Identification of Cannabielsoin, a New Metabolite of Cannabidiol Formed by Guinea-Pig Hepatic Microsomal Enzymes, and Its Pharmacological Activity in Mice

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Metabolism of cannabidiol (CBD), one of the major components of marihuana, was studied in the guinea pig both *in vitro* and *in vivo*. Analyses of metabolites by gas chromatography and gas chromatography-mass spectrometry proved that cannabielsoin (CBE) was formed from CBD as a novel metabolite, and that the amount was about one-sixth of 7-hydroxy-CBD, which was the most abundant metabolite under *in vitro* conditions in the presence of microsomal monooxygenase (cytochrome P-450). CBE was also found in the liver of the guinea pig that was given CBD (100 mg/kg) intraperitoneally 1 h before sacrifice. The effects of CBE on pentobarbital-induced sleep and body temperature were assessed in the mouse; CBE possessed little activity in either case.

Keywords — cannabidiol; cannabielsoin; *in vitro*–*in vivo* metabolite; monooxygenated metabolite; pentobarbital-induced sleep prolongation

Introduction

Cannabidiol (CBD), one of the major constituents of marihuana, is known to be nonpsychoactive but to have anticonvulsant activity especially against electrically induced seizures.^{1,2)} Although accumulated knowledge on the anticonvulsive properties of CBD supports the possibility of CBD as an antiepileptic^{3,4)} and CBD also has an inhibitory effect on the hepatic drug-metabolizing enzyme system, the action mechanism of CBD remains to be elucidated.^{5–7)} For this, it is important to study the metabolism of CBD in more detail. Many workers^{8,9)} have reported that CBD is converted to a number of products. We have previously described the epoxidation of the double bond in the terpene moiety of tetrahydrocannabinol.^{10,11)} However, no papers have dealt with the epoxidation of CBD. In the present study, we reexamined the CBD metabolism, with special emphasis on the epoxidation, using hepatic microsomes of the guinea pig, and found that cannabielsoin (CBE) was formed from CBD as a novel metabolite. The pharmacological effect of CBE was also studied in mice.

Materials and Methods

Materials — Nicotinamide adenine dinucleotide phosphate (NADPH) and glucose-6-phosphate (G-6-P) were purchased from Boehringer Mannheim GmbH (Darmstadt, FR Germany); G-6-P dehydrogenase (type V, EC1.1.1.49) and Fast Blue BB salt from Sigma Chemical Co. (St. Louis, Mo); Sephadex LH-20 from Pharmacia Fine Chemicals Co. (Uppsala, Sweden); silica gel (Wakogel B-5) for thin layer chromatography (TLC), trimethylsilylimidazole (TMSI), *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and trimethylchlorsilane (TMCS) from Wako Pure Chemical Ind. (Osaka, Japan). Other chemicals and solvents used were of the highest quality commercially available. CBD was isolated and purified from cannabis leaves by the method previously reported.¹²⁾ CBE was prepared as follows: CBD (4.6 mmol) was acetylated with acetic anhydride in pyridine to give CBD-2',6'-diacetate (4.4 mmol). To the acetylated CBD dissolved in 5 ml of dichloromethane, *m*-chloroperbenzoic acid (4.4 mmol) in 5 ml of dichloromethane was added at 0 °C. One hour later, the reaction was stopped by adding sodium sulfite. The reaction medium was successively washed with 5%

