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

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Six acidic metabolites were isolated from rat faeces and identified by gas chromatography–mass spectrometry and proton magnetic resonance. Cannabinol-7-oic acid was the most abundant acidic metabolite isolated. Others present in decreasing order of prominence were 1″-hydroxy-, 4″-hydroxy-, 3″-hydroxycannabinol-7-oic acid, cannabinol-3″-one-7-oic acid and 2″-hydroxycannabinol-7-oic acid.

Previous *in vivo* metabolic studies of cannabinol (CBN) have shown that CBN is transformed into a variety of neutral metabolites. Burstein & Varanelli (1975) isolated from mice 7-hydroxy-CBN together with two metabolites with characteristics of side-chain hydroxylation. From the rat, in addition to 7-hydroxy-CBN, we have isolated CBN-7-al, various metabolites monohydroxylated on the side chain as well as 1″,7-dihydroxy- and 4″,7-dihydroxy-CBN (Yisak, Widman & others, 1977). Two acidic metabolites of CBN have been reported so far, namely CBN-7-oic acid from mice (Burstein & Varanelli, 1975) and man (Wall, Brine & Perez-Reyes, 1976) ,and 2″,3″,4″,5″-tetranor-CBN-1″-oic acid from mice (Burstein & Varanelli, 1975).

We now report the identification of six acidic *in vivo* metabolites of CBN isolated from rat faeces.

MATERIALS AND METHODS

^{14}C-CBN (Widman, Nilsson & others, 1971) with a chemical 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} was administered in 70% ethanol via the tail vein to 12 Sprague-Dawley rats. The rats (200–250 g) received a single dose of 100 mg kg^{-1} of CBN. The urine and faeces were collected for six days and the samples assayed for radioactivity (Yisak & others, 1977).

Isolation of metabolites

Lyophilized and finely ground faeces were extracted successively with light petroleum (1000 ml) for 15 h, then diethyl ether (1000 ml) for another 15 h using a soxhlet extractor; with methanol (3 × 750 ml) with a separatory funnel at room temperature (20°) and finally 15 h with methanol (1000 ml) in a soxhlet extractor. The ether extract was passed through a column of Florisil (160 g; 1·5 × 115 cm) and the column was eluted successively with 500 ml each of the following solvents: 20% v/v ether–light petroleum; 50% ether–light petroleum; 100% ether; 5% methanol–ether; 20% methanol–ether and 100% methanol (Yisak & others, 1977).

The 100% methanol fraction was esterified using diazomethane and chromatographed on Sephadex LH-20 (1 × 70 cm) by eluting with light petroleum–chloroform–ethanol (10 : 10 : 1) as described by Widman, Nordqvist & others (1974). Precoated Silica gel F plates (Merck, 0·25 mm thickness, 5 × 10 cm) developed in 60% ether–light petroleum were used for final purification of the metabolites (Yisak & others, 1977).

Instrumental methods

A Packard Tricarb Model B 2450 spectrometer with external standardization was used for scintillation counting. The scintillation fluid was a mixture of Permablend III (Packard) in toluene (5·5 g litre^{-1}).

For gas chromatography a Varian Aerograph Model 2100 equipped 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}. The glass column (1·8 m × 2 mm i.d.) was packed with 2% SE-30 ultraphase on Gas-Chrom Q (125–150 mesh).

An LKB 9000 gas chromatograph-mass spectrometer (LKB, Bromma, Sweden), operated at 20 eV was used to record the mass spectra (ms). The glass column (1·4 m × 2 mm i.d.) was packed with 3% SE-32 ultraphase on Gas-Chrom Q (100–120 mesh) and the oven temperature was 200°. Before g.c. and ms analysis the esterified metabolites were further

§ Correspondence.

derivatized by silylation with *N,O*-bis(trimethylsilyl)-acetamide in dry acetonitrile. ^{1}H-nuclear magnetic resonance spectra were recorded on a Varian 100 MHz instrument ($CDCl_3$, Fourier-Transform).

RESULTS AND DISCUSSION

Six days after the administration of CBN to the rats 70–75% of total radioactivity was recovered from the faeces. The 5–6% in the urine was not further investigated. Unchanged CBN was mainly extracted with light petroleum and the metabolites with ether and methanol (see also Yisak & others, 1977). The ether extract which contained 30% of the radioactivity was chromatographed on Florisil. The first eluate contained mainly CBN, the second and third yielded mono- and dioxygenated neutral metabolites (Yisak & others, 1977), the fourth and fifth were not further investigated because of their low amount of radioactivity and the sixth (100% methanol), which contained almost 50% of the radioactivity of the original ether extract, consisted of acidic metabolites which were methylated before purification on Sephadex LH-20. Six metabolites were isolated. Quantities, t.l.c., and g.c. properties and diagnostic ms data of the metabolities are shown in Table 1.

