-GMA appears to be stable to chemical[8] and biochemical[9] degradation, it is possible for dental materials to be contaminated with BPA that is originally present in the monomer bis-GMA as an impurity. Thus, we tried to detect BPA in the commercially available bis-GMA. A portion of bis-GMA (1 mg) was ap-

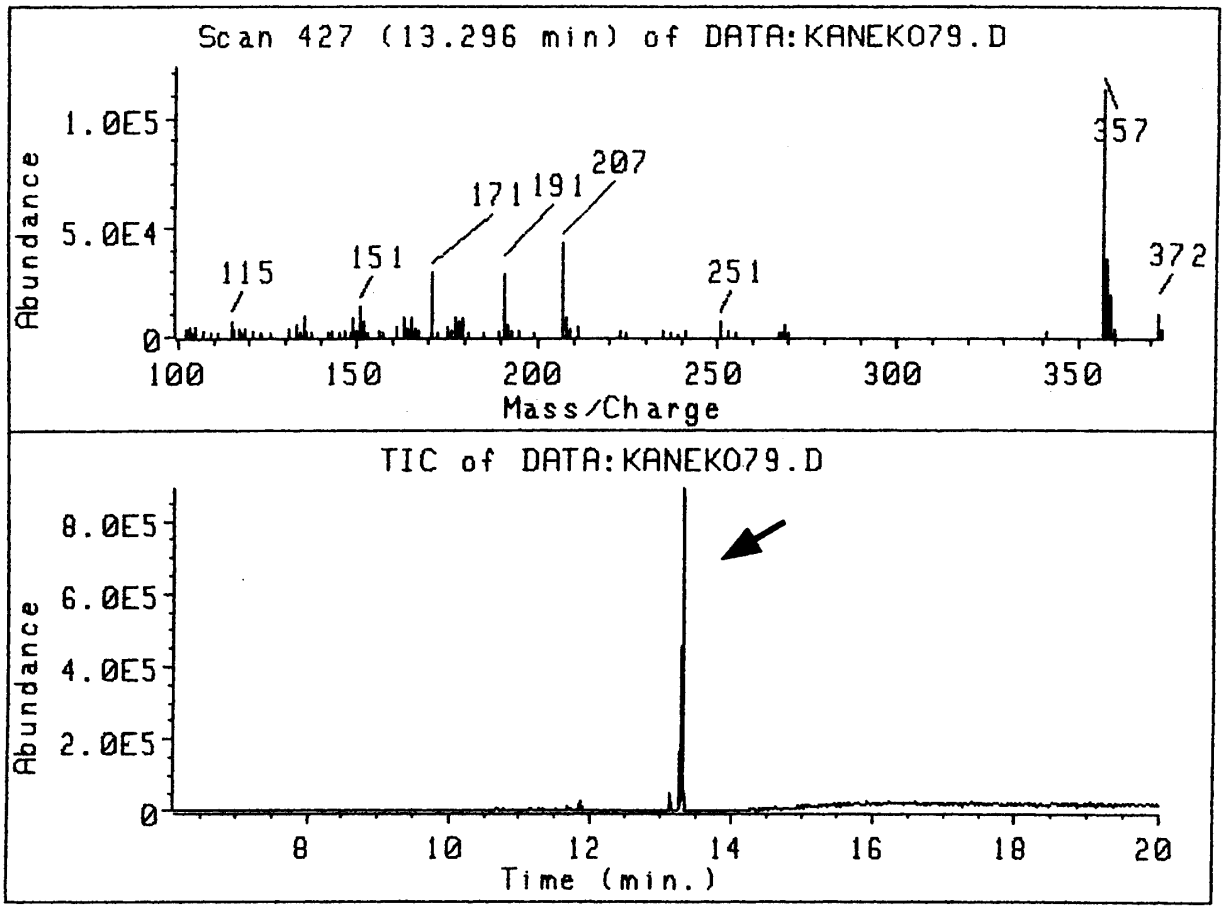

Fig. 3 Gas chromatography-mass spectrometry (GC-MS) profile of the trimethylsilyl derivative of BPA.
The authentic BPA reacted with *N,O*-bis(trimethylsilyl)trifluoroacetimide and 1% trimethylchlorosilane was introduced to GC-MS equipped with an HP-1 column (30 m × 0.25 mm, 0.25 μm film thickness). The oven temparature was programmed as follows; 100℃ (5 min), 100-300℃ (20℃/min), 300℃ (5 min). The total ion chromatogram and the electron impact mass spectrum of a peak labeled by an arrow (retention time at 13.30 min) are shown on the lower and the upper panel, respectively.

