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Urinary metabolites of cannabidiol in dog, rat and man and their identification by gas chromatography–mass spectrometry

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ABSTRACT

Urinary metabolites of cannabidiol (CBD), a non-psychoactive cannabinoid of potential therapeutic interest, were extracted from dog, rat and human urine, concentrated by chromatography on Sephadex LH-20 and examined by gas chromatography–mass spectrometry as trimethylsilyl (TMS), [$^2H_9$]TMS, methyl ester–TMS and methyloxime–TMS derivatives. Fragmentation of the metabolites under electron-impact gave structurally informative fragment ions; computer-generated single-ion plots of these diagnostic ions were used extensively to aid metabolite identification. Over fifty metabolites were identified with considerable species variation. CBD was excreted in substantial concentration in human urine, both in the free state and as its glucuronide. In dog, unusual glucoside conjugates of three metabolites (4″- and 5″-hydroxy- and 6-oxo-CBD), not excreted in the unconjugated state, were found as the major metabolites at early times after drug administration. Other metabolites in all three species were mainly acids. Side-chain hydroxylated derivatives of CBD-7-oic acid were particularly abundant in human urine but much less so in dog. In the latter species the major oxidized metabolites were the products of β-oxidation with further hydroxylation at C-6. A related, but undefined pathway resulted in loss of three carbon atoms from the side-chain of CBD in man with production of 2″-hydroxy-*tris,nor*-CBD-7-oic acid. Metabolism by the epoxide-diol pathway, resulting in dihydro-diol formation from the Δ-8 double bond, gave metabolites in both dog and human urine. It was concluded that CBD could be used as a probe of the mechanism of several types of biotransformation; particularly those related to carboxylic acid metabolism as intermediates of the type not usually seen with endogenous compounds were excreted in substantial concentration.

INTRODUCTION

Cannabidiol (CBD, I, Fig. 1) is one of over sixty cannabinoids found in the plant *Cannabis sativa* L. [1], the source of the drug marihuana. Although non-psychoactive, the compound has been shown to possess anticonvulsant [2] and neurological properties [3] and has received attention as a candidate clinical drug. Metabolism of CBD has been studied in several animal species, particularly *in vitro* [4–9], but there is little information available on urinary metabolites. This

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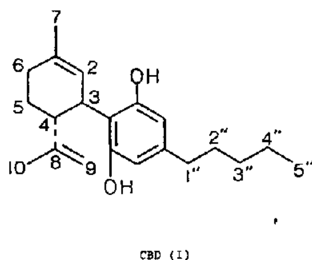


Fig. 1. Structure of cannabidiol (I).

paper describes the identification by gas chromatography–mass spectrometry (GC–MS) of urinary metabolites of this drug in three species with particular reference to species differences.

EXPERIMENTAL

Materials

CBD was obtained from the National Institute on Drug Abuse (NIDA) and from Makor Chemicals (Jerusalem, Israel). β-Glucuronidases and N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) were obtained from Sigma (Poole, U.K.). "Diazald", for the preparation of diazomethane, was obtained from Aldrich (Gillingham, U.K.) and methoxyamine hydrochloride from BDH (Poole, U.K.).

Drug treatment

Dogs. Three mongrel dogs (18–26 kg) were treated (in the Jerusalem Labs.) with CBD (90 mg in 2 ml of 70% ethanol) by injection into the cephalic vein. Urine was collected *via* an indwelling catheter from the urinary bladder at 0, 1, 2, 3, 4, 5, 6, 8, 10, 12, 16, 22, 26 and 30 h after drug administration and stored at −20°C.

Rats. Three male rats (250–350 g) were treated with CBD (5 mg in 0.5 ml of 70% ethanol) by injection into the tail vein. Urine was collected at 6, 10, 20 and 26 h after dosing.

Human. Urine (1.5 l) was collected over 24 h from a dystonic patient treated chronically with CBD (600 mg daily) at the Neurology Department, Hadassah-Hebrew University Hospital (Jerusalem, Israel) [10].

Extraction of metabolites

Metabolites were extracted from the urine (2-ml samples from dogs and rats) with ethyl acetate (3 × 2 ml for dogs and rats, 300 ml for human), both before hydrolysis with β-glucuronidase (Type VII from *Escherichia coli* at pH 6.8, and additionally with Type HP-2 from *Helix pomatia* at pH 5 for the dog urine) for 3 h at 37°C. The solution was dried over magnesium sulphate and evaporated to

dryness. Half of the extract was converted directly into derivatives for GC–MS as described below. The other half was chromatographed on Sephadex LH-20 (5 g packed into a 10 mm I.D. column) in chloroform and chloroform–methanol mixtures as described earlier [11] in order to concentrate the metabolites. The fraction eluted with a 10% solution of methanol in chloroform was collected and evaporated to dryness under reduced pressure.

Preparation of derivatives

Trimethylsilyl (TMS) derivatives. Samples, of about 100 μg, of both the unhydrolysed and hydrolysed ethyl acetate extract were heated with BSTFA (10 μl) for 10 min at 60°C.

[$^2H_9$]TMS derivatives [12]. These were prepared as for the TMS derivatives with [$^2H_{18}$]bis(trimethylsilyl)acetamide ([$^2H_{18}$]BSA) replacing the BSTFA.

Methyl ester–TMS derivatives. Methanol (10 μl) was added to the dried sample from the ethyl acetate extract followed by a fresh, ethereal solution (0.5 ml) of diazomethane (prepared from Diazald). The mixture was well stirred and allowed to stand at room temperature for 2 min. The reagent and solvents were removed with a stream of nitrogen (the human sample was first centrifuged to remove the precipitate) and the residue was converted into TMS derivatives as described above.

