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Metabolism and disposition of n-butyl glycidyl ether in F344 rats and B6C3F1 mice

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a) **Running title:** Metabolism and disposition of n-butyl glycidyl ether

b) 21 text pages

3 tables

2 figures

11 references

190 words in the *Abstract*

392 words in the *Introduction*

858 words in the *Discussion*

c) Abbreviations used are: BGE, n-butyl glycidyl ether; ESI, electrospray ionization; GSH, glutathione; LSC, liquid scintillation counter; MS, mass spectrometry; MS/MS, tandem mass spectrometry; MW, molecular weight; NTP, National Toxicology Program; RT, retention time

Abstract

The disposition of [^{14}C]-labeled n-butyl glycidyl ether (BGE, 3-butoxy-1,2-epoxypropane) was studied in rats and mice. The majority of a single oral dose (2-200 mg/kg) was excreted in urine (rats, 84-92%; mice, 64-73%) within 24 h. The rest of the dose was excreted in feces (rats, 2.6-7.7%; mice 5.3-12%) and in expired air as $^{14}CO_2$ (rats, 1.5%; mice 10-18%), or remained in the tissues (rats, 2.7-4.4%; mice, 1.5-1.7%). No parent BGE was detected in rat or mouse urine. Fifteen urinary metabolites were identified, including 3-butoxy-2-hydroxy-1-propanol and its mono-sulfate or mono-glucuronide conjugates, 3-butoxy-2-hydroxypropionic acid, O-butyl-N-acetylserine, butoxyacetic acid, 2-butoxyethanol, and 3-butoxy-1-(N-acetylcystein-S-yl)-2-propanol, the mercapturic acid metabolite derived from conjugation of GSH with BGE at the C-1 position. Some of these metabolites underwent further ω-1 oxidation to form a 3'-hydroxybutoxy substitution. One urinary metabolite was from ω-oxidation of 3-butoxy-1-(N-acetylcystein-S-yl)-2-propanol to yield the corresponding carboxylic acid. Oxidative deamination of 3-butoxy-1-(cystein-S-yl)-2-propanol gave the corresponding α-keto acid and α-hydroxy acid metabolites that were present in mouse urine but not in rat urine. An in vitro incubation of BGE with GSH showed that the conjugation occurred only at the C-1 position with or without the addition of glutathione S-transferase.

Introduction

Epoxy resins are used in electronics, coatings and a variety of other applications. They are generally prepared from a phenol, commonly bisphenol A and a reactive epoxide such as epichlorohydrin. The properties of the resin are modified by including other phenols or epoxides. n-Butyl glycidyl ether (BGE, 3-butoxy-1,2-epoxypropane) is one of the modifiers used to reduce the viscosity of the resin (Kirk-Othmer, 2005). BGE is a high production volume chemical with production of more than a million pounds produced yearly in the US (National Toxicology Program (NTP), 2004). It has been selected for study by the NTP as an example of the aliphatic glycidyl ethers class.

The acute toxicity of BGE is low. The oral LD50 is reported to be 1.53 and 2.26 g/kg in mice and rats respectively. Inhalation exposure to as much as 200 ppm for 8 hours appeared to affect the CNS, cause congestion of the central zones of the liver, and result in pneumonitis and irritation of the lungs. BGE is positive in the Ames test without the addition of S9 (NTP, 2004). There are no chronic toxicity studies reported for BGE, however two-year studies on an olefinic and an aromatic glycidyl ether have been conducted. Chronic exposure to 5 or 10 ppm allyl glycidyl ether by inhalation resulted in some evidence (male mice), equivocal (male rat, female mice), or no (female rats) evidence of carcinogenic activity (NTP, 1990). Chronic exposure to diglycidyl resorcinol ether by gavage administration resulted in increases in benign and malignant lesions of the forestomach in male and female mice (50 or 100 mg/kg) and rats (25 or 50 mg/kg) (NTP, 1986).

The absorption, tissue distribution, metabolism, and excretion (ADME) of BGE in rats have been investigated, but the data are limited. Male rats excreted 87% of an oral dose ([^{14}C]-BGE, 20 mg/kg) in urine within 24 h (Eadsforth et al., 1985). TLC analysis of the urine revealed

more than ten metabolites, although only three major metabolites, including 3-butoxy-2-hydroxypropionic acid (9%), O-butyl-N-acetylserine (23%) and butoxyacetic acid (10%) were identified in that study (Eadsforth et al., 1985). The present studies were designed to identify the remaining metabolites and investigate potential sex and species differences in BGE metabolism and disposition in support of NTP bioassays in male and female F344 rats and B6C3F1 mice. Three doses (2, 20, 200 mg /kg) were used in the studies.

Materials and Methods

Chemicals

[n-Butyl-1'-^{14}C]-BGE (specific activity 55 mCi/mmol, radiochemical purity 99.7 %) was obtained from Moravek Biochemicals (Brea, CA). Unlabeled BGE (purity 95%), 2-butoxyethanol, GSH, glutathione S-transferase (75 % pure) from rat liver, and β-glucuronidase from bovine liver (10,000,000 units/g solid) were purchased from Sigma-Aldrich Corp. (St. Louis, MO).

Instruments

^{1}H NMR spectra were acquired on a Varian Gemini 300 MHz NMR spectrometer (Palo Alto, CA). Chemical shifts are reported in ppm relative to D_2O at 4.80 ppm. HPLC analysis of urine (10-20 µL) was carried out on a Beckman System Gold 126 Solvent Module pump, a 168 photodiode array detector, and an IN\US (Tampa, FL) β-RAM flow detector equipped with a liquid cell (500 µL) for radiochemical detection. Liquid scintillation fluid (In-Flow ES, IN\US, Tampa, FL) was delivered in 3:1 scintillator/eluant ratio. A Varian (Walnut Creek, CA) Inertsil C18 5µm column (4.6 x 250 mm) was used for all studies. The solvent system consisted of solvent A: 0.1% trifluoroacetic acid in H_2O and solvent B: CH_3CN and a gradient from 100%A and 0%B to 65%A and 35%B over 28 min, then held at 35% B for 5 min at a flow rate of 1.5 mL/min. This HPLC system was used for detection of radiolabeled peaks and for isolation of the metabolites, and will be referred to as HPLC in the Results and Discussion. Electrospray ionization mass spectra (ESI-MS) were obtained on a Thermo Finnigan LCQ Advantage Max ion trap mass spectrometer (Riviera Beach, FL). Tandem mass spectra (ESI-MS/MS) were produced by collision-induced dissociation of selected parent ions with helium in the mass