(w/v) aqueous sodium bicarbonate and water. The organic layer was evaporated *in vacuo* and the residue was applied to a silica gel column using a solvent system of *n*-hexane-ethyl acetate (4:1). Fractions containing a major product were combined and the product was further purified by silica gel column chromatography with dichloromethane as a solvent to give 1*S*,2*R*-epoxy-CBD-2',6'-diacetate (1.3 mol). To ethanolic solution of 1*S*,2*R*-epoxy-CBD-2',6'-diacetate (0.32 mmol), 0.5 N sodium hydroxide (0.5 mmol) was added and the mixture was stirred for 20 min at room temperature. The reaction mixture was then extracted with diethylether to give CBE (0.30 mmol). MS *m/z*: 330 (M^+ , see Fig. 2). Proton nuclear magnetic resonance ($^1\text{H-NMR}$ (CDCl_3 , δ)) 6.18 (2H, s, 3'- and 5'-H), 5.29 (1H, br s, 2'-OH), 4.38 (2H, s, 9-H₂), 3.70 (1H, d, $J = 5.9$ Hz, 2-H), 3.20 (1H, dd, $J = 5.1, 11.3$ Hz, 3-H), 2.35 (2H, t, $J = 7.8$ Hz, 1''-CH₂), 1.64 (3H, s, 7-CH₃), 1.34 (3H, s, 10-CH₃), 0.88 (3H, t, $J = 4.6$ Hz, 5''-CH₃). The purity of the cannabinoid was found to be above 98% by gas chromatography (GC) and high performance liquid chromatography (HPLC).

Animals — Male Hartley guinea pigs (350–450 g body weight) and male ddN mice (23–25 g body weight) were obtained from Hokuriku Experimental Animals Lab. (Kanazawa, Japan). The animals were kept in air-conditioned rooms with a 12 h light and dark cycle and were given food and water *ad libitum*.

***In Vitro* Metabolism of CBD** — After starvation for 24 h, guinea pigs were killed by decapitation and the livers were perfused with ice-cold physiological saline. Microsomal suspension was prepared by the method previously reported.⁷⁾ CBD (500 μg) was incubated with microsomes (1 g liver equivalent) in medium consisting of potassium phosphate buffer (pH 7.4, 530 μmol), NADP (5 μmol), G-6-P (50 μmol), MgCl_2 (90 μmol), nicotinamide (27 μmol) and G-6-P dehydrogenase (5 units) in a final volume of 9.0 ml. Incubation at 37 °C was initiated by addition of the microsomes and continued for 1 h. Metabolites formed were extracted with ethyl acetate (25 ml $\times$ 2) and analyzed by silica gel TLC with a solvent system of *n*-

hexane-acetone-diethylamine (20:10:1, v/v). The remaining sample was subjected to silica gel preparative TLC under the same conditions and metabolites isolated were converted to trimethylsilyl (TMS) derivatives by heating with BSTFA, TMCS and TMSI.¹³⁾ The metabolites were then examined by GC and gas chromatography-mass spectrometry (GC-MS). Approximate amounts of metabolites were calculated from peak areas on the total ion chromatograms in GC-MS.

***In Vivo* Metabolism of CBD** — CBD (100 mg/kg in 0.9% (w/v) NaCl containing 1% (v/v) Tween 80) was intraperitoneally injected into a male Hartley guinea pig (360 g body weight) and the animal was killed by decapitation 1 h later. The liver was removed and homogenized in three volumes of acetone with a Polytron. (Kinematica GmbH, Lucerne, Switzerland). The homogenate was further extracted twice with the same volume of acetone. The combined extract was evaporated *in vacuo*, and the residue was dissolved in a small amount of chloroform. The sample was applied to a Sephadex LH-20 column equilibrated with chloroform. Metabolites were eluted stepwise by increasing concentration of methanol (from 2 to 16% (v/v)) in chloroform. Each fraction (2 ml) was checked by TLC, and fractions containing a metabolite with a *R_f* value corresponding to that of authentic CBE on TLC were combined and evaporated *in vacuo*. The residue dissolved in a small amount of acetonitrile was converted to the TMS derivative, and analyzed by GC and GC-MS.

Conditions for GC and GC-MS — GC: A Shimadzu GC-5A gas chromatograph equipped with a hydrogen flame ionization detector and a column of 5% (w/w) SE-30 on Chromosorb W (60–80 mesh, 3 mm i.d. $\times$ 1.5 m) was used. Column temperature, 250 °C; detector temperature, 270 °C; carrier gas, N_2 50 ml/min.