Methyloxime–TMS derivatives. The dried sample was dissolved in pyridine (0.1 ml) and methoxyamine hydrochloride (about 100 μg) was added. The mixture was heated at 60°C for 1 h, cooled, diluted with water (1 ml) and the metabolites were extracted with ethyl acetate (3 × 1 ml). The combined ethyl acetate extracts were washed with water (1 ml) and saturated sodium chloride solution (2 × 1 ml) and evaporated to dryness with a stream of nitrogen. The residue was reacted with BSTFA as described above.

Reduction with lithium aluminium deuteride. An aliquot of the dried ethyl acetate extract form both hydrolysed and unhydrolysed samples was dissolved in dry (sodium) ether (1.0 ml) and heated at reflux temperature with an excess of lithium aluminium deuteride for 1 h. After destruction of the excess of reagent with ethyl acetate, the aluminium salts were dissolved in 0.5 *M* sulphuric acid (1 ml) and the products were extracted with ethyl acetate (three times) and washed with water and saturated aqueous sodium chloride. The solvent was removed with a stream of nitrogen and the residue was converted into TMS derivatives.

Gas chromatography

GC retention data and the relative concentration of the metabolites were measured with a Hewlett-Packard 5890A gas chromatograph fitted with a 50 m × 0.3 mm I.D. OV-1 bonded-phase fused-silica capillary column (film thickness 0.52 μm) (Hewlett-Packard). Helium at 2 ml/min was used as the carrier gas with a split ratio of 10:1. The injector and detector (flame ionisation detector) temperatures were both 300°C and the column oven was temperature-programmed from

130 to 350°C at 2°C/min. Data were recorded with a Servoscribe flat-bed recorder and with a Hewlett-Packard 3390A recording integrator.

Gas chromatography–mass spectrometry

GC–MS data were recorded with a VG 70/70F double-focussing mass spectrometer connected to a Varian 2440 gas chromatograph. The column was a 30 m × 0.2 mm I.D. DB-1 bonded-phase fused-silica capillary (film thickness 0.33 μm) (J. & W. Scientific) terminating 1 cm inside the ion source. Helium at 1 ml/min (measured in the absence of the vacuum) was used as the carrier gas. The injector was an SGE split–splitless system used in the split mode with a split ratio of 10:1. The column oven was temperature programmed from 220 to 320°C at 2°C/min. Other operating conditions were: injector, transfer line and ion source temperatures, 300, 300 and 250°C, respectively; electron energy, 70 eV; trap current, 1 mA; accelerating voltage, 4 kV. An accelerating voltage of 3 kV was used to record spectra of the metabolites with molecular weights in excess of 700 Da. The instrument was scanned repetitively at 1 s per decade under the control of the VG 11/250 data system.

RESULTS

Identification of metabolites

Metabolites were identified by GC–MS using data from the literature and by a comparison of spectra from the different derivatives; these were used to identify the functional groups. Thus, comparison between the mass shifts produced by [$^2H_9$]TMS derivatives [12] and the masses observed from the TMS derivatives allowed the number of TMS groups and thus the number of hydroxy and acid groups to be determined. Carboxylic acids were identified by preparation of methyl ester–TMS derivatives and by their reduction to alcohols with lithium aluminium deuteride. The extent of deuterium incorporation in the latter experiment allowed differentiation of the alcohols produced by reduction from those occurring naturally [13]. Ketones were identified in a related manner by derivatization as their methyloximes.

Fragmentation of CBD and its metabolites under electron impact (EI) produced several very abundant, diagnostic ions which readily allowed the structures of the compounds to be determined [14]. Thus, the retro-Diels–Alder cleavage of the terpene ring (ion *a*, Fig. 2) resulted in loss of the five carbon atoms, together with their substituents, and localized the metabolically added group either to the eliminated fragment or to ion *a*. This ion was often the base peak. The mass of the tropylium ion (*b*) further differentiated metabolites modified in the pentyl side-chain [m/z 337 with no substitution, m/z 425 with one hydroxy group (TMS derivatives)]. The presence of a substituent at C-7 invariably led to loss of C-7 and its substituent (ion *c*), allowing ready identification. In the spectra of metabolites with a 7-oic acid group, this fragmentation was more prominent in the spectra of

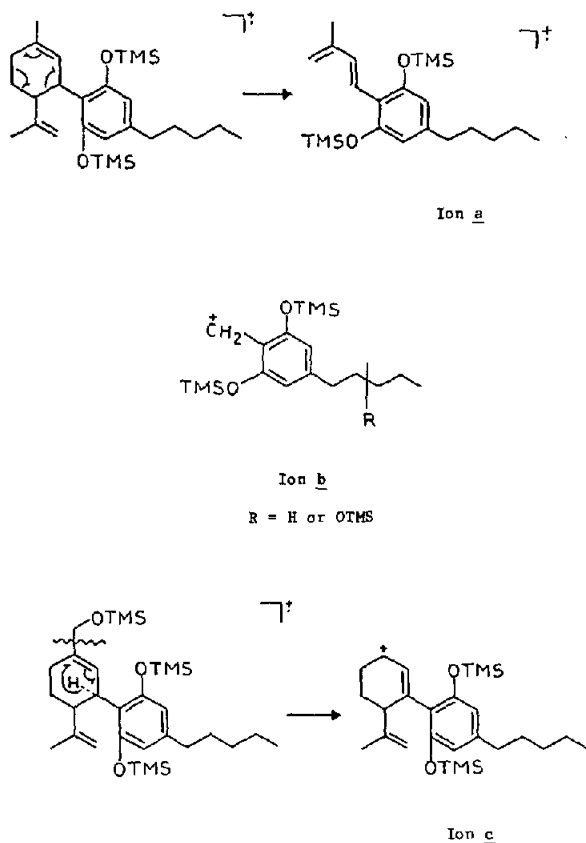


Fig. 2. Structures of ions *a*, *b* and *c*.

