METABOLISM OF (R)-(+) -MENTHOFURAN IN FISCHER-344 RATS: IDENTIFICATION OF SULFONIC ACID METABOLITES

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(Received June 6, 2003; accepted July 8, 2003)

ABSTRACT:

(R)-(+) -Menthofuran is a metabolite of (R)-(+) -pulegone, the chief constituent of pennyroyal oil. Menthofuran has been shown to account for a significant percentage of pulegone toxicity through further metabolism to a reactive intermediate, an enonal (2-Z-(2''-keto-4''-methylcyclohexylidenepropanal). Hydration of the enonal followed by a 1,4-dehydration and rearrangement gives rise to diastereomeric (-)-mintlactone and (+)-isomintlactone (mintlactones). We have conducted disposition studies on pulegone as part of the National Toxicology Program initiative in herbal medicines and dietary supplements, and have reported previously unknown urinary metabolites of pulegone. Comparative metabolism studies of 14C-labeled menthofuran in Fischer-344 (F344) rats were carried out to determine urinary metabolites of pulegone that are derived from the menthofuran pathway. Three sulfonic acid metabolites, namely, hexahydro-3,6-dimethyl-1-(2-sulfoethyl)-2H-indol-2-one, hexahydro-3,6-dimethyl-7a-sulfo-2(3H)-benzofuranone, and 2-sulfomenthofuran, were identified in urine of treated rats. Formation of these metabolites may be derived from reactions of the enonal with taurine or glutathione (GSH) (or sulfite ion). Other identified urinary metabolites of menthofuran could be attributed to further metabolism of mintlactones. Further hydroxylation of menthofuran could give 7a-hydroxymintlactone and 6,7a-dihydroxymintlactone. Glucuronidation or reduction of 7a-hydroxymintlactone could give rise to the major metabolites 7a-hydroxymenthofuran glucuronide and 2-[2''-keto-4''-methylcyclohexyl]propionic acids. Glucuronidation or repeated hydroxylation/dehydration of 2-[2''-keto-4''-methylcyclohexyl]propionic acids could result in formation of hexahydro-3,6-dimethyl-7a-hydroxy-2(3H)-benzofuranone glucuronide and 2-[2''-hydroxy-4''-methylphenyl]propionic acid. 2-(Glutathione-S-y1)menthofuran, a GSH conjugate of the enonal that has been partially characterized in bile of rats dosed with pulegone, is at most a minor biliary metabolite of menthofuran in rats.

{Pennyroyal oil, which contains chiefly pulegone, is a flavoring agent in foods and beverages as well as an abortifacient (Hall and Oser, 1965; Tyler, 1993). Ingestion of large doses (up to 30 ml) of pennyroyal oil has resulted in severe toxicity or death (Anderson et al., 1996). Menthofuran, which is also present in pennyroyal oil, is a microsomal metabolite of pulegone and has been detected in plasma of rats dosed with pulegone (Gordon et al., 1982, 1987; Thomassen et al., 1988). Both pulegone and menthofuran are hepatotoxic in mice and rats, and their metabolites covalently bind to tissue proteins extensively (Gordon et al., 1982; Thomassen et al., 1988; McClanahan et al., 1989). Up to half of the pulegone hepatotoxicity was estimated to arise from menthofuran (Thomassen et al., 1988). An in vitro study showed that menthofuran was further metabolized to give 2-Z-(2''-keto-4''-methylcyclohexylidenepropanal (an enonal\(^1\)), which has been shown to bind to proteins (Thomassen et al., 1992). Hydration of the enonal to a dihydroxydihydromenthofuran, followed by a 1,4-dehydration first gives 2-hydroxymenthofuran, which then rearranges to diastereomeric mintlactones. The hydroxylations of pulegone and menthofuran are catalyzed by the cytochrome P450 enzymes (Khojaste-Bakht et al., 1999).

In vivo metabolism of pulegone is extremely complex, generating dozens of metabolites in urine and bile of treated animals (Thomassen et al., 1991; Chen et al., 2001). Previous studies of urinary metabolites of pulegone and menthofuran in rats at doses closer to the LD\(_{50}\) demonstrated characterization of 14 and 10 phase I metabolites, respectively, but no metabolites were common to both studies (Madyastha and Raj, 1992, 1993). We have identified previously unknown phase II urinary metabolites of pulegone in Fischer-344 (hereafter called F344) rats at an 80 mg/kg dose and reported a common urinary metabolite, 7a-hydroxymintlactone glucuronide, of pulegone and menthofuran from a comparative study on F344 rats dosed with 60 mg/kg menthofuran (Chen et al., 2001). It is still not clear what other pulegone metabolites are derived from the menthofuran pathway. The objective of the current study is identification of other metabolites of menthofuran in rats, especially phase II metabolites, to advance our understanding of the in vivo metabolism of menthofuran and to explore metabolites derived from conjugation of the enonal with biomolecules.

