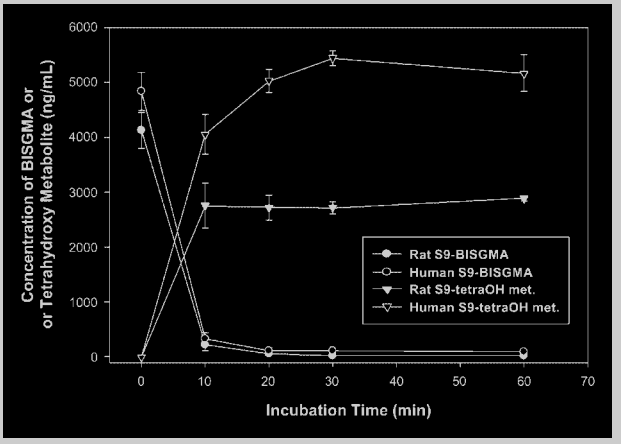
Full Paper: The stability and bioavailability of the biomaterial monomers, bisglycidyl methacrylate (BISGMA), bisphenol F diglycidyl ether (BFDGE), and bisphenol A dimethacrylate (BPADM) were investigated using in-vitro techniques. A reverse-phase high-pressure liquid chromatographic/mass spectrometric (HPLC/MS) system was developed to quantitate each monomer and its primary metabolite. Each monomer (10×10^{-6} M) was incubated at 37 °C under various conditions. Aliquots ($N = 3$) were removed at various time intervals and quantitated from a standard curve. The in-vitro transport of each parent monomer and its tetrahydroxy metabolite was measured in a Caco-2 system. BISGMA and BPADM were stable in aqueous solution at pH 1. However, BFDGE, was unstable. Plasma esterase of the rat rapidly hydrolyzed the ester compounds, but human esterase did not have a hydrolytic effect on BISGMA or BPADM. BFDGE disappeared in both rat and human plasma, but no tetrahydroxy metabolite was observed. All three parent compounds were unstable in human- and rat-hepatic fractions producing either tetrahydroxy metabolites or bisphenol A (BPA). The tetrahydroxy metabolites, however, were relatively stable under identical conditions, but BPA disappeared when incubated in hepatic-microsomal fractions. While BPADM metabolism produced BPA, an estrogen disrupter, BISGMA and BFDGE did not appear to produce BPA. These results suggest that the potential toxicity of leached dental monomers is more likely to be a result of the metabolite rather than the parent monomer. From Caco-2 transport studies, BFDGE and its tetrahydroxy metabolite both crossed the Caco-2 membrane at a low rate of transport in 2 h (approximately 3 and 5.2%, respectively). The BISGMA metabolite crossed at approximately 8%, indicative of a moderate rate of transport, and BPA crossed at approximately 10% in 1 h (high rate of transport). The transport of BPADM and BISGMA was unable to be determined due to nonspecific absorption to the acrylic vertical transport well. The transport of BFDGE tetrahydroxy metabolite is of particular interest as BFDGE is likely to be chemically hydrolyzed in stomach acid. It is well known that epoxies react with acids resulting in ring opening, so it is not surprising that BFDGE decomposes at pH 1. As a result, it is necessary to identify the decomposition (hydrolysis) products and test their bioavailability.



Mean (SD) stability of BISGMA (10×10^{-6} M) and bisphenol A tetrahydroxy metabolite in human- and rat-S9-hepatic fractions at 37 °C for 1 h.

In-Vitro Stability, Metabolism, and Transport of Dental Monomers Made from Bisphenol A and Bisphenol F

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Keywords: bioavailability; dental polymers; enzymatic stability; mass spectrometry; monomers

Introduction

It is well known that epoxies react with acids resulting in ring opening,[1,2] so it is not surprising that bisphenol F diglycidyl ether (BFDGE) decomposes at pH 1. Because it decomposes, it is necessary to identify the decomposition (hydrolysis) products and test their bioavailability.

In the mid 1950s, Buonocore laid the foundations for adhesive restoratives and prevention when he reported acid etching tooth enamel as a means of greater adhesion of resin to the tooth.[3] Historically, the oxirane resins,[4] (diglycidyl ether of bisphenol A) were replaced with methyl methacrylate esters with the invention of bisglycidyl methacrylate (BISGMA) monomers.[5] However, today BISGMA is made by the reaction of bisphenol A diglycidyl ether with methacrylic acid. Continuing improvements in dental restoration systems in the past four decades have included: coupling agents which allow BISGMA to bind to hydrophilic

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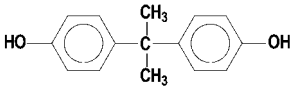
silica fillers;[6] removal of smear layer[7] and subsequent formation of a hybrid layer[8] resulting in increased dentin bonding;[9] increased shear bond strength;[10] viscosity adjustment with triethylene glycol dimethacrylate (TEDGMA);[6] adjustment of inorganic fillers;[11] changes in initiation of polymerization.[12]

Although reported shear bond strengths of resin composites are comparable with those for amalgams,[13,14] there is still a need for improvement. Polymeric shrinkage leading to microleakage[15,16] and the issue of reduced durability[17,18] are still problematic for clinical restoratives. It is now known that the reason why shrinkage leads to microleakage is because of polymerization stress, which depends upon polymeric shrinkage and the modulus of elasticity.

Bisphenol A (4,4′-isopropylidene-2-diphenol, BPA) is the core moiety of many dental monomers as shown in the formula below. A number of aromatic monomers have this core moiety, and the phenolic hydrogens may be substituted variously in these compounds.



Bisphenol A also has extensive commercial usage. Production in 1996 was approximately 1.6 billion pounds (7.3 billion kg). It is among those synthetic industrial monomers found in plastics used for polycarbonate plastics[19] and is a major component found in food can linings[20] and restorative dentistry.[21]

There are a number of concerns about toxicities induced by BPA. Although BPA demonstrated a weak response in uterotropic assays,[22] recently there has been a renewed interest devoted to the estrogenic potential of BPA.[23–30]

Numerous authors report finding leachable components from dental resins which may be a consequence of contamination from synthesis, changes in polymer structure,[31] and/or enzymatic degradation.[32,33] Consequently it is likely that BPA and its derivatives pose a potential deleterious effect in humans. Particularly relevant in this respect is the determination of the metabolic fate of common commercial monomers used in dental polymer restoratives based on the BPA structure. The purpose of this study was to investigate that aspect as well as the potential for transport of the related monomers and their metabolites.

Experimental Part

Chemicals

BISGMA was supplied by 3M Corporation (Minneapolis, MN) and purified using preparative-scale, normal-phase liquid chromatography as described previously.[34] BFDGE, bisphenol F bis[(2,3-dihydroxypropyl) ether], and bisphenol A bis[(2,3-dihyroxypropyl) ether] were purchased from Fluka (Milwaukee, WI). BPA and bisphenol A dimethacrylate (BPADM) were purchased from Aldrich (Milwaukee, WI). Primary reference solutions were prepared in acetonitrile (Allied Signal Burdick and Jackson, Muskegon, MI) to yield either 1 $mg \cdot mL^{-1}$ if used for standard curve preparation or a 10×10^{-6} M spiking solution when used for incubation preparations. All primary standards were stored at −20 °C until use. Incubation buffer consisted of 0.1 M potassium phosphate dibasic, adjusted to pH 7.4 with 0.1 M potassium phosphate monobasic (both purchased from Mallinkrodt Baker Paris, Kentucky) stored at 0–4 °C. Glacial acetic acid was also purchased (from Mallinkrodt Baker Paris, Kentucky). Working standards were prepared in 25% acetonitrile. Methyl-*tert*-butyl ether (MTBE) was also purchased (from Allied Signal Burdick and Jackson Muskegon, MI). ^{14}C-Mannitol was obtained from American Radiochemical (St Louis, MO). ^{14}C-Poly(ethylene glycol) 4 000 and ^{14}C-diphenylhydantoin-5,5-[4-^{14}C] (phenytoin) were obtained from Dupont New England Nuclear (Boston, MA). Midazolam, dextromethorphan hydrobromide, and dextrophan D-tartrate were purchased from Research Biochemicals International (Natick, MA).

Aqueous Stability Experiments

All three monomers, BISGMA, BFDGE, and BPADM (10×10^{-3} M) were tested for aqueous chemical stability in water, 0.1 M potassium phosphate buffer, and 1 M HCl at 37 °C for 1 h. BPADM was also tested under basic conditions using 0.1 M Na_2CO_3. Three replicate samples were incubated, and aliquots were removed at 0, 30, and 60 min intervals from each sample. Each aliquot was diluted with 100 µL of acetonitrile containing internal standard and analyzed by LC/MS. The 1 M HCl was used to simulate the hydrogen ion content of the stomach. A standard curve was prepared detectable over the range 25 to 6 000 $ng \cdot mL^{-1}$ by LC/MS analysis. Bisphenol A was not detectable using positive electrospray mass spectrometry and consequently it was derivatized so it could be detected. The dansyl chloride derivatization procedure required basic conditions, so it was necessary to determine the stability of BPADM under similar conditions. Derivatization was accomplished by the reaction of dansyl chloride with BPA in acetone at 60 °C for 1 h. Samples of 100 µL volume ($N = 3$) were removed at 0, 30, and 60 min intervals and diluted with 100 µL of 100% acetonitrile containing an internal standard. Samples were capped, inverted, and analyzed by positive electrospray LC/MS, monitoring the ammoniated adduct of the parent monomer. Subsequent experiments were repeated using BFDGE and BPADM, and, the respective metabolite was also quantitated by LC/MS analysis.