TMS derivatives than in those of their corresponding methyl esters. 6-Hydroxy metabolites were identified by the large retro-Diels–Alder ion and very weak molecular ion. The position of side-chain hydroxylation was identified by the production of the diagnostic ions at $[M - 57]^+$, m/z 145, $[M - 144]^{+\cdot}$ and m/z 117 from metabolites hydroxylated in the 1″-, 2″-, 3″- and 4″-positions, respectively [15]. In most cases, the losses of 57 and 144 mass units, diagnostic of 1″- and 3″-hydroxylation, respectively, were more prominent as losses from the retro-Diels–Alder ion (*a*) than from the molecular ion.

Metabolites dihydroxylated at the double bond in the isopropenyl group caused charge localization in this area of the molecule with the result that spectra were considerably different to those of the other metabolites. In particular, the charge distribution between the fragments produced by retro-Diels–Alder cleavage, as shown in Fig. 2, was partially reversed resulting in observation of both fragments. The ion at m/z 143, formed by direct cleavage of this fragment, was the base peak; this and other diagnostic ions were formed as shown in Fig. 3. The masses of the diagnostic ions are listed in Tables I and II.

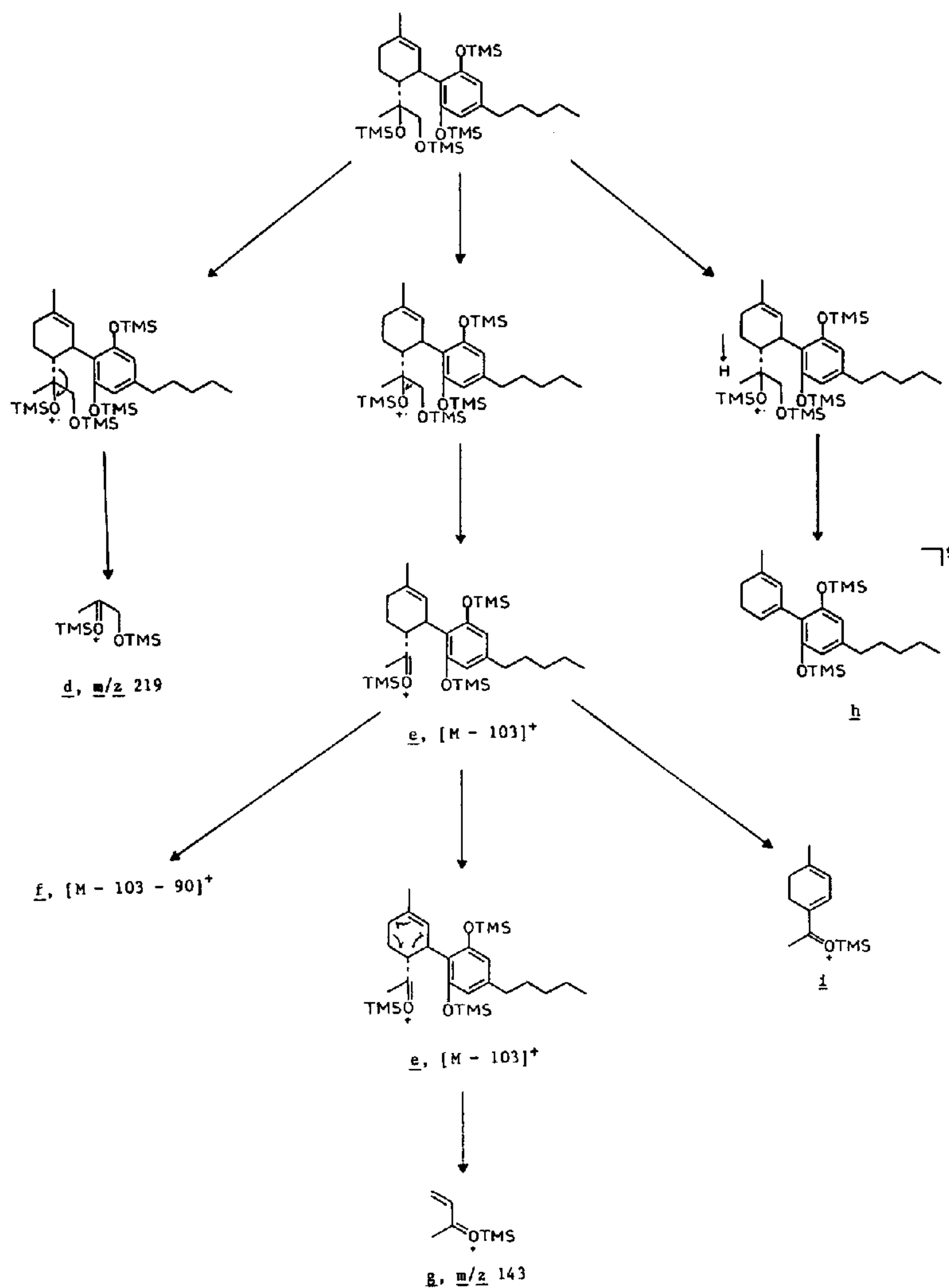


Fig. 3. Structures of the major diagnostic ions formed from the derivatives of dihydro-diol metabolites.

