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Short communication

In vitro and in vivo metabolism of myristicin in the rat

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Abstract

Myristicin [5-allyl-1-methoxy-2,3-(methylenedioxy)benzene] is a flavoring plant constituent and has been known to produce significant psychopharmacological responses as well as insecticidal activity. From in vitro and in vivo metabolism of myristicin, the two metabolites 5-allyl-1-methoxy-2,3-dihydroxybenzene and 1′-hydroxymyristicin were identified using GC–MS after derivatization of sample matrices with a mixture of BSTFA–TMCS. Those metabolites from in vitro study were also confirmed in urine after an oral administration of myrisitcin to rats, and enzymatic hydrolysis of urine suggested that these metabolites were excreted as conjugated forms as well. © 1998 Elsevier Science B.V.

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1. Introduction

Myristicin [5-allyl-1-methoxy-2,3-(methylenedioxy)benzene], which has insecticidal and strong synergistic properties [1], is a plant constituent found in parsley, carrot, black pepper, nutmeg, many essential oils and flavoring agents [2–5]. It produces significant psychopharmacological responses [6] and has been shown to induce glutathione *S*-transferase [7] and rat liver P450 1A1/2, 2B1/2 and 2E1 [8]. It inhibits benzo[*a*]pyrene-induced tumorigenesis [9], forms the hepatic DNA adducts in mice [10] but no genotoxicity has been reported [11].

As noted above, myristicin has some unusual properties and a wide occurrence in the environment has been reported. Tertiary aminopropiophenones were identified as urinary metabolites from rats and guinea pigs given myristicin [12] while Braun and Kalbhen [13] have reported that rat liver is capable of converting myristicin into 3-methoxy-4,5-methylenedioxyamphetamine. Kamienski and Casida [14] reported that the major metabolic pathway for myristicin in mice, after an oral administration, involves cleavage of methylenedioxyphenyl moiety and expiration of the methylene carbon as carbon dioxide. However, the in vitro/vivo metabolism of myristicin has not been studied in detail and the general analytical methods employed were the identification by mass spectrometry (MS) after the separation of corresponding metabolites by thin-layer chromatography (TLC) in addition to radioisotope study.

This paper describes the in vitro and in vivo metabolism of myristicin in rats and the identification of its metabolites with gas chromatography (GC)–MS after TMS derivatization of samples,

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which allows a more feasible and rapid analysis of the metabolite.

2. Experimental

2.1. Chemicals

Myristicin, NADP, glucose-6-phosphate, glucose-6-phosphate dehydrogenase and β-glucuronidase (Type X-A from *E. coli*) were obtained from Sigma (St. Louis, MO, USA). A mixture of bis-(trimethylsilyl)trifluoroacetamide (BSTFA) and trimethylchlorosilane (TMCS) (99:1) was purchased from Supelco (Bellefonte, PA, USA). All organic solvents (Burdick and Jackson, Muskegon, MI, USA) and the other chemicals were of the highest quality available.

2.2. Preparation of liver microsomes

Specific pathogen-free male Sprague–Dawley rats (160–180 g) were sacrificed by cervical dislocation before the livers were perfused with saline to remove excess blood and homogenized with four-volumes of ice-cold 1.15% potassium chloride solution. The liver homogenates were centrifuged at 9000 *g* for 10 min at 4°C and the resulting post-mitochondrial supernatants were centrifuged again at 105 000 *g* for 60 min at 4°C. The microsomal pellets were resuspended in 50 m*M* potassium phosphate buffer (pH 7.4) containing 0.1 m*M* EDTA. Aliquots of liver microsomes were stored at −70°C until use.

2.3. In vitro metabolism of myristicin with rat liver microsomes

Incubations of rat liver microsomes were carried out with 100 μ*M* of myristicin in 1 ml of 100 m*M* potassium phosphate buffer (pH 7.4). After preincubation for 3 min at 37°C, the reaction was initiated by adding a NADPH-generating system containing $NADP^+$, glucose-6-phosphate, 1 unit of glucose-6-phosphatedehydrogenase. After a 1 h incubation period the reaction was stopped by the addition of 2 ml of methylene chloride and the aqueous phase extracted three times with the same solvent (2 ml). The combined organic extract was evaporated under the stream of nitrogen gas and the residue was silylated with a mixture of BSTFA–TMCS (70 μl) by heating at 70°C for 10 min. On completion of the reaction, the mixture was evaporated to dryness under a nitrogen stream and the residue was redissolved in 0.5 ml of hexane for analysis.

2.4. Drug administration

Specific pathogen-free male Sprague–Dawley rats (160–180 g) were administered with myristicin dissolved in propylene glycol (100 mg/kg p.o.). The animals were placed in individual metabolic cages with water supply but no feed, and 0–24 h urine samples were collected. The samples were stored at −70°C until analyzed.

