Formation of a Predominant Metabolite of Hydroxydihydrocarvone Evaluated by a Biomimetic Oxidative Model and in Rat Liver Microsomes

Sara M. Thomazzi[1], Fernanda L. Moreira[2], Izabel C. C. Turatti[3], Juliana N. Paula e Souza[3], Luciana N. Andrade[1], Denise B. Silva[3], Anderson R. M. Oliveira[4], Damião P. De Sousa[5], Norberto P. Lopes[3]

[1] Departamento de Fisiologia, Universidade Federal de Sergipe, São Cristóvão, SE, Brazil
[2] Departamento de Ciências Farmacêuticas, Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, SP, Brazil
[3] NPPNS, Departamento de Físico-Química, Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, SP, Brazil
[4] Departamento de Química, Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, SP, Brazil
[5] Centro de Ciências da Saúde-Campus I, Universidade Federal da Paraíba, João Pessoa, PB, Brazil

Abstract

This paper reports the biomimetic oxidation of hydroxydihydrocarvone by iodosylbenzene using tetraphenyl-porphine iron(III) chloride as the catalyst in ethyl acetate. Mass spectrometry fragmentation maps of hydroxydihydrocarvone (obtained by gas chromatography-mass spectrometry analyses) allowed for the identification of the major product as 4-hydroxy-5-(2-hydroxypropan-2-yl)-2-methylcyclohex-2-en-1-one (4-hydroxy-hydroxydihydrocarvone). This compound was also observed in an *in vitro* metabolism assay that employed isolated rat liver microsomes, thereby validating the biomimetic procedure.

Key words

biomimetic oxidation · drug metabolism · hydroxydihydrocarvone · liver microsomal metabolism · oxidative metabolism · phase I metabolism

Abbreviations

CYP: cytochrome
CG-MS: Gas chromatography-mass spectrometry
CO: carbon monoxide
EI: electron ionization
EI-MS: electron ionization-mass spectrometry
ESI-MS: electrospray ionization-mass spectrometry
ESI-TOF: electrospray ionization – time-of-flight mass spectrometry
Fe(TPP)Cl: 5,10,15,20-tetraphenyl-21 H,23 H- porphine iron(III) chloride
HC: hydroxydihydrocarvone
m-CPBA: *meta*-chloroperbenzoic acid
Mn(salen): (S,S)-(+)-N,N′-Bis(3,5-di-*tert*-butylsalicylidene)-1,2-cyclohexane-diaminomanganese(III) chloride
NADP+: Nicotinamide adenine dinucleotide phosphate
NADPH: Reduced nicotinamide adenine dinucleotide phosphate
PhIO: iodosylbenzene
4-hydroxy-HC: 4-hydroxy-5-(2-hydroxypropan-2-yl)-2-methylcyclohex-2-en-1-one

Supporting information available online at http://www.thieme-connect.de/products

HC (**Fig. 1 A**) is a semisynthetic monoterpene obtained by hydration of the natural compound (*R*)-(−)-carvone [1]. Previous studies have demonstrated that this compound protects mice against pentylenetetrazol-induced convulsions, potentiates the pentobarbital sleeping time, and presents an antinociceptive effect in both the chemical and thermal nociception models [2,3]. This compound exerts anti-inflammatory action in rodents by inhibiting both increased plasma extravasation and leukocyte influx [4,5]. The subacute administration of HC did not lead to pharmacological tolerance and did not cause significant toxicological alterations in mice [6,7].

Several monoterpenoids, including 1,4- and 1,8-cineole, limonenes, menthols, α-thujone, and terpinen-4-ol, are metabolized by CYP450 enzymes in human liver microsomes [8–13]. However, there are no prior studies reporting the metabolism of HC. The oxidative metabolism has been investigated through biological simulation models, using perfused organs, isolated cells, or cell fragments (microsomes) [14], and through several biomimetic models [15]. In this context, the aim of this work was to investigate HC metabolism using biomimetic reactions and by employing rat liver microsomes.

The oxidation of HC, catalyzed by the porphyrin Fe(TPP)Cl or Jacobsen catalyst [Mn(salen)], was performed in the presence of PhIO or *m*-CPBA, employing either ethyl acetate or dichloromethane as solvents. The analysis indicated that a higher yield of oxidized product after 24 h was obtained when the reaction was catalyzed by Fe(TPP)Cl using PhIO as the oxidant in ethyl acetate (**Table 1**).