Hepatic Stability Conditions

Parent monomers and commercially available metabolites were separately incubated in rat- and human-hepatic-S9 fractions and microsomes (Xenotech, Kansas City, KS). A portion of forty microliters of each cofactor was added to each tube with 1 $mg \cdot mL^{-1}$ of S9 or 5 $mg \cdot mL^{-1}$ of microsome. The cofactors were glucose-6-phosphate (56.3 $mg \cdot mL^{-1}$),

$MgCl_2$ (9.4 $mg \cdot mL^{-1}$), glucose-6-phosphate dehydrogenase (30 $units \cdot mL^{-1}$), NAD (33.3 $mg \cdot mL^{-1}$), and NADP (15.3 $mg \cdot mL^{-1}$). The test monomer was added to each incubation vial (10×10^{-6} M; 10 μL added to 2 mL) and was incubated in triplicate at 37 °C. A 250 μL aliquot was removed at 0, 10, 20, 30, and 60 min. The reaction was quenched with an equal volume of 100% acetonitrile containing the internal standard and 5 mL of MTBE. Each sample was inverted for 15 min in a 13×100 mm^2 conical screw-cap test tube and centrifuged for 10 min at approximately 3 500 rpm. The upper organic layer was transferred into a second appropriately labeled 13×100 mm^2 conical test tube and evaporated to dryness under a gentle stream of nitrogen at 45 °C in a Turbovap (made by Zymark). The samples were reconstituted with 50 μL of acetonitrile, vortexed for 2 min followed by the addition of 75 μL of mobile-phase A, vortexed again, and after 5 min they were transferred to an injection vial. Samples were capped, and a 5 μL aliquot was analyzed by LC/MS for the parent monomer and commercially available metabolite. Each monomer and metabolite standard curve was prepared separately by adding 25 μL of working standards to 225 μL of 0.1 M potassium phosphate buffer and processed identically as the samples. Dextromethorphan and midazolam (10×10^{-6} M) were used as positive controls. Each substrate was added in duplicate to separate incubation vials, and aliquots were removed at identical times to that of the monomers. An equal volume of acetonitrile, containing internal standard, was also added to these samples and they were allowed to sit for 15 min after vortexing for 3 min. Samples were centrifuged for 10 min at approximately 3 500 rpm. Supernatant from each control sample was added to injection vials, capped, and stored frozen at −20 °C until analysis.

A standard curve of the positive controls dextromethorphan, dextrophan, midazolam, and 1′-hydroxymidazolam was prepared separately the day of analysis using the same acetonitrile containing internal standard. Analysis by LC/MS included monitoring the disappearance of the parent monomer and appearance of the metabolite of each control sample.

Plasma Stability Conditions

Unfrozen heparinized rat and human plasma from a single donor were purchased from Biological Specialities (Colmar, PA). Plasma was stored refrigerated until use. A portion of ten microliters of acetonitrile containing BISGMA, BFDGE, or BPADM was added to 2 mL of plasma ($N = 3$) yielding a 10×10^{-6} M concentration of monomer, and this was incubated at 37 °C for 1 h. A second set of 3 vials also contained 10×10^{-6} M of physostigmine (100×10^{-6} M), a known esterase inhibitor.[35] Duplicate incubation vials containing Procaine (10×10^{-6} M) were incubated as a positive control with and without physostigmine. BISGMA was additionally incubated with pooled frozen heparinized rat or human plasma. The parent monomer and metabolite were quantitated. Sample incubation preparation ($N = 3$) occurred on wet ice until after the T-0 aliquot (250 μL) was removed. All samples were then incubated at 37 °C for 1 h. Additional aliquots of 250 μL were removed at 5, 15, 30, and 60 min intervals, and the reaction was quenched with an equal volume of acetonitrile and 5 mL of MTBE. A standard curve of each monomer and metabolite was separately spiked into tubes containing acetonitrile, 5 mL of MTBE, and an equal volume of fresh blank plasma (225 μL of plasma and 25 μL of the working standard). All samples were extracted, evaporated, and analyzed by LC/MS to quantitate the appearance of the metabolite and the disappearance of the parent monomer.

Caco-2 Cell Seeding and Maintenance on Membranes

Caco-2 cells were obtained from the American Type Culture Collection (ATCC; Rockville, MD). Cells were grown in T-150 cell-culture flasks with the cells attaching to the bottom of the flasks forming a monolayer. Caco-2 cells were cultured in complete media, which contained Dulbecco's Modified Eagle Medium supplemented with 10% heat inactivated fetal bovine serum, penicillin/streptomycin solution (10 000 units each), glutamine (200×10^{-3} M), MEM nonessential amino acids (100×), sodium bicarbonate, and 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid (HEPES). The flasks of cells were incubated at 37 °C, with 5% CO_2/95% O_2, and approximately 95% humidity. The media in the flasks was removed and replaced with fresh complete media every other day.

The cells were split at approximately 80% confluency. A portion of ten milliliters of ethylenediaminetetraacetate (EDTA)/phosphate buffered saline (PBS) was added to the flask(s) of Caco-2 cells, and the cells were allowed to sit for 1–5 min. The solution was removed from the flasks followed by the addition of 5 mL of trypsin/EDTA, slowly sliding the solution over the cells. The solution was immediately removed allowing the cells to loosen from the flasks. The cells were mixed well and 1 mL of the cell suspension was added to new flasks. The flasks were placed in the incubator with lids slightly unscrewed. The remaining cell suspension was counted using a hemacytometer and diluted with complete media for the seeding on the polycarbonate membranes.

The Caco-2 cells seeded on polycarbonate membranes were grown and maintained for 21 d to form a confluent monolayer with tight cell-to-cell junctions and functional brush borders. Once reaching 100% confluency, the cells differentiate rapidly to become structurally and functionally similar to cells of the small intestine. Snapwell® (Costar) inserts with polycarbonate membranes were coated with 100 mL of diluted rat-tail collagen (1 to 3 with 60% ethanol) and allowed to dry. The membranes were then conditioned with incomplete media by adding 2.0 mL to the outer well (4 h minimum) and 0.5 mL (2 h, minimum) to the inner well and were then placed in an incubator at 37 °C. Incomplete media were removed from the outer well and replaced with 2.0 mL of complete media. The incomplete media were removed from the inner well and replaced with 0.5 mL of cell suspension in completed media. Snapwell® (Costar) inserts were seeded at 60 000 $cells \cdot cm^{-2}$ on the polycarbonate membranes. Cells were cultured in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum. Media were changed every other day (old media were removed and replaced with 0.5 mL of unused media to the inner insert and 2.0 mL to the outer well). The cells were maintained in an incubator at 37 °C with 5% CO_2 and approximately 95% humidity until used for transport studies between days 21 and 30 after seeding.

Caco-2 Cell-Transport Experimental Procedure

After Caco-2 cells were grown on the polycarbonate membranes for 21 d, the membranes were then ready for the transport experiments. The Costar transport apparatus was warmed and maintained at 37 °C with a circulator water bath. Snapwell® inserts with Caco-2 covered membranes were removed, rinsed three times with Hank's balanced salt solution (HBSS) at 37 °C, and placed in diffusion chambers. HBSS (4.5 mL) was added to the basolateral (receiver) side of the transport chambers. A 4.5 mL solution containing 40×10^{-3} M each of BISGMA, bisphenol A tetrahydroxy metabolite, BFDGE, and bisphenol F tetrahydroxy metabolite in 1% dimethyl sulfoxide (DMSO)/99% HBSS, was added to the apical (donor) side ($N = 3$). A gas mixture of 95% O_2/5% CO_2 was bubbled continuously through the HBSS. Basolateral samplings were taken at 0, 20, 40, 60, 80, 100, and 120 min after the addition of the test compound. Apical samples were taken at 0, 60, and 120 min. Sample volumes were 100–150 mL. Apical samplings were replaced with 100–150 mL of HBSS as appropriate. Control samples for liquid scintillation counting had 5 mL of scintillation cocktail added, and samples for LC/MS analysis were prepared by a 1 : 1 dilution with acetonitrile containing an internal standard. Apical samples were also diluted 1 : 4 prior to the addition of acetonitrile.

Method of Calculation

Percent transport across Caco-2 cells was calculated for the compounds at each time point. The corrected dpm or ng amount was divided by the initial apical concentration (C_o) $\times$ 100 to determine % transported across the Caco-2 barrier. The corrected concentration of either dpm or ng was calculated to adjust for background, sampling volume, and volume of buffer in the chambers.