2.5. Extraction of urinary metabolites

Five ml urine samples were saturated with powdered anhydrous ammonium carbonate and extracted two times with 10 ml of ethyl acetate. After centrifugation, the organic phases were combined, dried over anhydrous sodium sulfate and evaporated to dryness under a stream of nitrogen gas. The aqueous layer was acidified (pH 1) with sulfuric acid, extracted with 10 ml of ether twice and the ether layer was extracted two times with 10 ml of 5% sodium bicarbonate solution. The resulting aqueous layer was then acidified to pH 1 and twice extracted with 10 ml of ether. The combined ether layer was dried, evaporated, silylated as described in Section 2.3 and the residue was redissolved in 0.5 ml of hexane for analysis.

2.6. Enzymatic hydrolysis

Five ml of urine was adjusted to pH 6.8 with phosphate buffer and 0.1 ml of β-glucuronidase (1000 unit) added. After incubation for 16 h at 37°C, the metabolites were extracted as described in Section 2.5.

2.7. GC–MS

Confirmation of metabolites was performed on a Finnigan MAT GCQ-MS–MS (Finnigan, San Jose, CA, USA) (electron impact mode) with DB-5 column (30 m×0.25 mm I.D., 0.25 μm film thickness) and temperature programming starting at 80°C for 1

min, with a subsequent increase of 5°C/min to 290°C. Carrier gas (He) flow-rate was 40 cm/s and injection was made with 1 μl of sample (injector temperature: 250°C).

For mass spectrometry, 70 eV was used and the ion source temperature was 200°C.

3. Results and discussion

3.1. In vitro metabolism of myristicin

Myristicin and its two metabolites, M1 and M2, formed by rat liver microsomes were well separated by GC (Fig. 1A) with retention times of 16.39, 20.28 and 21.58 min, respectively.

A major metabolite, M1, was identified as 5-allyl-1-methoxy-2,3-dihydroxybenzene, based on a molecular ion at m/z 324 (TMS derivative) and fragment ions at m/z 309 ($M–CH_3$) and m/z 235 (M–OTMS) (Fig. 2). M2 was identified as 1′-hydroxymyristicin, based on a molecular ion at m/z 280 and fragment ions at m/z 190 (M–HOTMS), m/z 265 ($M–CH_3$) and m/z 249 ($M–OCH_3$) (Fig. 2). These metabolites were not previously reported however, owing to the lack of authentic standards, tentative identification was done on the basis of the mass spectra and comparison with the previous works on myristicin

