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Identification of monohydroxylated metabolites of cannabidiol formed by rat liver

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Cannabidiol (CBD) was metabolized *in vitro* by rat liver enzymes. Unchanged CBD and eight monohydroxylated metabolites were isolated and positively identified. As previously reported, 7-hydroxy-CBD was the major metabolite. The second most abundant metabolite was 6α-hydroxy-CBD; whereas only a trace amount of 6β-hydroxy-CBD was found. In addition hydroxylation occurred in all positions of the pentyl side chain, 4″-hydroxy-CBD being most abundant. 3″-Hydroxy-CBD was formed in half of the yield of 4″-hydroxy-CBD, while 1″-, 2″-, and 5″-hydroxy-CBD were each formed in approximately one fourth of the yield of 4″-hydroxy-CBD.

Recent evidence has shown cannabidiol (CBD) to exhibit potent anticonvulsant activity (Carlini, Leite, & others, 1973; Dyguerids & Tannhauser, 1973), to inhibit prostaglandin biosynthesis (Burstein, Levin, & Varanelli, 1973), and also to prolong barbiturate sleeping time (Paton & Pertwee, 1972) apparently due to inhibition of barbiturate metabolism by CBD. Likewise, CBD increased brain concentrations of Δ^1-tetrahydrocannabinol (Δ^1-THC) and its metabolite 7-hydroxy-Δ^1-THC apparently by interfering with their metabolism (Jones & Pertwee, 1972). CBD also blocked the excitatory effects of Δ^1-THC in animals but potentiated the depressant effects (Karniol & Carlini, 1973). There has therefore been increased interest in the metabolism of CBD. CBD has been reported to be converted by rat liver to predominantly 7-hydroxy-CBD and to a minor extent 3″-hydroxy-CBD (Nilsson, Agurell & others, 1973). A more thorough investigation was undertaken to determine a more complete metabolic pattern of CBD. Metabolites of CBD were identified after isolation from a rat liver incubation as described herein.

MATERIALS AND METHODS

Livers (229 g) from 20 male Sprague Dawley rats (pretreated with sodium pentobarbitone 40 mg kg^{-1} twice daily for 4 days) were homogenized in 450 ml of isotonic KCl, and the homogenate was centrifuged at 10 000 *g*. The resulting microsomal containing supernatant was enriched with appropriate cofactors (Jones, Widman & others, 1974). An emulsion of 125 mg of (−)-1″-[^{3}H]-CBD (0·15 mCi mM^{-1}) in 6·2 ml of Tween 80-phosphate buffer (1 : 25) was added to the liver microsomal preparation and incubated at 37° for 2 h and 45 min. A control experiment was carried out simultaneously by incubating ^{3}H-CBD with an aliquot of the microsomal containing supernatant heated to 100°.

Isolation of metabolites

The combined incubation mixtures were extracted with light petroleum (40–60°, 3 × 650 ml) followed by diethyl ether (3 × 500 ml). The diethyl ether (ether) extract was chromatographed on a Florisil column (115 g; 1·5 × 115 cm), and the radioactivity was eluted by increasing the polarity of the eluent as follows: 20% ether–light petroleum (640 ml), 50% ether–light petroleum (500 ml), 100% ether (500 ml), 5% methanol–ether (500 ml), 40% methanol–ether (500 ml), and 100% methanol (500 ml). The fractions from the Florisil column were rechromatographed on several Sephadex LH-20 columns (Fig. 1) according to the procedure of Widman, Nordqvist & others (1974). Metabolites were isolated from those fractions (Sephadex LH-20) by thin-layer chromatography.

Thin-layer chromatography and identification methods

Thin-layer chromatography (t.l.c.) was carried out on precoated silica gel F plates (Merck, 0·25 mm thickness, 5 × 10 cm) which were developed in 60% ether–light petroleum (unless stated otherwise) and visualized with 0·1% Fast Blue B salt in 2 N sodium hydroxide. Radioactivity was assayed in a Packard Tricarb model 3375 spectrometer with external

