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Liquid chromatographic determination of the glutathione conjugate and ring-opened metabolites formed from coumarin epoxidation

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Abstract

Species differences in the biotransformation of coumarin are thought to play an important role in its toxicity. Since the putative toxic metabolite is coumarin 3,4-epoxide (CE), methods to measure the metabolites of CE were developed. The glutathione (GSH) conjugate of CE (CE-SG) at the 3-position was purified by reversed-phase (RP)-high performance liquid chromatography (HPLC), and characterized by mass spectrometry (MS) and nuclear magnetic resonance spectroscopy (NMR). An RP-HPLC method was developed to quantify CE-SG in hepatic microsomal mixtures and a separate RP-HPLC method was also developed to quantify the three ring-opened coumarin metabolites; *o*-hydroxyphenylacetic acid (*o*-HPAA), *o*-hydroxyphenylethanol (*o*-HPE) and *o*-hydroxyphenylacetaldehyde (*o*-HPA) in hepatic microsomal mixtures. Detection limits for all four products of coumarin epoxidation exceeded 3.5 ng/ml and recovery from hepatic microsomal mixtures was essentially quantitative with RSD values less than 8%. Species differences in *o*-HPA detoxification were consistent with sensitivity to coumarin, thereby demonstrating that these methods have utility in addressing the fate of CE and its contribution to toxicity.

Keywords: Glutathione; Coumarin

1. Introduction

Coumarin (1,2-benzopyrone) is a natural product used in perfumes and detergents as a fixative and enhancing agent [1]. Although coumarin is widely recognized as a rat liver toxicant [1], humans are relatively resistant to its toxic effects [2]. Species differences in coumarin-induced hepatotoxicity are thought to be metabolism-dependent [1,3–5]; however, the specific mechanism leading to toxicity has yet to be determined. Since chronic administration of coumarin increases the incidence of liver tumors in rats and mice; an effect linked to the toxicity of this chemical [6], concern for the safety of coumarin in humans has been raised.

Coumarin metabolism in humans occurs predominately via 7-hydroxylation [3], whereas in mice and rats 3,4-epoxidation is favored [7]. 7-Hydroxycoumarin and its glucuronide and sulfate conjugates are relatively non-toxic and readily excreted [8,9]. In

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contrast, epoxidation leads to a reactive metabolite, coumarin 3,4-epoxide (CE), which loses CO_2 spontaneously with ring-opening and rearrangement to *o*-hydroxyphenylacetaldehyde (*o*-HPA) [10]; Fig. 1. The toxic effect of coumarin is likely due to the formation of both CE [1,4,5,8] and *o*-HPA, since these functional groups constitute a class of relatively reactive compounds that are known to cause toxicity [11].

In studying coumarin metabolism, 7-hydroxycoumarin can be readily detected as a highly fluorescent compound [12]. In contrast, direct measurement of CE in a biological matrix has been precluded by its short half-life (4 s) [10]. Consequently, the rate of epoxidation has been indirectly determined by measuring *o*-HPA formation [7]. *o*-HPA can be further metabolized by oxidation to *o*-hydroxyphenylacetic acid (*o*-HPAA) or reduction to *o*-hydroxyphenylethanol (*o*-HPE) [13]. Hence, coumarin epoxidation can yield three different products; *o*-HPAA and *o*-HPE which represent relatively nontoxic metabolites [8,14], and *o*-HPA which is hepatotoxic [15].

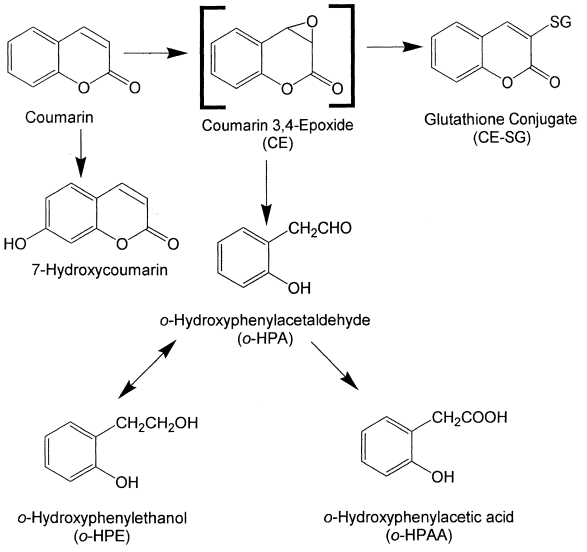


Fig. 1. Metabolic pathways of coumarin. Coumarin is metabolized predominately to coumarin 3,4-epoxide in mice and rats, whereas in humans 7-hydroxycoumarin is the major pathway. In the absence of glutathione (GSH), coumarin 3,4-epoxide loses CO_2 and rearranges to *o*-hydroxyphenylacetaldehyde which is subsequently oxidized or reduced to *o*-hydroxyphenylacetic acid or *o*-hydroxyphenylethanol, respectively.

There is direct evidence that CE is conjugated by glutathione (GSH) in vivo [16]. Furthermore, several investigators have determined the formation of CE indirectly by measuring GSH depletion [8,17,18]. Similarly, several investigators have reported separating *o*-HPA, *o*-HPAA and *o*-HPE, however these methods are either labor intensive, have lengthy separation times or use [^{14}C] coumarin and require on-line radioactivity detection [17,19,20].

Interest in coumarin metabolism and its role in toxicity has increased because of two new findings. First, evidence of carcinogenic activity in rodents [6] suggests the potential for a human hazard. Second, a known genetic polymorphism in cytochrome P450 2A6 (CYP2A6), the enzyme responsible for coumarin 7-hydroxylation, is suspected to increase the potential for coumarin toxicity in humans [21,22]. Therefore, methods to assess the metabolic activation of coumarin and the fate of CE are needed to evaluate human metabolism relative to laboratory animals. The purpose of the present work was (1) to synthesize CE-SG, (2) to develop a high-performance liquid chromatography (HPLC) method to measure its formation in hepatic microsomes, and (3) to develop an HPLC method for separating the ring-opened coumarin metabolites (*o*-HPAA, *o*-HPE and *o*-HPA) in hepatic microsomes.

2. Experimental

2.1. Chemicals

Coumarin, *o*-HPAA and *o*-HPE were purchased from Aldrich (Milwaukee, WI, USA). GSH, NAD^+, NADH, NADP, magnesium chloride, dimethylsulfoxide (DMSO), EDTA, potassium phosphate, glucose 6-phosphate, glucose 6-phosphate dehydrogenase, and trichloroacetic acid (TCA) were purchased from Sigma (St Louis, MO, USA). *o*-HPA was synthesized [15] according to the method of Bruce and Creed [23] and the purity exceeded 97%. 3-Hydroxycoumarin was previously synthesized [15] according to the method of Rajyalakshmi and Srinivasan [24]. Stock solutions of coumarin, *o*-HPAA, *o*-HPE, *o*-HPA and 3-hydroxycoumarin were prepared in DMSO. Working standards of *o*-HPAA, *o*-HPE, *o*-HPA and CE-SG were prepared in potas-

sium phosphate buffer (100 m*M*, pH 7.4). GSH was prepared in nitrogen purged water.