Materials and Methods

Chemicals. [2-\(^{14}\text{C}\)]Menthofuran (specific activity 55.2 mCi/mmol, radiochemical purity \(\pm 98\%\)) was obtained from ChemSyn Laborato-
ries (Lenexa, KS). Unlabeled menthofuran (purity 95%) was obtained from Acros Organics USA (Fairlawn, NJ). Trifluoroacetic acid, NaHSO₄, and taurine were purchased from Aldrich Chemical Co. (Milwaukee, WI). Reduced glutathione (GSH) (97% pure) was purchased from Fluka BioChemika (Milwaukee, WI). Mintlactones and 7a-hydroxymenthionolactone were prepared according to published procedures (Thomassen et al., 1992).

[3â′-14C]-2-[2-Keto-4′-methylcyclohexyl]propionic acids were synthesized from hydrolysis of [2-14C]-2-hydroxymenthofuran, which was prepared according to a published procedure except that [2-14C]menthofuran was used (Khojasteh-Bakht et al., 1999). HPLC analysis of the products showed that four isomers were present. 1H NMR (CDCl₃) of the methyl groups of three isomers: δ 1.21 (d, J = 6.9 Hz, 1-CH₃), 0.94 (d, J = 6.6 Hz, 4′-CH₃); δ 1.17 (d, J = 7.1 Hz, 1-CH₃), 1.03 (d, J = 6.3 Hz, 4′-CH₃); δ 1.13 (d, J = 7.2 Hz, 1-CH₃), 0.97 (d, J = 7.1 Hz, 4′-CH₃). UV of all four isomers: λmax < 190 nm.

NMR. 1H NMR spectra were acquired on a Varian 300 MHz NMR spectrometer (Varian Inc., Palo Alto, CA). The chemical shifts are reported in ppm relative to solvents.

HPLC. HPLC analyses were carried out with either of two systems. System A consisted of two Waters (Milford, MA) pumps, an automated system controller, and a model 481 UV detector. System B consisted of a Beckman Coulter (Fullerton, CA) System Gold 126 Solvent Module pump, a model 168 photodiode array detector, and Nouveau software. System B was connected with an IN/US (Tampa, FL) β-Ram flow detector for radiochemical detection. A MetaChem Technologies (Lake Forest, CA) Inertsil C18 5-μm column (4.6 × 250 mm) was used for all studies unless otherwise indicated. HPLC method 1 consisted of a linear gradient system from 100% 0.1% trifluoroacetic acid in H₂O to 100% CH₃CN over 28 min at a flow rate of 1.5 ml/min. Method 2 consisted of a linear gradient system from 100% 0.1% trifluoroacetic acid in H₂O to 100% CH₃CN over 28 min at a flow rate of 1.5 ml/min.

MS and LC/MS. Electrospray ionization mass spectra (ESI-MS) were obtained on a Thermo Finnigan LCQ DUO ion trap mass spectrometer (Thermo Finnigan, San Jose, CA). Tandem mass spectra (ESI-MS/MS) were produced by collision-induced dissociation of the selected parent ions with the He gas present in the mass analyzer. Samples were introduced to the mass spectrometer through direct infusion (2.5 μl/min). Most samples were dissolved in MeOH/H₂O (1:1) for negative ionization and in MeOH/H₂O (1:1) + 1% acetic acid for positive ionization. The heated capillary was maintained at 200°C and the source voltage at 4.5 kV unless otherwise indicated.

Liquid chromatography/positive ion electrospray ionization-mass spectra (LC/ESI(+)-MS) of [3â′-14C]-2-[2-keto-4′-methylcyclohexyl]propionic acids were acquired on a capillary HPLC system (HP1100; Agilent Technologies Inc., Wilmington, DE) coupled with the LCQ mass spectrometer. The heated capillary was maintained at 50°C and the source voltage at 4.5 kV. The authentic standards (as NH₄CO) were separated from Fluka BioChemika (Milwaukee, WI). Reduced glutathione (GSH) (97% pure) was purified at room temperature at time points ranging from 4 to 72 h after dosing.