analyzer. Samples were dissolved in CH_3OH-H_2O (1:1) and introduced into the mass spectrometer through direct infusion (12.5 µL/min) for either negative ionization (ESI(-)-MS or ESI(-)-MS/MS) or positive ionization (ESI(+)-MS or ESI(+)-MS/MS) analysis. The heated capillary was maintained at 250 °C and the source voltage at 5 kV. LC-MS of the collected urine (1- 4 µL) was carried out on an Agilent 1100 HPLC equipped with an Agilent G1315B DAD photodiode array detector and connected with the LCQ ion trap mass spectrometer. A Varian Polaris C18 3 µm column (2.0 x 100 mm) was used for all LC-MS analyses. The solvent system consisted of solvent A: 0.1% formic acid in H_2O and solvent B: 0.1 % formic acid in CH_3CN and a gradient from 95% A and 5% B to 65% A and 35% B over 30 min at a flow rate of 200 µL/min. The MS was run in the negative ionization mode (ESI(-)-MS or ESI(-)-MS/MS), the heated capillary maintained at 300 °C, and the source voltage at 5 kV. This HPLC system was used for identification of the BGE metabolites by MS analysis, and will be referred to as LC/MS in the Results and Discussion.

Animals and treatments

Male (11.5-12.5 weeks old, 233-276 g) and female (13 weeks old, 179-196 g) F344 rats were obtained from Charles River Laboratories (Kingston, NY). Male (8 weeks old, 24-27 g) and female (7-9 weeks old, 19-22 g) B6C3F1 mice were obtained from Charles River Laboratories (Raleigh, NC). Male rats and female mice (n = 4 or 5/treatment groups) were dosed with [^{14}C]-labeled BGE (2, 20, or 200 mg/kg, 37-56 µCi/kg) by gavage. Female rats and male mice (n = 4 or 5/treatment groups) were dosed with [^{14}C]-labeled BGE (200 mg/kg, 53-55 µCi/kg) by gavage. All oral dose solutions were in corn oil, 5 mL/kg for rats and 10 mL/kg for mice. Animals were housed individually in metabolism cages allowing for the collection of

urine, feces, and expired air, and provided with food (NIH #31) and distilled water *ad libitum*. Animals were euthanized by CO_2 asphyxiation 24 h after dosing. The Institutional Animal Care and Use Committee approved all animal procedures.

Sample collection and analysis

Urine and feces were collected at 24 h following dosing. The metabolism cages were rinsed with distilled water at the end of each study to increase urine recovery. Urine aliquots were added to EcoLume (ICN Research Products Division, Costa Mesa, CA) and counted for ^{14}C in a liquid scintillation counter (LSC) (Beckman LS 6500, Beckman Instruments, Fullerton, CA) and the remainder was stored at –20 °C. Feces were air dried, weighed, and ground to a powder using a ceramic mortar and pestle. The amount of BGE-derived radioactivity in expired air was determined for male rats receiving a single 20 mg/kg oral dose and in both male and female mice using methods previously described (Sanders et al. 1998). After the animals were euthanized by CO_2 asphyxiation, blood was drawn by cardiac puncture, and the following tissues were collected: liver, kidney, lung, pancreas, thyroid, thymus, adrenal glands, abdominal muscle, skin (ear pinna), fat (abdominal), brain, testes or uterus, forestomach, glandular stomach, small intestine, and large intestine. All tissue weights were determined gravimetrically, except blood, fat, skin, and muscle, which were estimated to be 8, 11, 16, and 50% of total body weight, respectively (Matthews and Anderson, 1975; Birnbaum et al., 1980). Tissue and feces aliquots (triplicate 50 - 100 mg samples from rats, up to 100 mg from mice) were oxidized in a Packard 307 Biological Sample Oxidizer (Packard Instruments Co., Meriden, CT) and counted in the LSC for determination of total ^{14}C.

Isolation and spectral analysis of urinary metabolites

Urine collected 24 h post-dosing from male rats dosed orally with BGE (200 mg/kg) was analyzed by the HPLC with radiochemical detection to reveal 13 radiolabeled peaks **1**-**13** (Figure 1A). The metabolites were not observed by UV detection so the isolation of these urinary metabolites was accomplished by collection of HPLC fractions at 1-min intervals between 8 and 28 min. The solvents were evaporated by a Speed-Vac (Thermo Savant, Holbrook, NY) and the metabolites were characterized by MS and ^{1}H NMR. The spectral data of metabolites **1**-**13** were obtained and **1**-**12** were identified as the structures show in Figure 2.

Metabolite **1** had an HPLC retention time of 8.7 min. ^{1}H NMR (D_2O): δ 3.98-3.83 (m, 2H, 2-CH and butoxy 3'-CH), 3.71-3.46 (m, 6H, 1-CH_2, 3-CH_2, and butoxy 1'-CH_2), 1.74 (q, J = 6.9 Hz, 2H, butoxy 2'-CH_2), 1.19 (d, J = 6.2 Hz, 3H, butoxy 4'-CH_3).

Metabolite **2** had HPLC retention times of 9.6 and 10.1 min. ESI(-)MS/MS: m/z 177 $[M - H]^-$, 133 $[M - H - CO_2]^-$, 89 [3'-hydroxybutoxide]$^-$, 87 [2-hydroxyacrylate]$^-$. ^{1}H NMR (D_2O) of the major isomer: δ 1.73 (q, 6.6 Hz, 2H, butoxy 2'-CH_2), 1.18 (d, J = 6.5 Hz, 3H, butoxy 4'-CH_3); the minor isomer: δ 1.62 (q, 7.5 Hz, 2H, butoxy 2'-CH_2), 1.11 (d, J = 7.2 Hz, 3H, butoxy 4'-CH_3), the other protons were not well resolved or overlapped with the signals of endogenous compounds.

Metabolite **3** had HPLC retention times of 10.9 and 11.1 min. ESI(+)MS/MS: m/z 220 $[M + H]^+$, 202 $[M + H - H_2O]^+$, 184 $[M + H - 2\ H_2O]^+$, 130 [2-acetylaminoacrylic acid + H]$^+$, 90 [3'-hydroxybutanol]$^+$. ESI(-)MS: m/z 218 $[M - H]^-$. ^{1}H NMR (D_2O) of one diastereomer: δ 1.18 (d, J = 6.9 Hz, 3H, butoxy 4'-CH_3); the other diastereomer: δ 1.17 (d, J = 7.2 Hz, 3H, butoxy 4'-CH_3), the other protons were not well resolved or overlapped with the signals of endogenous compounds.