2.2. Animals

Male F344 rats (210–220 g) and female B6C3F1 mice (20–25 g) were purchased from Charles River Laboratories (Portage, MI, USA). The selection of the strain and sex of the animals was based on the coumarin bioassay data [6]. These data indicated rat liver necrosis was more severe in males, and that female mice were more susceptible to coumarin-induced lung tumor formation. Animals were housed in humidity and temperature controlled rooms and allowed free access to a standard lab diet (Purina Laboratory Rodent chow, Ralston-Purina, St Louis, MO, USA) and water.

2.3. Human liver microsomes

Human hepatic microsomes (ID number H0017) were purchased from XenoTech LLC (Kansas City, KS, USA). This sample was used because it formed *o*-HPA at a relatively fast rate compared to other human microsomal samples evaluated [7].

2.4. Human liver cytosol

Pooled human hepatic cytosol ($n=20$/pool) (ID number H861, lot number 3) was purchased from GENTEST (Woburn, MA, USA). The cytosol was processed through a PD-10 desalting column manufactured by Amersham Pharmacia Biotech (Uppsala, Sweden) to remove GSH and soluble cofactors. The cytosol was stored at −80 °C until time of use.

2.5. Preparation of rodent hepatic microsomes and cytosol

Hepatic microsomes were prepared from untreated female B6C3F1 mice ($n=25$/pool) and untreated male F344 rats ($n=15$/pool) via differential centrifugation [25]. Hepatic cytosol from mice and rats was desalted as described for human cytosol, and both microsomal and cytosolic fractions were stored. at −80 °C until time of use. Microsomal and cytosolic protein was determined by the Bradford assay with bovine serum albumin as the standard [26].

2.6. Glutathione s-transferase (GST) activity

GST activity in hepatic cytosol was determined by measuring the rate of GSH conjugation to 1-chloro-2,4-dinitrobenzene (CDNB) [27,28].

2.7. CE-SG isolation, characterization and quantitation

Coumarin (2 m*M*) was incubated in a 300 ml mouse hepatic microsomal reaction mixture (0.25 mg/ml) containing potassium phosphate buffer (100 m*M*, pH 7.4), EDTA (1 m*M*), $MgCl_2$ (3 m*M*), and a NADPH regenerating system consisting of glucose 6-phosphate (5 m*M*), glucose 6-phosphate dehydrogenase (1 IU/ml) and NADP (1 m*M*). The reaction mixture also contained mouse hepatic cytosol (1 mg/ml) and GSH (5 m*M*). The 300 ml reaction was incubated in a 1-l Erlenmeyer flask for 120 min in a 37 °C shaking water bath. The reaction mixture was stopped by the addition of 75 ml of ice-cold 15% TCA, followed by centrifugation at 16,000 *g* for 10 min. The supernatant was concentrated to 25 ml after approximately 72 h in a CentriVap Centrifugal Concentrator manufactured by Labconco (Kansas City, MO, USA).

CE-SG was isolated from the 25 ml concentrated reaction by HPLC using a Waters 2690 separation module, and a Waters 2487 dual channel UV detector (Waters, Milford, MA, USA). Separation of CE-SG was performed with a Zorbax guard column (SB-C_{18}, 4.6×12.5 mm, 5 μm), and a Zorbax analytical column (SB-C_{18}, Rapid Resolution, 4.6×75 mm, 3.5 μm) (Agilent Technologies, Wilmington, DE, USA). The isolation was performed at 35 °C with a flow-rate of 1.5 ml/min, and an isocratic mobile phase composition of 85% of 1% formic acid and 15% methanol. Following a 50 μl injection volume, the eluant was monitored at 332 nm (λ_{max} of CE-SG) and the CE-SG peak eluted at approximately 11.5 min. Approximately 500 injections from the 25 ml reaction were separated by HPLC. CE-SG peaks were collected from each injection and the final volume of all CE-SG fractions was approximately 600 ml. The isolated fractions of CE-SG were then lyophilized and a portion of the dried material was dissolved in water (50 μg/ml) to make a reference

standard. The CE-SG reference standard was placed in long-term storage at −80 °C.

Solutions of coumarin, GSH, and CE-SG were characterized by collisionally induced disassociation MS-MS using a Sciex API–III triple quadrupole mass spectrometer (Toronto, Canada). Mass spectra were obtained with electrospray ionization (ESI) in the negative ion mode. Negative ions were formed at a spray voltage of 4000 V, extracted with a source orifice of 70 V and fragmented with a collision energy of 35 V using argon as the collision gas. The samples were in a 1:1 methanol–water solvent containing 0.1% formic acid, and were infused at a flow-rate of 5 μl/min. The resulting spectra were used to identify m/z values for the CE-SG molecular ion ($m/z = 450$) and a characteristic fragment ion ($m/z = 177$).

The chemical structure of CE-SG isolated by HPLC was confirmed by flow injection mass spectrometry (FI–MS) on a P/E Sciex API 165 mass spectrometer (Toronto, Canada). Conditions were similar to the MS-MS experiments, with the exception that a sample volume of 10 μl was injected into a solvent flow-rate of 70 μl/min. Molecular ions were examined at a low source voltage (20 V) and in-source decay MS-MS spectra were obtained at a source voltage setting of 100 V.

^{1}H NMR chemical shift assignments for the isolated CE-SG product was aided by a series of NMR experiments including proton, carbon, double quantum filtered correlation, heteronuclear multiple-bond connectivity, heteronuclear single quantum correlation, and distortionless enhancement by polarization transfer conducted on coumarin, GSH and CE-SG. All NMR experiments were performed on a Varian 600 MHz instrument (Palo Alto, CA, USA).

The concentration of the CE-SG standard solution was determined by ^{1}H NMR relative to a phenol internal standard (Mallinckrodt, Paris, KY, USA) with D_2O as a spin lock. Spectra were acquired with suppression of the water signal, and a delay of 10 s to enable estimation of the CE-SG concentration relative to the phenol internal standard.

Metabolically-formed CE-SG was separated and quantitated by reversed-phase (RP)-HPLC on a Waters 2690 system using a Waters 2487 dual channel UV detector (332 nm) and a Zorbax analytical column (SB-C_{18}, 2.1×100 mm, 3.5 μm). A gradient mobile phase was employed with a flow-rate of 0.35 ml/min, an injection volume of 10 μl, and a column temperature of 35 °C. An initial mobile phase composition of 85% A (1% formic acid) and 15% B (methanol) was held for 3 min, followed by a 12-min linear gradient to a final composition of 77% A and 23% B. The column was allowed to equilibrate under initial conditions for at least 10 min between injections. CE-SG had a retention time of approximately 12 min.

2.8. RP-HPLC separation of o-HPAA, o-HPE and o-HPA

The ring-opened coumarin metabolites were separated and quantitated at 275 nm on a similar system to that above. The mobile phases were 1% formic acid (buffer A) and 100% methanol (buffer B). The isocratic mobile phase conditions were 92% A and 8% B. A volume of 40 μl was injected and the analytes were eluted at 35 °C at a flow-rate of 0.35 ml/min. The column was then washed with 50% A and 50% B for 5 min and re-equilibrated at initial mobile phase conditions. The retention times were approximately 9.3, 9.8 and 11.7 min for *o*-HPAA, *o*-HPE and *o*-HPA, respectively. The total run time between injections was 30 min.

