*Agric. Chem. Biotechnol.* **44**(4), 180-184 (2001)

# Metabolism and Disposition of Myristicin in the Isolated Perfused Rat Liver

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Received September 21, 2001

**To investigate the hepatic metabolism of myristicin isolated rat livers were perfused under single-pass conditions with perfusate containing myristicin. In outflow perfusate and bile, 5-allyl-1-methoxy-2,3-dihydroxybenzene (M1), M1-sulfate, and M1-glucuronide conjugates were identified as the metabolites of myristicin. HPLC method with UV detection was applied to investigate the hepatic disposition of the compounds. The concentration of myristicin, M1, and M1-conjugates in the outflow perfusate reached steady-state levels within 20 min after commencing the perfusion of 4.5 μM myristicin. At steady-state, the mean (± S.D.) extraction ratio of myristicin was 0.49 (± 0.16) and clearance was 13.7 (± 4.5) ml/min. M1 accounted for 44.0 ± 5.3% of eliminated myristicin and was recovered as unchanged M1, M1-sulfate, and M1-glucuronide in the bile and outflow perfusate.**

**Key words:** *myristicin, metabolism, isolated perfused rat liver.*

Myristicin, 1-allyl-3,4-methylenedioxy-5-methoxybenzene, has been found in nutmeg, mace, parsley, carrot, black pepper, processed tobacco, and many essential oils.[1-4] Nutmeg and mace are extensively used as flavorings in various foods. Randerath *et al.* [5] suggested that cola drinks contained myristicin as well as a small amount of safrole, and that these compounds elicited the hepatic adduct formation in mice fed cola drinks. Myristicin, which produces psychotropic effects in human at high dose levels,[6] became a drug of abuse after the Second World War. It has been shown to induce glutathione S-transferase and rat liver P450 1A1/2, 2B1/2, and 2E1.[7,8] Myristicin inhibits benzo[a]pyrene-induced tumorigenesis and no genotoxicity has been reported.[9,10]

In spite of the wide spread occurrence and use, studies on the metabolism and pharmacokinetics of myristicin are limited.[11-15] Tertiary aminopropiophenones were identified as urinary metabolites from rats and guinea pigs treated with myristicin,[11] while Braun and Kalbhen[12] have reported that rat liver converted myristicin into 3-methoxy-4,5methylenedioxy amphetamine. Kamienski and Casida[13] reported that the major metabolic pathway for myristicin in mice involves the cleavage of methylene-dioxyphenyl moiety and expiration of the methylene carbon as carbon dioxide. In a previous paper,[14] myristicin was shown to metabolize into 1-allyl-3,4-dihydroxy-5-methoxybenzene (M1), 1'-hydroxymyristicin, and their conjugates in rats.

The purpose of this study was to investigate the hepatic metabolism and disposition of myristicin and a major metabolite M1 using isolated perfused rat liver under controlled conditions without the influences of other organs and tissues.

## Materials and Methods

**Materials.** Myristicin, β-glucuronidase (type VIII from *E. Coli*), sulfatase (type VI from Aerobacter aerogenes), taurocholic acid, and lactate dehydrogenase (LDH) assay kits were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Pentobarbital sodium was obtained from Daehan Pharm. Co. (Seoul, Korea). Organic solvents were purchased from Burdick & Jackson, Inc. (Muskegon, MI, USA), and all other reagents were the highest purity available.

**Instruments.** HPLC was applied to determine myristicin and M1 in inflow and outflow perfusates. HPLC system consisted of a SpectraSystem P4000 pump (Thermo Separation Products, CA, USA), a Spectra System UV 3000 multi-scanning detector, a TSP A3500 auto-sampler, and a PC 1000 software for data handling. Myristicin and M1 were separated on Capcell pak UG120 column (150 × 2.0 mm i.d.) using 45% acetonitrile in 50 mM phosphate buffer (pH 4.5) at a flow rate of 0.2 ml/min. The effluent was monitored at 245 nm, and UV spectra of myristicin and M1 were obtained in the range of 210-300 nm for identification.

**Synthesis of 1-allyl-3,4-dihydroxy-5-methoxybenzene (M1).** M1 was synthesized from myristicin through a cleavage reaction of the methylenedioxyl moiety with lead tetraace-

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**Abbreviations:** LDH, lactate dehydrogenase.

tate in benzene.[16] A solution of myristicin (80 mg) and lead tetraacetate (222 mg) in dry benzene (5 m*l*) was stirred at 60°C for 7 h, then diluted with ether. The reaction mixture was washed with water, dried over sodium sulfate, and concentrated to dryness under reduced pressure. The residue was purified through preparative thin layer chromatography (PLC) [hexane : ethylacetate (4 : 1)] to give acetoxylester of M1 (*Rf* = 0.58). A solution of acetoxylester of M1 in 80 % acetic acid was stirred at room temperature overnight. The mixture was concentrated to dryness, and the residue was purified via PLC [hexane : ethylacetate (7 : 3)] to give M1 (14 mg) as colorless oil. This compound was confirmed through $^{1}$H- and $^{13}$C-NMR.