Metabolite **4** had an HPLC retention time of 13.9 min. ESI(-)MS/MS: m/z 308 $[M - H]^-$, 179 [3-(3'-hydroxybutoxy)-2-hydroxy-1-propanethiol - H]$^-$, 128 [2-acetylaminoacrylate]$^-$. ^{1}H NMR (D_2O): δ 4.45 (dd, J = 7.3, 4.6 Hz, 1H, Cys α-CH), 3.96-3.90 (m, 2H, 2-CH and butoxy 3'-CH), 3.66-3.48 (m, 4H, 3-CH_2 and butoxy 1'-CH_2), 3.09 (dd, J = 13.8, 4.5 Hz, 1H, cys β-CH_a), 2.92 (dd, J = 13.6, 7.6 Hz, 1H, cys β-CH_b), 2.79 (dd, J = 13.8, 5.3 Hz, 1H, 1-CH_a), 2.64 (dd, J = 13.8, 6.7 Hz, 1H, 1-CH_b), 2.06 (s, 3H, $COCH_3$), 1.74 (q, J = 6.9 Hz, 2H, butoxy 2'-CH_2), 1.19 (d, J = 6.6 Hz, 3H, butoxy 4'-CH_3).

Metabolite **5** had an HPLC retention time of 14.3 min. ESI(-)MS/MS: m/z 322 $[M - H]^-$, 219 [M - 4-hydroxybutanoic acid]$^-$, 193 [M - H - 2-acetylaminoacrylic acid]$^-$, 175 [M - H - 2-acetylaminoacrylic acid - H_2O]$^-$, 128 [2-acetylaminoacrylate]$^-$, 103 [4-hydroxybutanoate]$^-$. ^{1}H NMR (D_2O): δ 3.93 (br. s, 1H, 2-CH), 3.63-3.50 (m, 4H, 3-CH_2 and butoxy 1'-CH_2), 3.09 (dd, J = 12.6, 3.7 Hz, 1H, cys β-CH_a), 2.92 (dd, J = 13.3, 8.0 Hz, 1H, cys β-CH_b), 2.79 (dd, J = 14.6, 4.3 Hz, 1H, 1-CH_a), 2.63 (dd, J = 14.5, 8.2 Hz, 1H, 1-CH_b), 2.44 (t, J = 7.1 Hz, 2H, butoxy 3'-CH_2), 2.06 (s, 3H, $COCH_3$), 1.95-1.82 (m, 2H, butoxy 2'-CH_2).

Metabolite **6** had an HPLC retention time of 16.2 min. ESI(-)MS/MS: m/z 227 $[M - H]^-$, 153 [M - H - butanol]$^-$, 97 $[HSO_4]^-$, 81 $[HSO_3]^-$. ^{1}H NMR (D_2O): δ 4.49 (quintet, J = 4.8 Hz, 1H, 2-CH), 3.87-3.55 (m, 6H, 1-CH_2, 3-CH_2, and butoxy 1'-CH_2), 1.56 (quintet, J = 7.4 Hz, 2H, butoxy 2'-CH_2), 1.34 (sextet, J = 7.4 Hz, 2H, butoxy 3'-CH_2), 0.90 (t, J = 7.4 Hz, 3H, butoxy 4'-CH_3).

Metabolite **7** had an HPLC retention time of 17.3 min. ESI(-)MS/MS: m/z 323 $[M - H]^-$, 305 [M - H - H_2O]$^-$, 287 [M - H - 2 H_2O]$^-$, 175 [glucuronide - H_2O]$^-$, 157 [glucuronide - 2 H_2O]$^-$, 147 [M - H - 176 (Gluc)]$^-$ or [3-butoxy-2-hydroxy-1-propanol - H]$^-$. ^{1}H NMR (D_2O): δ 4.51 (d, J = 7.7 Hz, 1H, Gluc 1"-CH), 1.06-3.96 (m, 1H, 2-CH), 3.92-3.86 (m, 1H, Gluc 5"-CH), 3.77-3.50

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(m, 8H, 1-CH_2, 3-CH_2, butoxy 1'-CH_2, and Gluc 2"-CH, 4"-CH), 3.36 (t, J = 8.2 Hz, 1H, Gluc 3"-CH), 1.56 (quintet, J = 7.2 Hz, 2H, butoxy 2'-CH_2), 1.34 (sextet, J = 7.5 Hz, 2H, butoxy 3'-CH_2), 0.89 (t, J = 7.4 Hz, 3H, butoxy 4'-CH_3).

Metabolite **8** had an HPLC retention time of 19.4 min. ESI(-)MS/MS: m/z 161 $[M - H]^-$, 115 $[M - H - HCOOH]^-$, 73 $[butoxide]^-$. ^1H NMR (D_2O): δ 4.30 (dd, J = 4.9, 3.1 Hz, 1H, 2-CH), 3.83-3.67 (m, 2H, 3-CH_2), 3.64-3.49 (m, 2H, butoxy 1'-CH_2), 1.55 (quintet, J = 7.3 Hz, 2H, butoxy 2'-CH_2), 1.33 (sextet, J = 7.4 Hz, 2H, butoxy 3'-CH_2), 0.89 (t, J = 7.3 Hz, 3H, butoxy 4'-CH_3).

Metabolite **9** had an HPLC retention time of 21.7 min. ESI(-)MS/MS: m/z 202 $[M - H]^-$, 160 $[M - H - COCH_2]^-$, 128 $[2\text{-acetylaminoacrylate}]^-$, 84 $[2\text{-acetylaminoacrylate} - CO_2]^-$. ^1H NMR (D_2O): δ 4.58 (t, J = 5.0 Hz, 1H, 2-CH), 3.88-3.78 (m, 2H, 3-CH_2), 3.62-3.48 (m, 2H, butoxy 1'-CH_2), 2.06 (s, 3H, $COCH_3$), 1.59 (quintet, J = 7.2 Hz, 2H, butoxy 2'-CH_2), 1.32 (sextet, J = 7.4 Hz, 2H, butoxy 3'-CH_2), 0.88 (t, J = 7.3 Hz, 3H, butoxy 4'-CH_3).

Metabolite **10** had an HPLC retention time of 22.4 min. ESI(-)MS: m/z 131 $[M - H]^-$. This ion did not fragment upon MS/MS analysis. ^1H NMR (D_2O): δ 3.89 (s, 2H, CH_2CO), 3.52 (t, J = 7.3 Hz, 2H, butoxy 1'-CH_2), 1.57 (quintet, J = 8.2 Hz, 2H, butoxy 2'-CH_2), 1.34 (sextet, J = 7.6 Hz, 2H, butoxy 3'-CH_2), 0.89 (t, J = 7.7 Hz, 3H, butoxy 4'-CH_3).