Male rats (n = 3) with cannulated bile ducts were dosed intravenously in a tail vein with 0.6 mg of [2-14C]menthofuran/kg, 40 μCi/kg, in water (80%), Emulphor (10%), and ethanol (10%) (1 ml/kg). Bile was collected on ice for 6 h.

Aliquots (20 μl) of urine and bile were counted in Ecolume (ICN Research Products Division, Costa Mesa, CA) in a Beckman Coulter LS 6500 liquid scintillation counter for determination of total 14C. The remainder of the samples were stored at −20°C until analysis by HPLC. The Institutional Animal Care and Use Committee approved all animal procedures.

Identification and Quantification of Urinary Metabolites. Urine samples (0–4, 4–8, and 8–24 h) from rats dosed at 60 mg/kg were centrifuged at 5,000 rpm (Beckman Coulter Microfuge 18 centrifuge) before separation by HPLC (system A) to yield single metabolites. Similar HPLC methods for isolation of pulegone metabolites were used (Chen et al., 2001). During HPLC separation, a small portion of the isolated metabolites was added to Ecolume and counted for 14C in the liquid scintillation counter to ensure that radiolabeled peaks were being collected. The purified metabolites were identified based on 1H NMR, MS, and HPLC (System B) analyses, or independent synthesis. For quantification, composite urine samples (0–24 h, 100 μl) were centrifuged at low gravity and injected onto HPLC (system B) to determine 14C content in major radiolabeled peaks. Reactions of [14C]α′-Dimethoxydihydropemthofuran with N-Acetylcysteine, GSH, NaHSO₄, or Taurine. [14C]α′-Dimethoxydihydropemthofuran was synthesized from [2-14C]menthofuran according to the method described by McClanahan et al. (1989). 2-(4-Acetylcystein-S-yl)menthofuran was prepared as described before (Chen et al., 2001).

[14C]α′-Dimethoxydihydropemthofuran was reacted with GSH in CH₃CN/H₂O (3:1) at room temperature overnight according to a published procedure (Thomassen et al., 1991). The major product was separated by HPLC. Its spectral properties were as follows: negative ion ESI-MS/MS: m/z 454 (M − H), 436 (M − HO₂), 272 (M − 2-thionymethofuran − H), 254 (M − 2-thionymethofuran − HO₂). 1H NMR (D₂O): δ 4.36 (dd, J = 8.9, 4.3 Hz, 1H, Cys α-CH), 3.81 (s, 2H, Gly CH₂), 3.78 (t, J = 6.5 Hz, 1H, GSH α-CH), 3.18 (dd, J = 13.9, 4.4 Hz, 1H, Cys β-CH₂), 2.98 (dd, J = 13.9, 9.3 Hz, 1H, Cys β-CH₂), 2.64 (dd, J = 16.5, 4.4 Hz, 1H), 2.51 (J = 7.2 Hz, 2H, Glu γ-CH₂), 2.38–2.28 (m, 2H), 2.14 (q, J = 7.4 Hz, 2H, Glu β-CH₂), 1.93 (s, 3H, 3-CH₃), 1.92–1.78 (m, 2H), 1.38–1.26 (m, 1H), 1.04 (d, J = 6.6 Hz, 3H, 6-CH₃); the signal of 1H on the six-membered ring overlapped with the signal of Glu β-CH₂. UV: λmax 255 nm. The data are consistent with formation of 2-(glutathion-S-yl)menthofuran.

2-Sulfomenthofuran was synthesized by reacting α′,α′-dimethoxydihydropemthofuran (31.4 mg, 0.15 mmol) with 10 Eq of NaHSO₄ (150.2 mg, 1.4 mmol) in CH₃CN/H₂O (1:1) (8 ml) at 47°C under N₂ overnight. The major product (2-sulfomenthofuran) was purified by HPLC. Its spectral properties were as follows: negative ion ESI-MS/MS: m/z 229 (M − H), 187 (M − C₂H₅ − H, retro-Diels-Alder), 165 (M − H − SO₃), 137 (M − H − SO₃ − CO), 80 (SO₃). 1H NMR (D₂O): δ 2.70 (dd, J = 16.5, 4.9 Hz, 1H), 2.46–2.34 (m, 2H), 2.20 (dd, J = 17.0, 9.1 Hz, 1H), 2.12 (s, 3H, 3-CH₃), 2.03–1.94 (m, 1H), 1.91–1.83 (m, 1H), 1.45–1.32 (m, 1H), 1.08 (d, J = 6.9 Hz, 3H, 6-CH₃); UV: λmax 236 nm. The data are consistent with the structure assigned.