**Liver perfusion.** Specific pathogen-free male Sprague-Dawley rats (240-270 g) were obtained from Experimental Animal Laboratory of KRICT (Taejeon, Korea). The perfusions were carried out as described in our previous report.[17] Following an overnight fast, the rats were anaesthetized with sodium pentobarbital (40 mg · kg$^{-1}$ i.p.) before surgery. To collect bile, the bile duct was cannulated by PE 10 tubing and the flow rate of the bile was determined gravimetrically assuming a specific gravity of 1. The portal vein and vena cava were catheterized, and the livers were isolated and placed in a perfusion cabinet. Freshly prepared Krebs-Henseleit bicarbonate buffer (pH 7.4) containing glucose (2.5 mM), calcium chloride (2.5 mM), and taurocholic acid (0.1 mM), equilibrated with an $O_2/CO_2$ mixture (95 : 5 by volume), was delivered into the portal vein at a constant flow rate of 28 m*l*/min. Temperatures of the perfusion cabinet and perfusion medium were thermostatically controlled at 37°C. Single-pass perfusions were performed, and the effluent perfusate was collected via a cannula secured in the vena cava. Initial stabilization period of 20 min was allowed before adding myristicin to the inflow perfusion medium. The viability of perfused rat livers was assessed through bile flow (>0.3 m*l*/h), percent recovery of inflow perfusate (>95%), and the gross appearance of the organ. LDH activity in the outflow perfusate was monitored for liver viability. Livers were weighed at the end of each experiment.

To define the metabolic profile of myristicin and assess the time needed to reach steady-state with respect to the concentration of myristicin and its metabolites in outflow perfusate and bile, four isolated rat livers were perfused with a constant inflow concentration of myristicin (4.5 μM) for 100 min. Inflow and outflow perfusate samples (10 m*l*) and bile were taken at 10 min intervals after the addition of myristicin up to 100 min for the measurement of myristicin and its metabolites.

In another series of experiments, additional four isolated rat livers were perfused with two different inflow concentrations of myristicin (2.3 and 4.5 μM), each for 30 min, in a balanced cross-over manner. Perfusate samples were collected at 20, 25, and 30 min (period 1) and 50, 55, and 60 min (period 2). Bile samples were collected at a 5-min interval throughout each perfusate collection period.

**Extraction and analysis.** Perfusate (2 m*l*) and bile samples were extracted with methylene chloride ( 4m*l*) containing eugenol (internal standard) and were centrifuged. The organic phase was evaporated to dryness using a nitrogen gas stream, and the residue was dissolved in mobile phase (100 μ*l*). Aliquot (5 μ*l*) was analyzed via HPLC. Typical retention times for M1, eugenol (internal standard), and myristicin were 3.9, 7.7, and 2 0min, respectively. The recoveries of myristicin and M1 in the concentration range of 0.1-10 μM were 94.5 ± 5.4 and 93.2 ± 4.5 %, respectively.

To investigate the extent and type of M1 conjugation, M1-glucuronide and sulfate were determined as M1 equivalents after complete hydrolysis with β-glucuronidase or β-glucuronidase/sulfatase. After 1 h incubation of outflow perfusate (1 m*l*) and bile samples with β-glucuronidase (100 units) and β-glucuronidase (100 units)/sulfatase (2.5 units) at 37°C, respectively, myristicin and M1 were extracted and analyzed as described above.

**Data analysis.** Model-independent pharmacokinetic parameters were calculated using data recorded after the steady-state was reached. Inflow and outflow molar concentrations were averaged before inclusion into the following pharmacokinetic equations.

Availability of myristicin (F):

$$F = C_{outflow} / C_{inflow} \quad (1)$$

where $C_{outflow}$ and $C_{inflow}$ are the concentrations of myristicin in the outflow and inflow perfusates, respectively.

Clearance of mMyristicin (CL, m*l*/min):

$$CL = E \times \text{perfusate flow rate} \quad (2)$$

where E is the hepatic extraction ratio, 1- F.