The pmr spectra of the metabolites I–VI (Fig. 1) were similar to that of CBN (Petrzilka, Haefliger & Sikemeier, 1969) with a few exceptions, the major one being the lack of the C-7 methyl signal at $\delta = 2{\cdot}38$ ppm in all the metabolites. Instead there was a three proton signal around $\delta = 3{\cdot}94$ ppm consistent with a methylated carboxylic group at the C-7 position. The marked downfield shift of the protons at C-2 and C-6 from $\delta = 8{\cdot}02$ ppm and $\delta = 7{\cdot}10$ ppm to $\delta = 9{\cdot}1$ ppm and $\delta = 7{\cdot}9$ ppm, respectively, also supported the presence of a carboxylic group at the C-7 position (cf. Nordqvist Agurell & others, 1974).

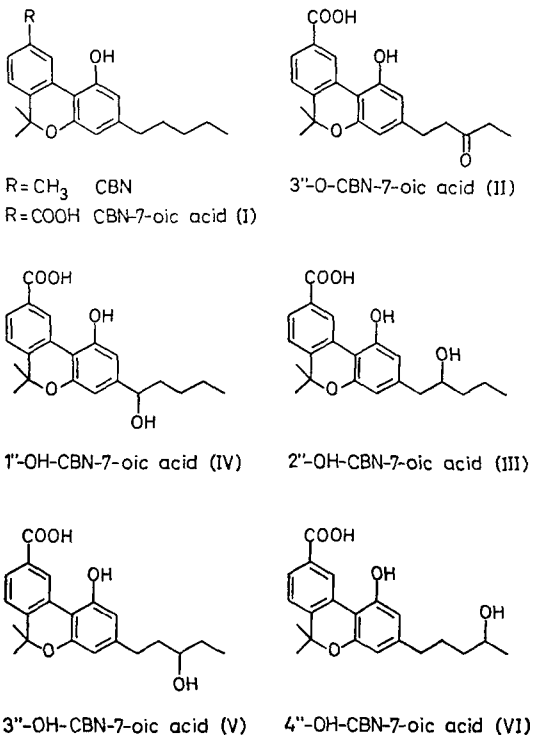

FIG. 1. Structures of acidic *in vivo* metabolites of CBN isolated from rat faeces.

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

Metabolite	Quantity[a] (μg)	T.l.c.[b] (R_F)	G.c.[c] Rt (min)	M$^{+\cdot}$	M.s.[d] other diagnostic ions		
CBN-7-oic acid (I)	2420	0·76	7·1	426 (26)	411 (100)	396·5*	370 (3)
3″-*O*-CBN-7-oic acid (II)	110	0·46	23·1	440 (30)	425 (100)	410·5*	383 (8)
1″-OH-CBN-7-oic acid (IV)	1497	0·32	8·9	514 (21)	499 (27)	484·4*	457 (100)
2″-OH-CBN-7-oic acid (III)	75	0·27	9·6	514 (5)	499 (6)	442[e] 427[e]	370 145 (21) (100)
3″-OH-CBN-7-oic acid (V)	178	0·21	13·0	514 (26)	499 (79)	484·4*	370 (100)
4″-OH-CBN-7-oic acid (VI)	427	0·17	13·2	514 (44)	499 (100)	484·4*	370 117 (28) (9)

[a] Metabolites quantitated after purification according to the specific activity of CBN.
[b] Developed in 60% ether–light petroleum. R_F — value for methylated metabolite.
[c] Retention time for methylated and silylated metabolite on 2% SE-30 (250°).
[d] Ms of methylated and silylated derivative at 20 eV. Relative intensity within parentheses. * = metastable ion.
[e] 442 = M$^{+\cdot}$ − C_4H_8O (Harvey & Paton, 1976) and 427 = M$^{+\cdot}$ − C_4H_8O − CH_3.
Relative intensity could not be determined due to interference by ions from other sources.

Metabolite I (Fig. 1) showed by ms, as a methylated/silylated derivative, a molecular ion at *m/e* 426 which was consistent with the presence of a carboxylic group in the CBN-molecule. The pmr (see below) provided evidence that the carboxylic group was located at the C-7 position as discussed above. Furthermore, metabolite I showed identical t.l.c., g.c., and ms properties with CBN-7-oic acid prepared from $\Delta^1(^6)$-THC-7-oic acid and precipitated sulphur (cf. Widman & others, 1971). Thus, metabolite I was assigned the structure of CBN-7-oic acid.