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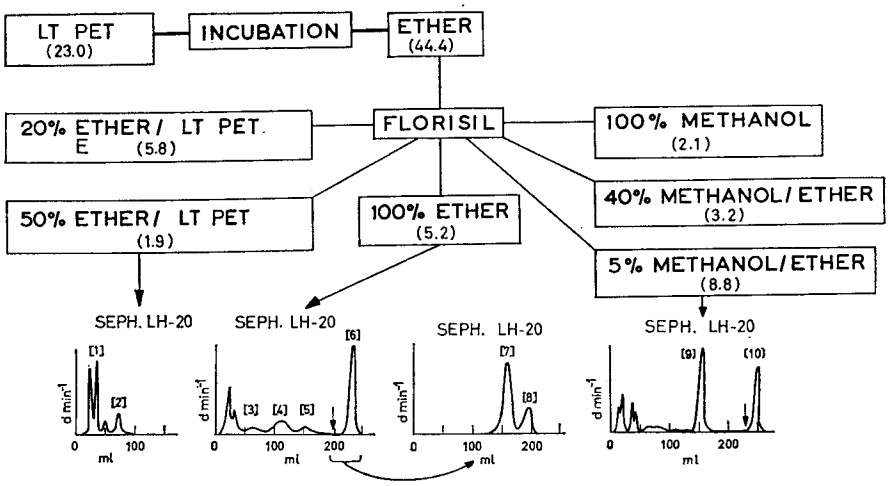

FIG. 1. Isolation of CBD metabolites from rat liver incubation. Numbers in parenthesis indicate percent of total radioactivity while the numbers in brackets represent the fractions described in the text. Arrows show beginning of methanol wash. Disintegrations per minute (d min^{-1}).

standardization. The scintillation fluid was a mixture of Permablend 111 (Packard) in toluene (5·5 g $litre^{-1}$) A Varian Aerograph Model 2100 with a flame ionization detector was used with: oven 250°, injector/detector 270°, carrier gas (N_2) 25 ml min^{-1}, H_2 25 ml min^{-1}, O_2 200 ml min^{-1}. (g.l.c.). The column was 2% SE-30 on Gas-Chrom Q (180 × 2 mm glass columns). An LKB 9000 gas chromatograph–mass spectrometer was used to record mass spectra (m.s.). The column was 3% SE-30 on Gas-Chrom Q (190°). M.s. were recorded at 70 eV except for silylated derivatives which were determined at 20 eV. ^{1}H-Nuclear magnetic resonance (^{1}H-nmr) spectra were recorded on a Varian 100 MHz instrument ($CDCl_3$, Fourier Transform).

Synthesized materials

(−)-1″-[^{3}H]-CBD was synthesized according to Agurell, Gustafsson & others (1973) and diluted with non-labelled (−)-CBD to a final specific activity of 0·15 mCi mM^{-1}. Its chemical and radio-chemical purity was 98% according to g.l.c. and t.l.c. (20% ether–light petroleum). Metabolites were identified by comparison with authentic samples whenever possible (Fig. 2). The synthesis of 7-, 10-, 6α-, and 6β-hydroxy-CBD was described by Lander, Ben-Zvi & others (1976). Synthesis of 1″- and 2″-hydroxy-CBD has previously been reviewed (Agurell, Dahmén & others, 1972) while that of 5″-hydroxy-CBD will be discussed elsewhere. The authenticity of the reference standard has been established by Binder, Agurell & others (1974). Trimethylsilyl (TMS) derivatives of reference compounds and metabolites were prepared as described by Binder & others (1974).

RESULTS

Greater than 90% of the radioactivity in the light petroleum and ether extracts of the control experiment corresponded to unchanged starting material when analysed by t.l.c. (15% ether–light petroleum). Of the viable incubation mixture 29% of the total radioactivity was extractable as unchanged CBD. The light petroleum extraction removed most of the CBD, while the ether extraction (44% of the total radioactivity) removed metabolites plus CBD (4:1). The ether fraction was chromatographed on a Florisil column, and the first eluate (20% ether–light petroleum) contained CBD by t.l.c. analysis in 15% ether–light petroleum. The 50% ether–light petroleum, 100% ether, and 5% methanol–ether fractions were further chromatographed on Sephadex LH-20 columns. The Sephadex LH-20 fractions which exhibited high radiopurity were Nos. 1 to 10 in Fig. 1. The other fractions were not investigated.

Fraction 1 contained predominantly one metabolite (I) which was slightly more polar than CBD on t.l.c. and g.l.c. but much less polar than the monohydroxylated reference compounds. The m.s. gave *m/e* (% abundance) of 312 (55, M^+), and the trimethylsilyl (TMS) derivative showed the introduction of only one TMS group [*m/e* 384, M^+ (61)]. The structure of metabolite I was not elucidated.