Hexahydro-3,6-dimethyl-1-(2-sulfooethyl)-2-indol-2-one was synthesized by reacting α,α′-dimethoxydihydropemthofuran (31.5 mg,
TABLE 1
Menthofuran-derived radiolabeled peaks in cumulative 24-h urine (mean ± S.D. of four rats) after a single oral administration

<table>
<thead>
<tr>
<th>Peaks</th>
<th>Male Rats</th>
<th>Female Rats</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6 mg/kg</td>
<td>60 mg/kg</td>
</tr>
<tr>
<td>% total dose excreted in urine</td>
<td>41.5 ± 1.6</td>
<td>48.7 ± 2.1</td>
</tr>
<tr>
<td>% total radioactivity injected onto an HPLC</td>
<td>5.2 ± 1.5</td>
<td>5.2 ± 1.0</td>
</tr>
<tr>
<td>d</td>
<td></td>
<td>Trace</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>e</td>
<td>11.5 ± 0.9</td>
<td>10.1 ± 0.7</td>
</tr>
<tr>
<td>f</td>
<td>13.9 ± 0.4</td>
<td>6.7 ± 0.5</td>
</tr>
<tr>
<td>g</td>
<td>7.3 ± 0.7</td>
<td>9.5 ± 1.3</td>
</tr>
<tr>
<td>h</td>
<td>2.2 ± 0.5</td>
<td>1.7 ± 0.6</td>
</tr>
<tr>
<td>i</td>
<td>0.9 ± 0.1</td>
<td>1.0 ± 0.3</td>
</tr>
<tr>
<td>j</td>
<td>5.0 ± 1.0</td>
<td>5.5 ± 0.4</td>
</tr>
<tr>
<td>k</td>
<td>6.4 ± 1.4</td>
<td>4.3 ± 0.5</td>
</tr>
</tbody>
</table>

A *representative HPLC radiochromatogram is shown in Fig. 1A. x and y in the same row are significantly different from each other (p < 0.05).

0.15 mmol) with 2.5 Eq of taурine (47.7 mg, 0.38 mmol, not totally soluble) in CH₃CN/H₂O (3:1) (8 ml) at 37°C overnight. The desired product (a minor product) was purified by HPLC. Its spectral properties were as follows: negative ion ESI-MS/MS: m/z 272 (M – H⁻), 257 (M – H⁺ – CH₃), 164 (M – CH₃CH₂SO₃H), 107 (CH₃ = CHSO₃⁻). H NMR (D₂O): δ 4.08–3.95 (m, 2H, CH₂SO₃H), 3.71–3.61 (m, 1H, 7a-H), 3.28–3.09 (m, 2H, NCH₂), 2.82 (br d, J = 12.6 Hz, 1H, 4-H), 2.48 (dd, J = 9.6, 4.8 Hz, 1H), 2.24 (td, J = 13.1, 4.5 Hz, 1H), 1.97 (br d, J = 12.7 Hz, 1H), 1.77 (s, 3H, 3-CH₃), 1.61–1.71 (m, 1H), 1.08–0.94 (m, 1H), 0.99 (d, J = 6.6 Hz, 3H, 6-CH₃), 0.75 (q, J = 11.8 Hz, 1H). UV: λmax 225 nm. The data are consistent with the structure assigned.

Statistics. Statistical analysis used JMP Software (SAS Institute Inc., Cary, NC) and consisted of an analysis of variance followed by pairwise comparison using a Tukey-Kramer test. Values were considered statistically significant at p < 0.05.

Results

Identification and Quantification of Urinary Metabolites.
Twenty-four hours after single oral administration of [14C]-menthofuran (6 or 60 mg/kg), the radioactivity excreted in urine of treated rats ranged from 36 to 49% of the dose (Table 1). Urine samples (0-24 h) analyzed by HPLC contained several major radiolabeled peaks (a–k) (Fig. 1A). This study focused on characterization of major metabolites within these radiolabeled peaks (a–k). The structures of identified metabolites are shown in Fig. 2.

Peak a appeared predominantly in urine of male rats; only a trace was detected in urine of females. This peak contained a metabolite with the following spectral properties: positive ion ESI-MS/MS [m/z 345 (M – H⁺)] of [2-14C]menthofuran in corn oil; B, bile collected 0 to 6 h from a male rat receiving a single i.v. dose (0.6 mg/kg, 40 μCi/kg) of [2-14C]menthofuran in water (80%), Emulphor (10%), and ethanol (10%); and C, co-injection of the bile sample from B with [2-14C]-2-γ-glutathionyl-menthofuran (↓).

