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Neutral *in vivo* metabolites of cannabinol isolated from rat faeces

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The *in vivo* transformation of cannabinol (CBN) in the rat has been studied. Unchanged CBN and nine neutral mono-oxygenated and dioxygenated CBN metabolites have been identified. In the mono-oxygenated series the metabolites occurred in decreasing order of prominence as follows: 7-hydroxy-CBN, 4″-hydroxy-CBN, 1″-hydroxy-CBN, 2″-hydroxy-CBN, 3″-hydroxy-CBN, 5″-hydroxy-CBN and CBN-7-al. In the dihydroxylated metabolite series only 1″,7-dihydroxy-CBN and 4″,7-dihydroxy-CBN were found with the former as the more prominent metabolite.

Although the main psychoactive principle of cannabis is Δ^1-tetrahydrocannabinol (Δ^1-THC) as shown by Mechoulam, Shani & others (1970) there are indications that cannabinol (CBN), being one of the major components in cannabis, is of importance for the overall effect of cannabis. It has been reported that CBN blocks the depressant effect of Δ^1-THC in mice (Krantz, Berger & Welch, 1971) and in rats (Fernandez, Schabarek & others, 1974). It has also been shown that CBN administered intravenously to man produces slight psychoactive effects (Perez-Reyes, Timmons & others, 1973). It has also been suggested that CBN and CBN-derivatives may be formed as metabolites of Δ^1-THC in rhesus monkeys (Ben-Zvi, Bergen & Burstein, 1974; Ben-Zvi, Bergen & others, 1976) and rats (Widman, Nordqvist & others, 1974; McCallum, Yagen & others, 1975).

In vitro metabolic studies of CBN in rats have shown that CBN is metabolized to a large variety of products. Previously we have reported five mono-hydroxylated (Widman, Dahmén & others, 1975) and four dihydroxylated metabolites of CBN (Fonseka & Widman, 1977). The *in vivo* metabolic pattern of CBN seems to be similar to the one found *in vitro* but so far only a couple of neutral and acidic metabolites have been identified (Burstein & Varanelli, 1975; Wall, Brine & Perez-Reyes, 1976). We have now examined the *in vivo* metabolism of CBN in rats to establish a more complete metabolic pattern.

MATERIALS AND METHODS

^{14}C-CBN, synthesized as described earlier (Widman, Nilsson & others, 1971) had a radiochemical purity greater than 97% according to thin-layer chromatography (t.l.c.) and gas chromatography (g.c.) and a specific activity of 8·0 μCi mmol^{-1}. The compound was dissolved in 79% aqueous ethanol and administered to 12 male Sprague-Dawley rats (200–250 g) via the tail vein. Each rat received 100 mg kg^{-1}.

Determination of radioactivity

Excreted radioactivity was assayed in a Packard Tri-Carb Model B 2450 spectrometer with external standardization. Urinary samples (0·2 ml) were counted in 10 ml of Insta-Gel (Packard). Faeces samples were lyophilized and ground into a fine powder. Twenty mg of each sample was combined with 0·1 ml of water and incubated with 1·0 ml of Soluene 350 (Packard) at 50° for 10–14 h. The samples were cooled to room temperature and isopropanol (0·5 ml) and 30% H_2O_2 (0·2 ml) were added before a final 2 h incubation at 50°. Fifteen ml of Insta-Gel-0·5 M HCl (9 : 1) was added before counting.

Isolation of metabolites

The combined lyophilized and finely ground faeces was successively extracted with light petroleum (b.p. 40–60°, 1000 ml) and diethyl ether (1000 ml) using soxhlet extractors. The residue was then extracted with MeOH (3 × 750 ml at 20°; cold MeOH), finally with MeOH (1000 ml) (soxhlet; hot MeOH).

The ether extract was chromatographed on a Florisil column (160 g; 1·5 × 115 cm), eluted with the following solvents (500 ml): 20% v/v ether-light petroleum, 50% ether-light petroleum, 100% ether, 5% MeOH-ether, 20% MeOH-ether and 100% MeOH. The 50% ether-light petroleum and the 100% ether fractions eluted from the Florisil column were then chromatographed on Sephadex LH-20 according to Widman & others (1974).

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Before nuclear magnetic resonance (nmr) and gas chromatography-mass spectrometry (g.c.-ms) metabolites were purified by t.l.c. on precoated Silica gel F plates (Merck, 0·25 mm, 5 × 10 cm) (prewashed with benzene–MeOH, 1 : 1) developed in 60% ether-light petroleum unless otherwise stated.