Fe(TPP)Cl can catalyze a wide range of CYP-mediated reactions, including epoxidation, aliphatic and aromatic hydroxylation, and the oxidation of heteroatoms [15,16]. PhIO is considered a standard oxidant for metalloporphyrin systems [17,18].

The reaction was monitored by GC-MS, revealing a mass spectra profile in EI-MS that could be assigned as an oxidation product from HC (**Fig. 1 S (A)**, Supporting Information). As expected, the molecular ion was not observed in EI, but ESI-TOF confirmed the molecular formula of the oxidative product at high resolution (**Fig. 2 A**). The detailed analysis of the fragmentation map of the oxidative product was fully consistent with allylic oxidation. Normally, electron loss is more prone to occur at the oxygen atom of the carbonyl group than at the oxygen atom of the hydroxyl group. In this case, oxidation at the allylic position induces a resonance structure (**Fig. 1**) that distributes the possibility of losing an electron between the two oxygen atoms [19]. Thus, the two major pathways begin. In the first case (**Fig. 1 B**), after electron abstraction occurs, a classical 6-member mechanism of water elimination affords a stable allylic carbocation [19]. In this case, after the neutral elimination of CH_2COHCH_3, the base peak at *m/z* 108 is observed. The sequential ions are formed by CO or radical eliminations. Pathway B begins by the cyclic ketones mechanism, and after the neutral elimination of 70 u, the se-

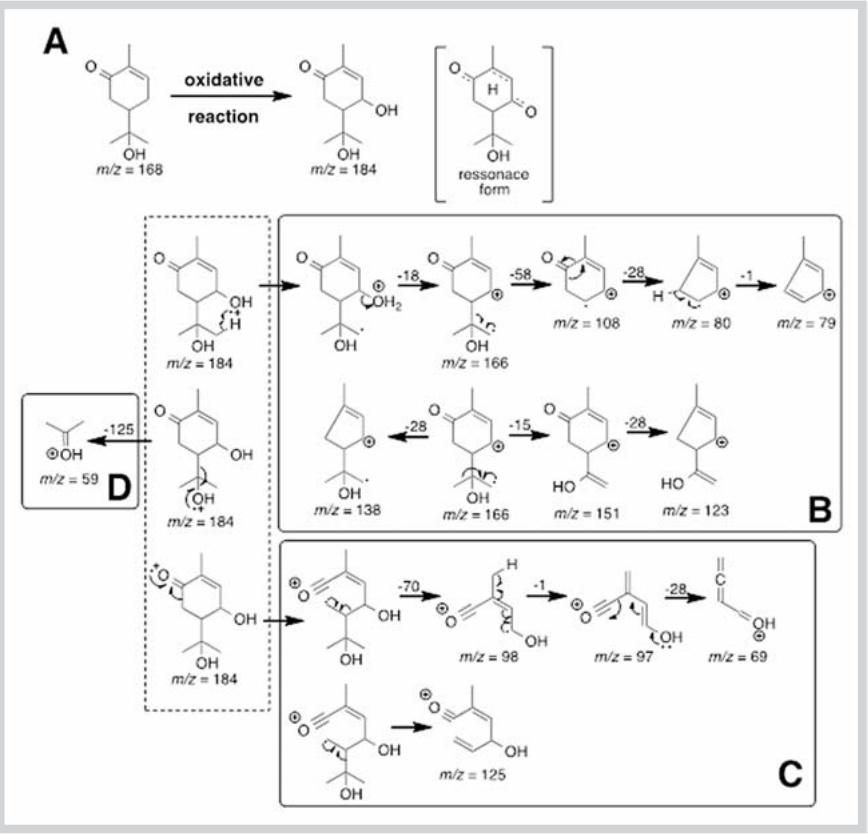

Fig. 1 Fragmentation pathway of 4-hydroxy-HC.

		Predominant metabolite[a]	
Catalyst	**Solvent**	**PhIO**	***m*-CPBA**
Fe(TPP)Cl	Ethyl acetate	52.31	Product not detected
	Dichloromethane	427.42	Product not detected
Mn(salen)	Ethyl acetate	2812.77	Product not detected
	Dichloromethane	1839.30	Product not detected

[a] Ratio between the HC area and predominant metabolite area determined by GC-MS analysis after 24 h of incubation

Table 1 Oxidation of hydroxydihydrocarvone in biomimetic reactions.

quential ions are formed (**Fig. 1C**). Finally, the minor pathway that starts with the direct abstraction of one electron from the hydroxyl group at the side chain affords a key ion at *m/z* 59 (**Fig. 1D**). Taken together, this information confirms the proposed structure.

