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Journal of Chromatography B, 793 (2003) 291–296
JOURNAL OF CHROMATOGRAPHY B
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High-performance liquid chromatographic method for the determination of (−)-verbenone 10-hydroxylation catalyzed by rat liver microsomes

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Received 6 December 2002; received in revised form 28 March 2003; accepted 10 April 2003

Abstract

A sensitive assay for the determination of (−)-verbenone 10-hydroxylation catalyzed by rat liver microsomes was developed using high-performance liquid chromatography. Verbenone was incubated in vitro with liver microsomes of untreated rats and rats treated with phenobarbital and the products thus formed were extracted with CH_2Cl_2 and the extracts were separated by HPLC with a C_{18} 5-μm analytical column. Elution was conducted with 40% methanol containing 20 m*M* $NaClO_4$ and the detection of UV absorbance was done at 251 nm. Product formation was dependent on the incubation time at least up to 30 min and the microsomal protein concentration between 0.01 and 0.1 mg protein/ml. The limit of detection of (−)-10-hydroxyverbenone with the HPLC was found to be about 40 pg, indicating that this method is about 100-fold sensitive than the GC–MS method. Optimized pH for the reaction was at 7.4 when examined with 100 m*M* potassium phosphate buffer in different pHs. Kinetic analysis showed that K_m values for liver microsomes of untreated and phenobarbital-treated rats were 206 and 41 μ*M* and V_{max} values were 5.8 and 44 nmol/min/mg protein, respectively. Thus the present results provided a sensitive and useful method for the determination of verbenone 10-hydroxylation catalyzed by rat liver microsomes.

Keywords: Verbenone; (−)-10-Hydroxyverbenone

1. Introduction

Verbenone has been reported to be a component of the essential oil from rosemary species such as *Rosmarinus officinalis L.*, *Verbena triphylla*, and *Eucalyptus globulus* that are used for a herb tea, a spice, and a perfume [1,2]. (−)-Verbenone has a spicy odor and camphoraceous fragrance. It has not been precisely determined how this terpene derivative is metabolized by mammalian tissues, until recently we have reported that human liver microsomes catatyze (−)-verbenone to 10-hydroxylated metabolite [3]. In that study we used gas chromatography–mass spectrometry to detect the hydroxylated metabolites of (−)-verbenone. A problem exists that this GC–MS method is not sensitive enough to

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1570-0232/03/$ – see front matter
doi:10.1016/S1570-0232(03)00329-5

detect low levels of (−)-verbenone metabolites and it is required to develop a sensitive method for the detection.

Liver microsomes contain multiple forms of cytochrome P450 (P450 or CYP)[1] that catalyze oxidation of numerous xenobiotic chemicals such as drugs, toxic chemicals, and carcinogens as well as endobiotic chemicals including steroids, fatty acids, prostaglandins, and vitamins [4,5]. Most of the xenobiotic chemicals have been reported to be catalyzed mainly by 1–3 families of P450s and the individual P450 enzymes have considerable, but overlapping, substrate specificities [5,6]. Our previous studies have demonstrated that several natural monoterpenoids present in plants, such as 1,4-cineole, 1,8-cineole, and (+)- and (−)-limonenes, are catalyzed by different forms of P450 enzymes to their respective oxidation products in rat and human liver microsomes [7–10].

In order to identify which P450 enzymes are involved in the metabolism of (−)-verbenone, we developed in this study a sensitive method for the detection of 10-hydroxylated metabolite using HPLC. Verbenone was metabolized by rat liver microsomes in the presence of an NADPH-generating system and the products were analyzed with HPLC using a C_{18} 5-μm column. Elution was conducted with 40% methanol containing 20 m*M* $NaClO_4$.

2. Experimental

2.1. Chemicals

(−)-Verbenone and its 10-hydroxylated metabolite (Fig. 1) were synthesized as described previously [3]. $NADP^+$, glucose 6-phosphate, and glucose 6-phosphate dehydrogenase were purchased from Sigma (St. Louis, MO, USA). Other reagents and chemicals used were obtained from sources as described previously or of the highest qualities commercially available [9–14].

[1]Abbreviations: P450 or CYP, cytochrome P450; HPLC, high-performance liquid chromatography.

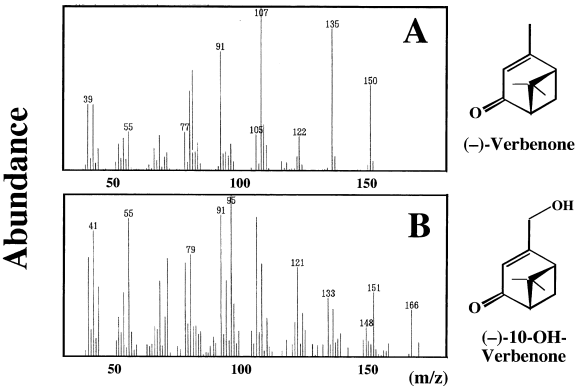

Fig. 1. Mass pattern of (−)-verbenone and 10-hydroxylverbenone.