TABLE I

MASSES OF THE DIAGNOSTIC IONS IN THE SPECTRA OF OXIDIZED CBD METABOLITES

Metabolite type	$M^{+\cdot}$	*a*	*b*	*c*	1″[a]	3″[a]
Mono-OH	546	478	337/425	443	421	334
Di-OH	634	566	337/425	531	509	422
Tri-OH	722	654	337/425	619	597	510
Mono-COOH	560	492	337/439	443	435	348
Mono-OH-mono-COOH	648	580	337/439	531	523	436
1 stage of β-oxidation[b]						
Mono-COOH	532	464	411	–	–	–
Mono-OH-mono-COOH	620	562	411	517	–	–
Di-OH-mono-COOH	708	650	411	605	–	–
Keto-mono-COOH	546	478	411	–	–	–
Di-COOH	634	576	411	517	–	–
2 stages of β-oxidation[c]						
Mono-COOH	504	436	383	–	–	–
Mono-OH-mono-COOH	592	534	383	489	–	–
Di-OH-mono-COOH	680	622	383	577	–	–
Di-COOH	606	548	383	489	–	–

[a] Ions diagnostic of hydroxy substitution at the 1″- and 3″-positions. These correspond to losses of 57 and 144 mass units respectively from ion *a* as discussed in the Results section.
[b] Loss of C_2H_4 from the pentyl chain to leave C_2H_4COOH.
[c] Loss of C_4H_8 from the pentyl chain to leave COOH.

To obtain the total metabolic profile, reconstructed ion chromatograms were plotted using the ions in the range *m/z* 330–800 in order to reduce the background produced by endogenous urinary constituents. Most ions from the metabolites, with the exception of some from the dihydro-diols (*m/z* 143, etc.) and the ion at

TABLE II

MASSES OF DIAGNOSTIC IONS IN THE SPECTRA OF 8,9-DIHYDRO,8,9-DIHYDROXY METABOLITES (TMS DERIVATIVES)

Metabolite type	MW	Ions								
		a	*b*	*c*	*d*	*e*	*f*	*g*	*h*	*i*
Mono-OH	724	478	337/425	621	219	621	531	143	504	297
Mono-COOH	738	492	337	621	219	635	545	143	518	311
Di-OH	812	566	337/425	709	219	709	619	143	592	385

m/z 145 from the 2″-hydroxy-containing metabolites had an m/z ratio of over 330. Further "clean-up" of samples was achieved with the spectrum-subtraction programs of the data system. Single-ion plots of diagnostic ions such as the tropylium ions for the various side-chain-modified metabolites were useful, as aids for identifying compounds with common functional groups.

Human

Fig. 4 shows the limited ion chromatogram of metabolites of CBD found in unhydrolysed human urine. Peaks are identified in Tables III – V. One of the major excreted compounds (peak 3, 12.1% of the total excreted cannabinoids as determined by GC) was identified as unmetabolized CBD (I) by the identity of its mass spectrum (TMS derivative) with that of an authentic sample. This compound was also excreted as its O-glucuronide (peak 49, 13.3%) which was identified by comparison of its mass spectrum (TMS and methyl ester–TMS derivatives) with published data [8,16]. The presence of the aglycone ion at m/z 458, corresponding to the molecular ion of CBD TMS ether, indicated that the compound was an O- and not a C-glucuronide [16].

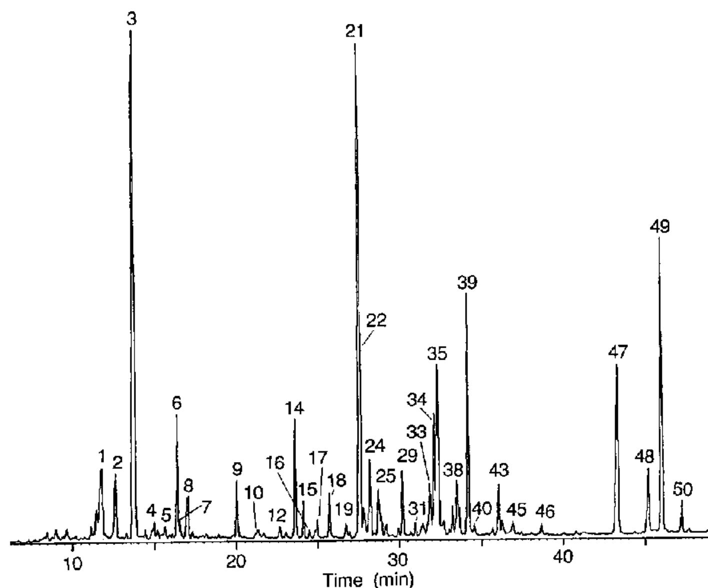
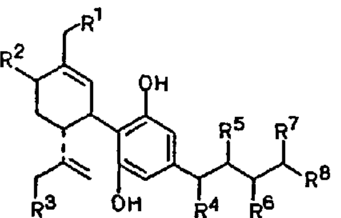


Fig. 4. Reconstruced ion chromatogram (m/z 330–700) of the metabolites (TMS derivatives) extracted from human urine. Separation was made with a 25 m × 0.2 mm I.D. OV-1 fused-silica capillary column operated as described in the Experimental section. Most peaks were produced by metabolites as identified in Tables III–V and in the text. Major peaks not identified are urinary constituents. Peak heights differ from those cited in the text as measured by GC as the GC and GC–MS (m/z 330–700) response factors differ.