The sample volume taken was 0.1 mL, and 4.5 mL was the buffer volume in the chamber. The apparent permeability coefficient (P_{app}, which was the rate of transport across the Caco-2 monolayers in cm $\cdot$ sec^{-1}, was calculated for all of the compounds using Equation (1).[36]

$$P_{app} = \frac{\Delta Q}{\Delta T \times 60\,\mathrm{s} \cdot \mathrm{min}^{-1} \times A \times C_o} (\mathrm{cm} \cdot \mathrm{s}^{-1}) \qquad (1)$$

In Equation (1), $\Delta Q/\Delta T$ was the permeability rate (dpm $\times$ min^{-1} or ng $\times$ min^{-1}), C_o was the initial concentration in the apical or donor chamber (dpm $\cdot$ mL^{-1} or ng $\cdot$ mL^{-1}), and A was the surface area of the membrane (1.13 cm^2). The permeability rate was determined by plotting the corrected dpm or ng of each compound on the basolateral side through the 240 min time course. The slopes (permeability rate) were then determined from these plots.

All transport calculations were performed on a Macintosh IIci computer (Apple Computer Inc., Cupertino, CA) using Lotus 1-2-3 (Lotus Development Corp., Cambridge, MA) software, and Microsoft Excel (Microsoft Corp., Redway, WA). Graphs were prepared using the DeltaGraph Professional (Deltapoint Inc., Monterey, CA) graphics software.

Analytical Measurements

High-pressure liquid chromatographic/mass spectrometric (LC/MS) instrumentation consisted of the following: HP 1100 binary pump, Finnigan TSQ-700 mass spectrometer, Digital Unix work station, Finnigan software ICIS ver. 8.3, Perkin Elmer autosampler, and an Eppendorf TC-50 column heater. Chromatographic separation was achieved on a Metachem precolumn filter, a 2×4 mm^2 C18 guard column, and a 2×30 mm^2 C18 3µ Luna analytical column from Phenomenex at 40 °C. A binary gradient system at 400 µL $\cdot$ min^{-1} consisted of mobile phase A (95% deionized (DI) water/5% acetonitrile) and mobile phase B (95% acetonitrile/5% DI water). Mobile phases were buffered by the addition of 5 mL of glacial acetic acid and 1.2 mL of ammonia hydroxide per L^{-1} of mobile phase. The gradient was as follows: (a) 5% B for 0.25 min; (b) 0.25–1.5 min increase to 95% B and hold until 3.0 min; (c) decrease to 5% B from 3.0–3.1 min. The mass spectrometer ionization mode was positive electrospray. The manifold pressure was 2×10^{-6} Torr. The ESI spray voltage was 4.5 kV, and the current was 10 µA. The capillary temperature was 200 °C. The electron multiplier was set at 1 600 V. The charge injection device (CID) argon gas pressure was 1.7 mTorr, and the CID offset was −18.0 V. The injection volume was 20 µL, and the split ratio was 1 : 3.

For sample quantitation, chromatograms consisting of appropriately integrated peaks were printed, and areas of each peak were entered into an Excel spreadsheet. Standard curve samples were generated the day of analysis and used to quantitate the study samples (''unknowns'') using a quadratic 1/$\times$ weighted regression curve from the ratio of the area of the internal standard to the area of the peak of interest. A similar quantitative process was used for each compound in each experiment. A curve was generated for each positive control to quantitate experimental control samples. Sample concentration values were then inserted into SigmaPlot 4.0 to generate the linear graphs for each compound from each experiment.

Results and Discussion

Analytical Instrumentation

It was observed that BISGMA analogues were transparent at submicrogram quantities using positive electrospray LC/MS conditions. In the current research a more sensitive assay resulted. The detection was a result of spontaneous adduct formation in the source of the mass spectrometer occurring via ammonia (molecular weight of 17), sodium (molecular weight of 23), and potassium (molecular weight of 39) ions with BISGMA analogues. The detection of the ammonium adduct by LC/MS was chosen as ammonium acetate was present in the mobile phase. Consequently, it was always in excess and was not a factor in affecting sensitivity. Bisphenol A, however, did not form adducts and had to be derivatized with dansyl chloride. Once derivatized, BPA could be analyzed by LC/MS.

The LC/MS conditions represent selected ion monitoring (SIM) of the ammoniated adduct ($M + 17 + H^+ = 530$) of

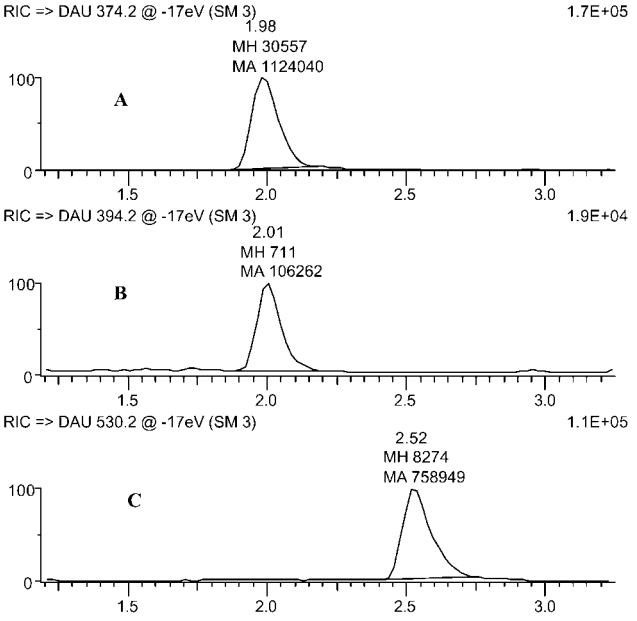


Figure 1. Selected ion monitoring (SIM) of 500 ng · mL^{-1} of each compound. A. Internal standard; B. BISGMA tetrahydroxy metabolite (394); C. BISGMA (530).

BISGMA and the ammoniated adduct ($M + 17 + H^+ =$ 394) of bisphenol A tetrahydroxy metabolite as chromatographically shown in Figure 1. In Scheme 1a is shown the structure of BISGMA (530) and the structure of the bisphenol A tetrahydroxy metabolite (394). The structure of BFDGE ($M + 17 + H^+ = 330$) and the structure of bisphenol F tetrahydroxy metabolite ($M + 17 + H = 366$) are shown in Scheme 1b. Scheme 1c depicts the structure of BPADM ($M + 17 + H^+ = 382$), the structure of BPA (228), and the dansyl chloride derivatized BPA (695). Bisphenol A did not form an adduct in the source.

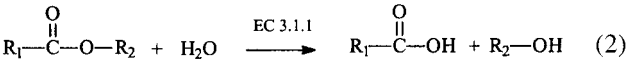
Aqueous Chemical Stability

All three monomers were stable in water and 0.1 M potassium phosphate buffer at 37 °C for 60 min. Monomers were incubated in triplicate and quantitated from separate standard curves. The aqueous stability of the monomers in hydrochloric acid was determined, as HCl could simulate gastric pH in humans. BISGMA and BPADM were stable in acidic conditions at 37 °C for 60 min. However, BFDGE, an oxirane, underwent chemical hydrolysis in 1 M HCl to the tetrahydroxy metabolite as shown in Scheme 1b. It is well known that epoxies react with acids resulting in ring opening,[1,2] so it is not surprising that BFDGE decomposes at pH 1. Stability samples were quantitated for the presence of BFDGE and its tetrahydroxy metabolite from separate standard curves. BFDGE disappeared, and the appearance of the metabolite was immediate indicating a rapid and complete chemical hydrolysis of the oxirane in acidic media. If orally ingested, the oxirane would likely be unavailable for gastrointestinal (GI) absorption because of conversion into the tetrahydroxy metabolite. Alkaline stability was determined for BPADM at 37 °C for 60 min. Early analytical investigation demonstrated the lack of detection of BPA using positive electrospray LC/MS conditions. Derivatization of BPA with dansyl chloride and subsequent detection indicated harsh alkaline conditions.[37,38] It was deemed necessary to evaluate the aqueous stability of BPADM under similar conditions. After 30 min incubation at 37 °C, complete hydrolysis of BPADM occurred forming bisphenol A.

Although we report the hydrolysis of oxiranes in acid and esters in base, Olea et al. reported the presence of bisphenol A by hydrolysis of resins and oligomers in alkaline (pH 13) and acid media (pH 1) after heating to 100 °C for 30 min.[39] Ester linkages are not as stable to hydrolysis as most ether linkages, therefore the result is explainable.