Metabolite II (Fig. 1) showed a poor ms however, the molecular ion at *m/e* 440 indicated an aldehyde or ketone function to be present besides a carboxylic group. The fragment *m/e* 383 ($M^{+\cdot}-57$) was consistent with a ketone located at the C-1″ position (benzylic cleavage) or C-3″ position (α-cleavage between C-2″ and C-3″). The pmr signals of metabolite II were similar to those of CBN-7-oic acid except for the side chain. The protons from C-4″ and C-5″ appeared as a sharp quartet and triplet at $\delta = 2{\cdot}43$ and $\delta = 1{\cdot}06$ ppm, respectively, which are in agreement with the pmr data of synthesized CBN-3″-one (Widman, Dahmén & others, 1975). The protons at C-1″ and C-2″ afforded, due to coupling to each other, two triplets at chemical shifts so close to each other that they appeared to be almost superimposed. On the basis of pmr and ms we identified metabolite II as CBN-3″-one-7-oic acid.

The metabolites III–VI (Fig. 1) were all identified as side chain hydroxylated CBN-7-oic acid based on their pmr and ms data. As derivatized compounds they all showed molecular ions at *m/e* 514 consistent with the presence of both a carboxylic and a hydroxylic group in the CBN-molecule. Their pmr suggested C-7 carboxylation. The position of the hydroxyl group was determined both by pmr and ms according to Binder, Agurell & others (1974) and Binder (1976).

Metabolite III was identified as 2″-hydroxy-CBN-7-oic acid. The 2″-hydroxylation was indicated by the broad multiplet pmr signal at $\delta = 3{\cdot}90$ ppm due to the proton at C-2″. The protons at C-1″ position appeared at $\delta = 2{\cdot}4$–$2{\cdot}7$ ppm as a complex multiplet as a result of an AB system that was further split by different couplings to the proton at C-2″. Furthermore, the ms provided the fragment *m/e* 145 as a base peak which is typical of 2″-hydroxy cannabinoids.

Metabolite IV was assigned the structure of 1″-hydroxy-CBN-7-oic acid. The 1″-hydroxylation was characterized by the disappearance of the signals at $\delta = 2{\cdot}52$ ppm for the C-1″ protons and the appearance of one proton signal at $\delta = 4{\cdot}55$ ppm. Its ms was also consistent with 1″-hydroxylation due to the base peak at *m/e* 457 ($M^{+\cdot}-C_4H_9$). Metabolite V was identified as 3″-hydroxy-CBN-7-oic acid. The 3″-hydroxylation was suggested by the multiplet pmr signal at $\delta = 3{\cdot}45$–$3{\cdot}7$ ppm for the proton at C-3″ which was coupled to the protons at C-2″ and C-4″. The protons at C-1″ appeared as a multiplet in the range of $\delta = 2{\cdot}5$–$2{\cdot}8$ ppm as a result of couplings to non-equivalent protons at C-2″. The ms showed a base peak at *m/e* 370 ($M^{+\cdot}-144$) which is consistent with the loss of the side chain by McLafferty rearrangement and is characteristic of 3″-hydroxylation. Metabolite VI afforded a broad multiplet pmr signal at $\delta = 3{\cdot}71$–$3{\cdot}99$ ppm for the proton at C-4″. The three protons at C-5″ were also slightly shifted downfield to $\delta = 1{\cdot}20$ ppm which appeared as a sharp doublet showing the presence of only one proton at C-4″. The ms also supported 4″-hydroxylation by the characteristic fragment at *m/e* 117 [$C_2H_4O^+Si(CH_3)_3$]. Thus, metabolite VI was assigned the structure of 4″-hydroxy-CBN-7-oic acid.

The oxidation of CBN to CBN-7-oic acid via CBN-7-al previously isolated (Yisak & others, 1977) seems to be a major route of biotransformation in the rat. Side-chain hydroxylation is also prominent especially at the C-1″ and C-4″ positions. Δ^1-THC also yields side-chain hydroxylated 7-oic acids in the rabbit (Burstein, Rosenfeld & Wittstruck, 1972) and the mouse (Harvey & Paton, 1976). We have not observed in the rat any CBN metabolites with degraded side chain which was found for CBN in the mouse (Burstein & Varanelli, 1975) and for Δ^1-THC in the mouse (Martin, Harvey & Paton, 1976), and the rabbit (Nordqvist & others, 1974). However, the CBN-3″-one-7-oic acid might be a precursor to an acid with a side chain cleavage similar to the one found for Δ^1-THC in the rabbit (Nordqvist & others, 1974).