Metabolic clearance of myristicin via M1 ($CL_{M1}$, m*l*/min) :

$$CL_{M1} = \text{Rate of M1 formation} / C_{inflow} \quad (3)$$

where the rate of M1 formation was assumed to be the sum of biliary excretion rate of M1 and its recovery rate in outflow perfusate.

Biliary excretion ratio of hepatically-generated M1 ($E_b$):

$$E_b = \text{Rate of biliary M1 excretion} / \text{Rate of M1 formation} \quad (4)$$

Data are presented as means (± S.D.). Analysis of variance was used to test for changes in the disposition of myristicin and its metabolite M1 with respect to the myristicin inflow concentration.

## Results and Discussion

In the present study, the isolated perfused rat liver was used to investigate the hepatic metabolism of myristicin and to characterize the nature of hepatically formed myristicin metabolites. From HPLC analysis of the outflow perfusate, M1 was identified as a major metabolite of myristicin in the isolated perfused rat liver. However, 1'-hydroxymyristicin, previously identified as a major metabolite following rat liver microsomal incubation,[14] was not detected in the outflow per-

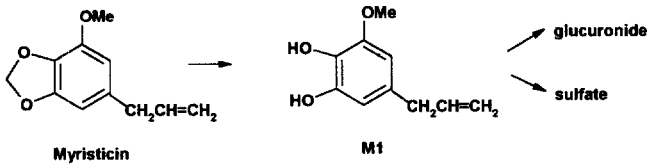


**Fig. 1. Proposed major metabolic pathway for myristicin.**

fusate or bile. Upon the incubation of outflow perfusate and bile samples with β-glucuronidase or sulfatase, the amount of M1 increased, indicating that hepatically generated M1 was conjugated to M1-glucuronide and M1-sulfate in the liver. Because authentic samples of M1-glucuronide and M1-sulfate were not available, the concentrations of M1 conjugates in the outflow perfusate or bile samples were determined as M1 equivalents after hydrolysis with β-glucuronidase or sulfatase. The metabolic pathway of myristicin is proposed based on the results in Fig. 1.

Results of experiments, in which isolated rat livers were perfused with a constant myristicin concentration (4.5 μM), indicated that less than 20 min was required before steady-state was reached with respect to the concentration of myristicin, M1, M1-glucuronide, and M1-sulfate in outflow perfusate. LDH activity in the outflow perfusate was not changed by myristicin treatment suggesting that the liver was not damaged at the concentration (4 .5μM) used in this study.

Pharmacokinetic parameters describing the disposition of myristicin in isolated perfused rat livers (n = 4) at a nominal inflow concentration of 4.5 μM are shown in Table 1. Pharmacokinetic analysis was performed using equations 1-4 applying data from samples collected during the steady-state period (20-100 min after the commencement of perfusion). Myristicin was found to have a low availability (0.42 ± 0.11), high extraction ratio (0.58 ± 0.11), and clearance (16.2 ± 3.0 m*l*/min). Unchanged myristicin accounted for 0.3 ± 0.08% of the dose in the bile. The biliary extraction ratio of hepatically generated M1, which was defined as the rate of recovery of M1 in bile relative to total rate of M1 recovery via perfusate plus bile, was 0.15 ± 0.03. These results suggest that the disposition of myristicin in rats may be characterized by the extensive metabolism, high hepatic extraction, and low bioavailability after oral administration. They support the previous results in which area under the serum concentration-time curve (AUC,

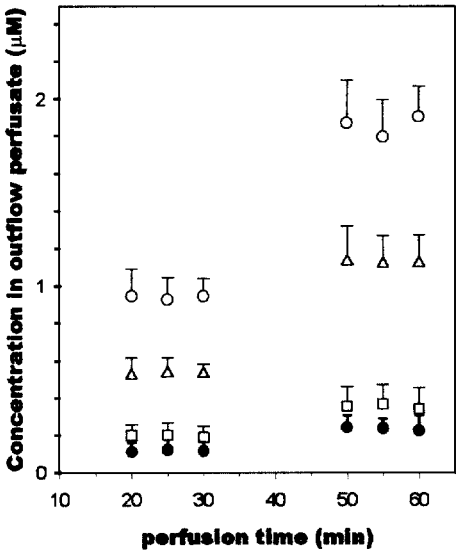


**Fig. 2. Concentrations of myristicin ( ○), M1 ( ●), M1-glucuronide (□), and M1-sulfate (△) in outflow perfusate during perfusion of four isolated rat livers with perfusate containing 2.3 μM (period 1; 0-30 min) and 4.5 μM (period 2; 30-60 min) of myristicin.**

1324 ng h/m*l*) and the peak serum concentration ($C_{max}$, 782 ng/m*l*) of myristicin were lower after a single oral administration of myristicin (10 mg · $kg^{-1}$).[15]