2.9. Coumarin metabolism in hepatic microsomal incubations with and without hepatic cytosol

Coumarin (100 μ*M*) metabolism was studied in a 1 ml reaction containing mouse, rat or human hepatic microsomal mixtures (0.25 mg/ml). Metabolism of CE and *o*-HPA was evaluated in the 1 ml microsomal reaction mixture containing mouse, rat or human hepatic cytosol mixtures normalized to GST activity (1 μmol of CDNB conjugated to GSH/min). The hepatic cytosol mixture also contained NAD^+ (1 m*M*), NADH (0.5 m*M*) and GSH (5 m*M*). Following a 2 min pre-incubation in a 37 °C shaking water bath, NADP was added at a final concentration of 1 m*M* to initiate the reaction. The samples were incubated for 30 min and the reaction was terminated by the addition of 0.25 ml of ice-cold 15% TCA. After centrifugation at 16,000 *g* for 10 min, the supernatant was analyzed by RP-HPLC for CE-SG followed by a separate analysis for *o*-HPAA, *o*-HPE

and *o*-HPA. Metabolism of 3-hydroxycoumarin (2 m*M*) was evaluated using these same conditions.

2.10. Method accuracy and reproducibility

The range of accuracy for each analyte was determined by spiking *o*-HPAA, *o*-HPE and *o*-HPA at a final concentration of 0.25, 6.25 and 50.0 μ*M*, and CE-SG at a final concentration of 0.05, 2.0 and 18.1 μ*M* into a mouse, rat or human hepatic microsomal reaction mixture (0.25 mg/ml) containing respective mouse, rat or human hepatic cytosolic mixtures and coumarin (2 m*M*) in a final volume of 1 ml. Prior to the 30-min incubation at 37 °C, 0.25 ml of ice-cold 15% TCA was added to preclude metabolism. Following incubation, samples were centrifuged and analyzed by HPLC. A sample blank without metabolites was also analyzed for each species to determine whether there was endogenous material interfering with the analysis.

2.11. Limit of detection (LOD) and limit of quantification (LOQ)

Determination of LOD was based on the American Chemical Society guidelines and was equal to the concentration which gave a response three times σ above the average (four replicates) blank signal [29]. The LOD for *o*-HPAA, *o*-HPE and *o*-HPA was determined to be approximately 3.5 ng/ml (1 pmol on column), whereas the LOD for CE-SG was determined to be approximately 2.3 ng/ml (0.05 pmol on column). The LOQ for each analyte was calculated by multiplying the LOD by 10 and was equal to approximately 35 ng/ml (10 pmol on column) for *o*-HPAA, *o*-HPE and *o*-HPA, whereas the LOQ for CE-SG was determined to be approximately 23 ng/ml (0.5 pmol on column).

3. Results

CE-SG was successfully synthesized in a mouse hepatic microsomal reaction containing cytosol, and structural confirmation was determined by MS, MS-MS and NMR. The CE-SG molecular ion $[M-H]^-$ was observed at $m/z = 450$ in the mass spectrum (Fig. 2). CE-SG structural information was obtained by fragmentation in a MS-MS experiment giving a characteristic product ion at $m/z = 177$ corresponding to the $[\text{coumarin-S}]^-$ product ion (Fig. 3). This product ion, $[\text{coumarin-S}]^-$ was absent in the MS-MS spectrum of GSH (Fig. 4), indicating the response at $m/z = 450$ is CE-SG.

The ^{1}H NMR spectra shown in Fig. 5 confirmed the CE-SG structure and identified the 3-carbon as the only site of GSH conjugation. The CE-SG spectrum was also used to establish that the concentration of the standard CE-SG solution was 112 μ*M* based on relative peak areas to phenol. Following characterization, CE-SG was stored frozen at −80 °C for up to 1 year with no change in concentration or chromatographic integrity.

Fig. 6A shows a representative separation of an injection of the CE-SG standard in phosphate buffer. A representative chromatogram of an injection of a sample from a mouse microsomal blank mixture containing mouse cytosol is shown in Fig. 6B. Injections of mixtures containing rat or human microsomes with respective rat or human cytosol gave comparable results (data not shown) indicating no endogenous material co-eluted with CE-SG in any of the three matrices. A representative chromatogram derived from a mouse microsomal reaction mixture containing mouse cytosol is shown in Fig. 6C. Although CE-SG formation can occur in the absence of cytosol (data not shown), this chromatogram demonstrates the enzymatic formation of CE-SG by cytosolic GST. Injections from mouse microsomal reaction mixtures containing rat or human cytosol were similar to those with mouse liver cytosol. However, the rate of CE-SG formation is substantially less in mouse microsomal reactions containing human liver cytosol. Early experiments demonstrated that *o*-HPAA and *o*-HPE could be measured under the chromatographic conditions used for the conjugate. However, *o*-HPA could not be quantified under these conditions due to interfering material. Therefore, this method was not used to measure the ring-opened metabolites. Since the ring-opened metabolites eluted between 5 and 7 min under these chromatographic conditions, they do not interfere with measuring CE-SG, which elutes at 12 min.

Fig. 7A shows a representative chromatogram of an injection of *o*-HPAA, *o*-HPE and *o*-HPA standards in phosphate buffer. A representative chro-