Negative ion ESI-MS analysis of peak d gave two metabolites with m/z equal to 345 and 359. Metabolite d1 (m/z 345) had the following properties: negative ion ESI-MS/MS: m/z 345 (M – H⁺), 327 (M – H₂O), 193 (glucuronide ion), 175 (glucuronide ion – H₂O), 169 (8-hydroxyemethylenone anion), 157 (glucuronide ion – 2 H₂O). H NMR (D₂O): δ 1.00 (d, J = 7.3 Hz, 3H), 0.82 (d, J = 7.0 Hz, 3H); the other signals were not resolved. UV: λmax < 190 nm. The spectral properties of metabolite d1 of menthofuran are the same as metabolite G1 of pulegone, and they comigrated upon HPLC analysis. Pulegone metabolite G1 was assigned as 7-hydroxyemethylenone glucuronide (Chen et al., 2001). Isolation of metabolite d1 (G1) from menthofuran puts this assignment in doubt, because conversion of menthofuran to a monohene seems unlikely on chemical grounds. Metabolite d1 (G1) is more likely to be one of the octahydro-3,6-dimethyl-7a-hydroxybenzofuran glucuronides, which could exist as several isomers including pulegone metabolite E2 (Chen et al., 2001). Metabolite d2 (m/z 359) has been purified before as the metabolite G2 of pulegone (Chen et al., 2001). Its spectral properties were as follows: negative ion ESI-MS/MS: m/z 359 (M – H⁺), 193 (glucuronide ion),
183 ([2'-keto-4'-methylcyclohexyl]propionic ion), 175 (glucuronide ion - H2O). UV: \( \lambda_{\text{max}} < 190 \text{ nm} \). Metabolite d2 (G2) was tentatively identified as hexahydro-3,6-dimethyl-7a-hydroxy-2(3H)-benzofuranone glucuronide.

Peak e contained two metabolites with \( m/z \) equal to 272 and 247. Metabolite e1 (\( m/z \) 272) had the following spectral properties: negative ion ESI-MS/MS: \( m/z \) 272 (M-\( H \)), 257 (M-\( H \)-CH3), 208 (M-\( H \)-SO2), 164 (M-CH3CH2SO2H), 107 (CH3 = CHSO3-), 81 (HSO3-), 80 (SO3). 1H NMR (D2O): 1.77 (s, 3H, 3-CH3), 1.00 (d, \( J = 5.7 \text{ Hz} \), 3H, 6-CH3); the other signals were not well resolved. UV: \( \lambda_{\text{max}} \) 220 nm. To assign some of the MS fragments of e1, negative ion ESI-MS/MS analysis of taurine was carried out: \( m/z \) 124 (M-\( H \)), 107 (CH3 = CHSO3-), 81 (HSO3-), 80 (SO3). The result confirmed that taurine is part of e1. We assigned e1 as hexahydro-3,6-dimethyl-1-(2-sulfoethyl)-2H-indol-2-one, the dehydrated taurine adduct of the reactive enonal. To prove the structure assigned, we independently synthesized an authentic standard by reacting taurine with 2-(N-acetylcyestein-S-sulfo) menthofuran (Fig. 3). 1H NMR and mass spectra as well as the HPLC chromatograms of the metabolite and the authentic standard were identical. Metabolite e2 (\( m/z \) 247) had the following spectral properties. Negative ion ESI-MS/MS: \( m/z \) 247 (M-\( H \)), 183 (M-\( H \)-SO2), 81 (HSO3-). 1H NMR (D2O): \( \delta \) 2.90 (dt, \( J = 11.5, 7.0 \text{ Hz} \), 1H, 3a-H), 2.37 (br d, \( J = 15.1 \text{ Hz} \), 1H, 7eq-H), 1.99–1.89 (m, 1H, 3-H), 1.70 (dd, \( J = 14.8, 12.4 \text{ Hz} \), 1H, 7ax-H), 1.56–1.46 (m, 1H), 1.14 (d, \( J = 7.4 \text{ Hz} \), 3H, 3-CH3), 0.99 (d, \( J = 6.3 \text{ Hz} \), 3H, 6-CH3); there was one proton overlapping with 7ax-H and possibly three protons overlapping with the CH2 groups. UV: \( \lambda_{\text{max}} < 190 \text{ nm} \). MS analysis demonstrated that e2 could undergo hydrolysis to give a fragment with \( m/z \) 183, likely an anion of 2-[2'-keto-4'-methylcyclohexyl]propionic acids (mol. wt. 184). Its 1H NMR and UV spectra are similar to those of 2-[2'-keto-4'-methylcyclohexyl]propionic acids, consistent with e2 being a sulfonato-lactone of 2-[2'-keto-4'-methylcyclohexyl]propionic acids. The mechanisms proposed in Fig. 2 show that addition of GSH (or sulfite ion) to the ketone or aldehyde would eventually end up with formation of e2 and k. Based on the spectral data and the proposed mechanisms, we assign e2 as hexahydro-3,6-dimethyl-7a-sulfo-2(3H)-benzofuranone. An attempt to independently synthesize e2 by reacting 2-[2'-keto-4'-methylcyclohexyl]propionic acids with NaHSO3 was unsuccessful.