Identification methods

Metabolites were identified by g.c., ms and t.l.c. comparison with reference compounds if available; otherwise, combined nmr and g.c.-ms data were used.

Gas chromatography was on a Varian Aerograph Model 2100, with a flame ionization detector, using a 2% SE-30 column (Ultraphase/Gas-Chrom Q, 125–150 mesh, 180 × 2 mm glass column) at 250°.

Mass spectra [LKB 9000 g.c.-ms with a 2% SE-30 column (Ultraphase/Gas-Chrom Q, 100–120 mesh) at 190°] of non-silylated metabolites were determined at 70 eV and those of silylated metabolites at 20 eV. For silylation *N,O*-bis(trimethylsilyl)acetamide in dry acetonitrile was used (Binder, Agurell & others, 1974).

Nmr spectra were recorded on a Varian 100 MHz instrument ($CDCl_3$, Fourier transform).

RESULTS

Excretion of radioactivity

Six days after the administration (i.v.) of CBN to rats 5–6% of total radioactivity was recovered in urine whereas 70–75% was recovered in faeces (cf. Agurell, Nilsson & others, 1969) which pooled after freeze drying weighed 92·9 g. The % distribution of radioactivity in the various extracts were: 19% (light petroleum), 30% (ether), 25% (cold MeOH) and 5% (hot MeOH). About 60% of the light petroleum extract was to unchanged CBN.

Isolated metabolites

When the ether extract was chromatographed on the Florisil column the percentage distribution of radioactivity in the various fractions was 5% (20% ether-light petroleum), 24% (50% ether-light petroleum), 16% (100% ether), 4% (5% MeOH–ether), 1% (20% MeOH-ether) and 47% (100% MeOH). The 20% ether-light petroleum fraction contained mainly unchanged CBN. The 50% ether–light petroleum fraction and the 100% ether fraction were the only fractions that were further purified on Sephadex LH-20. The more polar eluents contained essentially no mono- or dihydroxylated derivatives. Fig. 1 shows the elution on Sephadex LH-20 of the 50% ether–light petroleum fraction.

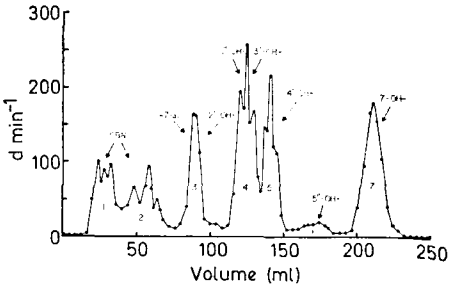

FIG. 1. Elution pattern on Sephadex LH-20 of CBN metabolites; 50% ether–light petroleum fraction (Florisil) of the ether extract of rat faeces. Two ml fractions were collected and eluted radioactivity was determined in aliquots of 100 μl as shown. Ordinate—Eluted radioactivity (d min^{-1}). Abscissa—Elution volume (ml).

Fractions 1 and 2 (Fig. 1) contained mainly unchanged CBN together with fat impurities.

Fraction 3 was also heavily contaminated with non-polar impurities, but after two purifications by preparative t.l.c. it provided two metabolites (I, II). Metabolite I showed by m.s. prominent peaks at *m/e*: 324 ($M^{+\cdot}$, 17%), 309 (100), 268 (5) and 252 (23). The ms of its silylated derivative exhibited fragments at *m/e*: 396 ($M^{+\cdot}$, 30%), 381 (100), 366·6 (9, metastable) and 340 (6). Reduction of metabolite I with Al/Li hydride gave a product with identical g.c., ms and t.l.c. properties as 7-hydroxy-CBN (Widman & others, 1971). Quantity, g.c. and t.l.c. data are given in Table 1. On the basis of this evidence metabolite I was assigned the structure of CBN-7-al (see Fig. 2). Metabolite II was identified as 2″-hydroxy-CBN (Fig. 2) by g.c., ms and t.l.c. comparison with reference 2″-hydroxy-CBN (Widman & others, 1975). The ms of its silylated derivative (cf. Binder, Agurell & others, 1974) is shown in Table 1 together with quantity, g.c. and t.l.c. properties. A small amount (45 μg) of metabolite II was also isolated from the light petroleum extract.