2.2. Enzyme preparation

Male Sprague–Dawley rats (weighing about 200 g) obtained from Nihon Clea (Osaka, Japan) were used throughout this study. Liver microsomes from untreated rats and rats treated with phenobarbital (80 mg/kg, daily for 3 days) rats were prepared as described and suspended in 10 m*M* Tris–HCl buffer (pH 7.4) containing 1.0 m*M* EDTA and 20% glycerol (v/v) [13,15].

P450 was estimated spectrally by the Omura and Sato method [16]. Protein concentrations were estimated by the Lowry et al. method [17].

2.3. Incubations

The standard incubation mixture consisted of rat liver microsomes (0.05 mg protein) and 200 μ*M* (−)-verbenone (dissolved in dimethyl sulfoxide) in a final volume of 0.50 ml of 100 m*M* potassium phosphate buffer (pH 7.4) containing an NADPH-generating system (0.5 m*M* $NADP^+$, 5 m*M* glucose 6-phosphate, and 0.5 unit of glucose 6-phosphate dehydrogenase/ml). Incubations were carried out at 37 °C for 20 min and terminated by adding 1.5 ml of CH_2Cl_2. The mixture was mixed vigorously and centrifuged at 2000 rpm for 4 min. The organic layer was evaporated to dried under nitrogen atmosphere and the residues were dissolved in small amounts of 40% methanol containing 20 m*M* $NaClO_4$ (pH 2.5).

2.4. HPLC system

The aliquot (usually 25 μl) of the extracts described above was used for HPLC analysis with a LC-CCPS system (Tosoh, Tokyo) with a spectrometer UV-8020 (Tosoh, Tokyo). Separation was done with a C_{18} 5-μm analytical column (Mightsil RP-18, 150×4.6 mm, Kanto Chemical, Tokyo) equipped with a C_{18} 5-μm guard column (Mightsil RP-18, 5–4.6 mm, Kanto Chemical). The eluent consisted of a mixture of 40% methanol containing 20 m*M* $NaClO_4$ (pH 2.5). The flow-rate was 1.0 ml/min and the UV detection was done at 251 nm (Fig. 2). Peak areas thus obtained were integrated with a Chromatopac Instrument (C-R6A Chromatopac, Shimadzu, Kyoto).

2.5. Kinetic analysis

Kinetic parameters for verbenone 10-hydroxylation by liver microsomes were estimated using a computer program (KaleidaGraph program from Synergy Software, Reading, PA, USA) designed for nonlinear regression analysis of a hyperbolic Michaelis–Menten equation.

3. Results

3.1. HPLC analysis of (−)-verbenone 10-hydroxylation catalyzed by liver microsomes of phenobarbital-treated rats

(−)-Verbenone was incubated for 5, 10, 20, and 30 min with liver microsomes of phenobarbital-treated rats in the presence of an NADPH-generating system and the products thus formed were analyzed with HPLC (Fig. 3). We examined which solvents were appropriate for the separation of verbenone and its metabolites and finally found that 40% methanol containing 20 m*M* $NaClO_4$ (pH 2.5) was of use for the analysis of verbenone and its 10-hydroxylated metabolite. The peak at about 5.1 min of retention time corresponding to the (−)-10-hydroxyverbenone was increased with incubation time. The parent verbenone was eluted around 19 min in this elution condition. In this assay condition, other metabolites rather than 10-hydroxylated verbenone were not detected, except that two very small peaks of retention time at 3.3 and 3.8 min were observed at the longer incubations of 20 and 30 min. The limit of detection of (−)-10-hydroxyverbenone was found to

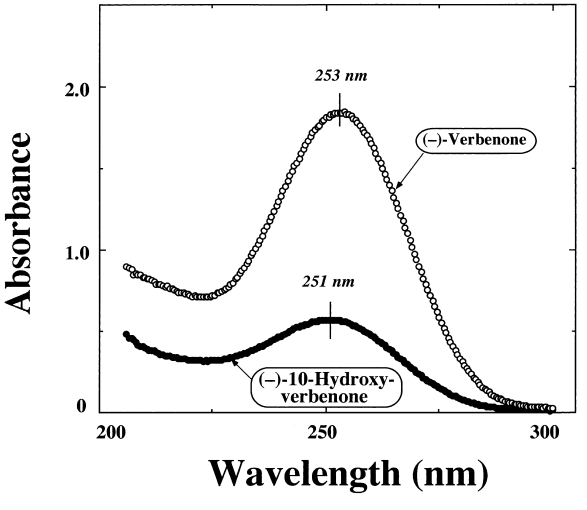

Fig. 2. UV spectra of (−)-verbenone and 10-hydroxylverbenone. Concentrations of these chemicals examined were 60 and 20 m*M*, respectively.

