In Vitro Characterization of Borneol Metabolites by GC–MS Upon Incubation with Rat Liver Microsomes

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Abstract

The metabolism of borneol is studied by the analysis of incubations of in vitro-prepared rat liver microsomes. A sensitive gas chromatography (GC)–mass spectrometry (MS) method is developed for the identification of borneol and its metabolites. Four novel metabolites, which have not previously been reported, are isolated and confirmed by comparison of the GC–MS method. The biotransformation pathway of borneol in rat liver microsomes is proposed based on the in vitro results.

Introduction

Borneol (BN) (Figure 1), a monoterpenoid alcohol, is an important component used in traditional Chinese medicine. Because of its high pharmacological activity, low toxicity, and rare complications, BN plays an important role in the clinical therapy of cardiovascular and cerebrovascular diseases (1–4).

BN is classified as synthetical borneol (SBN) and natural borneol (NBN). SBN includes borneol and isoborneol (IBN), and NBN contains only borneol. A review of the literature (5,6) showed that there were no differences between SBN and NBN in terms of their pharmacodynamics. Because of its low price and ease of availability, SBN is widely used in clinical therapy. The present study is mainly focused on the pharmacodynamic action, pharmacology, and pharmacokinetics of BN. The studies of Liu et al. and Chen et al. (7–9) suggest that BN can promote free movement between the blood-brain barriers, enhance the distribution of drugs in brain tissue, and improve the oral bioavailability of some drugs. The studies of Mi et al. and Wang et al. (10–11) indicated that the pharmacokinetics of BN can be described with a two-compartment open model of extravascular administration. The concentration of the drug in blood tended to reach a maximum level after 0.5 h. The half-life of a single dose was 2.53 h, while the half-life of multiple doses was 9.22 h. However, no in vitro or in vivo studies have been performed on BN phase I metabolisms. Thus, in this study, the in vitro metabolism of BN in rat liver microsomes was investigated. Metabolites observed were identified using a gas chromatography (GC)–mass spectrometry (MS) method, and a metabolic pathway of BN in rat liver microsomes was proposed.

Experimental

Chemicals and reagents

BN and IBN were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Nicotinamide-adenine dinucleotide phosphate (NADPH) was purchased from Roche (Guangzhou, China). Ethyl acetate was purchased from Merck (Darmstadt, Germany). Water was double-distilled and deionized. All other chemicals were obtained from the standard commercial sources. Organic solvents were high-performance liquid chromatography grade and were filtered through a 0.45-µm membrane before use.

Preparation of rat liver microsomes

Male Sprague-Dawley rats, aged approximately 50 days and weighing 230–250 g, were obtained from the Experimental Animal Center of Guangdong Province, P.R. China, and the studies were approved by the Animal Ethics Committee of Guangdong Province. The rats were housed in steel cages at 22–26°C and 50–60% humidity, and they were fed a control diet for 1 week. The animals were decapitated, and the livers were quickly taken and perfused with cold 0.9% NaCl solution (Guangzhou Chemical Industry, China). Rat liver microsomes

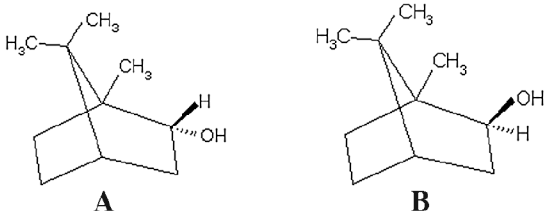

Figure 1. Structures of borneol (A) and isoborneol (B).

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were prepared as described by Yang et al. (12) with some modifications. Briefly, the liver samples were homogenized in a hand-held Teflon-glass homogenizer in three volumes of homogenization buffer, which contained 0.25M sucrose, 0.15M KCl, and 0.1M potassium phosphate buffer at pH 7.4. The homogenized tissue was centrifuged at 15,000 × *g* at 4°C for 20 min. Then the supernatant was decanted into a new polyamide tube, and 0.1 mL of an 88mM solution of $CaCl_2$ per mL of supernatant was added, followed by shaking for 5 min. The mixture was then centrifuged at 27,000 × *g* at 4°C for 20 min. The final microsmal pellet was suspended in 0.1M Tris buffer (pH 7.4) containing 20% glycerol and stored at –80°C. The protein concentration of microsomes was measured by the method of Lowry et al. (13). The total cytochrome P450 (P450) content of liver microsomal fractions was measured with P450 difference spectrum analysis (14).