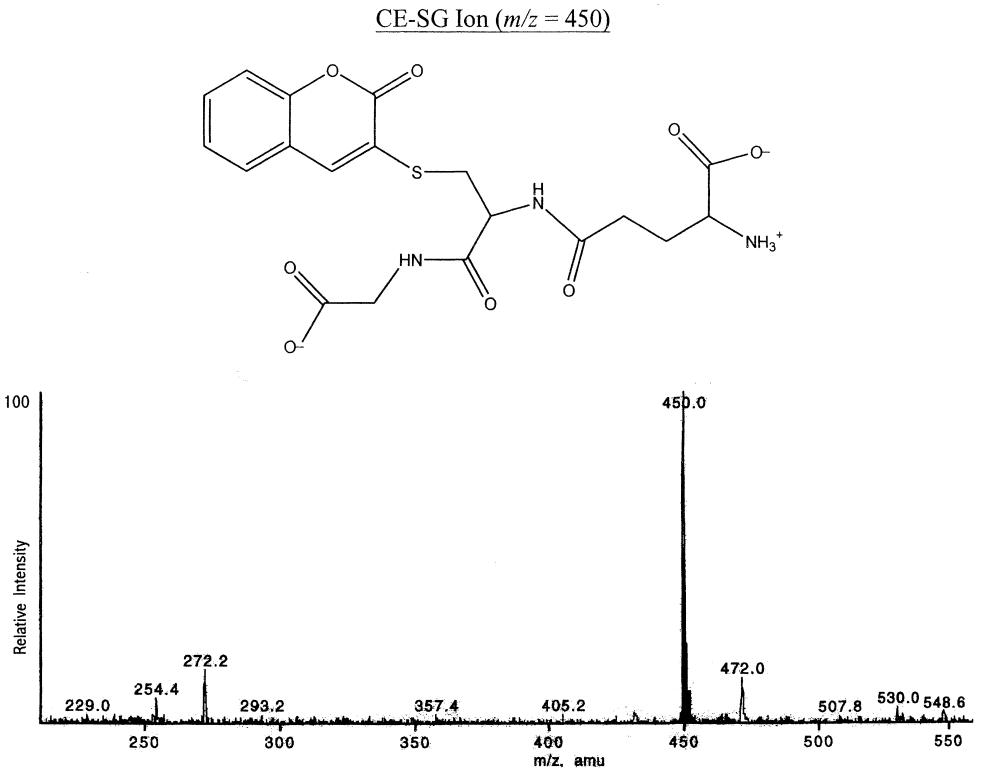


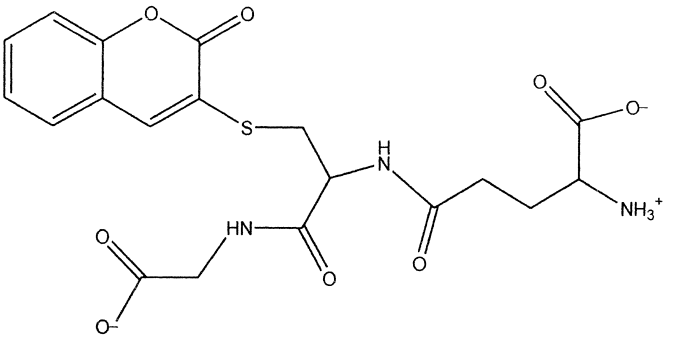
Fig. 2. Mass spectrum of CE-SG isolated by HPLC. A low source voltage spectrum showing CE-SG $[M-H]^-$ ion at $m/z = 450$ and a sodium adduct ion at $m/z = 472$, $[M+Na-2H]^-$.

matogram of an injection of a mouse microsomal blank mixture with cytosol is shown in Fig. 7B. Rat and human microsomal blank mixtures gave comparable results (data not shown), indicating that no endogenous material co-eluted with *o*-HPAA, *o*-HPE or *o*-HPA in any of the three matrices. A representative chromatogram obtained from a mouse microsomal reaction mixture without cytosol is shown in Fig. 7C. In reaction mixtures containing only mouse microsomes, the major product is *o*-HPA, and *o*-HPAA and *o*-HPE are virtually non-detectable. Similar results were obtained in reaction mixtures containing rat or human microsomes (data not shown). Although *o*-HPA is the predominate metabolite in this chromatogram, its formation would be reduced in the presence of exogenous GSH due to the rapid formation of CE-SG.

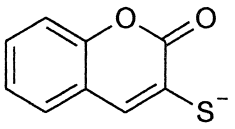
A representative chromatogram of an injection of a mouse microsomal reaction mixture containing mouse cytosol is shown in Fig. 8. When cytosol was added to the microsomal reaction mixture, *o*-HPA was metabolized to either *o*-HPAA or *o*-HPE representing the action of aldehyde or alcohol dehydrogenase, respectively. The chromatography was similar when rat or human cytosol was added to the mouse microsomal reaction mixture as shown in Fig. 8. In reactions containing human cytosol, CE was detected predominately as *o*-HPAA, whereas only 50% of CE was converted to *o*-HPA and further oxidized or reduced to *o*-HPAA or *o*-HPE, respectively, when rodent cytosol was present. Since exogenous GSH was present in these reaction mixtures, it is likely that a substantial portion of CE is conjugated with GSH in rodents. CE-SG elutes in the column wash under the HPLC conditions used for these analyses.

A standard curve of *o*-HPAA, *o*-HPE and *o*-HPA (0.250, 0.625, 1.25, 2.50, 12.5 and 50.0 μ*M*), and CE-SG (0.224, 0.448, 2.24, 8.96 and 22.4 μ*M*) was prepared on three different days and analyzed in

CE-SG Ion ($m/z = 450$)



[Coumarin-S]$^-$ Product Ion ($m/z = 177$)



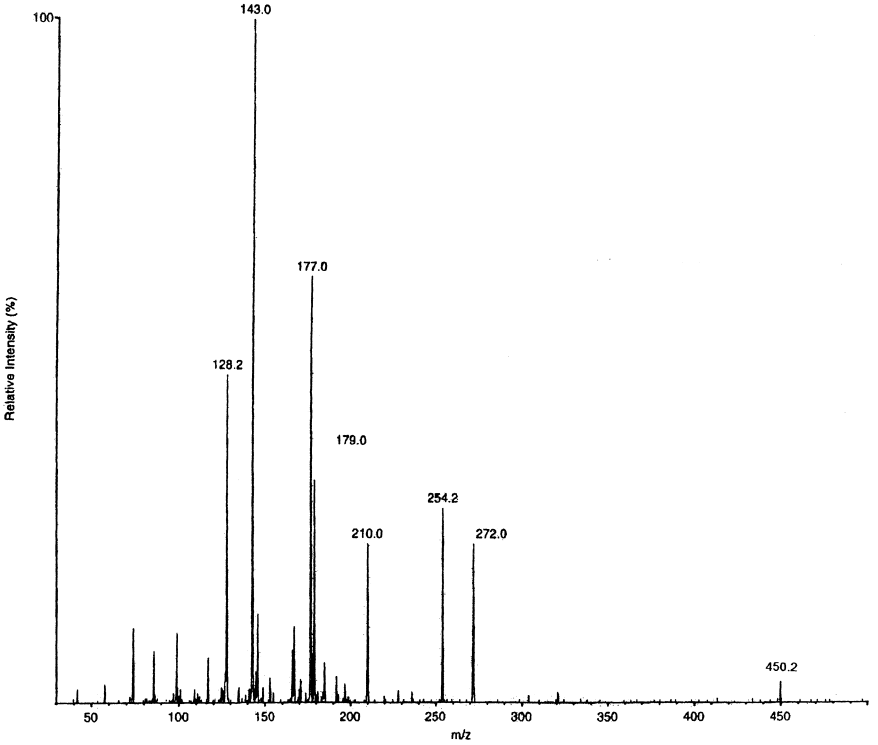


Fig. 3. MS-MS of CE-SG isolated by HPLC. An ESI–MS-MS product ion spectrum of an infused standard solution of CE-SG ($m/z = 450$). Many of the product ions originate from fragmentation of the glutathione moiety. Ions at these masses were also observed in the full scan spectrum of glutathione. A single ion, at $m/z = 177$, differentiated the product ion scans of CE-SG ($m/z = 450$) from the spectrum of a glutathione standard ($m/z = 306$) [Fig. 4].