Metabolite **11** had an HPLC retention time of 23.9 min. ESI(-)MS/MS: m/z 292 $[M - H]^-$, 250 $[M - H - COCH_2]^-$, 163 [3-butoxy-2-hydroxy-1-propanethiol - H]$^-$, 128 [2-acetylaminoacrylate]$^-$. ^1H NMR (D_2O): δ 4.59 (dd, J = 7.7, 4.7 Hz, 1H, Cys α-CH), 3.95 (quintet, J = 5.7 Hz, 1H, 2-CH), 3.60-3.47 (m, 4H, 3-CH_2 and butoxy 1'-CH_2), 3.13 (dd, J = 14.1, 4.5 Hz, 1H, cys β-CH_a), 2.96 (dd, J = 13.8, 8.0 Hz, 1H, cys β-CH_b), 2.81 (dd, J = 14.1, 5.4 Hz, 1H, 1-CH_a), 2.65 (dd, J = 13.8, 7.2 Hz, 1H, 1-CH_b), 2.06 (s, 3H, $COCH_3$), 1.57 (quintet, J = 7.3

Hz, 2H, butoxy 2'-CH_2), 1.34 (sextet, J = 7.5 Hz, 2H, butoxy 3'-CH_2), 0.89 (t, J = 7.4 Hz, 3H, butoxy 4'-CH_3).

Metabolite **12** had an HPLC retention time of 26.1 min. ^{1}H NMR (D_2O): δ 3.73 (t, J = 4.6 Hz, 2H, 1-CH_2), 3.57 (t, J = 5.6 Hz, 2H, 2-CH_2), 3.52 (t, J = 8.8 Hz, 2H, butoxy 1'-CH_2), 1.55 (quintet, J = 7.1 Hz, 2H, butoxy 2'-CH_2), 1.33 (sextet, J = 7.3 Hz, 2H, butoxy 3'-CH_2), 0.89 (t, J = 7.4 Hz, 3H, butoxy 4'-CH_3).

Metabolite **13** had an HPLC retention time of 27.0 min. ^{1}H NMR (D_2O): δ 4.21 (quintet, J = 4.5 Hz, 1H, 2-CH), 3.86-3.70 (m, 3H, 3-CH_2 and 1-CH_a), 3.61-3.50 (m, 3H, butoxy 1'-CH_2 and 1-CH_b), 1.57 (quintet, J = 7.4 Hz, 2H, butoxy 2'-CH_2), 1.35 (sextet, J = 7.7 Hz, 2H, butoxy 3'-CH_2), 0.90 (t, J = 7.3 Hz, 3H, butoxy 4'-CH_3).

HPLC analysis of urine of female mice administered a 200 mg/kg oral dose revealed metabolites with similar retention times as those present in rat urine (**1**-**13**) and three additional radiolabeled peaks (**14**-**16**) (Figure 1B). The HPLC retention times of **14**-**16** were as follows: **14** (17.7 min), **15** (23.3 min), and **16** (29.0 min). Metabolite **15** had an LC/MS retention time at 20.8 min and an ESI(-)-MS/MS spectrum as follows: m/z 251 $[M - H]^-$, 233 $[M - H - H_2O]^-$, 163 [3-butoxy-2-hydroxy-1-propanethiol - H]$^-$, 87 [2-hydroxyacrylate]$^-$. Metabolite **16** had an LC/MS retention time at 25.7 min and an ESI(-)-MS/MS spectrum as follows: m/z 249 $[M - H]^-$, 205 $[M - H - CO_2]^-$, 163 [3-butoxy-2-hydroxy-1-propanethiol - H]$^-$.

Incubation of BGE with GSH and glutathione S-transferase

Incubations of ^{14}C-BGE (1 mM) with GSH (5 mM) were conducted in a 0.1 M potassium phosphate buffer (pH 7.4) in the presence of glutathione S-transferase (0.4 mg/mL). [^{14}C]-BGE was added as a CH_3CN solution (100 mM, 0.25 mCi/mmol, 10 µL). The final volume was 1 mL

and the mixture was incubated at 37 °C in capped vials for 1 h. Control experiments omitting either GSH or glutathione S-transferase or both were included. The incubation mixtures were then placed on ice and analyzed by HPLC without further treatment. HPLC analysis of the incubation mixture containing BGE, GSH, and GST revealed one radiolabeled peak eluting at 18.7 min. The product was isolated by HPLC and its spectral data were as follows: ESI(-)MS/MS: m/z 436 $[M - H]^-$, 418 $[M - H - H_2O]^-$, 307 [M - H - 129 (glutamate)]$^-$, 272 [glutathione - H - $H_2S]^-$, 254 [glutathione - H - H_2S - $H_2O]^-$. ^{1}H NMR (D_2O): δ 4.59 (dd, J = 8.7, 4.0 Hz, 1H, Cys α-CH), 3.95 (quintet, J = 5.8 Hz, 1H, 2-CH), 3.91 (s, 2H, Gly α-CH_2), 3.78 (t, J = 6.5 Hz, 1H, Glu α-CH), 3.70-3.46 (m, 4H, 3-CH_2 and butoxy 1'-CH_2), 3.09 (dd, J = 14.5, 5.5 Hz, 1H, cys β-CH_a), 2.90 (dd, J = 13.5, 7.4 Hz, 1H, cys β-CH_b), 2.79 (dd, J = 13.0, 4.7 Hz, 1H, 1-CH_a), 2.63 (dd, J = 14.6, 7.4 Hz, 1H, 1-CH_b), 2.53 (td, J = 8.2, 2.3 Hz, 2H, Glu γ-CH_2), 2.15 (quartet, J = 7.1 Hz, 2H, Glu β-CH_2), 1.55 (quintet, J = 7.3 Hz, 2H, butoxy 2'-CH_2), 1.33 (sextet, J = 7.3 Hz, 2H, butoxy 3'-CH_2), 0.89 (t, J = 7.4 Hz, 3H, butoxy 4'-CH_3).

Statistics

Statistical analysis used JMP Software (SAS Institute, Cary, NC) and consisted of an ANOVA followed by pair-wise comparison using a Tukey-Kramer test. Values were considered statistically significant at $p \leq 0.05$.