Fig. 2B shows the time-dependent profile of HC oxidation by PhIO due to the predominant metabolite formation. At time zero, before the addition of PhIO, only *m/z* 168 (HC) is observed, eluting at 25.06 min with a base peak at *m/z* 59. After 24 h of analysis, semiquantitative data indicate that the product obtained is a metabolite (*m/z* 184) eluting at 30.74 min.

The rat liver microsomes model has been extensively used by our group in the study of the metabolism of natural products [20,21]. To establish a correlation between the oxidation reaction and the biological processes of drug metabolism, HC was subjected to *in vitro* metabolism employing rat liver microsomes. GC-MS analysis revealed that the same predominant metabolite obtained from the biomimetic oxidation reaction was observed in the *in vitro* biological study (base peak at *m/z* 108; t_R = 30.72 min) (**Fig. 1S (B)**, Supporting Information).

In conclusion, the Fe(TPP)Cl-catalyzed oxidation of HC resulted in the formation of a predominant metabolite. The same metabolite was observed in the *in vitro* metabolism assay using rat liver microsomes, thus confirming the chemical model of drug metabolism. In addition, these results represent the first report on the *in vitro* oxidative metabolism of HC.

Materials and Methods

Chemicals and materials

Fe(TPP)Cl, Mn(salen), *m*-CPBA, $NADP^+$, glucose-6-phosphate, and glucose-6-phosphate dehydrogenase were purchased from Sigma-Aldrich, as was iodosylbenzene diacetate, which was used to generate the oxidizing agent PhIO [22], reaching a purity of 85–90% determined by iodometric titration. The ethyl acetate and dichloromethane were of analytical grade (Malinckrodt Pharmaceuticals). Hydroxydihydrocarvone was synthesized as previously described [1].

Biomimetic oxidation

All reactions were performed at room temperature, with air and light excluded, in a glass vessel containing screw caps and equipped with a magnetic stirring bar. A catalyst [Mn(salen) or Fe(TPP)Cl, 1 µmol] and the substrate (HC, 30 µmol) were dis-

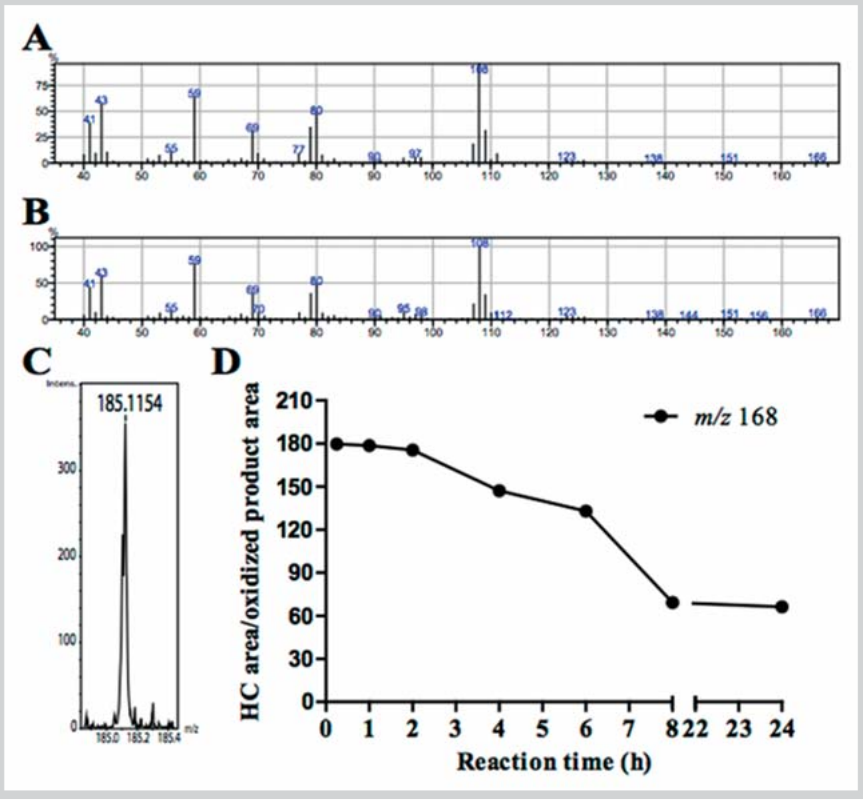

Fig. 2 **A** ESI-TOF in high resolution. **B** Time-dependent profile of HC oxidation using a biomimetic model.

solved in ethyl acetate or dichloromethane to a total volume of 4.0 mL, and the oxidizing agent *m*-CPBA or PhIO (30 µmol) was then added. The products after 0.0, 0.25, 1.0, 2.0, 4.0, 6.0, 8.0, and 24 h were analyzed by GC-MS and identified by mass spectrometry. Control and blank reactions were conducted in the absence of catalyst and substrate, respectively.