Fraction 2 CBD and metabolite II separated on t.l.c. (40% ether–light petroleum). After purification

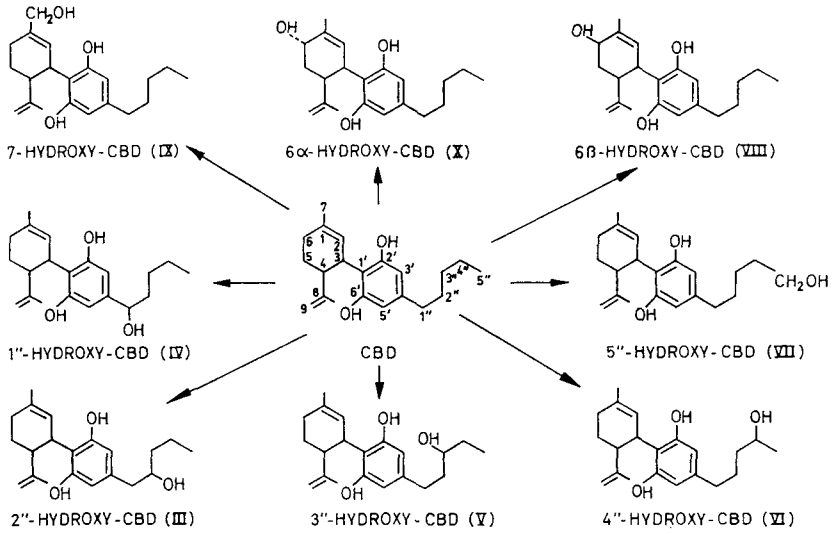

FIG. 2. Structures of CBD and its *in vitro* metabolites.

II (155 μg) had R_F = 0·56, retention time (Rt) = 4·15 min, and m.s. data of *m/e* 330 (M^+, 43) and 205 (100). Metabolite II was not assigned a structure, although ^{1}H-nmr indicated a structural alteration at C-9 or C-10 (loss of C-10 proton signal at δ = 1·66 and shift downfield of C-9 proton singlets. Hydroxylation at position 10 was ruled out after g.l.c. and m.s. comparisons with synthetic 10-hydroxy-CBD [Rt = 9·45 min, *m/e* 231 (100)].

Fraction 3 was predominantly one metabolite (III) that was purified on t.l.c. to yield 113 μg. Its structure was established as 2″-hydroxy-CBD by comparison with the reference compound [R_F = 0·51 Rt = 7·10 min; *m/e* 330 (M^+, 33), 262 (45), 258 (18), 247 (100), 190 (27), and 175 (27)]. Also, m.s. of the TMS derivative was characteristic of 2″-hydroxy-CBD (Table 1).

Fraction 4 was a mixture of three metabolites (IV–VI) which were separated on t.l.c. (60% ether–light petroleum, developed twice). Metabolite IV was identical to synthetic 1″-hydroxy-CBD by t.l.c. (R_F = 0·60), g.l.c. (Rt = 7·40 min), and m.s. [330 (M^+, 12), 262 (32), 247 (100), 206 (8), 205 (19), and 159 (10)]. M.s. of the TMS derivative was unique for 1″-hydroxy-CBD (Table 1). The quantity of 1″-hydroxy-CBD (98 μg) was calculated after doubling the amount of radioactivity, since hydroxylation would presumably remove half of the ^{3}H on C–1″. The isomers of authentic 1″-hydroxy-CBD could be separated on t.l.c. after developing three times in 40% ether–light petroleum (R_F = 0·57 and 0·52). The 1″-OH metabolite appeared to be a single compound corresponding to the more polar isomer.

Metabolite V (189 μg) had R_F = 0·35 and Rt = 8·10 min. M.s. indicated monohydroxylation [330, M^+ (27)] in the side chain [247 (100)], specifically at position 3″[301 (5), 258 (48), 242 (16), and 190 (55)]. M.s. of the TMS derivatives is in Table 1. The structure of 3″-hydroxy-CBD was further supported by ^{1}H-nmr: δ($CDCl_3$) 6·26 (s, 2H, C–3′, C–5′), 5·56 (br, s, 1H, C–2), 4·68 (s, 1H, C–9), 4·56 (s, 1H, C–9), 3·48 (p, 1H, C–3″), 2·52 (m, 2H, C–1″), 1·80 (s, 3H, 6–7), 1·66 (s, 3H, C–10), and 0·92 (t, 3H, C–5″).