Results

Excretion

The disposition of BGE in rats and mice 24 h after a single oral dose is shown in Table 1. Most of the dose (2-200 mg/kg) administered to male and female rats was excreted in urine (84-92%) within 24 h. The rest of the dose was excreted in feces (2.6-7.7%), expired air (0.1% volatiles, and 1.5% CO_2, 20 mg/kg), or remained in the tissues (1.8-4.4%). There is no obvious dose effect on disposition among the range of doses (2-200 mg/kg) in male rats. Female rats have less of the dose remaining in tissues compared to males. Most of the dose given to male and female mice was also excreted in urine (64-73%). Mice appeared to excrete a larger percent of the dose in feces (5.3-12%), but separation of urine and feces in mouse metabolism cages was not complete and contamination of urine with feces occurred in some experiments. This resulted in an underestimation of the recovery from urine and overestimation of recovery from feces. As a result of the contamination some of the urine samples were not useful for metabolic profile analysis. Mice excreted a larger percent of the oral dose in expired air as $^{14}CO_2$ (10-18%) and had less remaining in the tissues (1.5-1.7%) than rats within 24 h**.** Mice that received a 200 mg/kg dose excreted more $^{14}CO_2$ than the lower dose treatment (2 and 20 mg/kg). The total recovery was 93% to 98% for rats and 88% to 97% for mice**.**

Tissue distribution

The BGE-derived radioactivity remaining in tissues 24 h after dosing with 2-200 mg/kg accounted for less than 5% of the dose (Table 1). Table 2 summarizes the tissue distribution data 24 h after a single oral dose (200 mg/kg) to male and female rats and mice. The concentration of BGE-derived radioactivity in glandular and forestomach is high in mice. Tissue distribution data

were obtained for 2 and 20 mg/kg doses for male rats and female mice (data not shown). The distribution is generally dose-proportional and with the exception of forestomach, the data for the 200 mg/kg dose adequately describe the distribution of lower doses. Forestomach concentration after an oral dose of 2, 20 and 200 mg/kg was 42, 530, and 1770 nmol/g in female mice and 9.6, 90 and 323 nmol/g in male rats. Tissues listed in Materials and Methods, but not appearing in Table 2, contained 0.01% or less of the dose for both species and sexes.

Identification of urinary metabolites in rats

The urinary metabolite profile from male rats dosed orally with BGE (200 mg/kg) is shown in Figure 1A, which adequately describes the urinary metabolite profiles from male rats dosed with 2 and 20 mg/kg and female rats dosed with 200 mg/kg. The spectral data for these thirteen metabolites (designated **1**-**13** in Figure 1A) were obtained and reported in the Materials and Methods and **1**-**12** were identified as the structures show in Figure 2. Some of these metabolites exist as stereoisomers. C-2 of BGE is an asymmetric center, so BGE exists as two enantiomers. There are up to 3 chiral centers present at BGE metabolites, so in theory **8** and **9** could include two enantiomers, **1**-**3** could include two sets of enantiomers that are diastereomeric, **5** and **11** could include two diastereomers, and **4** could include four diastereomers. However, NMR analysis of these metabolites either could not distinguish the diastereomers or show only the major isomer. The HPLC radiochromatograms in Figure 1 demonstrate more than one isomer for some of these metabolites, but the exact numbers of isomers cannot be established because these stereoisomers are not always adequately separated.

Metabolite **1** did not ionize upon ESI-MS analysis so we were not able to obtain the molecular weight (MW). The NMR data clearly indicate ω-1 oxidation of the butoxy group and

eight protons on carbons attached to O. The difference in retention times between the other ω-1 oxidation products and the primary metabolites is: 9.6 min (**2** and **8**), 10.7 min (**3** and **9**), and 10.0 min (**4** and **11**). We suspect that metabolite **1** is the ω-1 oxidation product of 3-butoxy-2-hydroxy-1-propanol (**14**), the hydration product of BGE, because (1) **14** had an HPLC retention time at 17.7 min, 9.0 min difference in retention times from **1**, (2) **14** contains seven protons on carbons attached to O, ω-1 oxidation would give a product with eight protons on carbons attached to O, and (3) both **14** and **1** are Phase I metabolites that do not ionize readily. Metabolite **1** is tentatively identified as 3-(3'-hydroxybutoxy)-2-hydroxy-1-propanol.

The two isomeric metabolites **2** have a MW of 178, 16 Da higher than those of 3-butoxy-2-hydroxypropionic acid (**8**) and the NMR result indicates ω-1 oxidation of the butoxy group. The MS fragmentation is consistent with 3'-hydroxybutoxy- and 2-hydroxypropionic acid being parts of the molecule. Metabolite **2** is identified as 3-(3'-hydroxybutoxy)-2-hydroxypropionic acid.

The two isomeric metabolites **3** have a MW of 219, 16 Da higher than those of O-butyl-N-acetylserine (**9**) and the NMR result indicates ω-1 oxidation of the butoxy group. The MS fragmentation is consistent with 3'-hydroxybutoxy- and 2-acetylaminopropionic acid being parts of the molecule. Metabolite **3** is identified as O-(3'-hydroxybutyl)-N-acetylserine.

Metabolite **4** has a MW of 309, 16 Da higher than that of 3-butoxy-2-hydroxy-1-(N-acetylcystein-S-yl)-2-propanol (**11**) and the NMR result indicates ω-1 oxidation of the butoxy group. The MS fragmentation is consistent with 3-(3'-hydroxybutoxy)-2-hydroxy-1-propanethiol and 2-acetylaminopropionic acid being parts of the molecule. The NMR spectrum shows two sets of CH_2 groups as AB quartets with chemical shifts around 3 ppm, consistent with two CH_2 groups attached to S and therefore, the attachment of the mercapturic acid is at the C-1

position. Metabolite **4** is generated from ω-1 oxidation of **11,** and identified as 3-(3'-hydroxybutoxy)-2-hydroxy-1-(N-acetylcystein-S-yl)-2-propanol.

Metabolite **5** has a MW of 323, 30 Da higher than that of 3-butoxy-1-(N-acetylcystein-S-yl)-2-propanol (**11**), indicating oxidation of a methyl group to a carboxylic acid. The MS fragmentation is consistent with 4-hydroxybutanoic acid and 2-acetylaminopropionic acid being parts of the molecule. The NMR result confirms the absence of the butoxy 4'-CH_3 group in metabolite **5** and show two sets of CH_2 groups as AB quartets with chemical shifts around 3 ppm, consistent with two CH_2 groups attached to S and therefore, the attachment of the mercapturic acid is at the C-1 position. Metabolite **5** is generated from ω-oxidation of the butoxy group in **11**.