GC-MS: A JEOL JMS DX-300 mass spectrometer equipped with a JMS mass data system and a column packed with 5% (w/w) SE-30 on Chromosorb W (60–80 mesh, 1.5 mm i.d. $\times$ 2 m) for GC were used. Column temperature, 250 °C; injector temperature, 270 °C; carrier gas, He 40 ml/min; ionization current 0.3 mA;

Fig. 1. Identification of CBE as an *in Vitro* Metabolite of CBD in the Guinea Pig Using Liver Microsomes

A metabolite whose *R_f* value on TLC corresponded to that of synthetic CBE was isolated by silica gel preparative TLC, converted to the TMS derivative and submitted to GC and GC-MS under the conditions described in Materials and Methods.

ionization energy, 70 eV.

Pharmacological Activity — Effect on Body Temperature: Two groups of mice ($n = 10$ each) were intravenously given CBE (10 mg/kg) or the vehicle (1% Tween 80-saline), and the rectal temperature was measured just before and at 10, 20, 30, 45, 60, 90 and 120 min after the injection as previously reported.¹⁴⁾

Effect on Pentobarbital-Induced Sleep: Five groups of mice ($n = 10$ each) were intravenously injected with CBE or the vehicle, and intraperitoneally challenged with sodium pentobarbital (40 mg/kg) 20 min later. The duration of loss of righting reflex was measured as the sleeping time.¹⁵⁾

Statistics — Statistical significance of differences was calculated by the use of Student's *t* test.

Results

In Vitro Metabolism of CBD

Figure 1 shows a gas chromatogram and mass spectra of the trimethylsilylated metabolite fraction of CBD formed with guinea pig liver microsomes. A peak with a retention time of 3.4 min appeared on the chromatogram. A molecular ion was seen at m/z 474 and prominent fragment ions appeared at m/z 390, 303, 277, 197, 130 and 108 (a base ion). The retention time and fragment ions of this metabolite were in good agreement with those of a synthetic sample of CBE. In the control experiment under the same conditions except for the use of boiled microsomes instead of fresh ones, no spot appeared on TLC and no peak on GC corresponding to the authentic CBE. Table I summarizes various oxidative metabolites formed by liver microsomes of guinea pigs and their approximate amounts calculated from the peak areas on the total ion chromatogram of GC-MS. The most abundant metabolite was 7-hydroxy-CBD (7-OH-CBD, 100%) followed by 6-OH-CBD (mixture of 6 α - and 6 β -OH-CBD, 52%), 4''-

TABLE I. Trimethylsilylated Metabolites of CBD Identified by GC-MS

Metabolites	t_R (min)	Ratio (%) ^{a)}	Fragment ion (m/z) ^{c)}
3TMS-7-OH-CBD	4.0	100	546 (M^+ ,10), 478 (77), 433 (100), 337 (45)
3TMS-6-OH-CBD ^{b)}	3.6	52	546 (M^+ ,1), 478 (100), 337 (10)
3TMS-4''-OH-CBD	3.8	34	546 (M^+ ,20), 478 (100), 117 (25)
2TMS-CBE	3.4	16	474 (M^+ ,12), 391 (13), 130 (79), 108 (100)
3TMS-2''-OH-CBD	3.5	15	546 (M^+ ,10), 478 (9), 145 (100)

The metabolites except for CBE were tentatively identified by comparison with the reported fragment profile.^{17, 19)}

^{a)} Ratio of peak areas on the total ion chromatogram in GC-MS.

^{b)} Mixture of 6 α - and 6 β -OH-CBD.

^{c)} Numbers in parentheses are relative intensities.

OH-CBD (34%), CBE (16%) and 2''-OH-CBD (15%).

In Vivo Metabolism of CBD

An *in vivo* metabolite extract was applied to a column of Sephadex LH-20, and the effluent was checked by TLC with Fast Blue BB salt as a color reagent. Fractions containing a metabolite with an R_f value of 0.42 (corresponding to that of synthetic CBE) were combined and examined by GC and GC-MS. Figure 2 shows the gas chromatogram and fragment ions in GC-MS of synthetic and biologically formed CBE. The retention time (3.6 min) and profile of fragmentation of the metabolite corresponded well to those of the synthetic standard. As in the case of *in vitro* metabolism, the most abundant metabo-

lite was 7-OH-CBD (100%) followed by 6-OH-CBD (mixture of α - and β -forms, 34%), but the amount of CBE (5%) calculated from the peak areas of the total ion chromatogram was much smaller than that under the *in vitro* conditions.