TABLE III

STRUCTURES AND QUANTITIES PRESENT IN URINE OF THE METABOLITES OF CBD CONTAINING AN INTACT SIDE-CHAIN



Compound	No.	R^1	R^2	R^3	R^4	R^5	R^6	R^7	R^8	Concentration[a]			Peak		
										Dog	Rat	Man	Figs. 11 and 12	Fig. 13	Fig. 4
7-OH	II	CH_2OH	H	H	H	H	H	H	CH_3	+	–	+	4	–	15
6-OH	III	CH_3	OH	H	H	H	H	H	CH_3	++	–	+	1	–	12
4",6-Di-OH	IV	CH_3	OH	H	H	H	H	OH	CH_3	+++	+	–	15	8	–
4",7.Di.OH	V	CH_2OH	H	H	H	H	H	OH	CH_3	+	–	–	17	–	–
5",6-Di-OH	VI	CH_3	OH	H	H	H	H	H	CH_2OH	+	–	–	18	–	.
6,7-Di-OH	VII	CH_2OH	OH	H	H	H	H	H	CH_3	+	–	–		–	–
2",6,7-Tri-OH	VIII	CH_2OH	OH	H	H	H	H	H	CH_2OH	++	–	–	19	–	–
4",6,7-Tri-OH	IX	CH_2OH	OH	H	H	H	H	OH	CH_3	+++	+	–	23	14	–
5",6,7-Tri-OH	X	CH_2OH	OH	H	H	H	H	H	CH_2OH	++	+	–		18	–
7-COOH	XI	COOH	H	H	H	H	H	H	CH_3	–	–	++	–	–	18
1"-OH, 7-COOH	XII	COOH	H	H	OH	H	H	H	CH_3	–	–	+++	–	–	21
1"-OH, 7-COOH	XIII	COOH	H	H	OH	H	H	H	CH_3	–	–	++	–	–	23
2"-OH, 7-COOH	XIV	COOH	H	H	H	OH	H	H	CH_3	+	–	+++	–	–	33
2"-OH, 7-COOH	XV	COOH	H	H	H	OH	H	H	CH_3			+++	–	–	34
3"-OH, 7-COOH	XVI	COOH	H	H	H	H	OH	H	CH_3	–	–	+++	–	–	37
3"-OH, 7-COOH	XVII	COOH	H	H	H	H	OH	H	CH_3	–	–	+++	–	–	38
4"-OH, 7-COOH	XVIII	COOH	H	H	H	H	H	OH	CH_3	++	+++	+++	22	13	39

(Continued on p. 308)

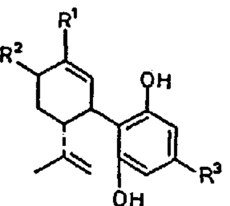
TABLE III *(continued)*

Compound	No.	R^1	R^2	R^3	R^4	R^5	R^6	R^7	R^8	Concentration[a]			Peak		
										Dog	Rat	Man	Figs. 11 and 12	Fig. 13	Fig. 4
5″-OH, 7-COOH	XIX	COOH	H	H	H	H	H	H	CH_2OH	–	++	–	–	17	–
10-OH, 7.COOH	XX	COOH	H	OH	H	H	H	H	CH_3	–	–	+++	–	–	35
7-OH, 5″-COOH	XXI	CH_2OH	H	H	H	H	H	H	COOH	+	–	+	26	–	44
6-OH, 5″-COOH	XXII	CH_3	OH	H	H	H	H	H	COOH	++	–	+	24	–	41
6-=O, 5″-COOH	XXIII	CH_3	=O	H	H	H	H	H	COOH	++	–	–	25	–	–
6,7-Di-OH, 5″-COOH	XXIV	CH_2OH	OH	H	H	H	H	H	COOH	+	–	+	33	–	–
5″,7-Di-COOH	XXV	COOH	H	H	H	H	H	H	COOH	++	+	+	32	21	–

[a] +++ = major metabolite; + = minor metabolite.

TABLE IV

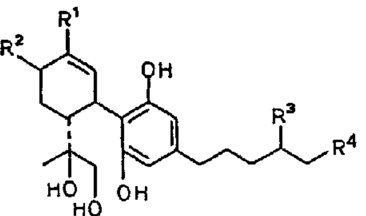
STRUCTURES AND QUANTITIES PRESENT IN URINE OF THE METABOLITIES OF CBD CONTAINING A SHORTENED SIDE-CHAIN