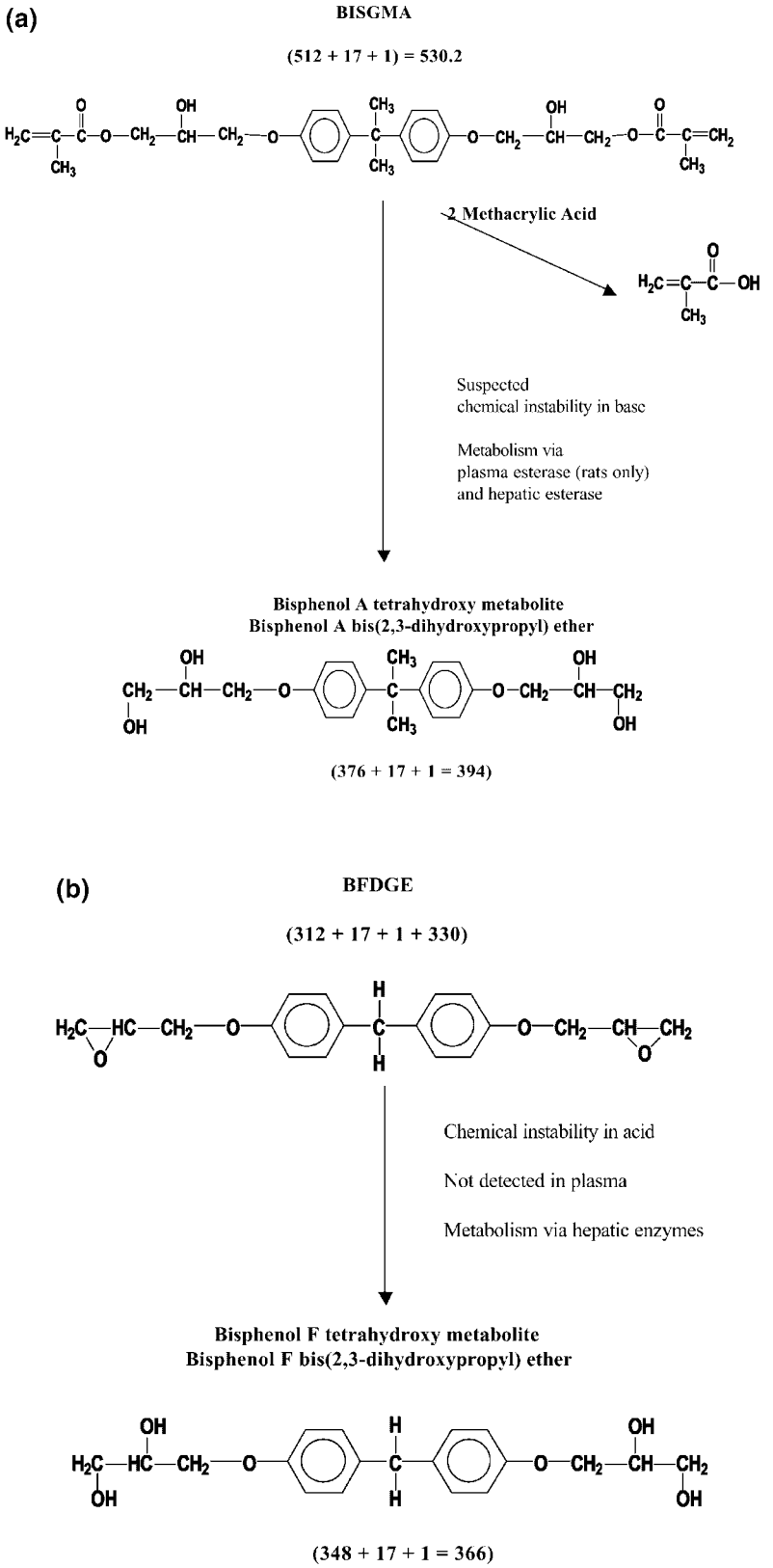
Humans are exposed to a wide variety of xenobiotic esters used in pesticides. Hydrolysis by esterases present in the liver, cytosol, and blood limit the activity of many esterified drugs and chemicals.[40] Esterase activity is ubiquitous. Carboxylesterase (EC 3.1.1) has been studied in a wide variety of vertebrate and nonvertebrate animals. Specifically, mammal carboxylesterase was studied in liver,[41] kidney,[42] small intestine,[43] pancreas,[44] serum,[45] plasma, red blood cell cytosol and membrane,[46] brain,[47] and skin.[48] Numerous studies have shown that a variety of xenobiotics are metabolized by these carboxyl ester hydrolases which exhibit broad substrate specificity and hydrolytic activities, which vary among species and individuals.[49]

Aldridge introduced the classification of xenobiotic esterases which is still used today, based on sensitivity to organophosphate inhibitors such as diethyl 4-nitrophenylphosphate.[50] Apart from aliphatic carboxylic acid esters, aromatic esters, and aromatic amides, thioesters are also substrates for these enzymes. They catalyze the hydrolysis of carboxylic acid esters to their corresponding free acids and alcohol as in Equation (2).

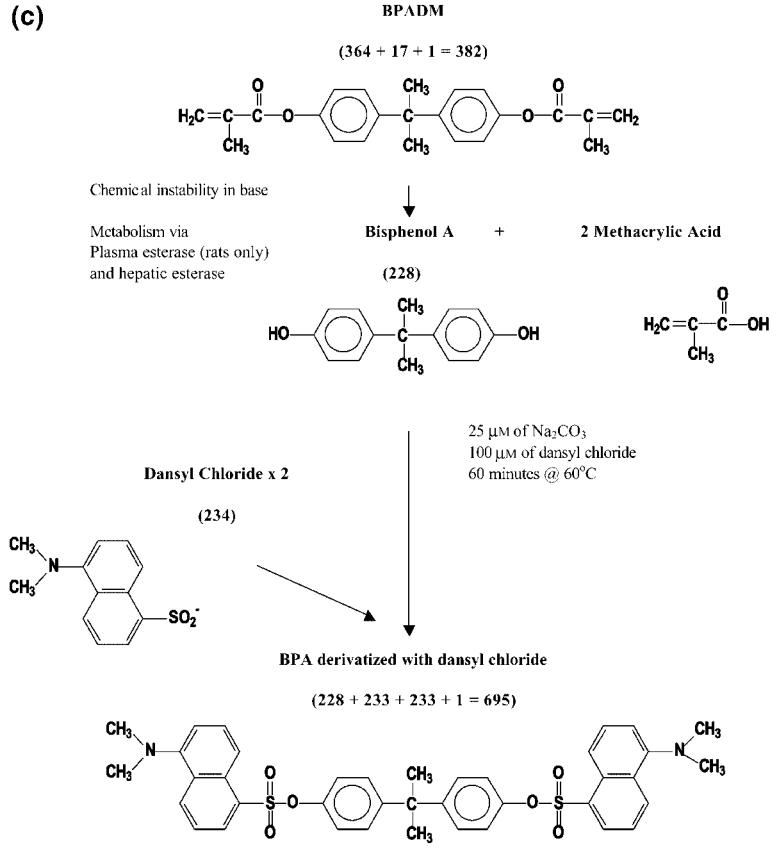


(2)

Plasma cholinesterase, EC 3.1.1.8 (also called pseudocholinesterase) cholinesterase, and butylcholinesterase are synthesized in the liver.[51] It is a tetrameric glycoprotein, containing four identical subunits each having one active catalytic site.[52] The physiological function of plasma cholinesterase has not been established. It is involved in the metabolism of succinylcholine, mivacurium, procaine, chlorprocaine, tetracaine, cocaine, heroin, and other drugs.[53] Human plasma contains cholinesterase but little carboxylesterase in contrast to rat plasma, which has high carboxylesterase levels.[54] Human-plasma hydrolysis of mebeverine was completely inhibited with physostigmine but only partially inhibited in rat plasma.[36]



Scheme 1. (a) Structures of BISGMA and its metabolites; (b) Structures of BFDGE and its metabolites; (c) Structures of BPADM, its metabolites, and its derivatives from Finnigan LC/MS SIM, positive electrospray.



Scheme 1. (*Continued*)

For BISGMA, the presence of the tetrahydroxy metabolite (Figure 2) was determined to be below the limit of quantitation of 25 $ng \cdot mL^{-1}$ indicating stability in fresh human plasma. The low T-0 values and high variance were thought to be due to the lack of dissolution of the monomer while on ice. Physostigmine had no effect on the analysis or monomer plasma stability. The positive control, procaine, was approximately 75% metabolized in the first 5 min in human plasma. However, in the presence of physostigmine, procaine was stable in human plasma. Physostigmine inhibited the enzymatic activity of human-plasma esterases.