Pharmacological Activity of CBE

The body temperature of the mice pretreated with CBE (10 mg/kg, i.v.) was not significantly different from that of the control mice given the vehicle (1% (v/v) Tween 80-saline) during the time period of the temperature measurement (just before and until 120 min after the injection).

Fig. 2. Identification of CBE as an *in Vivo* Metabolite of CBD in the Guinea Pig

At 1 h after CBD injection (100 mg/kg, i.p.), the liver was excised and metabolites were extracted with acetone. The extract was applied to a column of Sephadex LH-20. Isolated metabolites were analyzed by GC and GC-MS under the conditions described in Materials and Methods.

Fig. 3. Effects of CBE and CBD on Pentobarbital-Induced Sleep in the Mouse

Four groups of mice ($n = 10$ each) were pretreated with CBE or CBD (5 and 10 mg/kg, i.v.) and 20 min later they were given sodium pentobarbital (40 mg/kg i.p.). The control group received the vehicle instead of the cannabinoids. Each bar represents the mean value $\pm$ S.E. of 10 mice. ^{a)} Significantly different from the control ($p < 0.01$) in Student's t test.

tions) (data not shown). Figure 3 shows the sleeping times induced by sodium pentobarbital after the cannabinoids injections. The pentobarbital-induced sleeping time in mice tended to be prolonged by the treatment with CBD at a dose of 5 mg/kg (78 ± 5 min). Pretreatment of mice with CBD (10 mg/kg) significantly prolonged the sleep (188 ± 20 min) as compared with the control group (57 ± 6 min) given the vehicle (1% (v/v) Tween 80-saline) instead of CBD.

Discussion

Metabolism of CBD has been extensively studied in various animal species.^{16–20} In the guinea pig, Harvey *et al.* examined CBD metabolism *in vivo*.²¹ CBE was reported to be present in low concentration in cannabis and not to play an important part in pharmacological activity of cannabis.^{22, 23} However, no one has reported CBE formation in any animal species. In the present study, we found CBE as an *in vivo* and *in vitro* metabolite of CBD in the guinea pig for the first time. The amount of CBE formed *in vitro* was one-sixth of the most abundant metabolite (7-OH-CBD) in the guinea pig. On the other hand, the amount of CBE formed *in vivo* was much smaller than that formed by the microsomal fraction. It is not clear at present whether this smaller amount of CBE *in vivo* reflects a slight formation of CBE itself or further metabolism of CBE.

Uliss *et al.*²⁴ synthesized CBE from CBD and tentatively examined its pharmacological effect, observing little activity in the mouse. However, considering its structure, CBE may have cannabinoid-type action such as hypothermia, which is a typical pharmacological effect of psychoactive cannabinoids. Furthermore, CBD is known to prolong pentobarbital-induced sleep in mice through inhibition of the hepatic drug-metabolizing enzyme system.^{5, 25} It is also possible that CBE inhibits the enzyme system as an active metabolite of CBD. Thus we reexamined the pharmacological effects of CBE in mice by using these two indices. In the present study, however, CBE was found not to show any significant effect on the pentobarbital-induced

sleep or body temperature in mice. As previously reported,^{5,7,26} CBD prolonged the barbiturate-induced sleep in this study. From this result and our reported data,⁷ it is unlikely that CBE and its metabolites account for the inhibition of the hepatic drug-metabolizing enzyme system. CBE is assumed to be biotransformed from CBD *via* 1*S*, 2*R*-epoxy-CBD as an intermediate, and this is now under investigation. Moreover, CBE retains rather high lipophilicity as judged from its *R_f* value on TLC and retention time on the GC analyses. Considering these points, it seems desirable to perform a further systematic assessment of the pharmacological effect of CBE.

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