Compound	No.	R^1	R^2	R^3	Concentration			Peak		
					Dog	Rat	Man	Figs. 11 and 12	Fig. 13	Fig. 4
1″-COOH,2″,3″,4″,5″-*nor*	XXVI	CH_3	H	COOH	−	−	+	−	−	5
2″-COOH,3″,4″,5″-*nor*	XXVII	CH_3	H	CH_2COOH	−		+	−	−	
3″-COOH,4″,5″-*nor*	XXVIII	CH_3	H	$(CH_2)_2COOH$	−	−	+	−	−	13
4″-COOH,5″-*nor*	XXIX	CH_3	H	$(CH_2)_3COOH$	−	−	+	−	−	
6-OH-1″-COOH,2″,3″,4″,5″,-*nor*	XXX	CH_3	OH	COOH	+ + +	−	+	5	−	16
6,7-Di-OH-1″-COOH,2″,3″,4″,5″-*nor*	XXXI	CH_2OH	OH	COOH	+	−	−		−	−
7-OH,1″-COOH,2″,3″,4″,5″-*nor*	XXXII	CH_2OH	H	COOH	−	+ +	+	−	1	17
6-OH,3″-COOH,4″,5″-*nor*	XXXIII	CH_3	OH	$(CH_2)_2COOH$	+ + +	+ + +	+ +	12	4	25
7-OH,3″-COOH,4″,5″-*nor*	XXXIV	CH_2OH	H	$(CH_2)_2COOH$	+	+ +	+ + +	16	6	29
6,7-Di- OH,3″-COOH,4″,5″-*nor*	XXXV	CH_2OH	OH	$(CH_2)_2COOH$	+	+ +	−		12	−
6-=O,3″-COOH,4″,5″-*nor*	XXXVI	CH_3	=O	$(CH_2)_2COOH$	+ + +	−	−	14	−	−
3″,7-Di-COOH,4″,5″-*nor*	XXXVII	COOH	H	$(CH_2)_2COOH$	+ +	+ + +	−	20	11	−
7-OH,4″-COOH,5″-*nor*	XXXVIII	CH_2OH	H	$(CH_2)_3COOH$	−	−	+	−	−	
6-OH,4″-COOH,5″-*nor*	XXXIX	CH_3	OH	$(CH_2)_3COOH$	−	−	+ +	−	−	32
2″-OH,7-COOH,3″,4″,5″-*nor*	XL	COOH	H	CH_2CH_2OH	−	+ +	+ + +	−	3	21
2″,7-Di-OH,3″,4″,5″-*nor*	XLI	CH_2OH	H	CH_2CH_2OH	−	−	+	−	−	17
2″,6-Di-OH,3″,4″,5″-*nor*	XLII	CH_3	OH	CH_2CH_2OH	−	−	+	−	−	

TABLE V

STRUCTURES OF THE 8,9-DIHYDRO-8,9-DIHYDROXY METABOLITES OF CBD



Compound	No.	R^1	R^2	R^3	R^4	Concentration			Peak		
						Dog	Rat	Man	Figs. 11 and 12	Fig. 13	Fig. 4
7,8,9-Tri-OH	XLIII	CH_2OH	H	H	H	–	–	+	–	–	37
6,7,8,9-Tetra-OH	XLIV	CH_2OH	OH	H	H	++	–	–	29	–	–
4″,6,8,9-Tetra-OH	XLV	CH_3	OH	OH	H	++	–	–	31	–	–
5″,6,8,9-Tetra-OH	XLVI	CH_3	OH	H	OH	++	–	–	37	–	–
4″,7,8,9-Tetra-OH	XLVII	CH_2OH	OH	OH	H	+++	–	–	32	–	–
5″,7,8,9-Tetra-OH	XLVIII	CH_2OH	OH	H	OH	+		–	38	–	–
7-COOH,8,9-di-OH	XLIX	COOH	H	H	H	–	–	++	–	–	43

Other non-oxidised cannabinoids

The compounds producing peaks 6 (1.97%) and 8 (0.69%) were identified, by their mass spectra (TMS derivatives [14]) and retention times, to be Δ-8- and Δ-9-tetrahydrocannabinol (THC), respectively, and were presumably formed by cyclisation of CBD. The aromatic compound, cannabinol (CBN), was also identified as the compound producing peak 9 (0.6%).

Oxidised metabolites

Major metabolites in this sample were identified as side-chain hydroxylated derivatives (XII–XIX) of CBD-7-oic acid (Fig. 5) and were identified by the ions listed in Table I. All compounds contained the retro-Diels–Alder ion (*a*) at *m/z* 492 and the tropylium ion (*b*) at *m/z* 425. [A single-ion plot of the latter ion was useful in localizing these compounds (Fig. 6)]. The parent acid (XI) was identified as the compound giving peak 18. The resolution of the capillary column was such that the 1″- (XII and XIII), 2″- (XIV and XV) and 3″-hydroxy derivatives (XVI and XVII) were separated into two peaks corresponding, presumably, to the *R*- and *S*-forms of the alcohol. Other than one earlier report of the resolution of 1″-hydroxy isomers [6], this is the first time that evidence has been presented to show that both *R*- and *S*-alcohols are produced metabolically. The compound producing peak 35 was identified as 10-hydroxy-CBD-7-oic acid (XX), the first example of a CBD metabolite hydroxylated in the isopropenyl chain.

The compound producing peak 14 was a carboxylic acid with a mass spectrum very much like that of CBD-7-oic acid except that all peaks were 4 units lower in mass. The abundant ion of type *c* located the carboxylic acid group to C-7. The mass of the tropylium ion (*m/z* 333) showed that the metabolic change had occurred in the side-chain. Corresponding compounds in dog urine (see below) contained a hydroxy group at C-6 and it was noted that the metabolite was not reduced with lithium aluminium hydride. Only two empirical formulae can be written for the side chain, C_5H_7 (loss of four hydrogen atoms) and C_4H_3O. The latter formula can be accommodated by a furan substituted at its C-2 position but this does not appear biologically reasonable. This leaves the reduced side-chain as the most likely structure, but the positions of the double bonds were not determined.