plied to the Sep-Pak cartridge and the 40 to 70% methanol fraction was obtained. Extracts were further derivatized by silanized reagents and subjected to GC-MS analysis.

The TMS derivative of authentic BPA showed a single peak at 13.3 min on an apolar column (Fig. 3). The electron impact (EI) mass spectra of TMS-BPA indicated ions at m/z 372 as a molecular ion (M^+), m/z 357 as a base peak and other relative minor ions, such as m/z 171, m/z 191, and m/z 207. The TMS derivative of bis-GMA extracts showed a peak at 13.3 min on the total ion chromatogram, suggesting that EI mass spectra appeared to be identical to TMS-BPA in terms of the ion fragmentation and relative abundance (Fig. 4). These results indicate that bis-GMA actually contains a certain amount of BPA. The mass chromatography using the base peak ion revealed a single peak at 13.3 min indicating that BPA was only the component showing a fragment ion of m/z 357 in the bis-GMA extracts.

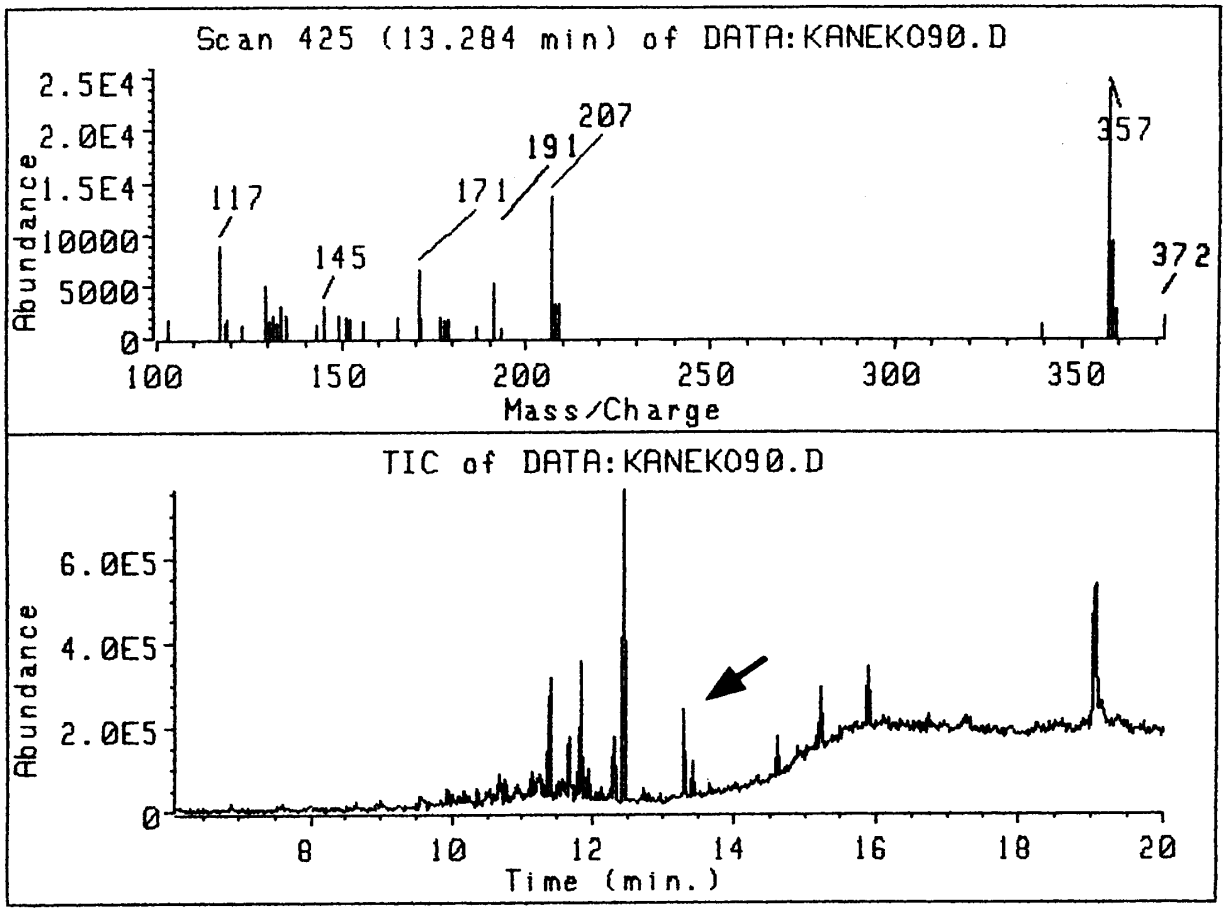

Fig. 4 Gas chromatography-mass spectrometry (GC-MS) profile of the trimethylsilyl derivative of bis-GMA extracts.
One mg of Bis-GMA was introduced into the Sep-Pak C18 cartridge and the 40-70% methanol fraction was obtained. The fraction was further reacted with *N,O*-bis (trimethylsilyl) trifluoroacetimide and 1% trimethylchlorosilane and analyzed by GC-MS. The upper panel shows the mass spectrum of a peak indicated by an arrow on the total ion chromatogram illustrated on the lower panel.

Detection of BPA in Dental Materials

We next attempted to determine whether BPA could be detected in unpolymerized dental materials. A typical result from GC-MS analysis of dental materials is shown in Fig. 5. No obvious peak was observed on the total ion chromatogram when a silanized sample of a fissure sealant was introduced to GC-MS (Fig. 5, lower panel). However, a peak having a retention time of 13.3 min, exactly same as that of authentic BPA standard, was observed on the mass chromatogram at m/z 357 (Fig. 5, upper panel), indicating that trace amounts of BPA are present in commercial dental materials. We also determined peaks of TEGDMA and 2-HEMA, components that may interfere with BPA detection by HPLC, on the total ion chromatogram (Fig. 5, lower panel). These peaks were well separated from BPA; thus, the present procedure using a capillary GC column is suitable for microanalysis of the xenoestrogen in dental materials.