Peak f contained a metabolite, which was converted to mintlactones when stored in the acidified solvents used for isolation. We suspected that the metabolite f was one of the previously identified menthofuran metabolites, 2-[2'-keto-4'-methylcyclohexyl]propionic acids (Madyastha and Raj, 1992). Dehydration of 2-[2'-keto-4'-methylcyclohexyl]propionic acids would give rise to mintlactones. Authentic [3,14C]-2-[2'-keto-4'-methylcyclohexyl]propionic acids were prepared and analyzed by HPLC to show four isomers with the same retention time.
as peaks f, g, i, and j in Fig. 1A in the following ratio: 6.3%, 33.4%, 36.0%, and 24.3%, respectively. LC/ESI(+)-MS analysis of peaks f and g to k (see below) gave metabolites f, g1, i, and j (as NH4+ salts) with the same retention times and spectra as those of authentic [3-14C]-2-[2’-keto-4’-methylcyclohexyl]propionic acids.

Peaks g to k were isolated in one fraction. Another metabolite, g2, present in peak g was identified as 7a-hydroxymintlactone since its 1H NMR and mass spectra were identical to those of the authentic compound. Further HPLC purification of this fraction gave rise to a metabolite h present in peak h with the same spectral properties as those of the metabolite J of pulegone, which is 2-(2’-hydroxy-4’-methylphenyl)propionic acid (Chen et al., 2001). Peak k contained a metabolite k with the following properties: negative ion ESI-MS/MS [in 10 mM NH4OAc in H2O-CH3CN (1:1)], m/z 229 (M – H+), 187 (M – C6H5 – H+), 165 (M – H+ – SO3), 137 (M – H+ – SO3 – CO), 108 (d, J = 6.6 Hz, 3H), and 92 (d, J = 4.2 Hz, 3H); the other signals were not well resolved. UV: λmax 236 nm. From the spectral data, we suspected that k is 2-sulfomenthofuran. To prove the structure assigned, we independently synthesized an authentic standard by reacting NaHSO3 with α,α’-dimethoxydihydromenthofuran (Fig. 3). 1H NMR and mass spectra as well as the HPLC chromatograms of the metabolite and the authentic standard were identical.

Authentic menthofuran and 2-(N-acetylcystein-S-yl)menthofuran prepared from reaction of α,α’-dimethoxydihydromenthofuran with N-acetylcysteine (Fig. 3) were analyzed by HPLC (method 2) to determine whether they were present in urine of treated rats. These chemicals were not eluted using HPLC method 1. Menthofuran (retention time = 26.0 min) was not detected in urine. There was a small radiolabeled peak (retention time = 19.1 min) in the 24-h urine of male rats treated with 60 mg/kg menthofuran (0.4% of the total radioactivity injected onto the HPLC) that coeluted with the 2-(N-acetylcystein-S-yl)menthofuran standard.

The percentage of each major peak a to k with each dosing method was calculated based on the 14C count in each peak compared with total 14C count in each injection (Table 1). Peak c is present as a significantly greater percentage of the total radioactivity in urine from female rats treated with 60 mg/kg compared with males treated with either 6 or 60 mg/kg. A significantly higher percentage of the total radioactivity was contained in peaks f and g in urine from female rats compared with males at 6 and 60 mg/kg, respectively. Peak k contains a greater percentage radioactivity in urine from low-dose males compared with high-dose rats of either sex. Differences in percentage of total radioactivity contained in other peaks are not significant.