Fraction 4 was a mixture of two metabolites (III, IV) which were separated by t.l.c. (60% ether-light petroleum, developed twice). Nmr of metabolite II showed signals at δ ppm: 8·17 (s, 1H, C-2), 7·3 (m, 1H, C-6), 7·1 (m, 1H, C-5), 6·34 (d, 1H, C-5′), 6·46 (d, 1H, C-3′), 5·1 (m, 1H, C-1″), 2·39 (s, 3H, C-7), 1·6 (s, 6H, C-9) and 0·95 (t, 3H, C-5″). The ms of the silylated metabolite exhibited fragments at *m/e*: 470 ($M^{+\cdot}$, 26%), 455 (100), 440·5 (6, metastable) and 413 (77). Metabolite II was assigned the structure of 1″-hydroxy-CBN (Fig. 2). Metabolite IV was identified as 3″-hydroxy-CBN (Fig. 2) by g.c., ms and t.l.c. comparison with reference 3″-hydroxy-CBN (Wid-

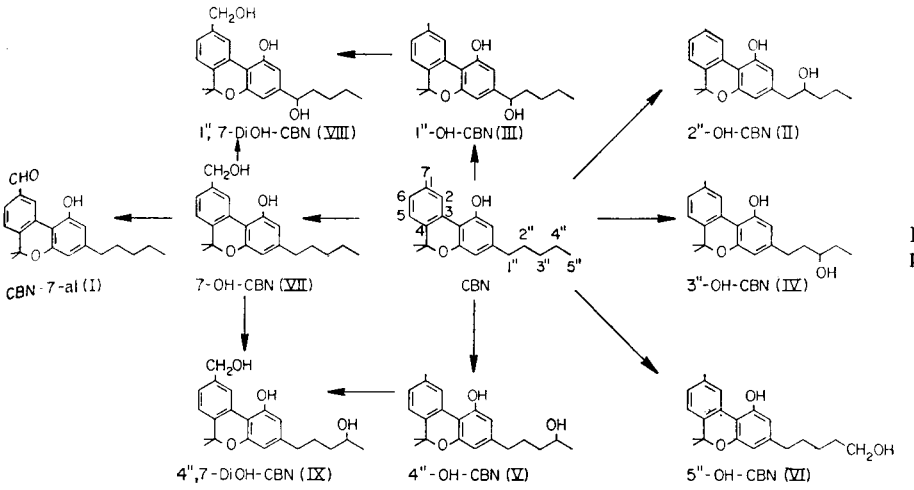

FIG. 2. Metabolic pathways for CBN.

man & others, 1975). The ms of the silylated metabolite (cf. Binder & others, 1974) together with other pertinent data are presented in Table 1.

Fraction 5 consisted predominantly of one metabolite (V; Fig. 2) which was shown by g.c., ms and t.l.c. (Table 1) to be identical to reference 4″-hydroxy-CBN (Widman & others, 1975).

Table 1. *Neutral* in vivo *metabolites of CBN.*

Metabolite	Quantity[a] (μg)	t.l.c.[b] (*Rf*)	g.c.[c] (min)	Ms of silylated derivatives[e] M+.	Other diagnostic peaks	
CBN-7-al	66	0·78	10·1	396 (30)	381 (100)	340 (6)
1″-OH-CBN (III)	555	0·51	8·1	470 (26)	455 (100)	413 (77)
2″-OH-CBN (II)	449	0·43	7·8	470 (8)	455 (17)	145 (100)
3″-OH-CBN (IV)	370	0·31	8·9	470 (25)	455 (100)	326 (90)
4″-OH-CBN (V)	580	0·27	9·0	470 (18)	455 (100)	117 (10)
5″-OH-CBN (VI)	157	0·22	12·2	470 (17)	455 (100)	
7-OH-CBN (VII)	1082	0·28	11·4	470 (17)	455 (100)	103 (3)
1″,7-diOH-CBN (VIII)	528	—	6·1[d]	558 (50)	543 (100)	501 (75) 103 (5)
4″,7-diOH-CBN (IX)	196	—	8·5[d]	558 (35)	543 (100)	414 (10) 117 (20)

[a] Account for the 50% ether–light petroleum and 100% ether fractions from Florisil.
[b] Developed in 60% ether–light petroleum.
[c] Retention time for non-silylated metabolite on 2% SE-30 (250°).
[d] Retention time for silylated metabolite on 2% SE-30 (250°).
[e] Ms at 20 eV; rel. intensity in parentheses.

Fractions 6 and 7 contained the metabolites VI and VII respectively with some overlap. After g.c., ms and t.l.c. (Table 1) comparison with references (Widman & others, 1975) the metabolites VI and VII were assigned the structures of 5″-hydroxy-CBN and 7-hydroxy-CBN (Fig. 2) respectively.