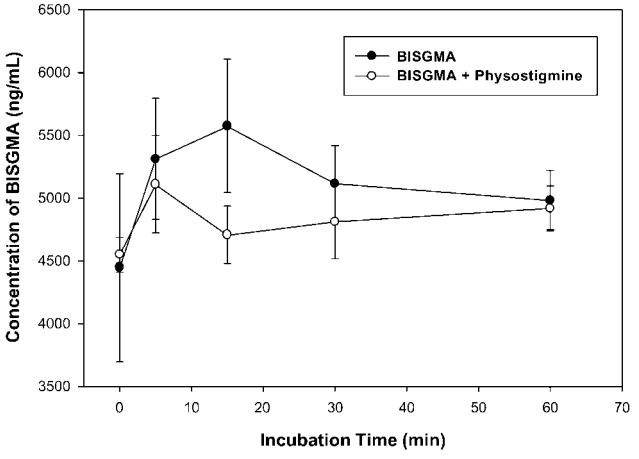


Figure 2. Mean (SD) stability of BISGMA (10×10^{-6} M) in fresh human plasma with and without physostigmine (100×10^{-6} M). The tetrahydroxy metabolite was below the limit of quantitation (25 $ng \cdot mL^{-1}$). Each value is a mean of 3 extracted samples.

The results were similar when using pooled, frozen, human heparinized plasma. The tetrahydroxy metabolite was not detected above 25 $ng \cdot mL^{-1}$, and the physostigmine had no effect on the assay or on the stability of the monomer. The pooled, frozen plasma also metabolized procaine. However, in the presence of physostigmine, the enzymatic activity of the plasma esterases was inhibited.

Studies of BISGMA (10×10^{-6} M) incubated at 37 °C for 60 min in fresh (Figure 3) and frozen, pooled heparinized rat plasma demonstrated the activity of rat-plasma esterase. Rat plasma rapidly hydrolyzed the BISGMA ester monomer to the tetrahydroxy metabolite. Physostigmine had no inhibitory effect on the rat-plasma esterase activity. Procaine was also incubated in the presence of fresh and frozen, pooled heparinized rat plasma (data not shown). Rat plasma hydrolyzed procaine in both instances. However, in the presence of physostigmine, rat-plasma esterase enzyme activity was marginally inhibited.

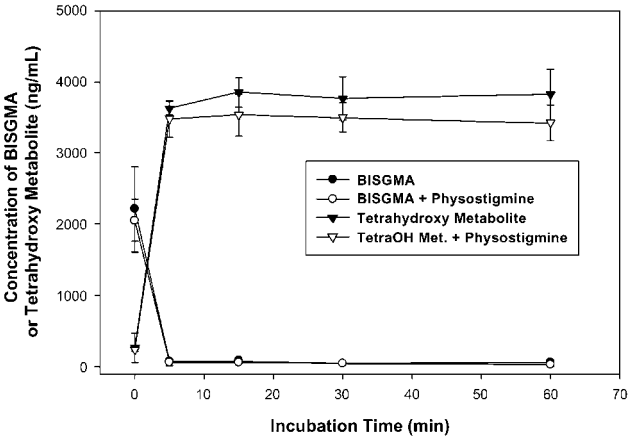


Figure 3. Mean (SD) stability of BISGMA (10×10^{-6} M) and the resulting bisphenol A tetrahydroxy metabolite in fresh human plasma with and without physostigmine (100×10^{-6} M). Each value is a mean of 3 extracted samples. Error bars are visually absent on time points where deviation from the mean is miniscule.

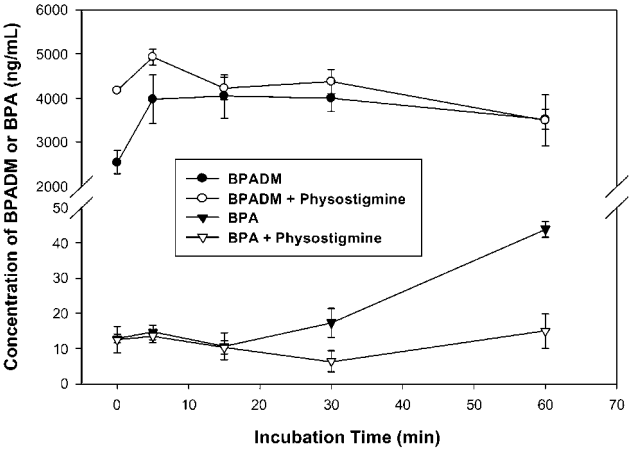


Figure 5. Mean (SD) stability of BISGMA (10×10^{-6} M) and the resulting bisphenol A metabolite in fresh rat plasma with and without physostigmine (100×10^{-6} M). Each value is a mean of 3 extracted samples.

The plasma stability of BFDGE (10×10^{-3} M) was also investigated in fresh, heparinized human and rat plasma. Samples for stability and standard curves were extracted identically, as described earlier. Figure 4 characterizes the plasma stability of BFDGE. Bisphenol F tetrahydroxy metabolite was analyzed by LC/MS. There was no detectable metabolite above the quantitation limit of 50 ng $\cdot$ mL^{-1} at $m/z = 366$. However, it was obvious that BFDGE disappeared by 46% in rat and 33% in human plasma. Procaine was also incubated and showed similar stability results to that of earlier experiments.

The stability of the BPADM ester was also determined in fresh human and rat heparinized plasma. The stability of BPADM in human (Figure 5) and rat (not shown) plasma, was very similar to that of BISGMA. BPADM appeared to be stable in human plasma but was metabolized in rat plasma to BPA. Physostigmine had no effect on the enzyme or assay. Procaine was incubated as the positive control.

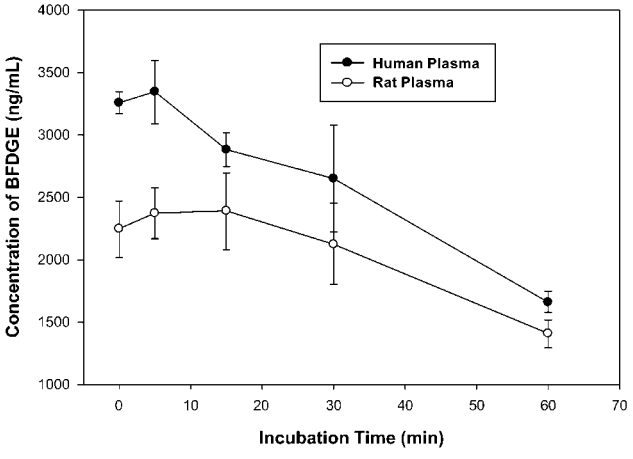


Figure 4. Mean (SD) stability of BISGMA (10×10^{-6} M) in frozen, pooled human plasma with and without physostigmine (100×10^{-6} M). The tetrahydroxy metabolite was not detected above the limit of quantitation (25 ng $\cdot$ mL^{-1}). Each value is a mean of 3 extracted samples.

As stated earlier, human plasma contains cholinesterase but little carboxylesterase. This is in contrast to rat plasma, which contains predominately carboxyesterase. There is a broad range of substrate specificity, and species differences have been reported earlier.[55] Ester monomers, BISGMA and BPADM, were unstable in rat plasma but stable in human plasma. Leung et al. demonstrated the enzymatic effects of pseudocholinesterase on commercial dental composites,[56] and Santerre et al. reported the degradation of dental composites by cholesterol esterase.[57] Procaine hydrolysis exhibited different results, being readily hydrolyzed in human plasma and hydrolyzed to a lesser extent in rat plasma. Procaine was incubated as a positive control in the presence and absence of physostigmine. Rat-plasma esterase activity was immune to the inhibitory effect of physostigmine when dental monomers containing ester linkages were substrates. Rat-plasma esterase also has a minimal effect on the hydrolysis of procaine. Hydrolysis of procaine in human plasma was completely inhibited in the presence of physostigmine. These results corroborate earlier findings by Dickinson et al.[35]

Oxiranes are not hydrolyzed to the tetrahydroxy metabolite in rat or human plasma at 37 °C in 60 min. The observed disappearance can possibly be explained by metabolism through a different pathway, but is likely a result of binding to plasma proteins. It has been well established that vinyl chloride and polyaromatic compounds such as aflatoxin B1 and benzo(a)pyrene are metabolically activated to oxiranes.[58] These electrophilic molecules alkylate nucleophilic positions of DNA suggesting the possible reactive nature of oxiranes with proteins.

Hepatic Stability

Olea et al. report that BPA and BPADM leach into saliva from treated patients.[39] This finding was confirmed by both Arenhold-Bindslev et al.[59] and Fung et al.[60] In addition to BPA,[61–63] BPADM and BISGMA were identified as leached monomers from polymerized resins.[61–66] Leaching of resin components may occur during polymerization of the resin and by degradation of the polymer.[67] Only the small portion of monomers that remain completely unreacted are extractable. Subsequent to leaching, determination of the presence of metabolites is of toxicological concern. BPADM can also be a source of BPA because of salivary esterases, pH, and porcine esterase.[68,69]

Knaak and Sullivan reported the metabolism of ^{14}C-BPA in rats. Collection of carbon dioxide, urine, and feces revealed no ^{14}C–CO_2; 56% of the dose was found in the feces, and 28% in the urine. After a single oral dose, in the urine, BPA is excreted primarily as the glucuronide conjugate. In the feces, 35% was free BPA, 35% was a hydroxylated product, and the remaining 30% was not identified.[70] These findings were confirmed by Pottenger et al. in F-344 rats[71] and by Synder et al. in female rats.[72]

Since BPA binds to estrogen,[73] it seemed prudent to determine the metabolic fate of BISGMA and related analogues. The stability of BISGMA in rat and human microsomes at 37 °C for 1 h is shown in Figure 6. Approximately 90% of the parent monomer disappeared within 10 min. The presence of the tetrahydroxy monomer was not determined in this initial experiment. Subsequent experiments did quantitate the formation of the tetrahydroxy metabolite. Midazolam and dextromethorphan were also incubated and analyzed for all hepatic experiments. The appearance of 1′-hydroxymidazolam and dextrophan was quantitated from individual standard curves. The disappearance of midazolam and dextromethorphan was also simultaneously quantitated from standard curves. In all experiments, these CYP 3A4[74] and 2D6[75] model substrates behaved as expected producing their respective metabolites.