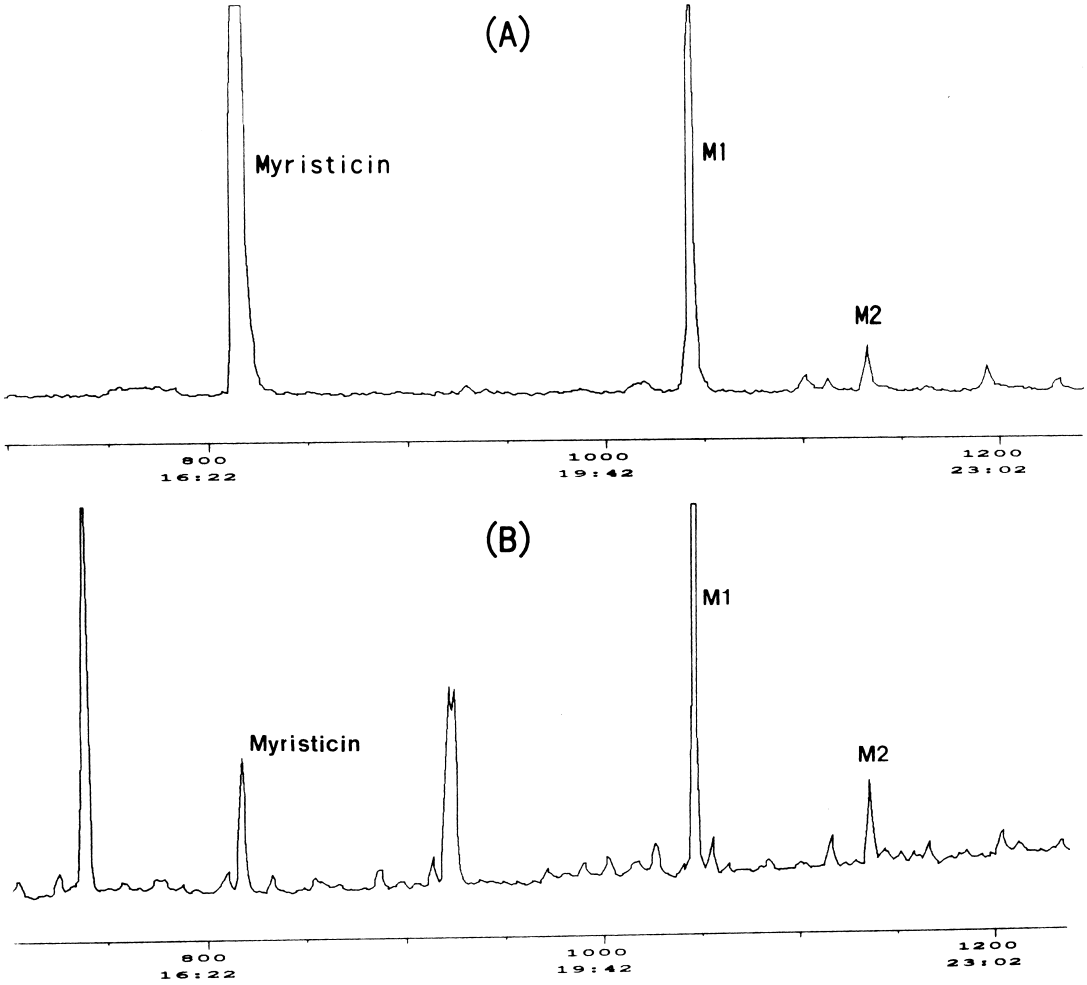

Fig. 1. Total ion chromatograms of myristicin and TMS derivatives of its metabolites (M1 and M2); (A) an incubation of rat liver microsome and (B) the neutral extract of myristicin-treated rat urine (before enzymatic hydrolysis).