Compounds containing side-chains with reduced numbers of carbon atoms

Peaks 5 and 13 (Fig. 3) were monocarboxylic acids as shown by their reactivity towards diazomethane. Their molecular weights, masses of ion *b* and GC retention times were consistent with their having lost four and two carbon atoms respectively from the side-chain to leave an acid group (compounds XXVI and XXVIII) (see Fig. 7). Various 6- and 7-hydroxylated analogues of these acids were also identified in a similar manner and are listed in Table IV. The major metabolite (peak 21) had a molecular weight and reactivity towards diazomethane and $[^2H_{18}]$BSA indicating that it was a hydroxy acid that had lost three

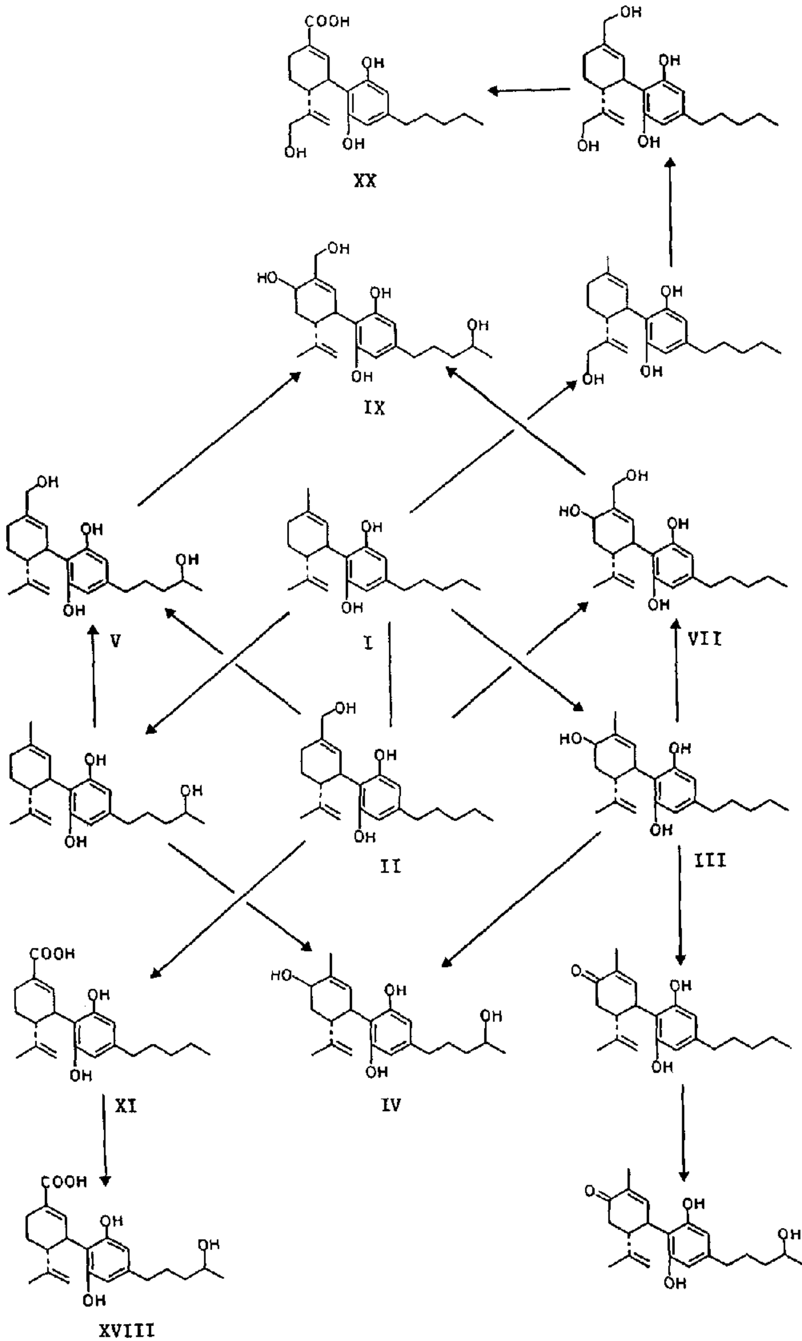


Fig. 5. Summary of metabolic routes of CBD involving hydroxylation and oxidation to carboxylic acids. The side-chain monohydroxylated metabolites were not observed in this study but have been identified in experiments with hepatic microsomes. The major pathway leading to the formation of polysubstitued metabolites has not been determined, thus pathways shown are speculative.

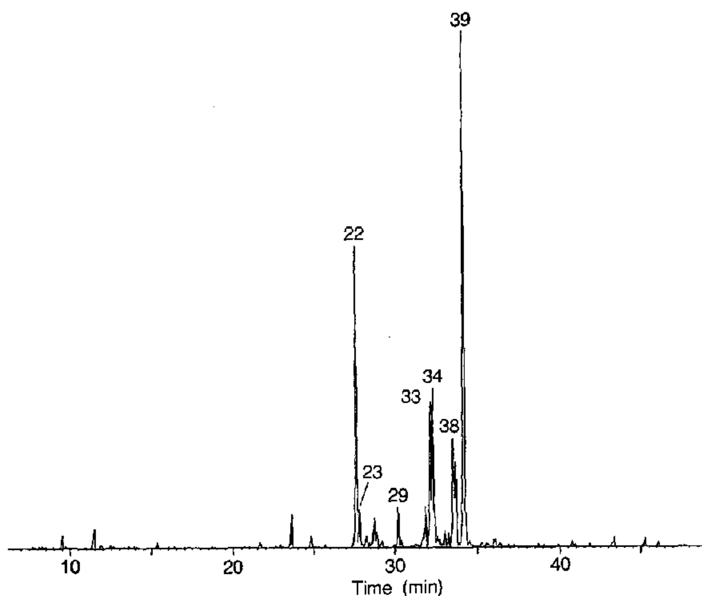


Fig. 6. Single-ion chromatogram of *m/z* 425 (ion *b*) from the experiment shown in Fig. 4. Peaks are numbered as in Fig. 4.

carbon atoms from the side-chain. The masses of ions *a* and *b* indicated that the acid group was at C-7 and the compound was identified as 2″-hydroxy-*tris,nor*-CBD-7-oic acid (XL) (Fig. 8). The 6- (XLII) and 7-hydroxy (XLI, peak 17) derivatives of 2″-hydroxy-3″,4″,5″-*tris,nor*-CBD were also identified.