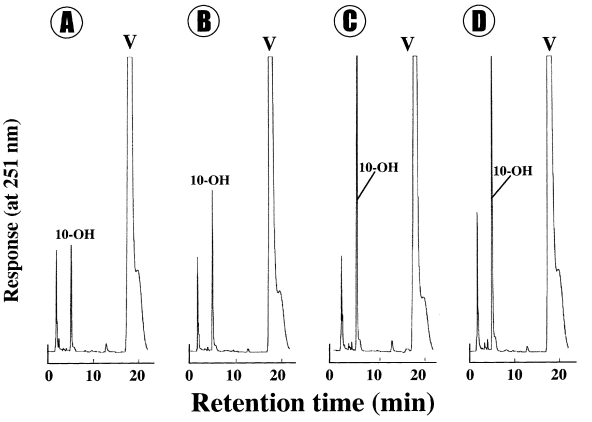

Fig. 3. Dependence of incubation time of verbenone 10-hydroxylation catalyzed by liver microsomes of phenobarbital-treated rats. Verbenone (200 μ*M*) was incubated with liver microsomes (0.10 mg protein/ml) for 5 min (A), 10 min (B), 20 min (C) and 30 min (D) in the presence of an NADPH-generating system and the products formed were determined by the method as described in Results section.

be about 40 pg in this HPLC condition. Our previous study has suggested that the limit of detection of this metabolite with GC–MS method is about 4 ng [3], indicating that the HPLC method was about 100-fold sensitive than the GC–MS method for the detection of (−)-10-hydroxyverbenone.

Verbenone 10-hydroxylation activity was found to increase with microsomal protein concentrations up to 0.10 mg/ml and with incubation time up to 20–30 min in rats treated with phenobarbital (Fig. 4). Thus we used a standard condition for the verbenone 10-hydroxylation using protein concentration of 0.10 mg/ml and incubation time of 20 min.

Effects of pH on the verbenone 10-hydroxylation catalyzed by liver microsomes of phenobarbital-treated rats were examined using different pHs in potassium phosphate buffer (Fig. 5). No catalytic activities were found in pH 4.5 and 5.5. The optical pH was around 7 and our results suggested that the highest activities were obtained at pH 7.4. The higher the pH, the lower the catalytic activity.

3.2. Kinetic analysis for the verbenone 10-hydroxylation catalyzed by liver microsomes of untreated and phenobarbital-treated rats

Verbenone 10-hydroxylation activities in liver microsomes of untreated and phenobarbital-treated rats were examined with substrate concentrations of 100, 200, 400, and 800 μ*M* (Table 1). Liver microsomes of phenobarbital-treated rats had higher catalytic activities than those catalyzed of untreated rats particularly at lower substrate concentrations. As results, the K_m value (40.6 μ*M*) of liver microsomes of phenobarbital-treated rats were 5.1-fold lower than that (206 μ*M*) of untreated rats. V_{max} value was about 7.5-fold higher in the former enzymes than those in the latter enzymes. V_{max}/K_m ratio (e.g., enzyme efficiency) was found to be 38-fold higher in phenobarbital-treated rats than untreated rats.

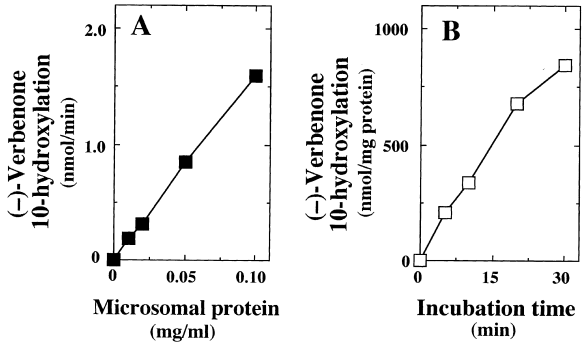

Fig. 4. Dependence of protein concentrations (A) and incubation time (B) on verbenone 10-hydroxylation catalyzed by liver microsomes of phenobarbital-treated rats. Incubation time used for A was 30 min and protein concentration used for B was 0.10 mg/ml.