Analysis of Biliary Metabolites. Male rats, with their bile ducts cannulated, excreted 40% of the total i.v. dose (0.6 mg/kg menthofuran) in bile within 6 h. Bile samples collected 0 to 6 h after dosing were analyzed by HPLC (Fig. 1B). Coinjection of bile and urine samples suggested that radiolabeled peaks c, d, f, and j in the urinary metabolic profile were also present in bile.

2-(Glutathion-S-yl)menthofuran has been partially identified by LC-MS in bile of rats dosed with pulegone (Thomassen et al., 1991). To determine whether it is a biliary metabolite of menthofuran, [2,14C]-2-(glutathion-S-yl)menthofuran was prepared from reaction of [14C]α,α’-dimethoxydihydromenthofuran with GSH according to a published method (Fig. 3; Thomassen et al., 1991). Coinjection of the standard and the bile sample (0–6 h) demonstrated that 2-(glutathion-S-yl)menthofuran is at most a minor biliary metabolite of menthofuran (Fig. 1C).

Discussion

The current study focused on characterization of urinary metabolites of menthofuran in rats. We identified 7a-hydroxymintlactone (g2), 6,7a-dihydroxymintlactone (a), 7a-hydroxymintlactone glucuronide (e), 2-[2’-keto-4’-methylcyclohexyl]propionic acids (f, g1, i, and j), octahydro-3,6-dimethyl-7a-hydroxybenzofuran glucuronide (d1), hexahydro-3,6-dimethyl-7a-hydroxy-2(3H)-benzofuranone glucuronide (d2), and 2-(2’-hydroxy-4’-methylphenyl)propionic acid (h) as menthofuran metabolites. We also characterized three sulfonic acid metabolites of menthofuran, namely, hexahydro-3,6-dimethyl-1-(2-sulfoethyl)-2H-indol-2-one (e1), hexahydro-3,6-dimethyl-7a-sulfo-2(3H)-benzo[furanone (e2), and 2-sulfomenthofuran (k) (Fig. 2). Unchanged menthofuran was not detected in urine. Female rats treated with 60 mg/kg menthofuran excreted a significantly greater percentage of 7a-hydroxymintlactone glucuronide (e) in urine than did male rats treated with 6 or 60 mg/kg within 24 h. This metabolite could account for a higher excretion of menthofuran-derived radioactivity in urine of female rats in 24 h (Table 1).

Some of the metabolites identified in this study have been previously characterized in rats dosed with pulegone. Menthofuran metabolites e, d1, d2, and h are identical to pulegone metabolites E3, G1, G2, and J (Fig. 2; Chen et al., 2001). Identification of common urinary metabolites from pulegone and menthofuran helps further define the position of menthofuran in the complex pulegone/menthofuran metabolic pathways. Other menthofuran metabolites were likely present in urine of rats dosed with pulegone but were not characterized due to low concentrations or different isolation procedures.

An in vitro study has shown that menthofuran is metabolized to mintlactones through the reactive enonal, which is capable of binding to proteins (Thomassen et al., 1992). In vitro microsomal metabolism of mintlactones gave 7a-hydroxymintlactone as one of the metabolites (unpublished observation). We have recently observed that mintlactones and 7a-hydroxymintlactone are among metabolites noncovalently bound to α-2u-globulin in kidney of male rats dosed with menthofuran (unpublished observation). Both in vitro and in vivo data support the sequential metabolism of menthofuran to mintlactones and then to 7a-hydroxymintlactone (g2) as shown in Fig. 2. However, we cannot totally rule out that g2 could also be generated through oxi-
dation of the hydrated enonal. Further hydroxylation of g2 could give rise to a, although the 6-CH3 group could have been hydroxylated first. Glucuronidation or reduction of g2 could give rise to the more abundant metabolites 7a-hydroxymintlactone glucuronide c (E3) and 2-[2'-keto-4'-methylcyclohexyl]propionic acids (f, g1, i, and j). Alternatively, f, g1, i, and j could also be produced through hydrolysis ofmintlactones followed by rearrangement. Glucuronidation or repeated hydroxylation/dehydration of 2-[2'-keto-4'-methylcyclohexyl]propionic acids (f, g1, i, and j) could result in formation of d2 (G2) and the aromatic metabolite, h (J). Some of the 2-[2'-keto-4'-methylcyclohexyl]propionic acids might be reduced to give alcohols followed by glucuronidation to give d1 (G1); however, we are not certain about the pathway leading to formation of d1 (G1) (Fig. 2).