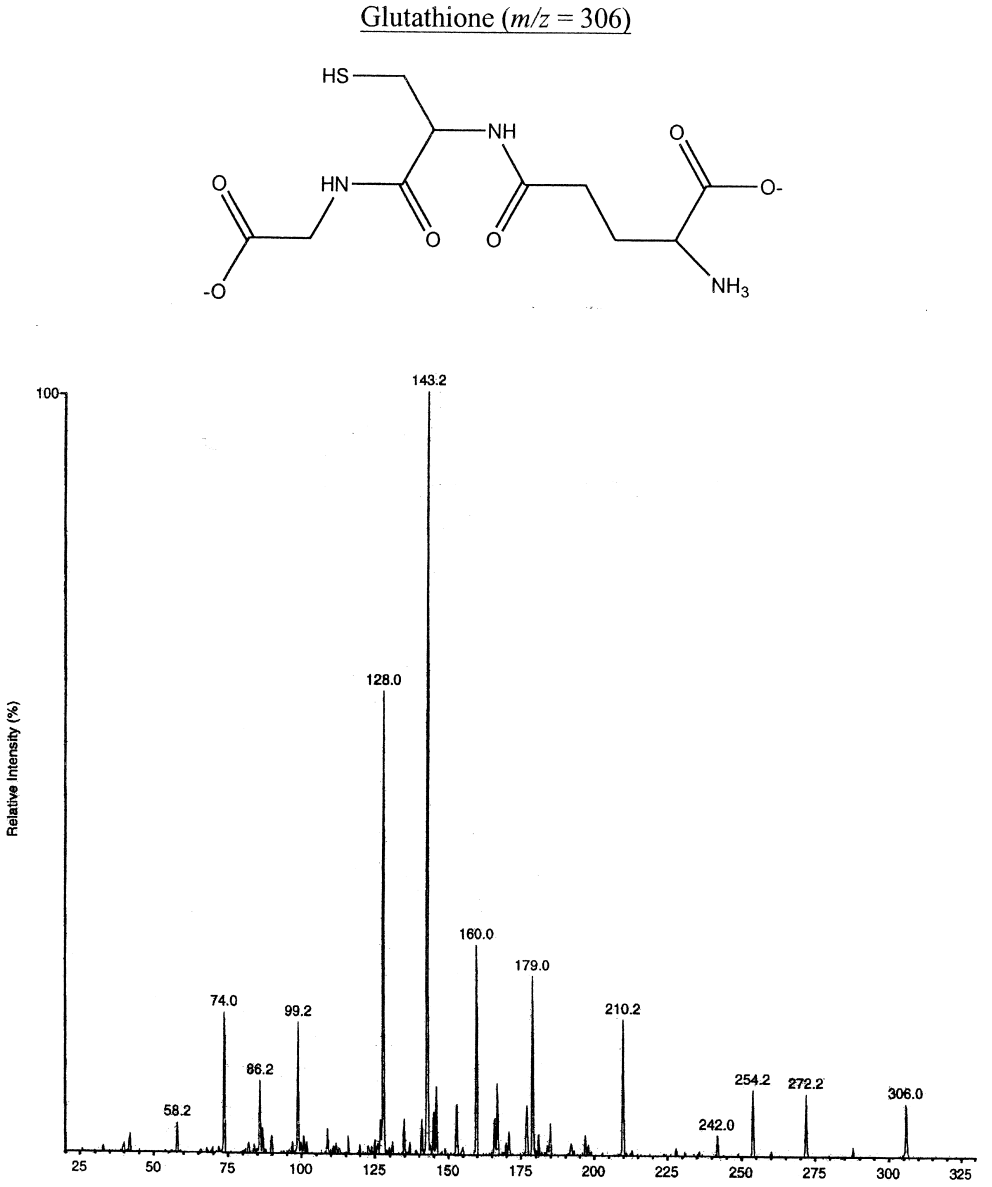


Fig. 4. Mass spectrum of glutathione. An ESI–MS-MS product ion spectrum of an infused standard solution of glutathione ($m/z = 306$). The MS-MS spectra of glutathione and the CE-SG conjugate have many common ions ($m/z = 74$, 86, 99, 128, 143, 179, 210, 254, and 272). The absence of $m/z = 160$, and the presence of $m/z = 177$ from the CE-SG product ions are keys for MS-MS verification that a response at $m/z = 450$ is actually CE-SG.

duplicate by HPLC. The mean linearity (r^2) of the three standard curves for each analyte was equal to or greater than 0.999. The mean slope (±S.E.M.) of the three standard curves for each analyte was 312.4 (5.33), 284.4 (4.23), 289.3 (4.98) and 2057 (5.79) peak area/pmol on column for *o*-HPAA, *o*-HPE, *o*-HPA and CE-SG, respectively. Recovery of *o*-HPAA, *o*-HPE, *o*-HPA and CE-SG from mouse, rat or human microsomal reaction mixtures containing cytosol is presented in Table 1. Recovery was

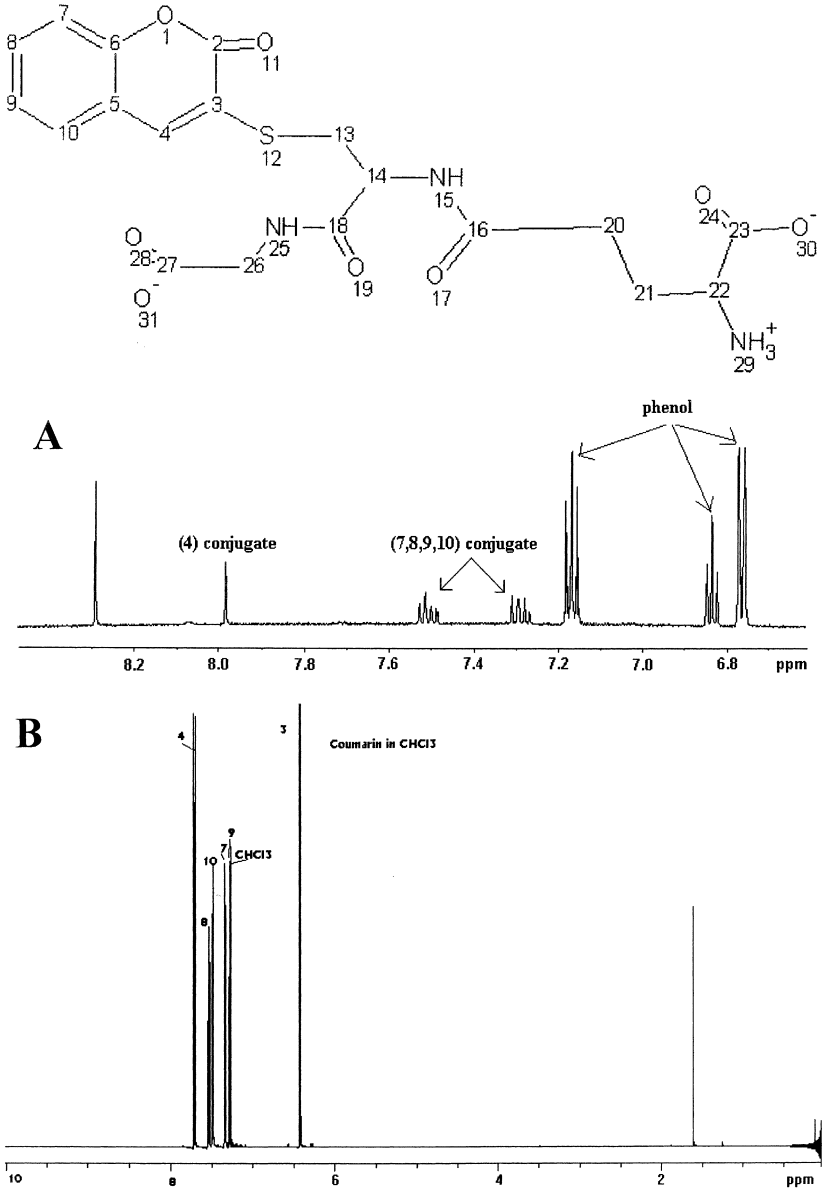


Fig. 5. NMR spectra of CE-SG. The structure of the CE-SG conjugated at the 3-position is shown above. The numbered protons correspond to the proton assignments in the ^{1}H NMR spectra. (Panel A) ^{1}H-NMR spectrum of CE-SG at the 3-position in deuterated water. Phenol was added as an internal standard to enable estimation of the absolute amount of CE-SG in the standard solution. (Panel B) ^{1}H-NMR spectrum of coumarin in chloroform illustrating the protons at the 3- and 4-position. 2-D NMR experiments (not shown) were used to assign the signals form the 3- and 4-positions. These assignments are consistent with the calculated chemical shifts obtained from the advanced chemical development ^{1}H-NMR simulation software (Toronto, Canada). (Panel C) ^{1}H-NMR spectrum of glutathione in DMSO.

essentially quantitative for each metabolite in all three species. Variability of the analysis for *o*-HPAA, *o*-HPE, *o*-HPA and CE-SG in mouse, rat or human microsomal reaction mixtures containing cytosol did not exceed 8% relative standard deviation (RSD) for quadruplicate determinations.