Metabolite VI (451 μg) was assigned the structure of 4″-hydroxy-CBD [R_F = 0·31; Rt = 8·25 min; nonsilylated m.s. *m/e* 330 (M^+, 24), 262 (45), 247 (100), 209 (18), and 122 (25); silylated m.s., see Table 1]. The structure was conclusively established by ^{1}H-nmr: δ($CDCl_3$) 6·30 (s, 2H, C–3′, C–5′), 5·58 (br, s, 1H, C–2), 4·68 (s, 1H, C–9), 4·58 (s, 1H, C–9), 3·84 (m, 1H, C–3), 2·50 (t, 2H, C–1″), 1·80 (s, 3H, C–7), 1·66 (s, 3H, C–10), and 1·19 (d, 3H, C–5″).

Fraction 5 contained metabolites VII and VIII. The more polar, VII, (130 μg) had a R_F = 0·33 and Rt = 11·05 min which were identical to those of 5″-hydroxy-CBD. M.s. was indistinguishable from that of 5″-hydroxy-CBD: *m/e* 330 (M^+, 29), 262 (45), 247 (100), and 209 (20). M.s. data of the TMS derivative are in Table 1. VIII (40 μg) corresponded to reference 6β-hydroxy-CBD by t.l.c. (R_F = 0·58), g.l.c. (Rt = 7·80 min), non-silylated m.s. [(330 (M^+, 0), 312 (77), 297 (25), 257 (100), 244 (21), and 193 (85)], and silylated m.s. (Table 1).

Table 1. *Mass spectral fragments characteristic of CBD metabolites (TMS derivatives).*

	Relative abundances (20eV) of OH CBD metabolites							
m/e	1″	2″	3″	4″	5″	6ß	6α	7
546	15	4	21	14	13	1	1	11
478	62	9	100	100	100	100	100	77
443								100
425	47	7	53	40	51			
421	100							
402			16					
338							3	
337						5	10	45
334	2	4	67	2	2			
268	2		48	3	5		1	
244	2		35	7	8			
159	10							
145		100						
117	4		14	25	2			

The remaining radioactivity on the Sephadex LH-20 column which contained the 100% ether fraction (Florisil, Fig. 1) was eluted with methanol. This methanol wash (fraction 6) was rechromatographed on a similar Sephadex LH-20 column to give fractions 7 and 8.

Fraction 7 (2·04 mg) was a single metabolite (IX) which was identified as 7-hydroxy-CBD by t.l.c. ($R_F = 0{\cdot}41$), g.l.c. (Rt = 10·75 min), and m.s. comparisons with an authentic sample.

Fraction 8 (700 μg) was also a pure metabolite (X) which was identified as 6α-hydroxy-CBD ($R_F = 0{\cdot}49$, Rt = 8·00 min) by comparison to the reference compound. The m.s. clearly established 6α-hydroxylation by *m/e* 330 (M^+, 4), 312 (45), 262 (100), 257 (29), 233 (39), and 193 (55). The m.s. of the TMS derivatives (Table 1) did not differentiate between 6α- and 6β-hydroxy-CBD to a great degree.

Fraction 9 (3·50 mg) was identified as 7-hydroxy-CBD as described above. Fraction 10 contained polar compounds which on t.l.c. did not move from the origin. M.s. of the TMS derivatives indicated a mixture of dihydroxy metabolites.

DISCUSSION

After incubation of ^{3}H-CBD with rat liver supernatant only 29% of the radioactivity was extractable as ^{3}H-CBD. The major portion of the unchanged CBD was extracted with light petroleum while the ether extraction which followed removed both metabolites and CBD. A total of eight metabolites were isolated and identified. The identity of 7-, 6α-, 6β-, 1″-, 2″-, and 5″-hydroxy metabolites was based on t.l.c., g.l.c., and m.s. comparisons to authentic samples. The structures of 3″- and 4″-hydroxy-CBD were assigned according to m.s. and ^{1}H-nmr data, since references were unavailable.

Determination of metabolite structures

The m.s. of both silylated and non-silylated compounds were essential in establishing the exact location of hydroxylation. Budzikiewicz & others (1965) described the fragmentation of non-silylated CBD by a Retro–Diels–Alder cleavage (RDA) with and without loss of the C–7 methyl group ($M^+ - 83$ and $M^+ - 68$, respectively), cleavage of the entire monoterpene moiety with the exception of C–3 ($M^+ - 121$), and benzylic cleavage of the side chain with transfer of the C–3 proton to the aromatic ring ($M^+ - 56$).