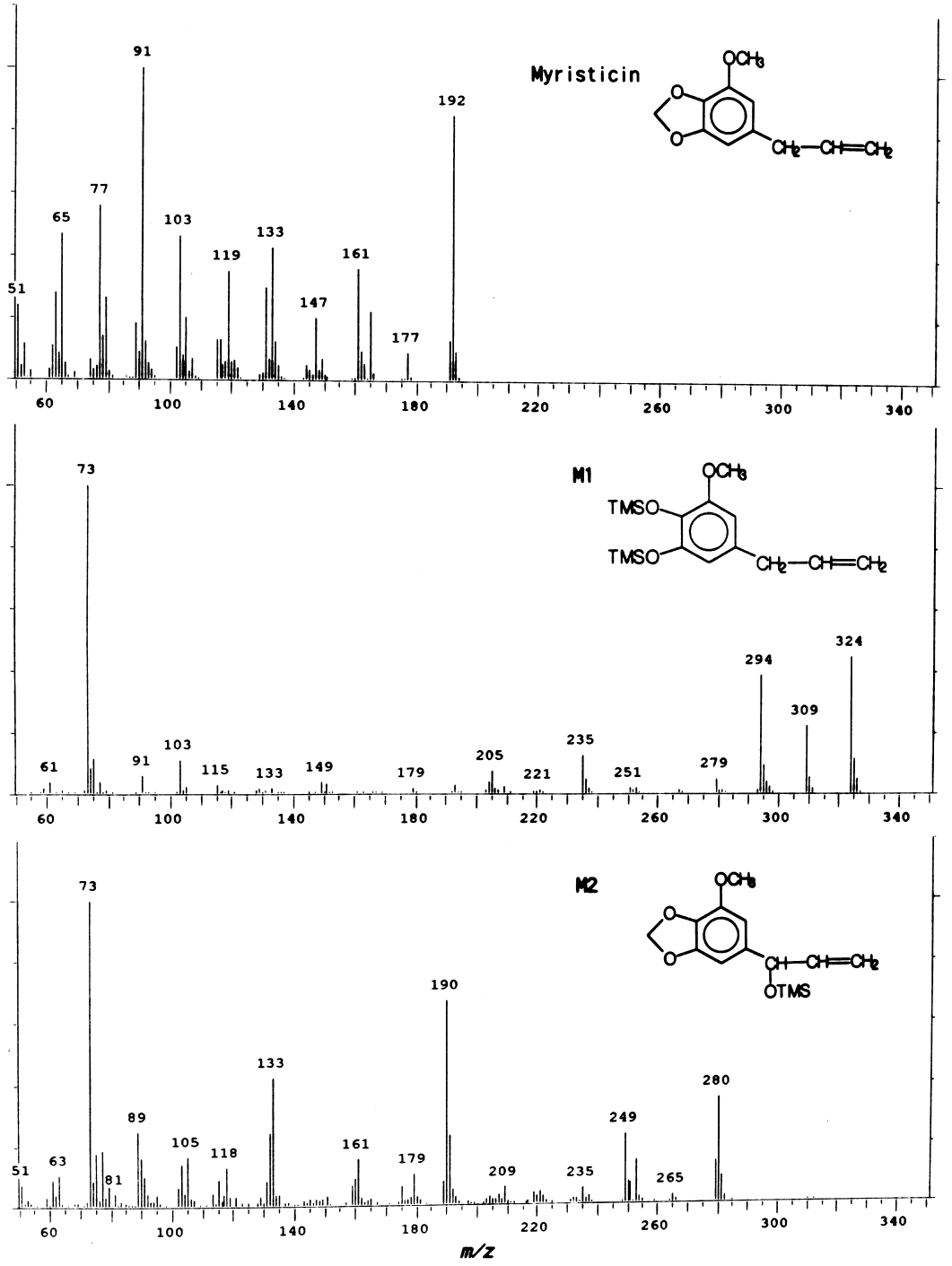

Fig. 2. Mass spectra (EI mode) of myristicin and its metabolites, M1 (5-allyl-1-methoxy-2,3-dihydroxybenzene) and M2 (1′-hydroxy-myristicin) as TMS derivatives.

and related allylbenzenes [12–17]. None of the above metabolites were observed with control samples in the absence of the NADPH-generating system.

Kamienski and Casida [14] reported that the methylene carbon was trapped as formate when myristicin was incubated with liver microsomal preparations. The catechol metabolite of myristicin formed by loss of methylene carbon was detected by TLC in their works, showing agreement with our findings. In the present work the catechol metabolite of myristicin was confirmed by GC–MS for the first time.

1′-Hydroxymyristicin, a new metabolite of myristicin, was observed with other allylbenzenes as Swanson et al. [15] reported that safrole and estragole were metabolized by hepatic microsomes from rats and mice to 1′-hydroxy derivatives and its 2′,3′-oxide.

Although myristicin and elemicin were converted to the corresponding amphetamine derivatives in the perfused liver or during incubation with liver homogenates [13], Solheim and Scheline [18] could not confirm the dimethoxyamphetamine derivative from in vivo study of 3,4-dimethoxyallylbenzene. In this study, any compounds indicating the presence of 1,2-methylenedioxy-4-methoxyamphetamine were not observed.

3.2. Urinary metabolites

The total ion chromatogram (TIC) profile of the neutral extract of myristicin-treated rat urine was similar to that from rat liver microsomal incubation (Fig. 1B), giving the unchanged myristicin, 1′-hydroxymyristicin and 5-allyl-1-methoxy-2,3-dihydroxy-benzene which was formed by oxidation of the methylenedioxy group [14].

However, in the neutral fraction after enzymatic hydrolysis of the urine with β-glucuronidase the presence of 1,2-dihydroxy-4-methoxy-5-allyl-benzene and 1′-hydroxymyristicin was greater than before the enzymatic hydrolysis, suggesting that these neutral metabolites were also excreted as conjugate forms. Particularly, the conjugation of 1′-hydroxymyristicin was more favored because it was found in larger excess than 1,2-dihydroxy-4-methoxy-5-allylbenzene in enzymatically hydrolyzed urine (Table 1). Unfortunately, it was not possible to quantitatively determine how much of the administered myrisitcin was recovered as urinary metabolites because of the volatility of myristicin and the lack of authentic metabolites.

In supporting the present results, Borchert et al. [16] identified 1′-hydroxysafrole and its conjugated form as urinary metabolites of safrole in rats, hamsters or guinea pigs. Stillwell et al. [17] have identified 1,2-dihydroxy-4-allylbenzene, 1′-hydroxysafrole, 1,2-methylenedioxy-4-(2,3-dihydroxypropyl)benzene, 1,2-dihydroxy-4-(2,3-dihydroxypropyl)benzene and 2-hydroxy-3-(3,4-methylenedioxyphenyl)propanoic acid as the urinary metabolites after an intraperitoneal administration of safrole.

From the acidic fraction of 24 h urines no metabolite was detected and the basic metabolites, the two nitrogen-containing metabolites isolated by Oswald et al. [12], were not investigated in the present study.

4. Conclusions

Myristicin was metabolized at different rates to 1,2-dihydroxy-4-methoxy-5-allyl-benzene and 1′-hydroxymyristicin by rat liver microsomes in the presence of a NADPH generating system. After an oral administration of myrisitcin to rats, the same metabolites were found in urine and enzymatic

Table 1
Peak intensity ratio of 1,2-dihydroxy-4-methoxy-5-allyl-benzene and 1′-hydroxymyristicin to myristicin before and after enzymatic hydrolysis of urine

Experiments	Myristicin[a]	1,2-Dihydroxy-4-methoxy-5-allyl-benzene	1′-Hydroxymyristicin
Before hydrolysis	1	10.5	0.6
After hydrolysis	1	12.0	42.6

[a] Peak height of myristicin was used as a reference value for comparison.

hydrolysis suggesting that these metabolites were also conjugated in different ratios.

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