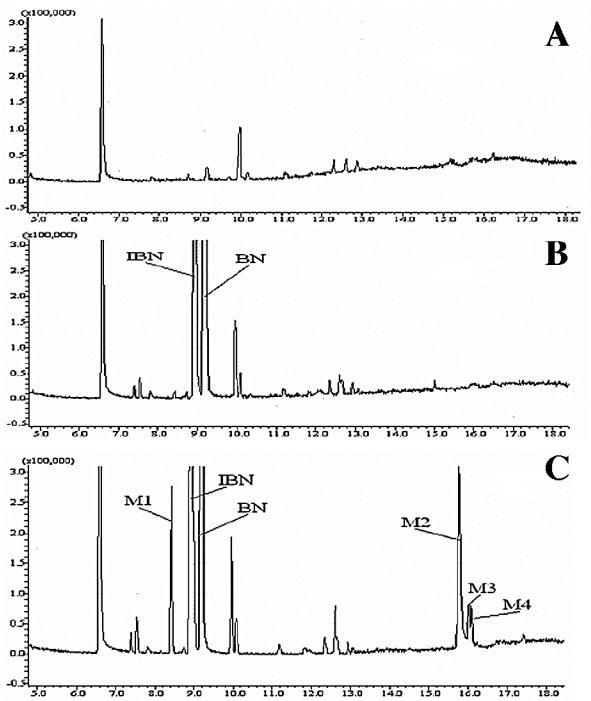

Figure 2. The total ion chromatogram of BN and its metabolites after incubation in rat liver microsomes: blank rat liver microsomes (A); incubation of 325 µM BN with inactivated rat liver microsomes (B); incubation of 325 µM BN with normal rat liver microsomes (C).

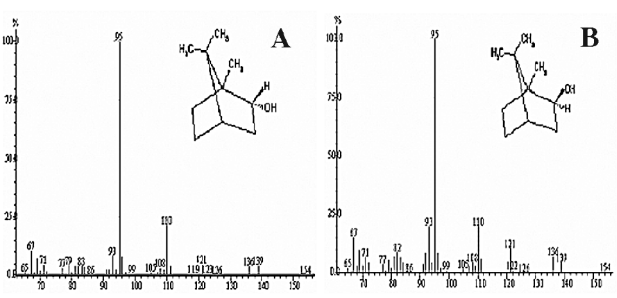

Figure 3. The mass spectra of borneol (A) and isoborneol (B) in rat liver microsomes.

In vitro incubation with rat liver microsomes

A typical incubation mixture consisted of 2.5 mg/mL rat liver microsmal protein, 0.1M potassium phosphate buffer (pH 7.4), 1mM NADPH, and 325µM BN with a final volume of 1 mL. BN was dissolved in methanol (final concentration in the reaction medium of < 1.0%). The reaction was initiated by the addition of the NADPH, and then the oxygen was quickly added with a syringe needle going into the middle of the mixtures for 40 s. Control incubations were performed using inactivated microsomes, which were boiled in 90°C water for 45 min. After incubation in a shaking water bath at 37°C for 30 min, the reaction was terminated by adding 2 mL ethyl acetate. The mixture was extracted for 10 min by shaking vigorously and then centrifuged at 15,000 × *g* for 10 min. The organic phases were directly injected into the GC–MS for analysis.

GC–MS analysis

GC–MS analysis was carried out in the negative ion chemical ionization (electron capture) mode, using a GC–MS-2010 (Shimadzu, Kyoto, Japan). GC separation was accomplished using a DB-5MS column (30 m lengths, 0.25 mm i.d., and 0.25 µm film thickness). The injector temperature was set at 220ºC. The carrier gas was helium at a flow rate of 1.0 mL/min. The column temperature program was started at an initial temperature of 90°C, held for 5.0 min, then increased to a temperature of 150°C at 5°C/min and held for 3.0 min. A volume of 1 µL of sample was injected into the GC. Approximately 10% of the sample was directed onto the column. Data was acquired from 4 to 20 min. Electron impact ionization (EI) was used with a nominal electron energy of 70 eV. The source temperature of the MS was 200°C and temperature of interface was 250°C. The mass range from *m/z* 60–500 was monitored.