Hydroxylation in the monoterpene moiety was determined by comparison with authentic 6α-, 6β-, 7-, and 10-hydroxy-CBD. The m.s. of the non-silylated references exhibited the following base peaks: 6β-hydroxy-CBD (*m/e* 257), 6α-hydroxy-CBD (*m/e* 262, RDA), 7-hydroxy-CBD (*m/e* 244, RDA with loss of H_2O), 10-hydroxy-CBD (*m/e* 231, RDA with loss of C–7 methyl group). M.s. of the silylated compounds (Table 1) also differentiated between 7- and 10-hydroxylation with base peaks of *m/e* 443 [loss of $-CH_2OSi(CH_3)_3$] and *m/e* 390 (RDA), respectively. Silylated derivatives of both 6α- and 6β-hydroxy-CBD had a common base peak of *m/e* 478 (RDA).

Side chain hydroxylation was established in the non-silylated m.s. by loss of a methyl group (metastable *m/e* 233) from the predominant fragment at *m/e* 262 (RDA) which gave rise to *m/e* 247 (base peak). Determination of the exact position of the OH group depended upon both silylated and non-silylated m.s. In the non-silylated m.s., only 1″- and 3″-hydroxy-CBD had unique fragmentation patterns. The distinguishing features of 1″-hydroxy-CBD were *m/e* 206 (RDA with loss of butene), *m/e* 205 (RDA plus benzylic cleavage), and *m/e* 159. The m.s. of 3″-hydroxy-CBD was characterized by *m/e* 301 (loss of ethyl group), *m/e* 258 (benzylic cleavage with proton transfer), and *m/e* 190 (RDA plus benzylic cleavage with proton transfer). Hydroxylation at position 3″ appeared to facilitate transfer of the C–3″ proton to the aromatic ring which resulted in benzylic cleavage more prominent than that associated with 2″-hydroxy-CBD.

As shown in Table 1, m.s. of the TMS derivatives could distinguish between each of the side-chain metabolites. 1″- and 2″-Hydroxy-CBD could be distinguished by their base peaks of *m/e* 421 (RDA plus benzylic cleavage) and 145 [$^+C_4H_8OSi(CH_3)_3$], respectively. Only 3″-hydroxy-CBD exhibited fragments *m/e* 402 (benzylic cleavage with proton transfer) and 334 (RDA from *m/e* 402). Fragment *m/e* 117 [$CH_3{}^+CHOSi(CH_3)_3$] was essential for the identity of 4″-hydroxy-CBD which also lacked the characteristic features of 3″-hydroxy-CBD. Although

the m.s. of 5″-hydroxy-CBD clearly indicated side-chain hydroxylation, it lacked all fragments characteristic of the other side-chain metabolites.

^{1}H-nmr was used to confirm the structures of 3″- and 4″-hydroxy-CBD, since authentic samples were not available. The ^{1}H-nmr of 3″-hydroxy-CBD was similar to that of CBD with the exception of a pentet at $\delta = 3{\cdot}48$ (C–3″ proton) as described by Binder & others (1974).

In the other spectra hydroxylation at position 4″ was supported by a doublet centered at $\delta = 1{\cdot}19$ which replaced the triplet at $\delta = 0{\cdot}87$ (C–5″ protons).

Metabolic comparison of CBD and other cannabinoids

The metabolism of CBD by rat liver was similar to that previously reported for other cannabis constituents. 7-Hydroxylation has been established as the major route of metabolism in the rat for CBD (Nilsson & others, 1973), Δ^1-THC (Wall, Brine, & others, 1970; Nilsson, Agurell, & others, 1970), Δ^6-THC (Burstein, Menezes, & others, 1970), and CBN (Widman, Nilsson, & others, 1971). Also Ben-Zvi, Burstein, & Zikopoulos (1974) identified 6α-hydroxy-Δ^1-THC from a rat liver incubation. As previously reported (Lander & others, 1976), CBD was converted to both 6α- and 6β-hydroxy-CBD and the 6α-isomer predominated. Maynard, Gurney & others (1971) were the first to note that hydroxylation could occur in the side chain. Using a dog liver incubate, they were able to identify 1″- and 3″-hydroxy-Δ^6-THC. Likewise, dog liver has been used to hydroxylate Δ^1-THC at positions 3″- and 4″ (Widman, Nordqvist & others, 1975b). Recently, rat liver supernatant was shown to convert CBN to 2″-, 3″- 4″-, and 5″-hydroxy-CBN (Widman, Dahmén, & others, 1975a) in a ratio comparable to that found for CBD side-chain metabolites. The primary difference appeared to be formation of a greater number of CBD metabolites.

A significant aspect of the CBD metabolism was the large number of products isolated and identified. Heretofore, a single *in vitro* experiment usually produced one or more mono-hydroxylated metabolites but failed to depict a pattern of metabolites as diverse as that of CBD.

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