The stability of BISGMA in rat- and human-hepatic-S9 fractions at 37 °C for 1 h is shown in Figure 7. After 10 min, greater than 90% of the initial BISGMA concentration had disappeared. The bisphenol A tetrahydroxy metabolite correspondingly appeared in both human- and rat-hepatic-S9 fractions.

The bisphenol A tetrahydroxy metabolite was spiked into both rat and human microsomes and hepatic-S9 fractions. The relative stability of this metabolite in rat and human microsomes is illustrated in Figure 8. Although there was a dissolution phenomena occurring between the T-0 and T-10 time point causing variability, overall the metabolite is relatively stable. In the rat there was as much as a 25% loss over 1 h compared with a 10% loss in human microsomes over the same time interval.

The relative stability of the tetrahydroxy metabolite in rat- and human-hepatic-S9 fractions is illustrated in Figure 9. The concentration of the metabolite decreased by 16% in rat-S9 fractions, and in the human-S9 fractions it remained relatively constant over the 1 h interval.

The stability of BFDGE in rat- and human-hepatic microsomes or S9 fractions at 37 °C for 1 h was determined under identical conditions to that of BISGMA. BFDGE was metabolized very rapidly in rat and human microsomes (Figure 10). After 10 min, virtually 100% of the parent monomer had disappeared.

The hepatic stability of BFDGE in rat- and human-S9 fractions is given in Figure 11. Similar to in the

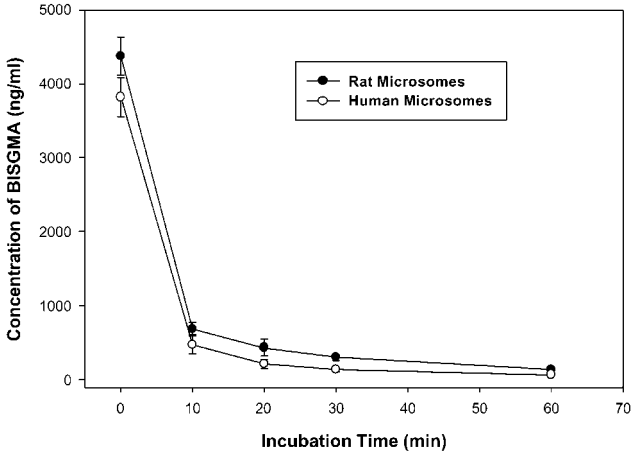


Figure 6. Mean (SD) stability of BISGMA (10×10^{-6} M) in human and rat microsomes at 37 °C for 1 h. Each value is a mean of 3 extracted samples. Error bars are visually absent on time points where deviation from the mean is miniscule.

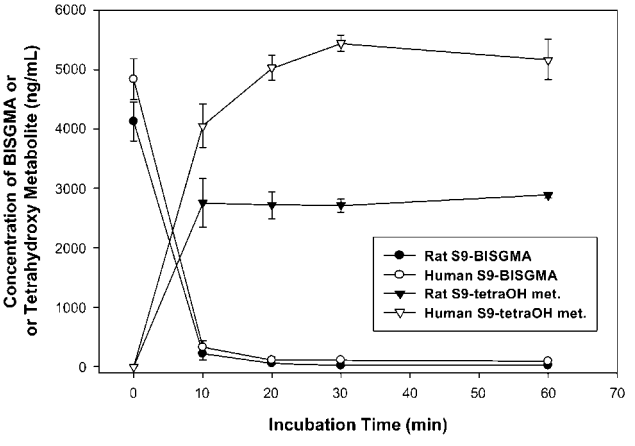


Figure 7. Mean (SD) stability of BISGMA (10×10^{-6} M) and bisphenol A tetrahydroxy metabolite in human- and rat-S9-hepatic fractions at 37 °C for 1 h. Each value is a mean of 3 extracted samples. Error bars are visually absent on time points where deviation from the mean is miniscule.

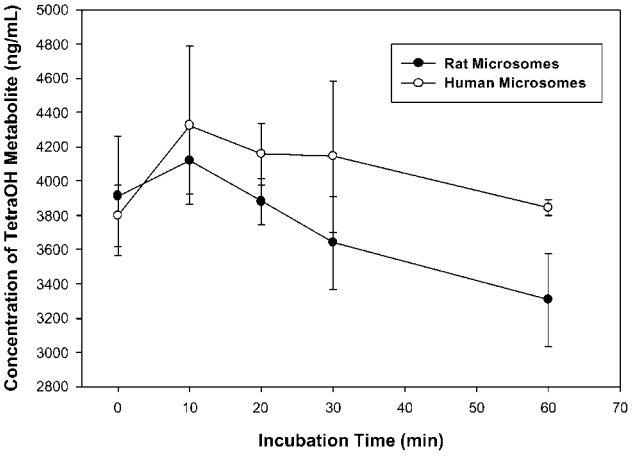


Figure 8. Mean (SD) stability of the BPA tetrahydroxy metabolite (10×10^{-6} M) in human- and rat-hepatic-microsomal fractions at 37 °C for 1 h. Each value is a mean of 3 extracted samples.

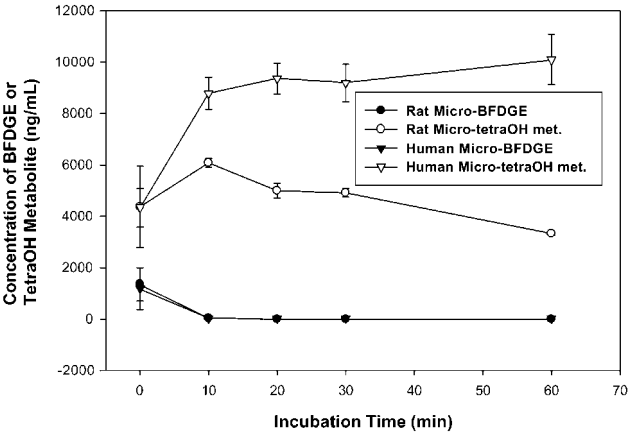


Figure 10. Mean (SD) stability of BFDGE (10×10^{-6} M) and the bisphenol F tetrahydroxy metabolite in human- and rat-hepatic-microsomal fractions at 37 °C for 1 h. Each value is a mean of 3 extracted samples. Error bars are visually absent on time points where deviation from the mean is miniscule.

microsomes, BFDGE was rapidly and completely metabolized. However, when bisphenol F tetrahydroxy metabolite (10×10^{-6} M) was incubated as the primary substrate at 37 °C for 1 h with rat- and human-hepatic microsomes (Figure 12) and S9 fractions, approximately 8% of the metabolite disappeared in rat microsomes but was unchanged in human microsomes. Approximately 12 and 9% of the bisphenol F metabolite disappeared in rat- and human-hepatic-S9 fractions, respectively.

The stability of BPADM and BPA, 10×10^{-6} M each, was investigated in a similar manner. The stability of BPADM in rat and human microsomes is shown in Figure 13. BPADM ester was metabolically unstable in rat or human microsomes producing at least the BPA metabolite. After 10 min at 37 °C approximately 50 and 70% of BPADM disappeared in the rat and human microsomes, respectively. In the human-hepatic-microsomal fraction, the accumulation of BPA continued. In rat-microsomal fractions, over the same time interval, BPA levels diminished. After 1 h incubation, BPA was almost completely absent.

The stability of BPADM in rat- and human-hepatic-S9 fractions was similar to that in the microsomes. BPADM was metabolically unstable in hepatic-S9 fractions, with approximately 66% of the parent monomer disappearing by T-10 min. The concentration of the BPA metabolite peaked at 10 min and disappeared in 60 min in the rat, whereas in the human-hepatic-S9 fraction, BPA reached a plateau over the 10–60 min incubation interval.