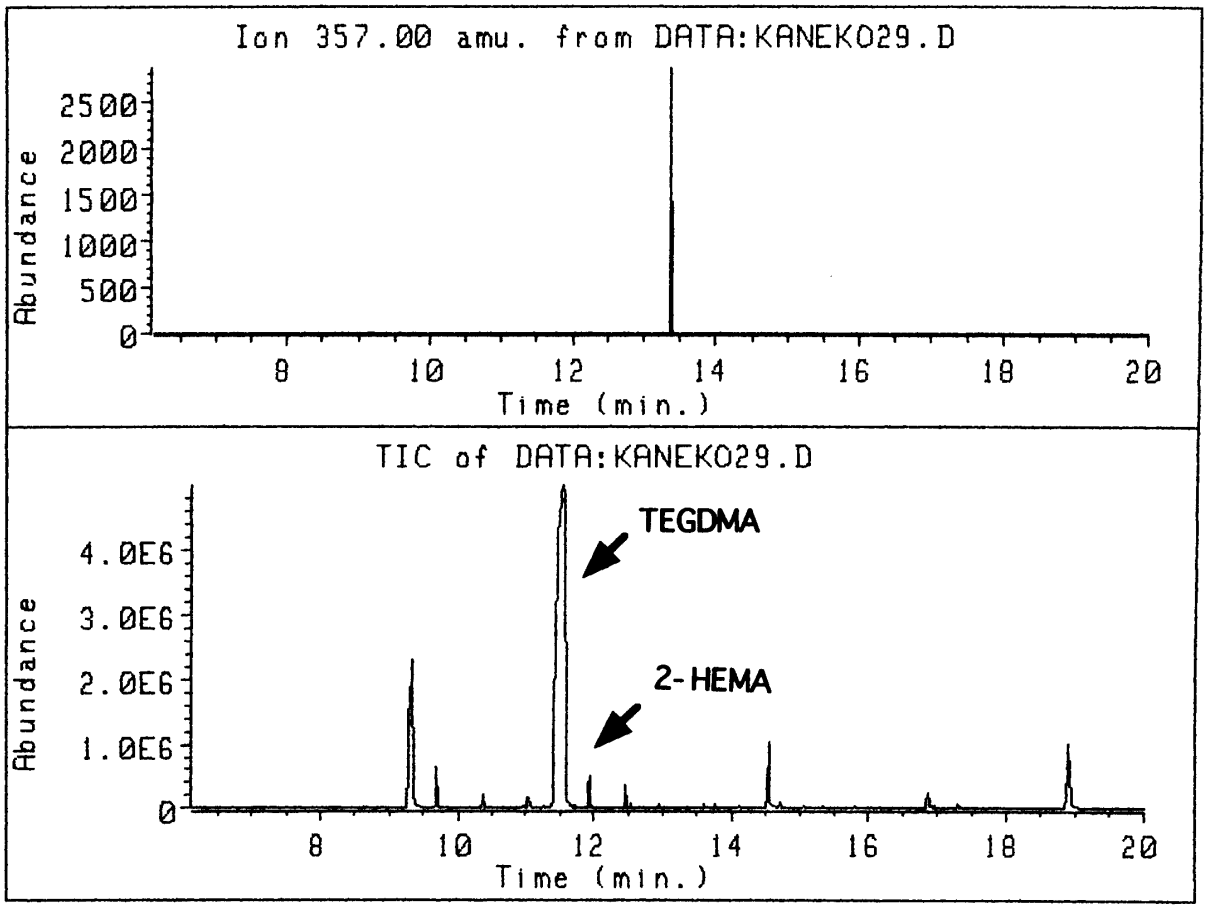

Fig. 5 Gas chromatography-mass spectrometry (GC-MS) profile of the trimethylsilyl derivative of a fissure sealant extracts.
One mg of Teeth Mate A was subjected to extraction with Sep-Pak C18 as described in the legend to Fig.4 and in the Materials and Methods. The TMS-derivative of the extracts was analyzed by GC-MS. The upper panel shows a mass chromatogram at m/z 357, which is a major fragment of TMS-BPA as shown in Fig. 4. The lower panel shows the total ion chromatogram.

No obvious evidence showing the presence of BPA in bonding agents or composite resins was detected by GC-MS analysis at the scan mode. However, selective ion monitoring (SIM) analysis using a BPA-derived specific ion, m/z 357, revealed a peak showing the same retention time as that of authentic TMS-BPA not only in the sealant but also in the other materials, such as composite resins and a bonding agent used in this study (not shown).

Quantitative Analysis of BPA in Dental Materials

To analyze the concentration of BPA in dental materials, we utilized D-BPA as an internal standard. The TMS derivative of D-BPA that eluted from the GC capillary column faster than the TMS-BPA, resulting in good separation of these compounds on the chromatogram. The ratio of the base peak to the molecular ion was similar when TMS-BPA and its deuterium labeled compound were compared. Thus, we used ions of m/z 357 and 368, the base peaks of TMS-BPA and TMS-D-BPA, respectively, in the following SIM analysis. A certain amount of authentic BPA and the fixed amount of the internal standard were dissolved in 40% methanol and subjected to the Sep-Pak extraction. Although TMS-derivatives of the standard revealed fluctuations in the absolute intensities of the signal, the ratio of BPA to D-BPA was apparently stable (variations were within 10% of mean values). In addition, the absolute intensity of the D-BPA derived peak in samples from which dental materials were extracted was higher than that in the standard alone. These results indicate an adsorptive property of BPA during the extraction procedure. On the other hand, D-BPA was shown to contain approximately 2.3% of BPA as an impurity, which was calculated from an absolute calibration curve of the authentic standard. Therefore, correction of the standard curve was carried out accordingly. The presence of BPA in the internal standard raised the question of reliable quantification. Therefore, a confidential limit of BPA in the present system was set tobe 1 ng/mg material; more than the amount derived from the internal standard, since 50 ng of D-PBA may contain 1.2 ng BPA.