Results

Following the incubation of BN with rat liver microsomes in the presence of NADPH for 30 min, four metabolites (M1, M2, M3, and M4) were observed in the incubation mixture, which were not present in the control incubations. The retention times of the four metabolites were: 8.42, M1; 15.77, M2; 16.02, M3; and 16.08 min, M4. Borneol eluted at 9.14 min and isoborneol at 8.88

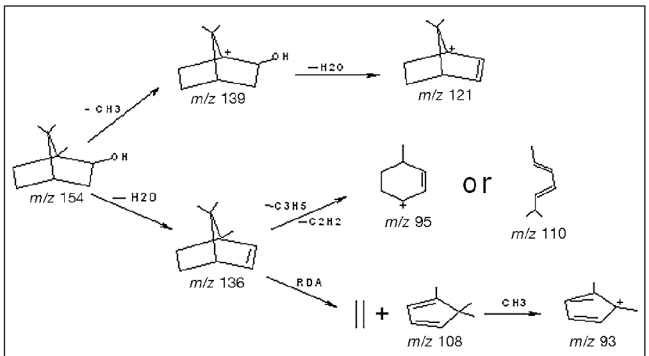

Figure 4. The fragmentation pathways of BN.

min. The total ion chromatograms are shown in Figure 2.

The EI mass spectra of BN and IBN are shown in Figure 3. Similar fragmentation patterns were obtained for these two compounds, demonstrating that they are isomers. The molecular ions of BN and IBN were observed at *m/z* 154 (1%, 1%). The formation of the main product ions were observed at *m/z* 139 (5%, 4%), 136 (4%, 8%), 121 (15%, 11%), 110 (22%, 20%), 108 (3%, 5%), 95 (100%, 100%), and 93 (9%, 20%). The proposed fragmentation pathway for BN is shown in Figure 4.

A molecular ion for M1 was observed at *m/z* 152 (19%). Other structural information of the ions was observed at *m/z* 137 (4%), 111 (2%), 109 (30%), 108 (45%), 95 (100%), 81 (72%), and 69 (39%) (Table I, and for the fragmentation pattern, see Figure 5). The characteristic parent ion was formed by the reduction of two mass units from the parent ion of BN. Using this information and the knowledge of the metabolism of monoterpenoid alcohol in rat liver microsomes (15), M1 was tentatively identified as a dehydrogenated metabolite of BN. The proposed structure for M1 is shown in Figure 6.

Table I. Mass Spectral Data for Metabolites of BN Isolated and Characterized from Incubations with Rat Liver Microsomes

Metabolite	$[M]^+$	Major fragment ion
M1	152 (19%)	137 (4%), 111 (2%), 109 (30%), 108 (45%), 95 (100%), 81 (72%), 69 (39%)
M2	122 (100%)	106 (72%), 94 (8%), 78 (98%)
M3	170 (2%)	155 (30%), 152(19%), 137 (25%), 119 (13%), 111 (79%), 110 (18%), 109 (100%), 108 (52%), 95 (46%), 93 (46%)
M4	170 (1%)	155 (20%), 152 (5%), 137 (12%), 119 (2%), 111 (100%), 110 (10%), 109 (68%), 108 (15%), 95 (25%), 93 (19%)

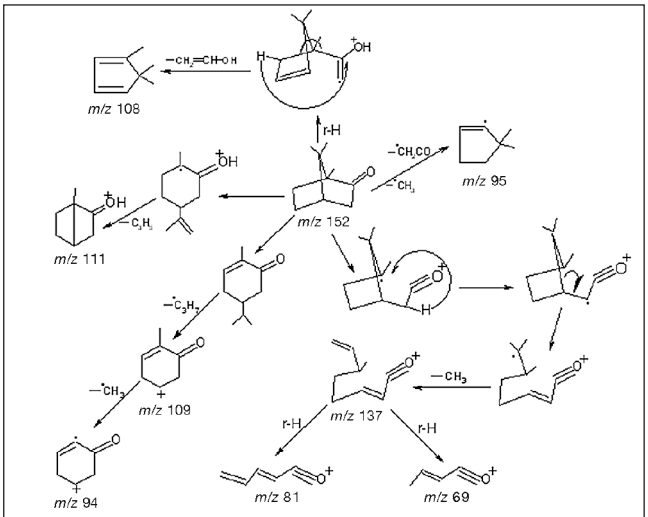

Figure 5. The fragmentation pathways of M1.

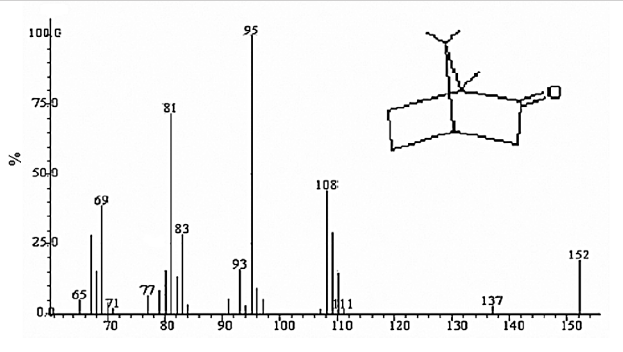

Figure 6. The structure and mass spectrum of M1 in rat liver microsomes.