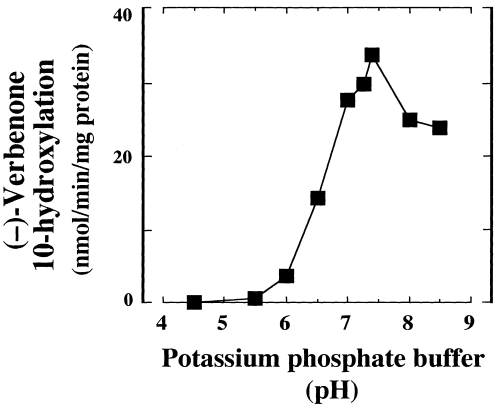

Fig. 5. Dependence of pH on verbenone 10-hydroxylation catalyzed by liver microsomes of phenobarbital-treated rats. Standard incubation condition was used except for potassium phosphate buffer in which different pHs were used.

4. Discussion

A sensitive assay for HPLC analysis of (−)-verbenone 10-hydroxylation catalyzed by rat liver microsomes was developed. After terminating the reaction containing liver microsomes and verbenone in the presence of an NADPH-generating system, the products were extracted with CH_2Cl_2 and applied to HPLC with a reversed-phase C_{18} 5-μm column. The elution was conducted with 40% methanol containing 20 m*M* $NaClO_4$ and the 10-hydroxylated metabolite had UV absorbance detected at 251 nm. Analysis of one sample was finished within 22 min and the assay was sensitive. In our present assay condition, we found that only 10-hydroxylated verbenone was a major metabolite of verbenone catalyzed by liver microsomes of phenobarbital-treated rats. We did not determine the structures of unidentified metabolites of retention time at 3.3 and 3.8 min were observed at

Table 1
Kinetic analysis of (−)-verbenone 10-hydroxylation by liver microsomes of untreated rats and rats treated with phenobarbital

(−)-Verbenone (μ*M*)	(−)-Verbenone 10-hydroxylation (nmol/min/mg protein)	
	Untreated rats	Phenobarbital-treated rats
100	1.9	31.0
200	2.7	37.0
400	4.0	40.3
800	4.5	40.7
K_m (μ*M*)	206±30	40.6±5.2
V_{max} (nmol/min/mg protein)	5.8±0.3	43.6±0.9
V_{max}/K_m (ml/min/mg protein)	0.028±0.005	1.07±0.06

longer incubation of 20 and 30 min. The limit of detection of (−)-10-hydroxyverbenone was found to be about 40 pg in this HPLC condition; this is about 100-fold sensitive than the GC–MS method as described previously [3].

Phenobarbital has been shown to induce several drug-metabolizing enzymes including CYP2B1, CYP2B2, CYP3A1, and CYP3A2 [5,18]. Verbenone 10-hydroxylation activities were increased in liver microsomes of phenobarbital-treated rats as compared with untreated rats. The K_m values for the verbenone 10-hydroxylation activities by liver microsomes of untreated and phenobarbital-treated rats varied 5.1-fold. These results indicated that different P450 enzymes play important roles in the hydroxylation of verbenone in rat liver microsomes and that the phenobarbital-induced forms of P450 are more active than P450s present in liver microsomes of untreated-rats. It remains unclear which P450 enzymes are actually involved in the hydroxylation of verbenone in liver microsomes of untreated rats. Our preliminary results with recombinant rat P450 enzymes in insect cells indicated that CYP2B1 was more active than other P450 enzymes present in the liver microsomes of phenobarbital-treated rats (Miyazawa et al., unpublished).

In our previous studies, three monoterpene compounds, 1,4-cineole, 1,8-cineole, and limonene, have been found to be catalyzed by different forms of P450 enzymes in rat and human liver microsomes [7–10,14]. The 1,4- and 1,8-cineoles have been shown to be mainly catalyzed by CYP3A enzymes in rat and human liver microsomes, whereas (+)- and (−)-limonene enantiomers are catalyzed by CYP2B1 and CYP2C11 in rat liver microsomes and CYP2C9 and CYP2C19 in human liver microsomes [7–10,14]. These monoterpene compounds have been reported to be ingested by humans through foods and cosmetics and thus it is important to examine how these chemicals are metabolized by these animal species and how these metabolites are excreted from the body after the metabolism. We are examining which P450 enzymes participate in the 10-hydroxylation of verbenone by rat and human liver microsomes.

In conclusion, the current study was designed to develop a sensitive method for the determination of verbenone 10-hydroxylation by liver microsomes of untreated and phenobarbital-treated rats using HPLC. The limit of detection (about 40 pg) with HPLC was about 100-fold higher than those with GC-MC method. The method thus developed was rapid, simple, and sensitive to detect 10-hydroxylated metabolite of verbenone. The method may be applicable to determine the verbenone 10-hydroxylation activities catalyzed by various animal species, including mice, rabbits, dogs, monkeys, and humans.

Acknowledgements

This work was supported in part by Grants from the Ministry of Education, Science, and Culture of

Japan, and the Ministry of Health and Welfare of Japan.

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