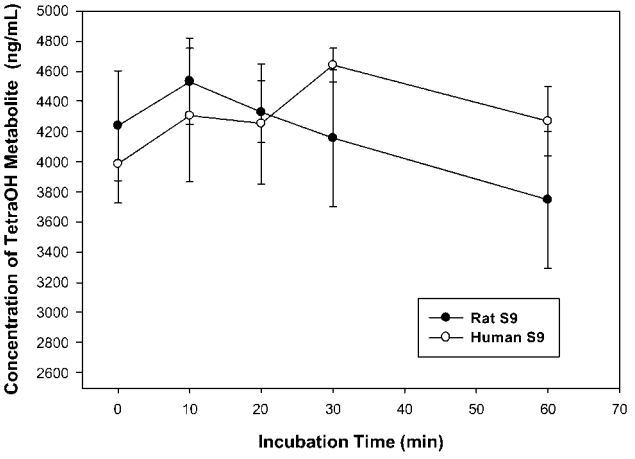


Figure 9. Mean (SD) stability of the BPA tetrahydroxy metabolite (10×10^{-6} M) in human- and rat-hepatic-S9 fractions at 37 °C for 1 h. Each value is a mean of 3 extracted samples.

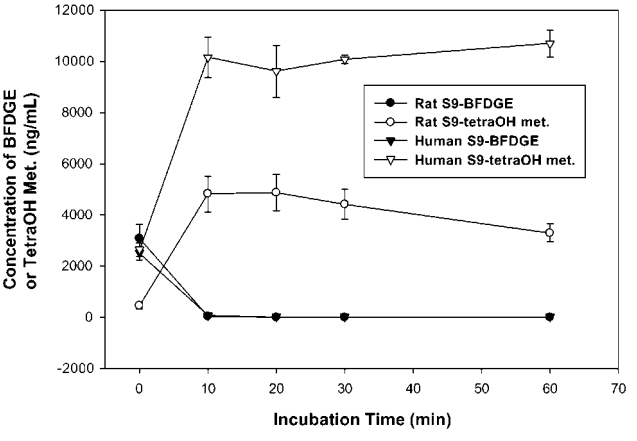


Figure 11. Mean (SD) stability of BFDGE (10×10^{-6} M) or the bisphenol F tetrahydroxy metabolite in human- and rat-hepatic-S9 fractions at 37 °C for 1 h. Each value is a mean of 3 extracted samples.

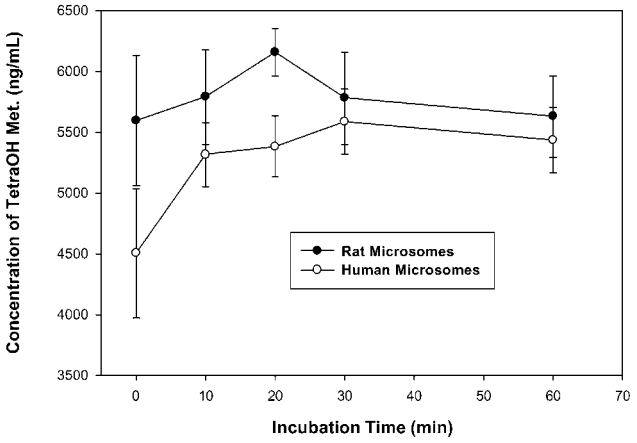


Figure 12. Mean (SD) stability of the bisphenol F tetrahydroxy metabolite (10×10^{-6} M) in human- and rat-hepatic-microsomal fractions at 37 °C for 1 h. Each value is a mean of 3 extracted samples.

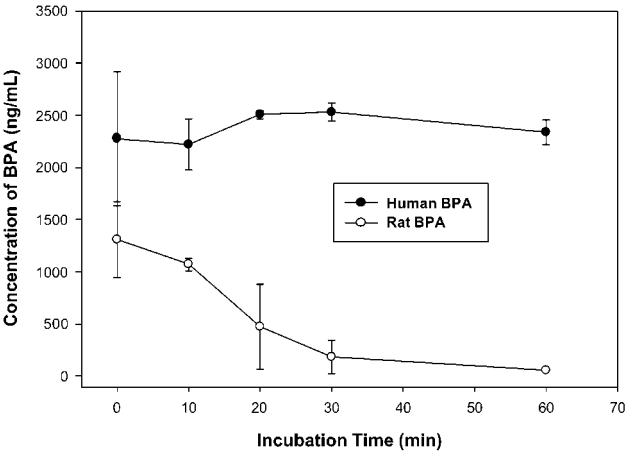


Figure 14. Mean (SD) stability of bisphenol A (10×10^{-6} M) in rat- and human-hepatic-microsomal fractions at 37 °C for 1 h. Each value is a mean of 3 extracted samples. Error bars are visually absent on time points where deviation from the mean is miniscule.

BPA (10×10^{-6} M) was also investigated as a primary substrate. BPA appeared to be unstable in rat fractions with 66 and 90+% disappearing in 30 min in the microsomal and S9 fractions, respectively. However, BPA appeared to be stable in human–hepatic-microsomal fractions as shown in Figure 14 and 15. These in vitro results demonstrate a rapid and complete metabolism of the parent monomer in each matrix.

Epoxide hydrolases (EHs; EC 3.3.2.3) are a family of enzymes that function to hydrate simple epoxides to vicinal diols and arene oxides to *trans*-dihydrodiols.[76] Five classes of mammalian EH have been characterized.[77–81] These classes are structurally and immunologically distinct.[78] The soluble form of EH participates in a xenobiotic metabolism with a preference for *trans*-substituted epoxides such as *trans*-stilbene oxide.[82] The soluble form and other forms of EH have been recognized for their roles in the metabolism of endogenous epoxides such as steroids and arachidonic acid derivatives[83,84] and leukotrienes.[85]

The microsomal form of EH is primarily associated with the metabolism of exogenous molecules and exhibits a broad range of activity. It has been implicated in both detoxification and bioactivation reactions. The bioactivation reactions include the metabolism of the procarcinogenic polyaromatic hydrocarbons to electrophilic and mutagenic bay-region diol-epoxides.[86,87] Although most

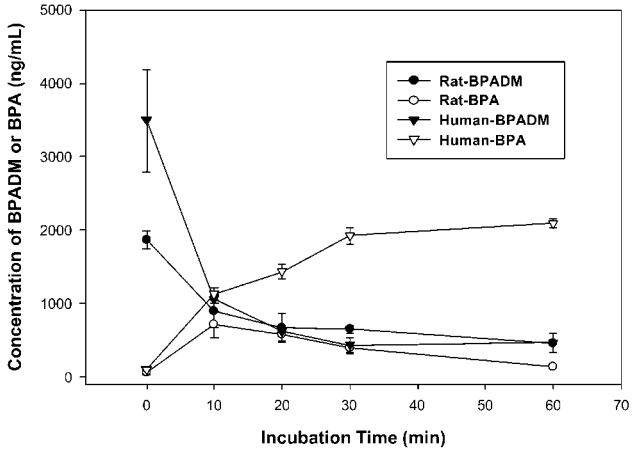


Figure 13. Mean (SD) stability of BPADM (10×10^{-6} M) and the bisphenol A metabolite in human- and rat-hepatic-microsomal fractions at 37 °C for 1 h. Each value is a mean of 3 extracted samples. Error bars are visually absent on time points where deviation from the mean is miniscule.

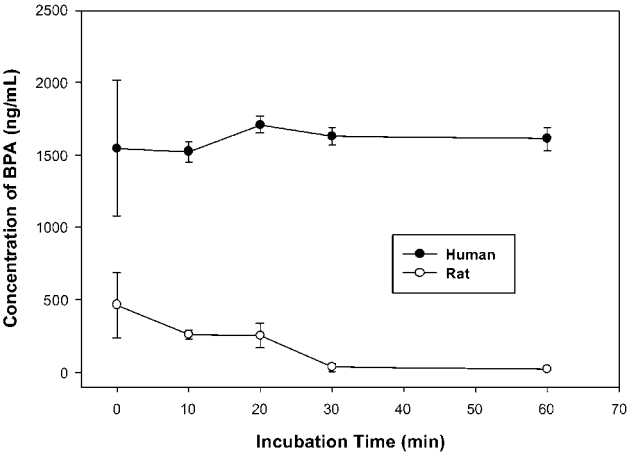


Figure 15. Mean (SD) stability of bisphenol A (10×10^{-6} M) in rat- and human-hepatic-S9 fractions at 37 °C for 1 h. Each value is a mean of 3 extracted samples. Error bars are visually absent on time points where deviation from the mean is miniscule.

epoxides are generated in situ by various oxidative enzymes,[88] exposure can occur directly as in the example of styrene oxide.[89] Microsomal EH appears to be expressed in all tissues and cell types.[90]

Earlier work by Climie et al. demonstrated that ^{14}C-bisphenol A diglycidyl ether is rapidly eliminated, mainly by excretion in the feces.[91] The metabolites and reactions identified include the tetrahydroxy metabolite, a reduction of a carboxylic acid forming the diol, and the methyl ester of the carboxylic acid. They also identified an esterified derivative, glyceraldehyde, glycidaldehyde, and a sulfonyl conjugate.[92] Climie et al.[92] proposed two metabolic possible pathways: initial oxidation resulting in glycidaldehyde and epoxide hydrolase and oxidation leading to glyceraldehyde. Based on the activity of epoxide hydrolyase, the glyceraldehyde would be more prevalent. Bentley et al. confirmed the apparent high affinity of BPADGE to epoxide hydrolase and also studied DNA adduct formation.[93] They suggested the formation of a glycidaldehyde–deoxyguanosine adduct. They further theorized that epoxide hydrolase was the primary route of metabolism. If this pathway was not available, then direct oxidation of the epoxide would occur producing glycidaldehyde. It could be hypothesized that in either route, the BPA analogue could undergo further *O*-dealkylation liberating BPA. The present in vitro results confirm the high affinity of the oxirane monomer to epoxide hydrolase. The lack of disappearance of the tetrahydroxy metabolite when incubated as the primary metabolite suggests no other oxidative metabolism occurs.