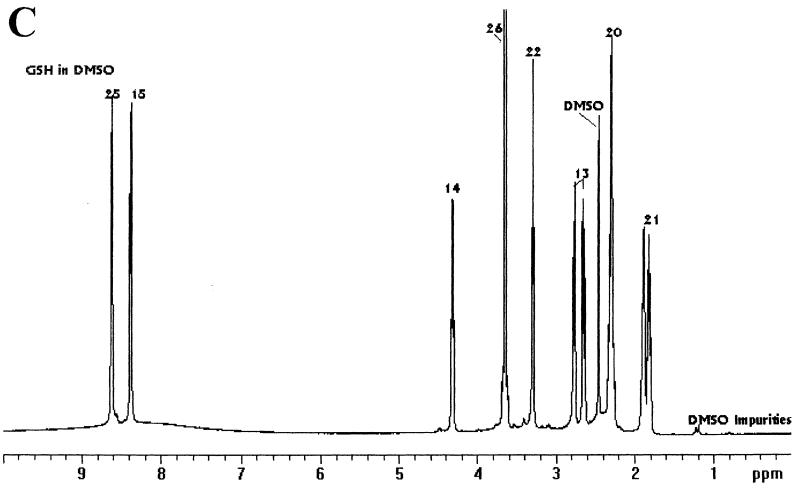


Fig. 5. (*continued*)

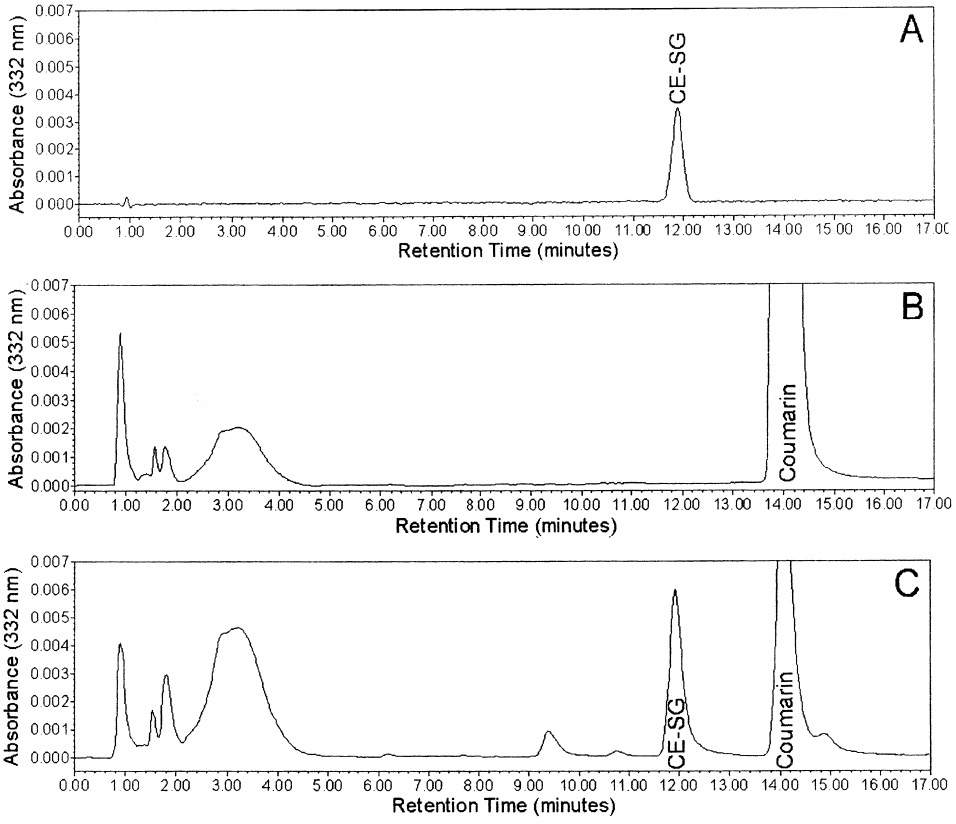


Fig. 6. RP-HPLC separation of CE-SG. (Panel A) A representative chromatogram of the CE-SG standard (25 pmol on column) with a retention time of 12 min. (Panel B) A representative chromatogram of an injection of a mouse hepatic microsomal blank reaction mixture with mouse liver cytosol and 2 m*M* coumarin showing no interfering peaks. (Panel C) A representative separation of CE-SG (57.5 pmol on column) with a retention time of 12 min in a mouse hepatic microsomal reaction mixture with mouse liver cytosol and 100 μ*M* coumarin following a 30 min incubation. The peak at 9.3 min is 7-hydroxycoumarin.