MS/MS demonstrated that **6** is a sulfate conjugate and its MW of 228 is equivalent to BGE + H_2O +SO_3. The NMR spectrum contained peaks for seven protons on carbons attached to O, which is in agreement with a diol structure. The chemical shift of the 2-CH is downfield compared to the corresponding proton in **1**, which implies that the sulfate conjugation is likely on 2-OH but the assignment is not certain. Metabolite **6** is identified as a mono-sulfate conjugate of 3-butoxy-2-hydroxy-1-propanol.

Metabolite **7** was hydrolyzed by β-glucuronidase to its aglycone (RT = 17.7 min), which had a similar retention time as that of 3-butoxy-2-hydroxy-1-propanol (**14**). MS/MS confirms that **7** is a glucuronide conjugate and its MW of 324, is equivalent to BGE + H_2O + glucuronide. NMR showed several protons on carbons attached to O, which is in agreement with a diol structure and conjugation with a glucuronide. Metabolite **7** is identified a mono-glucuronide conjugate of 3-butoxy-2-hydroxy-1-propanol with the conjugation on either 1-OH or 2-OH.

The MW (162) of **8** is consistent with that of a previously identified BGE metabolite, 3-butoxy-2-hydroxypropionic acid (Eadsforth *et al.* 1985). The NMR data are consistent with the proposed structure. Metabolite **8** is identified as 3-butoxy-2-hydroxypropionic acid.

The MW (203) of **9** is consistent with that of a previously identified BGE metabolite, O-butyl-N-acetylserine (Eadsforth *et al*. 1985). The NMR data are consistent with the proposed structure. Metabolite **9** is identified as O-butyl-N-acetylserine.

The MW (132) of **10** is consistent with that of a previously identified BGE metabolite, butoxyacetic acid (Eadsforth *et al*. 1985). The NMR data are consistent with the proposed structure. Metabolite **10** is identified as butoxyacetic acid.

Metabolite **11** has a MW of 193, equivalent to BGE + N-acetylcysteine. The MS fragmentation is consistent with 3-butoxy-2-hydroxy-1-propanethiol and 2-acetylaminopropionic acid being parts of the molecule. NMR shows two sets of CH_2 groups as AB quartets with chemical shifts around 3 ppm, consistent with two CH_2 groups attached to S, and therefore, the attachment of the mercapturic acid is at the C-1 position. Metabolite **11** is identified as 3-butoxy-1-(N-acetylcystein-S-yl)-2-propanol.

Metabolite **12** did not ionize upon ESI-MS analysis. NMR showed three CH_2O as triplets, implying that the metabolite might be 2-butoxyethanol. NMR of authentic 2-butoxyethanol showed an identical spectrum as that of **12**. Metabolite **12** is identified as 2-butoxyethanol.

Metabolite **13** did not ionize upon ESI-MS analysis. We were not able to identify this metabolite based on its NMR data, so **13** remains unidentified at this point.

Identification of urinary metabolites in mice

HPLC analysis of urine of female mice administered a 200 mg/kg oral dose revealed metabolites with similar retention times as those present in rat urine (**1**-**13**) and three additional radiolabeled peaks (**14**-**16**) (Figure 1B). The metabolite profile shown in Figure 1B adequately describes the urinary metabolite profiles from female mice dosed with 20 mg/kg and male mice dosed with 200 mg/kg. Metabolite **14** had a similar HPLC retention time (17.7 min) as that of the aglycone of **7** after β-glucuronidase hydrolysis. Microsomal incubation (no NADPH) of BGE also yielded a sole product that had a similar retention time as that of **14**, likely a diol from hydration of BGE catalyzed by epoxide hydrolase (data not shown). Metabolite **14** is identified as 3-butoxy-2-hydroxy-1-propanol. Female mouse urine was also analyzed by LC-ESI(-)-MS to reveal metabolites **2**-**9**, **11**, **15**, and **16**. Metabolite **15** has a MW of 252, consistent with an α-hydroxy acid generated from oxidative deamination of a cysteine conjugate of BGE, 3-butoxy-1-(cystein-S-yl)-2-propanol (MW 251), followed by reduction. The MS fragmentation is consistent with 3-butoxy-2-hydroxy-1-propanethiol and 2-hydroxypropionic acid being parts of the molecule (Figure 2). Metabolite **16** has a MW of 250, consistent with an α-keto acid generated from oxidative deamination of the cysteine conjugate of BGE, 3-butoxy-1-(cystein-S-yl)-2-propanol (MW 251). The MS fragmentation is consistent with 3-butoxy-2-hydroxy-1-propanethiol being a part of the molecule (Figure 2).

The radioactivity in each of the peaks in the urine metabolite profiles from rats and mice was quantified as a percent of the total radioactivity in all the peaks. The metabolites are products of two pathways, with the initial metabolism being either hydrolysis or GSH addition. Metabolites **1**-**3**, **6**-**10**, and **12** are hydrolysis pathway products. Metabolites **4**, **5**, **11**, **15**, and **16** arise from an initial addition of GSH. Table 3 presents the result of the total percentages of metabolites in each of the two pathways. The 2 mg/kg oral dose excreted in urine of female

mice was estimated entirely from the water rinse of the cage, so HPLC quantification of the radiolabeled peaks present in urine was not performed for this treatment.

Incubation of BGE with GSH and GST

The NMR and MS data of the sole product in the incubation demonstrate a GSH conjugate of BGE. Two sets of AB quartets with chemical shifts around 3 ppm are consistent with two CH_2 groups attached to S, indicating the addition of GSH occurred at the C-1 position of BGE. The product is identified as 3-butoxy-1-(glutathion-S-yl)-2-propanol. The product exists as two diastereomers with similar but not exactly the same NMR spectra. Therefore, the resonances from the two CH_2 groups attached to S are not well defined, due to the overlapping of the signals from two diastereomers.

Discussion

The disposition of BGE in male and female rats is similar to the previous study of BGE in male rats treated with a single 20 mg/kg oral dose (Eadsforth *et al*. 1985). Both this and the previous (Eadsforth *et al*. 1985) studies show that oral administration of BGE results in rapid absorption and metabolism with most of the dose excreted in urine within 24 h. Most of the dose given to mice was also excreted in urine. A major difference between rats and mice is that mice excreted more of the dose in expired air as $^{14}CO_2$ (10-18%, 2-200 mg/kg) than rats (1.5%, 20 mg/kg). Less than 5% of the dose remained in the tissues after 24 hours. After 10 consecutive daily 20 mg/kg doses tissue concentrations in nmol-eq BGE/g tissue were generally 2 to 4 times higher than after one dose (data not shown), indicating that there would be some accumulation of BGE and/or its metabolites in tissues during a chronic toxicity study. The highest concentration of BGE-derived radioactivity is in forestomach. This is also the only tissue where the concentrations may not be dose-proportional. It is a small tissue and the site of application, so non-linearity is not unexpected.