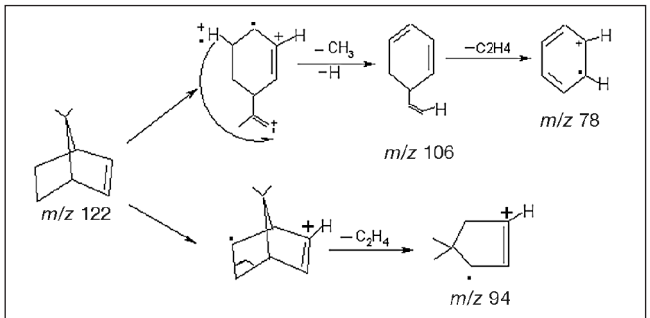

Figure 7. The fragmentation pathways of M2.

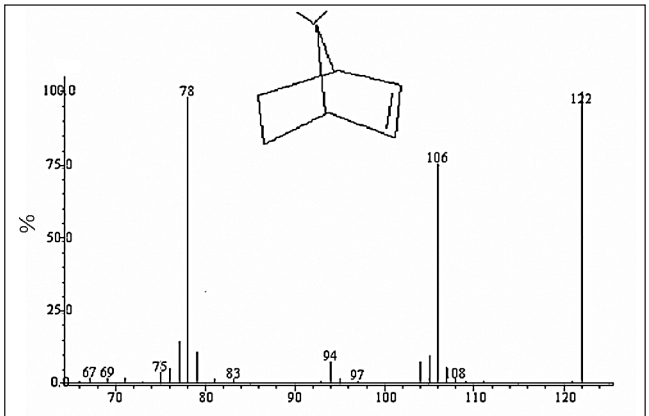

Figure 8. The structure and mass spectrum of M2 in rat liver microsomes.

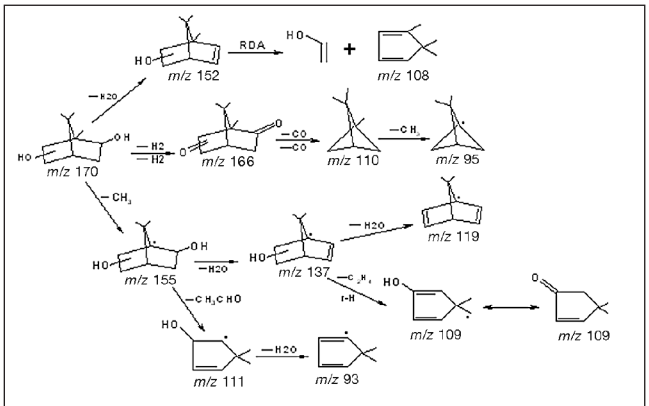

Figure 9. The fragmentation pathways of M3 and M4.

A molecular ion for M2 was observed at *m/z* 122 (100%). Other fragmentation ions were detected at *m/z* 106 (72%), 94 (8%), and 78 (98%) (Table I, for fragmentation patterns, see Figure 7). The characteristic parent ion was formed by the reduction 32 mass units from the parent ion of BN. Using this information and the knowledge of the metabolism of monoterpenoid alcohol in rat liver microsomes (15), M2 was tentatively identified as a de-methylated and de-hydrated metabolite of BN. The proposed structure for M2 is shown in Figure 8.

The molecular ion for M3 and M4 were observed at *m/z* 170 (2%, 1%). Other similar fragmentation ions were observed at *m/z* 155 (30%, 20%), 152 (19%, 5%), 137 (25%, 12%), 119 (13%, 2%), 111 (79%, 100%), 110 (18%, 10%), 109 (100%, 68%), 108 (52%, 15%), and 95 (46%, 25%) (Table I; for the fragmentation pattern, see Figure 9). The characteristic parent ions were both formed by the addition 16 mass units from the parent ion of BN. Using this information and the knowledge of the metabolism of monoterpenoid alcohol in rat liver microsomes (15), it was suggested that there were two possible positions for the hydroxylated metabolite of BN. The presumed structures for M3 and M4 are shown in Figure 10.