Gas chromatography-mass spectrometry assay

Chromatographic analyzes were performed using a Shimadzu GC-MS system (GCMS-QP2010) coupled with a Shimadzu autosampler (AOC-5000). The resolution of HC was achieved on a DB5-MS column of 0.25 µm film thickness, 30.0 m length, and 0.25 mm diameter (Agilent Technologies, Inc.). One µL of the samples was injected with a split ratio of 1 : 10 or splitless (1 min) for biomimetic oxidation or microsomal incubation, respectively, at an injector temperature at 250 °C. The initial oven temperature was set to 60 °C and increased by 3 °C/min to 150 °C, then increased by 15 °C/min to 290 °C and held for 5.0 min. The carrier gas was high-purity helium at 1.3 mL/min. The interface and ion source temperatures in the mass spectrometer were 290 °C and 250 °C, respectively. The ionization voltage was set to 70 eV, and positive-charge ions were analyzed in full-scan mode, applying a scan of *m/z* 40 to 500. Finally, to confirm the molecular mass, the metabolite was analyzed by high-resolution ESI-MS (ultrOTOFQ, Bruker Daltonics).

Animal and microsomal preparation

Male Wistar rats (180–220 g; n = 5) were obtained from the Faculty of Pharmaceutical Sciences of Ribeirão Preto-University of São Paulo (ethical approval 13.1.529.53.7 in 02/12/2013). The animals were fed under normal conditions and acclimatized to a 12-h light/dark cycle. The animals were sacrificed by decapitation, and the livers were removed and placed in ice-cold 0.05 mol/L Tris-HCl buffer (pH 7.4) containing 0.15 mol/L KCl. Microsomal preparation was performed as previously described [20] and stored at − 70 °C until use.

Microsomal incubation conditions

A cofactor solution (250 µL), substrate (HC, 2 mg/mL, 20 µL), and phosphate buffer (0.25 mol/L, pH 7.4, 578 µL) were prewarmed at 37 °C for 5 min in tubes with screw caps. The cofactor solution consisted of $NADP^+$ (0.25 mM), glucose-6-phosphate (5 mM), and glucose-6-phosphate dehydrogenase (0.5 units) in Tris-HCl buffer (Tris-HCl 0.05 mol/L-KCl 0.15 mol/L, pH 7.4). The reaction was then initiated by the addition of the rat liver microsomal preparation (2 mg/mL, 152 µL) to a total volume of 1.0 mL and incubated for 60 min (37 °C) using a shaking water bath. The reaction was terminated by the addition of ethyl acetate (4.0 mL).

To extract the HC and its metabolite from the rat liver microsomes, 4 mL ethyl acetate was used as the extractor solvent. The samples were shaken for 15 min at 1000 rpm (Vibrax VXR, IKA) and centrifuged for 5 min at 2860 × *g* (Hitachi CF16RXII, Himac). The supernatant (3 mL) was evaporated to dryness using a gentle stream of compressed air.

Each sample was pooled from ten single reactions and resuspended in 300 µL of ethyl acetate. Control incubations were performed in the absence of cofactor solution. A blank reaction was performed in the absence of cofactor solution and substrate. The difference between "with" and "without" NADPH was considered to indicate CYP450-mediated metabolism.

Supporting information

Mass spectra of the predominant metabolite obtained from biomimetic oxidation and rat liver microsomes are available as Supporting Information.

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Conflict of Interest

The authors declare no conflict of interest.