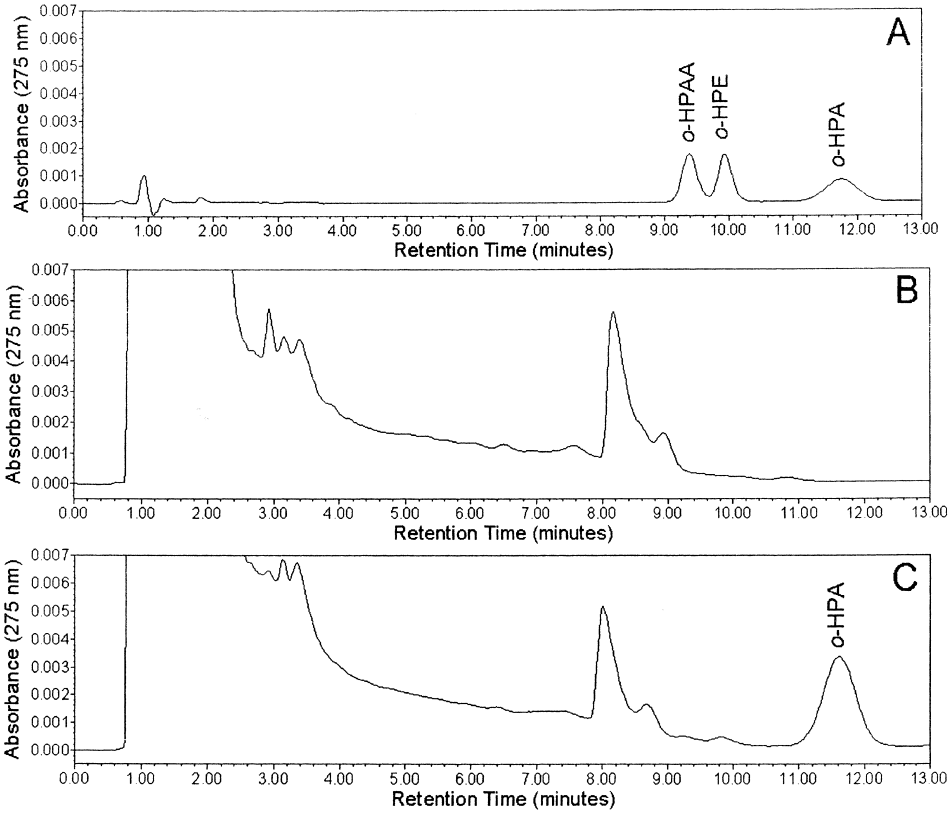


Fig. 7. RP-HPLC separation of *o*-HPAA, *o*-HPE and *o*-HPA. (Panel A) A representative chromatogram of *o*-HPAA, *o*-HPE and *o*-HPA standards (100 pmol of each metabolite on column) with retention times of 9.3, 9.8 and 11.7 min, respectively. (Panel B) A representative chromatogram of an injection of a mouse hepatic microsomal blank reaction mixture with mouse liver cytosol and 2 m*M* coumarin showing no interfering peaks. (Panel C) A representative separation of *o*-HPA (359 pmol on column) with a retention time of 11.7 min in a mouse hepatic microsomal reaction mixture (no cytosol) with 100 μ*M* coumarin following a 30 min incubation. In the absence of cytosol, some *o*-HPA is oxidized or reduced in liver microsomes, and these peaks are less than the LOQ for *o*-HPAA or equal to the LOQ for *o*-HPE.

As a final qualification of the two HPLC assays, the recovery of total CE derived metabolites was determined by measuring the ring-opened coumarin metabolites and CE-SG from reaction mixtures containing cytosol of each species examined. Since mouse microsomes had the highest coumarin epoxidation activity, they were incubated without cytosol for 30 min to generate CE, and the amount of CE was determined indirectly by measuring the rate of *o*-HPA formation. Separate mouse microsomal reaction mixtures were incubated for 30 min in which mouse, rat or human hepatic cytosol was added, after which *o*-HPAA, *o*-HPE, *o*-HPA and CE-SG were determined. As shown in Table 2, the sum of all metabolites yielded recoveries of 97%, 87% and 106% from reaction mixtures containing mouse, rat or human cytosol, respectively.

There is evidence indicating that administration of 3-hydroxycoumarin to rats results in excretion of *o*-HPAA [30]. In order to confirm that *o*-HPAA formation was exclusive to the epoxidation pathway, metabolites arising from the metabolism of 3-hydroxycoumarin were evaluated. No metabolite co-eluted with *o*-HPAA.

4. Discussion

The hepatotoxic and carcinogenic effects of coumarin, particularly in the rat, are metabolism-

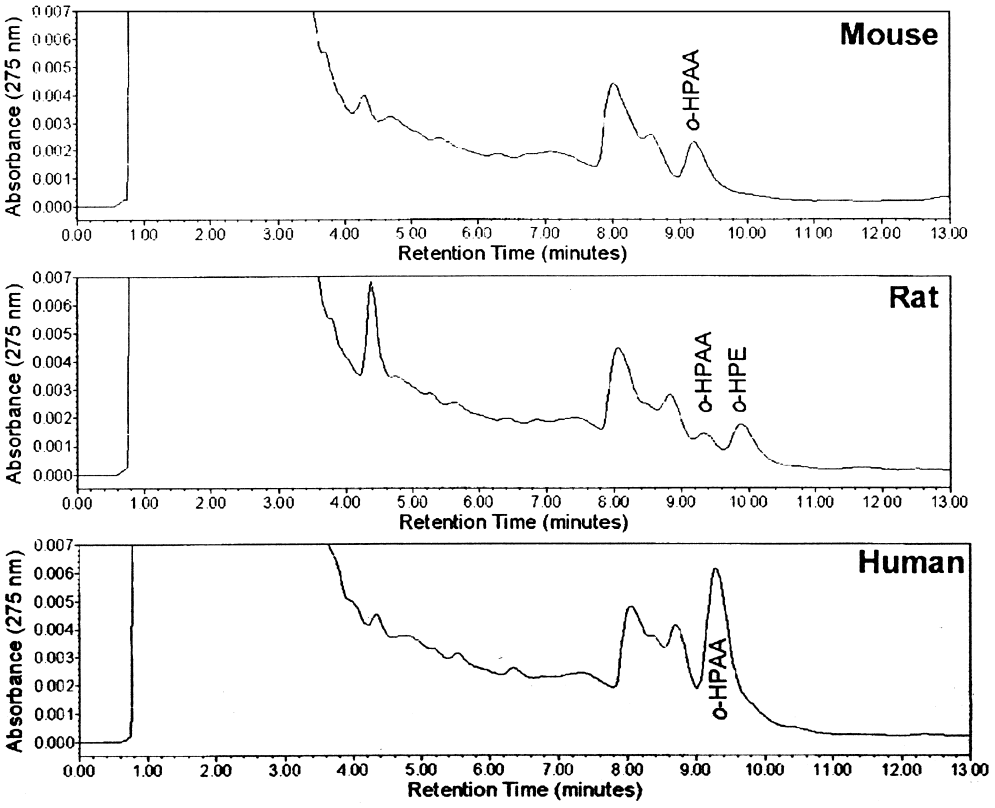


Fig. 8. RP-HPLC separation of *o*-HPAA and *o*-HPE. Coumarin (100 μ*M*) was incubated for 30 min in a mouse hepatic microsomal reaction mixture with mouse, rat or human liver cytosol. Representative chromatograms from mouse, rat and human injections are shown. In the mouse separation, *o*-HPAA (117 pmol on column) was the only ring-opened metabolite. Similarly, *o*-HPAA (361 pmol on column) was the only ring-opened metabolite in the human, whereas both *o*-HPAA (40 pmol on column) and *o*-HPE (87 pmol on column) formed in the rat cytosol. *o*-HPA (8.7 pmol on column) also formed in the rat, although it was below the LOQ.

mediated and are dependent on the formation of coumarin 3,4-epoxide [1,3–6,8,31]. Detoxification of CE occurs via conjugation with GSH [16], or through the spontaneous rearrangement of CE to a hepatotoxic ring-opened metabolite, *o*-HPA [10], followed by the oxidation or reduction of *o*-HPA to non-toxic *o*-HPAA or *o*-HPE, respectively [13]. Although several investigators have demonstrated that GSH conjugates CE [8,17,18], and a method exists to measure the excreted mercapturic acid [16], an authentic standard has not been available, and a method to measure the GSH conjugate of CE has not been developed until now.

Although several methods exist to separate *o*-HPAA, *o*-HPE and *o*-HPA [17,19,20], they are either labor intensive, have lengthy separation times or employ radioactivity detection. Some groups have only measured *o*-HPAA [21,32,33], however this does not allow the complete metabolic fate of CE to be evaluated. The two RP-HPLC methods presented here measure all products associated with coumarin epoxidation.

CE-SG formed by mouse liver microsomes was isolated and structural confirmation demonstrated that GSH conjugated CE at the 3-carbon. Consistent with this observation, previous investigators have identified that the excreted CE-SG product in rat urine is 3-mercapturic acid [16]. This purified CE-SG was used as an authentic standard to develop a RP-HPLC method which allows rapid quantification of CE-SG.