Three sulfonic acid metabolites of menthofuran (e1, e2, and k) were characterized in urine. There are two mechanisms proposed for the formation of e2 and k. The first one involves addition of glutathione to the ketone or the aldehyde of the enonal followed by further metabolism to give e2 and k (Fig. 2). A similar transformation has been observed in degradation of acetoclor S-glutathione conjugate in soil to give a sulfonic acid metabolite (Feng, 1991). A trace of 2-(N-acetylcystein-S-yl)menthofuran, a possible intermediate in the transformation, was detected in urine. The second mechanism involves direct addition of sulfite ion to the ketone or the aldehyde of the enonal, likely by the gut flora. Fecal metabolites from direct addition of sulfite ion to N-[4-chloro-2-fluoro-5-[1-(methyl-2-propynyl)oxy]phenyl]-3,4,5,6-tetrahydrophthalimide (S-23121) and its metabolites in the intestinal tract have been observed in rats (Yoshino et al., 1993). These authors showed evidence for direct incorporation of sulfite ion from coadministration of unlabeled S-23121 with 35S-labeled sodium sulfate, cysteine, or glutathione to give 11, 4, and 1%, respectively, incorporation of 35S into the sulfonic acid metabolites. Reduction of sulfate to sulfite by gut flora is required for formation of sulfonic acid metabolites of S-23121, a reduction process that is not feasible in mammalian species. Because e2 and k were observed in urine, absorption of e2 and k was necessary to complete the process. Addition of tuarine to the enonal could first give a diol, which could then be converted to e1 through a 1,4-dehydration and rearrangement. This conjugation is similar to reaction between N-acetyl-l-lysine and cis-2-butene-1,4-dial, a reactive metabolite of furan, to give a pyrrolin-2-one (Chen et al., 1997). In vivo metabolites of furan have never been characterized; therefore, it is not known whether similar metabolites can be formed between cis-2-butene-1,4-dial and glutathione or tuarine (Burka et al., 1991). Isolation of these sulfonic acid metabolites suggests that the covalent binding between the enonal and the thiol and amino groups of tissue proteins could have taken place.

These newly characterized urinary metabolites of menthofuran are mostly different from those reported in literature (Madyastha and Raj, 1992) except 2-[2'-keto-4'-methylcyclohexyl]propionic acids (f, g1, i, and j). The previous study identified 10 urinary metabolites in rats. Seven metabolites reported by Madyastha and Raj (1992) were formed as a result of the cleavage of the carbon-carbon double bond of the enonal to give propanaldehyde and methylcyclohexanones. Because of the position of the 14C label in [2,14C]menthofuran, methylcyclohexanones, if formed, would not have been detected. However, cleavage of the carbon-carbon double bond and subsequent metabolism should lead ultimately to expiration of radioiodized CO2 or volatiles, which turned out to be minor pathways in our study (0.5% of the 60 mg/kg oral dose in 24 h; unpublished observation). The difference in dose and isolation procedures might contribute to the difference in outcome of the metabolic studies.

The bile duct-cannulated male rats excreted 40% of the total i.v. dose (0.6 mg/kg) in bile within 6 h. This recovery is higher than what has been found in feces (10% of the dose) of male rats 24 h after receiving a single 0.6 mg/kg i.v. dose (unpublished observation). This result indicates that enterohpatic circulation occurs during metabolism of menthofuran. The biliary metabolites of menthofuran have not been characterized; however, based on HPLC coinjection of urine and bile samples, it is likely that some of the identified urinary metabolites are also biliary metabolites (Fig. 1B). 2-(Glutathion-S-yl)menthofuran, a GSH conjugate of the enonal that has been partially characterized in bile of rats dosed with pulegone, was synthesized and analyzed. It is at most a minor biliary metabolite of menthofuran (Fig. 1C). The majority of 2-(glutathion-S-yl)menthofuran formed in liver might have been transported to kidney for further metabolism to give k and only a small amount excreted in bile.

In summary, common urinary metabolites from rats dosed with pulegone and menthofuran were identified. The current studies are in agreement with the in vitro study that demonstrated formation of the reactive enonal from metabolism of menthofuran (Thomassen et al., 1992) and reveal the complex metabolic fate of the reactive intermediate in vivo. Menthofuran metabolites that are likely derived from reactions of the enonal intermediate with water, glutathione, and tuarine have been identified. Some of the urinary metabolites may have been the result of multiple rounds of metabolism due to enterohpatic circulation.

Acknowledgments. We acknowledge the assistance of Fred B. Lih and Dr. Kenneth B. Tomer for obtaining part of the LC-MS/MS data.

References