Epoxides may be carcinogenic via adduct formation with DNA by binding to the N-7 position of guanine, or as reported by Bentley et al.[93] by oxidation resulting in a glycidaldehyde adduct to DNA. The use of oxirane resins should be thoroughly evaluated. Ester hydrolysis produced BPA from BPADM and the tetrahydroxy metabolite from BISGMA. Based on the previous work of Climie et al.[92] and Bentley et al.[93] the same metabolic fate is possible for the BISGMA tetrahydroxy metabolite as for that of the BPADGE tetrahydroxy metabolite, i.e., the potential liberation of BPA and glyceraldehyde.

Although identification of potential metabolites in our study was not pursued, the disappearance of BPA when incubated confirms earlier results. Atkinson and Roy reported the metabolism of BPA to bisphenol-*o*-quinone[94] and later determined that bisphenol-*o*-quinone forms DNA adducts.[95] Pottenger et al. identified BPA glucuronides in rat,[71] while Yokota et al. identified the rat isoform responsible for conjugating glucuronic acid with BPA to form UGT2B1.[96]

Caco-2 Transport

The human adenocarcinoma cell line Caco-2 was originally isolated from human colon adenocarcinoma by Fogh et al.[97] It was developed as a model of the intestinal epithelium which exhibits morphological and biochemical characteristics similar to those of the human intestinal mucosa, such as brush-border marker enzymes, the presence of tight junctions, and the development of cell polarity.[98–100] Consequently, cultured Caco-2 cell monolayers have been extensively used to study the transcellular passive diffusion of lipophilic molecules and the paracellular flux of hydrophilic compounds.[101–103] Monolayers have also been employed to study the modulation of drug efflux due to the expression of p-glycoprotein,[104,105] to elucidate the mechanism of carrier- and receptor-mediated transport,[106–108] and the modulation of tight-junctional permeability to various solutes,[109] and monolayers are used a model system for determining cytoprotection.[110] However, no known studies have included the transport of dental resin monomers.

The transport of BISGMA and BPADM across Caco-2 cells could not be demonstrated. After considerable experimentation it was concluded that the ester monomers were adsorbed onto the acrylic diffusion chamber because of decreased apical BISGMA concentration, and, simultaneously, no BISGMA was detectable in the basolateral buffer. This adsorption prevented detection of transport across the monolayer of cells with these two ester monomers.

Approximately 8% of the bisphenol A tetrahydroxy metabolite crossed a Caco-2 cell monolayer at 37 °C over 2 h. The transport of BFDGE across the cell layer is illustrated in Figure 16. Approximately 3% of this monomer was transported from the apical to the basolateral side by the monolayer of cells in 2 h. Approximately 5% of the bisphenol F tetrahydroxy metabolite was transported across the Caco-2 cell membrane in 2 h (Figure 17). BPA (40×10^{-6} M) was incubated in a caco-2 cell system and demonstrated approximately 10% transport in 1 h.

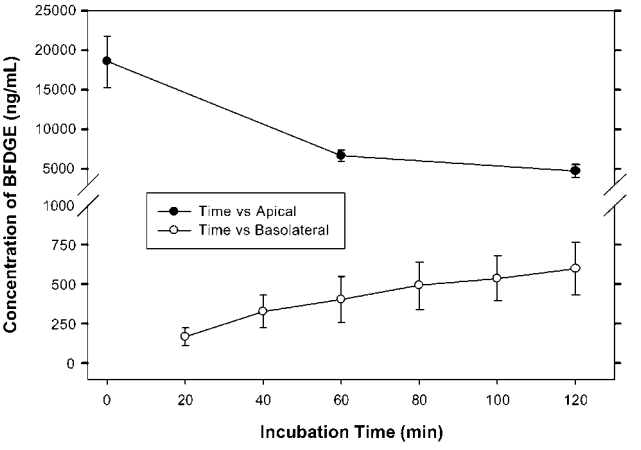


Figure 16. Mean (SD) transport of BFDGE (40×10^{-6} M) at 37 °C for 2 h. Samples were removed from the apical and basolateral sides. Each value is a mean of 3 analyzed samples. This monomer demonstrates approximately a 3% transport in 2 h.

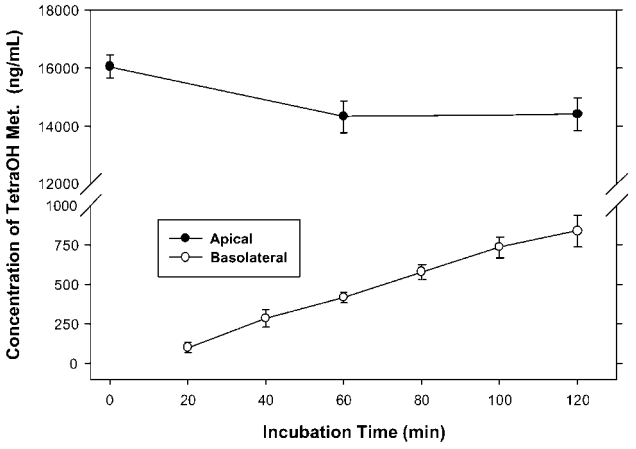


Figure 17. Mean (SD) transport of the bisphenol F tetrahydroxy metabolite (40×10^{-6} M) at 37 °C for 2 h. Samples were removed from the apical and basolateral sides. Each value is a mean of 3 analyzed samples. This monomer demonstrates approximately a 5% transport in 2 h.

Positive and negative controls were used in these transport studies. Phenytoin, a positive control, was a passively diffused, highly transported compound with the percent of transport varying between 15–30% in 2 h. Mannitol was used as a negative control with less than 1% transport across the cell membrane in 2 h.[111] Phenylalanine was a carrier-mediated, intermediate, positive control that showed approximately 5–15% transport in 2 h. The percentages of transport from each control were determined to be within acceptable ranges for cell passage for each experiment.

Metabolites of BISGMA and BPADM were transported across the cell layer. BISGMA tetrahydroxy metabolite demonstrated 8% transport in 2 h, while 10% transport in 1 h was observed for BPA. This is considered to be a high rate of transport for BPA.

BFDGE did not appear to bind to the acrylic chamber and demonstrated a 3% rate of transport in 2 h, and its metabolite demonstrated 5% in 2 h. The transport of the BFDGE tetrahydroxy metabolite was of particular interest because BFDGE was likely to be chemically hydrolyzed in stomach acid.

Conclusion

BISGMA was rapidly metabolized by human-hepatic fractions to a more water-soluble tetrahydroxy metabolite. It was also rapidly hydrolyzed in rat plasma and hepatic fractions. However, BISGMA was stable in human plasma and in the aqueous solutions tested. Results indicate that the tetrahydroxy metabolite is relatively stable. Hence, BPA is not likely to be a metabolite of dental restoratives containing BISGMA. BISGMA appeared to be adsorbed to the acrylic chamber of the Caco-2 system. However, the tetrahydroxy metabolite of BISGMA had a low to moderate rate of transport in the Caco-2 system.

BFDGE, an oxirane, was chemically hydrolyzed with 1 M hydrochloric acid to the corresponding tetrahydroxy metabolite. BFDGE was rapidly metabolized by hepatic fractions to the same tetrahydroxy metabolite. The tetrahydroxy metabolite showed minimal hepatic metabolism in our in-vitro model suggesting this compound does not form BPF. Both BFDGE and the tetrahydroxy metabolite were transported in the Caco-2 system implying intestinal absorption. If there were chemical hydrolysis in the stomach, the metabolite, not BFDGE, is likely to be absorbed. BFDGE disappeared in both rat and human plasma, but the tetrahydroxy metabolite was not present.

BPADM was rapidly metabolized to bisphenol A by rat plasma and in human- and rat-hepatic fractions. It was not hydrolyzed in human plasma. BPA could be transported by Caco-2 and demonstrated a high rate of transfer. Further metabolism did occur when BPA was incubated with hepatic fractions as a primary substrate.

BISGMA, BFDGE, and BPADM are likely to demonstrate a high-first-pass metabolism suggesting the tetrahydroxy metabolites of BISGMA and BFDGE should be candidates for future biocompatibility studies. BPA was a hydrolysis product of BPADM. BPA and potentially unidentified metabolites of BPA should also be candidates for further toxicological testing.

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