Metabolite I with an elution volume (Ve) of 41–66 ml on Sephadex LH-20 was identified as CBN-7-oic acid. Its methyl ester derivative provided pmr signals at δ ppm: 9·10 (d, 1H, C-2, J = 2Hz), 7·92 (dd, 1H, C-6, J_{2-6} = 2Hz, J_{5-6} = 8Hz), 7·31 (d, 1H, C-5, J = 8Hz), 6·44 (broad s, 1H, C-3′), 6·33 (broad s, 1H, C-5′), 3·94 (s, 3H, $-COOCH_3$), 2·52 (t, 2H, C-1″, J = 7Hz), 1·61 (s, 3H, C-9), 1·53 (s, 3H, C-10), 0·90 (t, 3H, C-5″, $J \simeq 7$Hz). Ms of the methyl ester of the metabolite showed fragments at *m/e* 354 ($M^{+\cdot}$, 100%), 339 (13%), and 282 (13%).

Metabolite II (Ve = 41–66 ml) was assigned the structure of CBN-3″-one-7-oic acid. The methyl ester derivative showed pmr signals at δ ppm: 9·08 (d, 1H, C-2, J = 2Hz), 7·93 (dd, 1H, C-6, J_{2-6} = 2Hz, J_{5-6} = 8Hz), 7·71 (d, 1H, C-5, J = 8Hz), 6·42 (d, 1H, C-3′, J ≃ 2Hz), 6·33 (d, 1H, C-5′, J ≃ 2Hz), 3·92 (s, 3H, $-COOCH_3$), 2·78 (overlapping triplets, 4H, C-1″ and C-2″, J ≃ 5Hz), 2·43 (q, 2H, C-4″, J = 7Hz), 1·61 (s, 3H, C-9), 1·55 (s, 3H, C-10), 1·06 (t, 3H, C-5″, J ≃ 7Hz).

Metabolite III (Ve = 41–66 ml) was identified as 2″-hydroxy-CBN-7-oic acid. Its methyl ester derivative showed pmr signals at δ ppm: 9·10 (d, 1H, C-2, J = 2Hz), 7·92 (dd, 1H, C-6, J_{2-6} = 2Hz, J_{5-6} = 8Hz), 7·31 (d, 1H, C-5, J = 8Hz), 6·49 (broad s, 1H, C-3′), 6·39 (broad s, 1H, C-5′), 3·93 (s, 3H, $-COOCH_3$), 3·90 (m, 1H, C-2″), 2·7–2·4 (m, 2H, C-1″), 1·65 (s, 3H, C-9), 1·57 (s, 3H, C-10), 0·95 (broad t, 3H, C-5″).

Metabolite IV (Ve = 67–106 ml) was assigned the structure of 1″-hydroxy-CBN-7-oic acid. Its methyl ester derivative provided pmr signals at δ ppm: 9·14 (d, 1H, C-2, J = 2Hz, 7·74 (dd, 1H, C-6, J_{2-6} = 2Hz, J_{5-6} = 8Hz), 7·31 (d, 1H, C-5, J = 8Hz), 6·58 (s, 2H, C-3′ and C-5′); 4·55 (t, 1H, C-1″), 3·95 (s, 3H, $-COOCH_3$), 1·62 (s, 3H, C-9); 1·60 (s, 3H, C-10); 0·90 (broad t, 3H, C-5″).

Metabolite V (Ve = 67–106 ml) was identified as 3″-hydroxy-CBN-7-oic acid. The pmr of its methyl ester afforded signals at δ ppm: 9·12 (d, 1H, C-2, J = 2Hz), 7·91 (dd, 1H, C-6, J_{2-6} = 2Hz, J_{5-6} = 8Hz), 7·30 (d, 1H, C-5, J = 8Hz), 6·44 (broad s, 1H, C-3′), 6·37 (broad s, 1H, C-5′), 3·94 (s, 3H, $-COOCH_3$), 3·70–3·45 (m, 1H, C-3″), 2·8–2·5 (m, 2H, C-1″), 1·85–1·45 (m, 4H, C-2″ and C-4″), 1·61 (s, 3H, C-9), 1·53 (s, 3H, C-10), 0·95 (t, 3H, C-5″, J ≃ 7Hz).

Metabolite VI (Ve = 67–106 ml) which was identified as 4″-hydroxy-CBN-7-oic acid exhibited as methyl ester pmr signals at δ ppm: 9·14 (d, 1H, C-2, J = 2Hz), 7·92 (dd, 1H, C-6, J_{2-6} = 2Hz, J_{5-6} = 8Hz), 7·56 (d, 1H, C-5, J = 8Hz), 6·44 (broad s, 1H, C-3′), 6·31 (broad s, 1H, C-5′), 3·99–3·71 (m, 1H, C-4″), 3·95 (s, 3H, $-COOCH_3$), 2·55 (t, 2H, C-1″, J ≃ 7Hz), 1·65 (s, 3H, C-9), 1·60 (s, 3H, C-10), 1·20 (d, 3H, C-5″, J = 7Hz).

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