Fifteen urinary metabolites of BGE were identified (Figure 2). The metabolism of BGE follows two major pathways: 1) hydration to give a diol (**14**), 2) conjugation with GSH. Diol formation is followed by further metabolism to sulfate (**6**) or glucuronide (**7**) conjugates or an α-hydroxy acid (**8**). Oxidation of the hydroxy acid to an α-keto acid provides a precursor for oxidative decarboxylation to butoxyacetaldehyde or transamination followed by acetylation to O-butyl-N-acetylserine (**9**). Butoxyacetaldehyde is further oxidized to butoxyacetic acid (**10**) or reduced to 2-butoxyethanol (**12**). The structural identification of the GSH conjugate of BGE from the in vitro experiment and the urinary mercapturic acid metabolites **4**, **5**, and **11** indicates that GSH conjugation with BGE occurred only at the C-1 position. Metabolites **8**, **9**, **11**, and **14**

undergo further ω-1 oxidation to form the corresponding 3-hydroxybutoxy-substituted metabolites **2**, **3**, **4**, and **1**. Metabolite **5** is generated from ω-oxidation of **11** to the corresponding carboxylic acid. The precursor to **11**, 3-butoxy-1-(cystein-S-yl)-2-propanol, undergoes oxidative deamination in mice only to the corresponding α-keto acid **16**, which is further reduced to an α-hydroxy acid **15**. Some of the BGE metabolic pathways are common biosynthetic pathways for amino acids and fatty acids, due to metabolism of BGE to an α-hydroxy acid and the long carbon chain in the structure.

Metabolites **7** and **14** were not totally separated by HPLC and therefore were quantified together, although the diol exists predominantly as an aglycone (**14**) in mice and as a glucuronide conjugate (**7**) in rats. Transamination and acetylation of the α-keto acid from oxidation of **14** to metabolite **9** is a major pathway in rats but not in mice, and as a result, the alternative metabolic pathway to **10** and **12** becomes dominant in mice, which might explain a larger amount of the dose excreted as CO_2 in mice. In comparison, oxidative deamination of 3-butoxy-1-(cystein-S-yl)-2-propanol to **15** and **16** occurs in mice only, especially in female mice. The ratio of metabolites formed via the hydrolysis pathway compared to the GSH pathway is dose-related, at least in male rats (Table 3). A linear decrease in GSH pathway products as the dose increases implies a dose-related GSH depletion. The hydrolysis/GSH ratio is similar for rats and male mice given a dose of 200 mg/kg. Female mice appear to require a higher dose to deplete GSH sufficiently to change the hydrolysis/GSH ratio (Table 3). The dose-related GSH depletion might explain that more of the higher dose (200 mg/kg) excreted in expired air (14%, male mice, and 18%, female mice) than the lower dose treatment (11%, 2 mg/kg, female mice and 10%, 20 mg/kg, female mice).

BGE is reported to cause CNS toxicity after acute intraperitoneal or intragastric administration, or inhalation exposure, but little BGE-derived radioactivity was detected in brain in this study. The epoxy group in BGE is reactive and may be responsible for the high forestomach/glandular concentration ratio, 4-5 in rats and 10 in mice, 24 h after a 200 mg/kg dose of BGE (Table 2). 2-Butoxyethanol is structurally similar to BGE, but does not have an epoxy group and is not a direct alkylating agent. Nevertheless 48 h after a 500 mg/kg dose of 2-butoxyethanol to male rats, the forestomach contains relatively high concentrations of 2-butoxyethanol-derived radioactivity, about 4 times the concentration found in the adjacent glandular stomach (Ghanayem, et al., 1987). Forestomach is a target tissue in the 2-year bioassay of 2-butoxyethanol in mice (NTP, 2000). Another target tissue associated with 2-butoxyethanol is erythrocytes. A metabolite, butoxyacetic acid, has been implicated in the erythrotoxictiy (Ghanayem et al., 1989). Butoxyacetic acid and 2-butoxyethanol are BGE metabolites. Blood/plasma ratios for BGE-treated animals are about 1 indicating no increased uptake of BGE radioactivity by erythrocytes. At the doses given in this study no erythrotoxicity was observed. It is likely that BGE will cause site-of-application toxicity in a chronic study. Small amounts of butoxyacetic acid and 2-butoxyethanol from BGE metabolism might not cause any significant toxicity.

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Footnotes

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Legends for Figures

Figure 1. Representative HPLC radiochromatograms of urine collected 24 h following a single oral dose (200 mg/kg) of BGE to (A) a male rat and (B) a female mouse. The urinary metabolites are designated **1**-**16**.

Figure 2. Proposed metabolic pathways of BGE in rats and mice.

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Table 1. Dispositionof BGE-derived radioactivity in excreta 24 h after a single oral dose or the last dose of 10 oral doses. (Percent dose ± SD, n = 4 or 5)

Dose	Male Rat 2 mg/kg	Male Rat 20 mg/kg	Male Rat 200 mg/kg	Female Rat 200 mg/kg
Urine	91.5 ± 1.3	84.3 ± 2.6	89.1 ± 2.1	87.1 ± 17.4
Feces[a]	2.6 ± 0.4	7.7 ± 2.0	3.0 ± 0.5	4.2 ± 4.4
Gut contents[b]	0.4 ± 0.2	0.4 ± 0.1	0.9 ± 0.1	0.2 ± 0.1
Tissues	2.7 ± 0.8	3.1 ± 1.4	4.4 ± 1.9	1.8 ± 0.1
Volatiles in expired air	ND[c]	0.1 ± 0.1	ND	ND
$^{14}CO_2$ in expired air	ND	1.5 ± 0.1	ND	ND
Total	97.3 ± 1.7	97.1 ± 2.8	97.9 ± 1.7	93.3 ± 13.6

Dose	Female Mouse 2 mg/kg	Female Mouse 20 mg/kg	Female Mouse 200 mg/kg	Male Mouse 200 mg/kg
Urine	73.2 ± 13.4	68.6 ± 5.5	71.0 ± 1.7	63.6 ± 5.1
Feces[a]	11.5 ± 9.2	7.7 ± 3.5	5.3 ± 2.3	12.2 ± 6.2
Gut contents[b]	0.1 ± 0.1	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
Tissues	1.6 ± 0.7	1.5 ± 0.1	1.7 ± 0.1	1.6 ± 0.1
Volatiles in expired air	0.2 ± 0.0	0.4 ± 0.1	0.2 ± 0.0	0.3 ± 0.2
$^{14}CO_2$ in expired air	10.8 ± 0.8	9.8 ± 1.2	17.9 ± 1.2	14.2 ± 0.8
Total	97.3 ± 5.9	88.0 ± 5.6	96.3 ± 2.8	92.0 ± 4.7

[a] Feces includes large intestine contents.