We analyzed the BPA content in 3 brands of the fissure sealant, 3 composite resins, and one dentin bonding agent. Unpolymerized dental materials showed a variety of BPA contents ranging from less than 1 ng/mg material to about 20 ng/mg material (Table 1). There was no correlation between the type of material and BPA content. In addition, different brands of fissure sealant from the same company showed obviously different BPA contents; specifically, the older brand, Teeth Mate A, was shown to contain about 20 ng/mg material, and less than 1 ng/mg material was observed in the newer brand, Teeth Mate F-1

Next we attempted to determine the BPA amount leached from polymerized dental materials. A resin composite and fissure sealants that showed relatively high concentrations of BPA were polymerized in glass tubes by visible light radiation for 1 min. Substances leaching out of these polymerized materials into PBS were subjected to extraction followed by GC-MS analysis (Table 2). Up to 91 ng BPA/g material was released *in vitro* during 24 h incubation. Further elution of BPA was observed when the polymerized resin composite was immersed in fresh PBS and incubated for

Table 1 Bisphenol-A (BPA) contents in unpolymerized dental materials detected by gas chromatography-mass spectrometry

Dental materials	BPA content*(ng/mg materials)
Dentin bonding agent	
Clearfil Photo Bond	18.5
Fissure sealants	
Concise	15.4
Teeth Mate A	20.2
Teeth Mate F-1	<1**
Resin composites	
Palfique Estelite	<1
Silux Plus	6.4
Clearfil Photo SC	<1

The unpolymerized dental material (1 mg) dissolved in 40% methanol was introduced into a Se-Pak C18 cartridge together with 200 ng of deutelium labeled BPA as an internal standard. The 40-70% methanol fraction was further reacted with *N,O*-bis(trimethylsilyl)trifluoroacetimide and 1 % trimethylchlorosilane and analyzed by GC-MS. *Data are the means of 2 or 3 indepentent experiments consisting of duplicate analysis. **A confidential limit of the BPA analysis was set to be 1 ng/mg material as described in the text.

Table 2 The leached bisphenol-A (BPA) amount out of polymerized dental materials *in vitro*.

Dental materials	BPA content*(ng/g materials)
Fissure sealants	
Concise	19.8
Teeth Mate A	55.5
Resin composite	
Silux Plus	91.4

Three pieces of the polymerized dental material (approximately 300 mg) were incubated in 10 ml phosphate buffered saline (PBS) for 24 h at 37℃. The incubation medium was introduced into a Se-Pak C18 cartridge together with 50 ng of the internal standard. The 40-70% methanol fraction was analyzed by gas chromatography-mass spectrometry after derivatization. *Data are the means of 2 or 3 independent experiments consisting of duplicate analysis and expressed as ng/g polymerized dental material.

another 24 h; however, the leachable amount decreased by 65% (not shown).

DISCUSSION

Conflicting reports by different laboratories may be the result of investigations with unreliable methodologies for BPA analysis in dental materials. In the previous paper[1],

BPA amount leaching from polymerized sealant into saliva was calculated based on the data from an HPLC system equipped with a UV detector. However, queries regarding selectivity arose from their studies in which BPA was observed adjacent to the solvent peak on the HPLC chromatogram[1]. Hence, concerning the selectivity and the sensitivity for BPA, we have demonstrated GC-MS analysis for the qualification and quantitation of xenoestrogen in the present study. The clean-up step is necessary for the microanalysis of BPA by GC-MS because the mass of BPA-related compounds, such as bis-GMA, present in dental materials may mask the trace amounts of BPA during GC separation. In addition, these compounds may cause contamination of the column and ion source of mass spectrometry, resulting in the appearance of ghost peaks and decreases in the sensitivity and the reproducibility. The present study demonstrated that BPA is separable from bis-GMA by solid phase extraction using a conventional Sep-Pak C18 cartridge. As bis-GMA is a major constituent in most dental materials, this extraction method was expected to be adequate for the microanalysis by GC-MS. However, it remains to be proved that TEGDMA co-extracted with BPA, as the present conditions prevented further sensitive analysis.