M1 was recovered as unchanged M1 (8.5 ± 3.1%), M1-glucuronide (12.7 ± 5.4%) and M1-sulfate (41.5 ± 8.9%) in the outflow perfusate, accounting for 62.6 ± 10.7% of the eliminated myristicin. However, in the bile, M1 was not recovered, but M1-glucuronide accounted for 5.2 ± 1.5% of the eliminated myristicin. The demethylenation pathway was found to form M1 accounting for 67.8 ± 11.4% of the eliminated myristicin during a single pass perfusion of myristicin. In addition, significant quantities of hepatically formed M1 were further metabolized to M1-sulfate and M1-glucuronide conjugates before M1 left the liver. The M1 formation is likely to be underestimated because M1 conjugates were analyzed via indirect approach devoid of authentic standards of M1-glucuronide and M1-sulfate. This result suggested that the conversion of myristicin to M1 was a major myristicin elimination route.

To determine whether livers were operating under linear

**Table 1. Mean kinetic parameters for myristicin and M1 in isolated perfused rat livers at steady-state.[a]**

| Parameter | Rat 1 | Rat 2 | Rat 3 | Rat 4 | Mean ± S.D. |
|---|---|---|---|---|---|
| Body weight (g) | 211 | 275 | 244 | 248 | 245 ± 26 |
| Liver weight (g) | 9.2 | 10.7 | 10.6 | 10.2 | 10.2 ± 0.7 |
| Extraction ratio (E) | 0.69 | 0.50 | 0.47 | 0.65 | 0.58 ± 0.11 |
| Availability (F) | 0.31 | 0.50 | 0.53 | 0.35 | 0.42 ± 0.11 |
| Clearance (CL, m*l*/min) | 19.3 | 14.0 | 13.2 | 18.2 | 16.2 ± 3.0 |
| Metabolic clearance to M1 ($CL_{M1}$, m*l*/min) | 12.3 | 9.4 | 11.1 | 10.5 | 10.8 ± 1.2 |
| Biliary extraction ratio of M1 ($E_b$) | 0.16 | 0.17 | 0.16 | 0.11 | 0.15 ± 0.03 |

[a]Single-pass perfusion of 4.5 μM myristicin.

**Table 2. Effect of inflow perfusate concentration of myristicin on the kinetics of flupyrazofos and M1 in isolated perfused rat livers.**[a]

| Parameters | Myristicin[b] | |
|---|---|---|
| | 2.3 μM | 4.5 μM |
| Availability (F) | 0.41 ± 0.12 | 0.42 ± 0.11 |
| Extraction ratio (E) | 0.59 ± 0.12 | 0.58 ± 0.11 |
| Clearance (CL, ml/min) | 16.5 ± 3.4 | 16.2 ± 3.0 |
| Metabolic clearance to M1 ($CL_{M1}$, ml/min) | 10.6 ± 1.4 | 10.8 ± 1.2 |
| Biliary extraction ratio of M1 ($E_b$) | 0.14 ± 0.05 | 0.15 ± 0.04 |

[a]Each liver was perfused at myristicin concentrations of 2.3 and 4.5 μM in a balanced and crossover manner.
[b]n = 4, mean ± S.D.

conditions with respect to the metabolism and disposition of myristicin and its metabolite M1, four isolated rat livers were perfused at two different myristicin inflow concentrations (2.3 and 4.5 mM) in a balanced and crossover manner. The concentration of myristicin and its metabolites in outflow perfusate was dose-dependently increased (Fig. 2). Availability, extraction ratio, clearance of myristicin, partial clearance of myristicin to M1, and the biliary extraction ratio of M1 were not influenced by myristicin inflow concentration (Table 2), suggesting the hepatic extraction of myristicin, the production of myristicin metabolites, and the biliary excretion of myristicin were essentially concentration-independent up to the inflow concentration investigated.

In conclusion, myristicin was absorbed and metabolized by isolated male rat livers perfused with erythrocyte-free and albumin-free medium. M1 was the major metabolite, accounting for 67.8% of eliminated myristicin, and hepatically generated M1 was extensively conjugated as glucuronide and sulfate (at least 87.7% of M1). Myristicin and M1 conjugates were excreted in bile as well as outflow perfusate. These results suggest that the isolated perfused rat liver could be a useful model for examining the oxidative metabolism of myristicin and the subsequent conjugation of the hepatically formed metabolite M1.

**Acknowledgments.** This work was supported by the Medicinal Resources Research Center, Wonkwang University sponsored by the Korea Science Engineering Foundation.

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