Discussion

The majority of xenobiotics require some metabolic transformation in order to convert them from lipophilic molecules (desirable for absorption) to more hydrophilic molecules (desirable for excretion). Biotransformation is regarded as a major cause of adverse drug reactions observed in clinical therapy (16–18). As a result of this, the interest of our research group during the last few years has been to focus on the possible intermediates and mechanisms involved in the metabolism of xenobiotics. Traditionally, the metabolism of xenobiotic has been classified as phase I and phase II, with phase I reactions generally considered to be "functionalization" and phase II reactions "conjugation" (19). Liver microsomes are an important tool for studying the in vitro metabolism of xenobiotics. Many metabolic enzymes are present in liver microsomal preparations, including various oxidase, reductase, dehydrogenase, and hydratase (15). Incubations with rat liver microsomes followed by GC–MS analysis are a powerful, useful tool for elucidating the metabolism of lipophilic chemicals with lower boiling points and lower molecular weights.

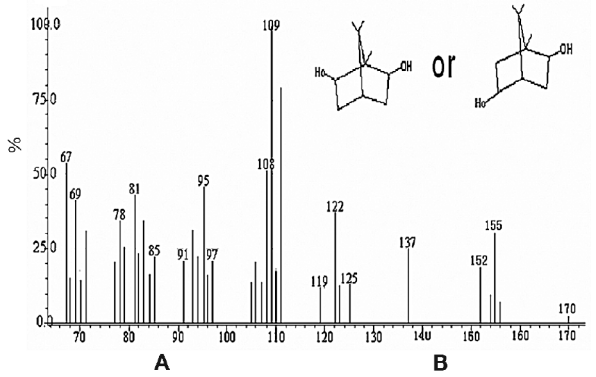

Figure 10. The structures and mass spectra of M3 and M4 in rat liver microsomes.

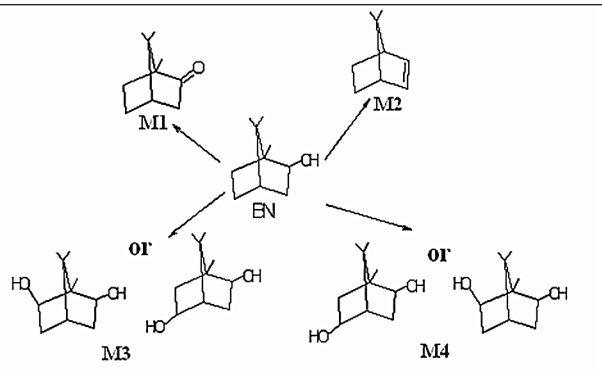

Figure 11. The metabolic pathway of BN in rat liver microsomes.

In the present study, BN was rapidly metabolized to four metabolites (M1, M2, M3, and M4) in incubations with normal rat liver microsomes in the presence of NADPH. Compared with the control incubations, these metabolites were not observed in the present inactivated rat liver microsomes and NADPH. This confirmed that all of the proposed metabolites are due to metabolism and not impurities or degradation products. Among these four metabolites of BN, M1 (*m/z* 152) has a molecular weight of two mass units less than BN. In addition, dehydrogenized metabolism may easily be reacted by the microsomes under the dehydrogenase effect. For these reasons, it was initially proposed that M1 was the de-hydrogenized metabolite of BN. Subsequently, M1 was confirmed as camphor by comparison with the standard mass spectrum library (NIST library, 95% similarity). The fragmentation pathways for M1 are shown in Figure 5. M2 (*m/z* 122) has a molecular weight of 32 mass units less than BN. We propose that this is the de-methylated and de-hydrated metabolite of BN. The main fragmentation pathways for M2 are shown in Figure 7. In view of the fact that de-methylation of monoterpenoid alcohols is rare and the mechanism of its formation is still unknown, further investigation is required to positively identify this metabolite. M3 (*m/z* 170) and M4 (*m/z* 170) have a molecular weight of 16 mass units more than BN. Both metabolites produce a similar fragmentation pattern. On account of the fact that hydroxylation is a common phase I reaction of xenobiotics to enhance the matter polarity, it was presumed that M3 and M4 were possibly two different positions of a hydroxylated metabolite of BN. The proposed fragmentation patterns for M3 and M4 are shown in Figure 9. However, the exact chemical structures have not provided enough information to discriminate between M3 and M4. Their separation and analysis could be accomplished through the use of various methods. Furthermore, based on the fact that the fragmentation ion at *m/z* 119 of M3 and M4 can also be formed from the fragmentation ion of BN, it was proposed that M3 and M4 are probably hydroxylated metabolite(s) of borneol. These will require further study to be certain.

In summary, little information is available about the metabolism of BN in vitro or in vivo. In our study, the metabolism of BN in the rat liver microsomes is significant, with at least four phase I metabolites being detected. The proposed metabolic pathway for BN is summarized in Figure 11.

Acknowledgments

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