References

1 *Büchi G, Wüest HJ.* New synthesis of *β*-agarofuran and of dihydroagarofuran. J Org Chem 1979; 44: 546–549
2 *De Sousa DP, Oliveira FS, Almeida RN.* Evaluation of the central activity of hydroxydihydrocarvone. Biol Pharm Bull 2006; 29: 811–812
3 *Oliveira FS, De Sousa DP, Almeida RN.* Antinociceptive effect of hydroxydihydrocarvone. Biol Pharm Bull 2008; 31: 588–591
4 *de Cássia da Silveira e Sá R, Andrade LN, De Sousa DP.* A review on anti-inflammatory activity of monoterpenes. Molecules 2013; 18: 1227–1254
5 *de Sousa DP, Camargo EA, Oliveira FS, Almeida RN.* Anti-inflammatory activity of hydroxydihydrocarvone. Z Naturforsch C 2010; 65: 543–550
6 *Guimarães AG, Quintans JS, Quintans jr. LJ.* Monoterpenes with analgesic activity-a systematic review. Phytother Res 2013; 27: 1–15
7 *Oliveira FS, Silva MVB, Sena MCP, Santos HB, Oliveira KM, Diniz MFFM, De Sousa DP, Almeida RN.* Subacute toxicological evaluation of hydroxydihydrocarvone in mice. Pharm Biol 2009; 47: 690–696
8 *Abass K, Reponen P, Mattila S, Pelkonen O.* Metabolism of *α*-thujone in human hepatic preparations *in vitro*. Xenobiotica 2011; 41: 101–111
9 *Haigou R, Miyazawa M.* Metabolism of (+)-terpinen-4-ol by cytochrome P450 enzymes in human liver microsomes. J Oleo Sci 2012; 61: 35–43
10 *Miyazawa M, Marumoto S, Takahashi T, Nakahashi H, Haigou R, Nakanishi K.* Metabolism of (+)- and (−)-menthols by CYP2A6 in human liver microsomes. J Oleo Sci 2011; 60: 127–132
11 *Miyazawa M, Shindo M, Shimada T.* Roles of cytochrome P4503 A enzymes in the 2-hydroxylation of 1, 4-cineole, a monoterpene cyclic ether, by rat and human liver microsomes. Xenobiotica 2001; 31: 713–723
12 *Miyazawa M, Shindo M, Shimada T.* Oxidation of 1, 8-cineole, the monoterpene cyclic ether originated from eucalyptus polybractea, by cytochrome P4503A enzymes in rat and human liver microsomes. Drug Metab Dispos 2001; 29: 200–205
13 *Miyazawa M, Shindo M, Shimada T.* Metabolism of (+)- and (−)-limonenes to respective carveols and perillyl alcohols by CYP2C9 and CYP2C19 in human liver microsomes. Drug Metab Dispos 2002; 30: 602–607
14 *Riley RJ, Grime K.* Metabolic screening *in vitro*: metabolic stability CYP inhibition and induction. Drug Discov Today Technol 2004; 1: 365–372
15 *Lohmann W, Karst U.* Biomimetic modeling of oxidative drug metabolismo – strategies, advantages and limitations. Anal Bioanal Chem 2008; 391: 79–96
16 *Sono M, Roach MP, Coulter ED, Dawson JH.* Heme-containing oxygenases. Chem Rev 1996; 96: 2841–2888
17 *Groves JT.* High-valent iron in chemical and biological oxidations. J Inorg Biochem 2006; 100: 434–447
18 *Mansuy D.* A brief history of the contribution of metalloporphyrin models to cytochrome P450 chemistry and oxidation catalysis. C R Chim 2007; 10: 392–413
19 *Pavia DL, Lampman GM, Kriz GS, Vyvyan JR.* Introdução à Espectroscopia, 4th edition. São Paulo: Cengage Learning; 2012
20 *Messiano GB, Santos RAS, Ferreira LS, Simões RA, Jabor VAP, Kato MJ, Lopes NP, Pupo MT, Oliveira ARM. In vitro* metabolism study of the promising anticancer agent the lignan (−)-grandisin. J Pharm Biomed Anal 2013; 72: 240–244
21 *Pigatto MC, Lima MCA, Galdino SL, Pitta IR, Vessecchi R, Assis MD, Santos JS, Costa TD, Lopes NP.* Metabolism evaluation of the anticancer candidate AC04 by biomimetic oxidative model and rat liver microsomes. Eur J Med Chem 2011; 46: 4245–4251
22 *Saltzmann H, Sharefkin JG.* Iodosylbenzene. Org Synth 1973; 5: 658

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Bibliography
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Correspondence
Prof. Norberto P. Lopes
Núcleo de Pesquisa em Produtos Naturais e Sintéticos
Department of Physics and Chemistry, Faculty of Pharmaceutical Sciences
University of São Paulo
Av. do Café, s/n,
4040–903 Ribeirão Preto, São Paulo
Brazil
Phone: + 55 16 36 02 41 68
npelopes@fcfrp.usp.br