Following CE-SG analysis, the ring-opened

Table 1
Method recovery and reproducibility

Metabolite	Mass on column (pmol)	Mouse		Rat		Human	
		Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
CE-SG	0.5	95.9 (3.9)	8.1	93.8 (3.0)	6.3	100.8 (1.1)	2.1
	20.0	100.9 (1.1)	2.2	99.7 (0.2)	0.4	100.4 (0.5)	0.9
	181.4	102.9 (0.6)*	1.0	102.3 (0.7)*	1.1	ND	ND
o-HPAA	10	81.1 (3.3)	8.2	82.9 (2.1)	5.0	83.6 (0.9)	2.1
	250	94.7 (0.5)	1.0	94.8 (0.7)	1.5	95.1 (0.8)	1.7
	2000	98.6 (1.3)	2.7	98.9 (0.8)	1.6	100.9 (0.3)	0.5
o-HPE	10	98.8 (2.6)	5.3	101.9 (1.1)	2.1	104.7 (1.1)	2.1
	250	99.5 (0.6)	1.2	99.1 (0.7)	1.4	99.5 (1.6)	1.6
	2000	101.9 (1.3)	2.5	102.2 (0.9)	1.8	104.0 (0.6)	1.1
o-HPA	10	96.6 (3.7)	7.7	99.9 (1.7)	3.4	88.2 (2.9)	6.6
	250	93.6 (0.6)	1.2	91.7 (1.2)	2.6	95.1 (1.0)	2.2
	2000	95.5 (1.2)	2.6	94.4 (0.6)	1.3	99.8 (0.2)	0.4

Recovery was determined by spiking *o*-HPAA, *o*-HPE, *o*-HPA and CE-SG into mouse, rat or human hepatic microsomal reaction mixtures containing respective mouse, rat or human liver cytosol. The mass on the column for each metabolite was calculated by using the standard curve for each analyte which was generated in phosphate buffer. The percent recovery for each metabolite was determined by dividing the measured value for each metabolite by the nominal mass on the column for each metabolite. The data represent the mean (±S.E.M.) of quadruplicate determinations unless otherwise stated. % RSD = (SD/mean) × 100. *$n = 3$. ND = not determined.

coumarin metabolites formed in the sample can be measured using the same column and solvent system without further sample preparation. Although *o*-HPA was completely resolved and accurately quantitated, *o*-HPAA and *o*-HPE were not fully baseline resolved. However, recovery of CE-SG, *o*-HPAA, *o*-HPE and *o*-HPA, representing all products of CE metabolism in hepatic microsomal reaction mixtures containing cytosol, was quantitative, indicating that with accurate peak integration the lack of baseline resolution does not preclude accurate assessment of these metabolites.

Previous investigators have suggested that CE can rearrange to 3-hydroxycoumarin, an intermediate in *o*-HPAA formation [8,30]. Although the paradigm of CE rearrangement to 3-hydroxycoumarin has been disproven [10], rats dosed with 3-hydroxycoumarin excrete *o*-HPAA [30] suggesting two divergent pathways, 3-hydroxylation and epoxidation, lead to the same metabolic fate; *o*-HPAA. Indeed, if 3-hydroxylation of coumarin leads to *o*-HPAA formation in vitro the recovery of the four metabolites through the epoxidation pathway would misrepresent the metabolism. Therefore, in order to demonstrate that *o*-

Table 2
Mass balance of CE formation and recovery

Species	CE	*o*-HPAA	*o*-HPE	*o*-HPA	CE-SG	Sum of metabolites	Mass recovery
Mouse	11.22 (0.23)	3.65 (0.04)	0.00	0.00	7.19 (0.10)	10.84 (0.14)	96.70 (1.22)
Rat	11.22 (0.23)	1.25 (0.03)	2.73 (0.01)	0.27 (0)	5.49 (0.06)	9.74 (0.06)	86.87 (0.52)
Human	11.22 (0.23)	11.29 (0.12)	0.00	0.00	0.58 (0.02)	11.87 (0.13)	105.85 (1.12)

CE formation was determined by measuring the rate of formation of *o*-HPA in mouse hepatic microsomal reaction mixtures with coumarin (100 μM) following a 30 min incubation. Further metabolism of CE was evaluated in a separate reaction by measuring the formation of *o*-HPAA, *o*-HPE, *o*-HPA and CE-SG in mouse hepatic microsomal reaction mixtures containing mouse, rat or human liver cytosol and coumarin (100 μM) following a 30 min incubation. The data represent the mean (±S.E.M.) of quadruplicate determinations. CE, *o*-HPAA, *o*-HPE, *o*-HPA and CE-SG are expressed in nmol of product formed in a 1 ml reaction. Mass recovery was determined by dividing the sum of metabolites (*o*-HPAA, *o*-HPE, *o*-HPA and CE-SG) by CE formed for each species, and multiplying by 100.

HPAA formation was exclusive to the epoxidation pathway, 3-hydroxycoumarin was incubated with mouse, rat and human liver microsomal mixtures containing respective mouse, rat or human hepatic cytosol. Since no *o*-HPAA could be detected it was determined that CE-SG, *o*-HPAA, *o*-HPE and *o*-HPA represent quantitative recovery of coumarin epoxidation and that 3-hydroxycoumarin was not an intermediate in *o*-HPAA formation in vitro. The observation that 3-hydroxycoumarin was not metabolized to *o*-HPAA has also been demonstrated in vitro by other investigators [13,34].

Since CE-SG, *o*-HPAA, *o*-HPE and *o*-HPA were shown to represent all the metabolites of CE, species differences in CE detoxification can be quantitatively compared. Distinct differences in the fate of CE in the mouse, rat and human hepatic cytosol were observed. Although CE-SG was the predominate metabolite in mouse and rat cytosol, it was not as readily formed in the human cytosol, suggesting this is not a major path of CE detoxification. In contrast, incubation mixtures containing human cytosol formed predominately *o*-HPAA, whereas those containing cytosol from mice and rats formed three- and nine-fold less *o*-HPAA. Consistent with this observation, quantitative recoveries of coumarin metabolites in humans shows *o*-HPAA to be the major ring-opened metabolite following coumarin epoxidation [1,3]. It is interesting to note that *o*-HPE and *o*-HPA were measured in mouse hepatic microsomal mixtures containing rat hepatic cytosol, yet they were not formed in mouse or human hepatic cytosol. Since rats are susceptible to coumarin-induced toxicity, these data support the hypothesis that injury is determined by the balance between CE formation and detoxification, as has been previously postulated [7].

In humans, the major pathway for coumarin biotransformation is 7-hydroxylation [3], and epoxidation is a minor pathway [7]. However, the enzyme responsible for coumarin 7-hydroxylation in humans, CYP2A6 is polymorphic, and individuals with low activity are thought to be more susceptible to coumarin-mediated hepatotoxicity [21,22]. The methods developed in the present work will allow for species differences in coumarin epoxidation, including the role of the human allelic variants, to be thoroughly evaluated.

In summary, two HPLC methods were developed and validated for measuring CE-SG and ring-opened coumarin metabolites in hepatic microsomal mixtures containing cytosol. These methods will be directly applicable to studying metabolism of coumarin in humans and to evaluate species differences in the fate and detoxification of CE.

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