[b] Stomach and small intestine contents.

[c] Not determined.

Table 2. Distribution (nmol-eq BGE/g tissue ± SD, n = 4 or 5) of BGE-derived radioactivity 24 h after a single 200 mg/kg oral dose.

Tissue[a]	Male Rat	Female Rat	Male Mouse	Female Mouse
Blood	39.4 ± 1.3	35.7± 3.5	18.1 ± 1.6	16.0 ± 2.3
Plasma	40.4± 2.9	38.7 ± 2.9	26.8 ± 4.8	18.6 ± 3.9
Liver	125± 9.5	150 ± 22	97.4 ± 15.2	75.9 ± 3.8
Kidney	97.2± 24.6	71.3 ± 5.0	128 ± 18	42.9 ± 5.5
Lung	69.3 ± 7.5	61.8 ± 2.8	44.1 ± 5.13	42.4 ± 4.2
Muscle	52.0 ± 40.9	11.7 ± 1.3	11.7 ± 1.4	15.0 ± 2.6
Skin	49.7 ± 3.5	36.7 ± 4.9	38.2 ± 3.4	38.2 ± 4.1
Fat	182 ± 95.7	40.0 ± 11.8	15.9 ± 2.3	32.9 ± 8.8
Brain	21.8 ± 2.2	16.8 ± 3.0	27.9 ± 36.5	11.5 ± 1.6
Forestomach	323 ± 29	394 ± 79	1750 ± 230	1770 ± 220
Glandular stomach	82.4 ± 18.4	74.3± 18.0	171 ± 21.6	184 ± 61
Small intestine	351 ±164	117 ± 9.8	80.0 ± 5.8	65.7 ± 5.7
Large intestine	351 ±154	175 ± 44	68.2 ± 6.1	50.1 ± 7.6

[a] Tissues listed in Methods, but not appearing in the Table contained 0.01% or less of the dose.

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Table 3. Percentage ± SD (n = 3-5) of urinary metabolites produced by hydration or GSH conjugation biotransformation pathways.

Sex, Species, Dose	Hydration Pathway	GSH Pathway
Male, Rat, 2 mg/kg	63.8 ± 1.2[a]	35.1 ± 1.6[b]
Male, Rat, 20 mg/kg	68.0 ± 2.2[a]	31.2 ± 2.5[b]
Male, Rat, 200 mg/kg	75.6 ± 1.0[a]	21.6 ± 1.4[b]
Female, Rat, 200 mg/kg	73.9 ± 0.9	22.3 ± 1.4
Male, Mouse, 200 mg/kg	73.0 ± 1.8	22.9 ± 1.5
Female, Mouse, 20 mg/kg	60.7 ± 1.4	38.0 ± 1.6
Female, Mouse, 200 mg/kg	65.9 ± 1.7[c]	31.4 ± 2.3[c]

[a, b] Significant dose-related differences, data with the same superscript are significantly different;

[c] Significantly different from other 200 mg/kg dose groups

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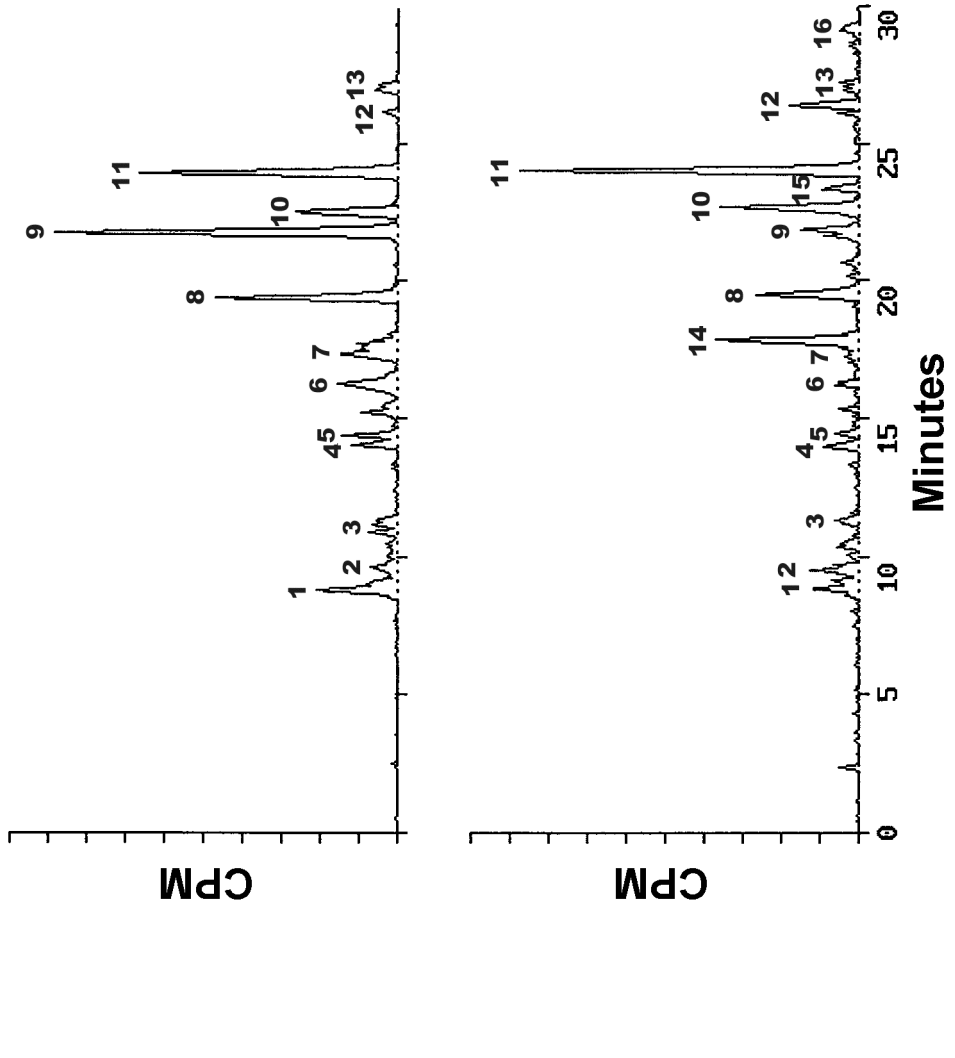

Figure 1

Figure 2