The absolute signal intensity obtained from the GC-MS analysis varied with the samples. In addition, the signals of the authentic standard were generally weaker than those of samples, indicating that components in dental materials behaved as a carrier and that BPA showed adsorptive property during the sample manipulation. Therefore, care must be taken in the quantitation of this compound. In the previous reports[1], although samples that might contain BPA were directly introduced to HPLC without extraction, the BPA amount was estimated based on the standard curve using absolute signal intensity. On the other hand, the extraction efficiency of the sample may differ from that of the standard. Thus, an internal standard method using a deuterium-labeled compound that should have similar extraction efficiency to BPA is obligatory to accurately quantitate the xenoestrogen amount in dental materials.

The present study demonstrated the presence of BPA in commercially aviable bis-GMA based on evidence that the retention time and mass spectrum were identical to those of the authentic standard. Furthermore, all the dental materials tested showed a peak corresponding with BPA on the ion chromatogram at m/z 357, although we failed to obtain clear mass spectra indicating a molecular ion. The results thus indicate that dental materials are contaminated with trace amounts of BPA. The internal standard method revealed varying amounts of BPA in unpolymerized materials, ranging from less than 1 ng/mg material to about 20 ng/mg material. It has been reported that a high percentage (25-50%) of methacrylate groups remains unreacted after polymerization[10]. Approximately one-tenth of these unreacted methacrylate groups is reportedly present as residual monomer[10]. Taken together, these previous observations in conjunction with the present results suggest that the xenoestrogen leaked into PBS originated from the residual monomer, which was contaminated with BPA present in the polymerized materials.

Repeated doses of 2μg/kg/d (2 ppb) of BPA to pregnant mice were shown to produce increases in the size of prostate and preputial glands and decreased the size of

epididymides in their male offspring[11,12]. Concern arose that such a BPA dosage was lower than the estimated amount of leakeable BPA based on the data demonstrated by Olea *et al.*,[1] who reported that as much as 931 μg of BPA was swallowed during the first hour after application of 50 mg of sealant, representing 13 ppb in a 70 kg adult. However, the present study demonstrated that up to 91 ng BPA migrated out of 1 g of dental material during 24 h-incubation *in vitro*. Such an amount of BPA (1.3 ppt in a 70 kg adult) was not only far beyond the reported one[1] but also 1500-times lower than the level showing xenoestrogenisity appeared *in vivo*[12] even if BPA eluted equally every 24 h.

On the other hand, biotransformation and/or degradation of bis-GMA to produce BPA *in vivo* also has raised concern[12]. The ester bond of dimethacrylate esters is a primary target of esterases[13]. However, the ether bond of bis-GMA appears to be stable in vivo since primary studies of the metabolism of bisphenol-A diglycidyl ether using its radiolabelled compound indicated no evidence of formation of BPA from the ether derivative[14,15]. Hence, it is strongly suggested that bis-GMA *in vivo* predominantly converts to bis-diol of bisphenol-A diglycidyl ether, which is further metabolized to carboxylic acids and respective conjugates[9], and is excreted quickly[14].

Our data presented herein support the view of Ruse[16], who reviewed the risk-benefit issue associated with the use of composites/sealants and concluded that there is no reason to change the indications for the clinical application of these dental materials at present. Leachable BPA out of polymerized materials might be negligible for xenoestrogenic effects *in vivo*. Nevertheless, dentists filling cavities with unpolymerized materials should use great care because of the irritation caused by these materials to the oral mucosa and gingiva. 2-HEMA and its bonding agent cause contact dermititis[17]. In addition, the dispersed dentin bonding agent blanches the mucosa and the gingiva in the oral cavity. This biological reaction indicates that unpolymerized monomers in the dentin bonding agent penetrate into the oral mucosa or gingiva. Therefore, in the case of filling with a resin composite or applying a dentin bonding agent to the cavity, operators should protect the oral mucosa or gingiva of patients with rubber dams. In addition, matrix tape would be useful to avoid residual monomer in the filled polymerized material.

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