The 100% ether fraction eluted from the Florisil column was further purified on Sephadex LH-20, using two solvent systems. First the column was eluted with light petroleum-chloroform-ethanol (10:10:1) to remove the monohydroxylated metabolites described above and then the remaining radioactivity (55%) was eluted with chloroform–MeOH (1:1). This last fraction was rechromatographed on Sephadex LH-20. Final purification by t.l.c. revealed the presence of several metabolites. Two major metabolites were isolated and identified as 1″,7-dihydroxy-CBN (VIII; Fig. 2) and 4″,7-dihydroxy-CBN (IX; Fig. 2) by g.c., ms and t.l.c. (Table 1) cf. references (Fonseka & Widman, 1977).

DISCUSSION

Previously the *in vivo* metabolism of CBN has been studied in mice (Burstein & Varanelli, 1975) and man (Wall & others, 1976). From these studies regarding neutral metabolites 7-hydroxy-CBN was definitely identified while 4″-hydroxy-CBN was assigned as a tentative structure. In addition, two dihydroxylated metabolites were described without definitive characterization. From *in vitro* metabolite studies of CBN using rat and rabbit liver we have isolated and identified 7-, 2″-, 3″-, 4″- and 5″-hydroxy-CBN (Widman & others, 1975) and 1″,7-, 2″,7-, 3″,7- and 4″,7-dihydroxy-CBN (Fonseka & Widman, 1977). These metabolites together with synthetic compounds have served as references in the identification of the reported metabolites except for CBN-7-al and 1″-hydroxy-CBN.

CBN-7-al was found in too low an amount (66 μg) to be characterized by nmr. However, the ms of the

derivatized (silylated) and nonderivatized compound showed molecular ions at *m/e* 396 and 324 respectively which are 14 mass units higher than that of CBN consistent with the conversion of a methyl to a carbonyl group. The fragments *m/e* 340 of the derivatized metabolite and 268 of the non-derivatized metabolite representing $(M\text{-}C_4H_8)^{+\cdot}$ indicated that C_2-C_5 of the side chain was not functionalized. The reduction of metabolite I to 7-hydroxy-CBN established that it was CBN-7-al. Ben-Zvi & Burstein (1974) have reported the isolation and identification of small amounts of Δ^1-THC-7-al formed by rat liver microsomes. CBN-7-oic acid, a major metabolite of CBN *in vivo* (Burstein & Varanelli, 1975; Wall & others, 1976; Yisak, Widman & others, to be published) is thought to be derived from the oxidation of 7-hydroxy-CBN with the CBN-7-al as an intermediate.

The structure of 1″-hydroxy-CBN was mainly determined by its ms and nmr. In the ms the molecular ion at *m/e* 470 is consistent with a silylated monohydroxylated CBN metabolite. The base peak at *m/e* 455 $(M\text{-}CH_3)^+$ and the prominent fragment at *m/e* 413 involving benzylic cleavage $(M\text{-}C_4H_9)^+$ strongly suggests that hydroxylation had occurred at the 1″-position (cf. Binder & others, 1974). The nmr spectra also supports that a hydroxyl group is situated at 1″-position, since the signal at $\delta = 2{\cdot}5$ (benzylic protons) disappeared to give a multiplet at $\delta = 5{\cdot}1$ which integrated for one proton.

In vivo 1″-hydroxy-CBN was in the rat excreted in the faeces as a major neutral metabolite while *in vitro* benzylic side chain hydroxylation was found only as 1″, 7-dihydroxy-CBN. *In vitro* (rat liver 10 000 *g* supernatant) 2″-, 3″-, 4″-, and 5″-hydroxy-CBN were formed as minor metabolites, together 2% of the converted material, while 7-hydroxy-CBN amounted to 70%. *In vivo* the side chain hydroxylated metabolites (see Table 1) were present in faeces in much higher concentrations relative to 7-hydroxy-CBN. *In vitro* the most prominent side-chain hydroxylated metabolite was 4″-hydroxy-CBN while *in vivo* 4″-hydroxy-CBN and 1″-hydroxy-CBN were present in slightly higher amounts than 2″- and 3″-hydroxy-CBN. 5″-Hydroxy-CBN was least favoured of all. The relatively low concentration of 7-hydroxy-CBN *in vivo* compared to its very high concentration *in vitro* is probably due to its further oxidation *in vivo* to metabolites such as CBN-7-oic acid. Furthermore, the *in vivo* pattern may also be modified by enterohepatic circulation, metabolism by organs other than liver, as well as the intestinal flora.

The slow excretion rate of CBN in the rat and the predominant elimination *via* faeces agrees with *in vivo* work with Δ^1-THC (cf. Widman & others, 1974).

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