Metabolites produced by the epoxide-diol pathway

These metabolites were all formed by reaction of the Δ-8 double bond to give a dihydro-diol structure (Fig. 9, Table V). Fragmentation of these compounds, as described above, differed from that of the metabolites discussed to date in that fragmentation occurred more favourably from the dihydroxy-diol moiety than from the terpene ring. Major fragment ions are detailed in Fig. 3 and Table II. The compound producing peak 43 was an acid derivative of 8,9-dihydro-8,9-dihydroxy-CBD (XLIX); the presence of ions *a* and *b* at 492 and 337, respectively, localised the acid group to C-7. Its 7-hydroxy analogue (XLIII) produced peak 37. Full mass spectral details of these compounds have been published [35].

Dog

Conjugates

Fig. 10 shows a limited ion chromatogram (masses 330–700) of the metabolites present in the ethyl acetate extract of the unhydrolysed urine taken at 3 h after

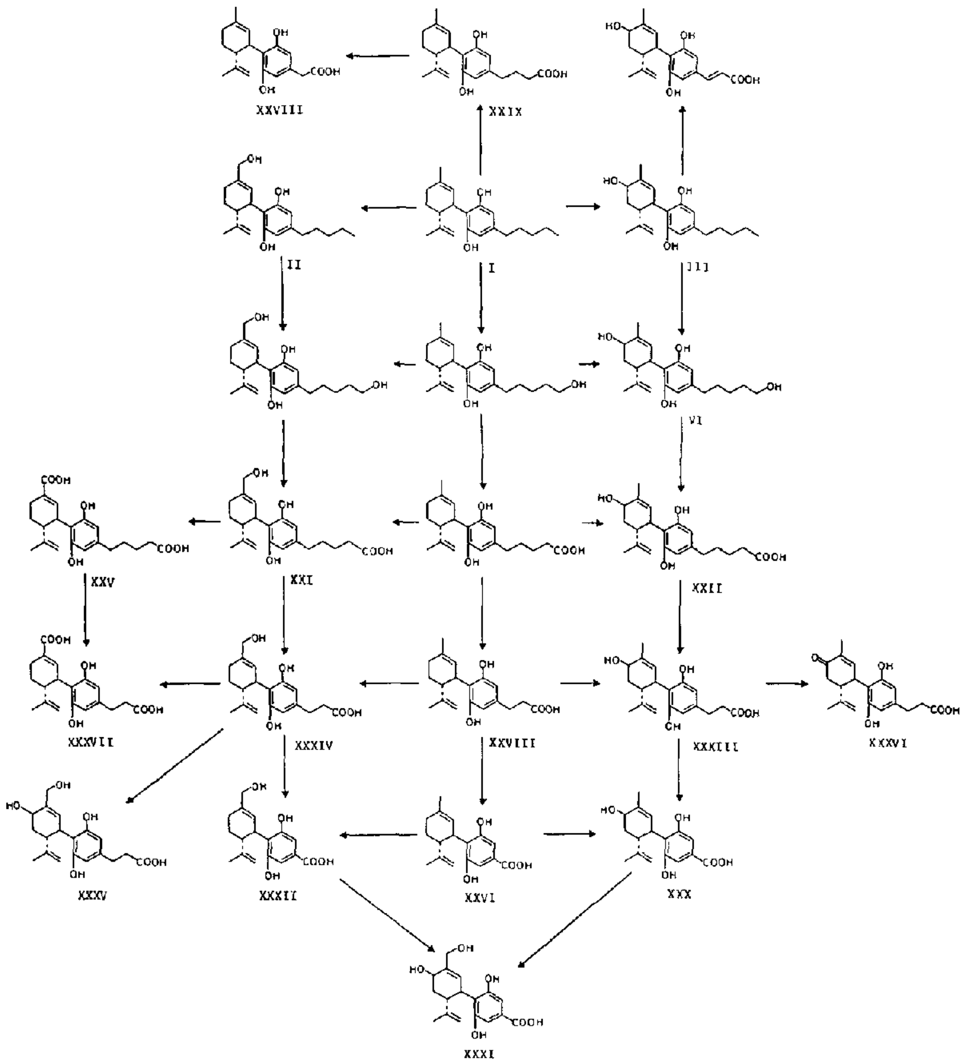


Fig. 7. Summary of metabolic routes of CBD involving β-oxidation. Pathways shown for the formation of polysubstituted metabolites are speculative.

dosing. The peaks eluting at around 30 min were also present in the fraction hydrolysed by β-glucuronidase Type VII but not in that hydrolysed by β-glucuronidase Type HP-2. This indicates that they are conjugates and this was confirmed by their mass spectra which showed typical sugar-derived ions [8,16] similar to those in the spectra of the glucuronide conjugates discussed above, but with some ions, particularly that at m/z 361 (base peak) at 14 mass units lower than their equivalents in the glucuronide spectra. The compounds did not react with diazomethane suggesting that they were glucosides. This was confirmed by identifica-

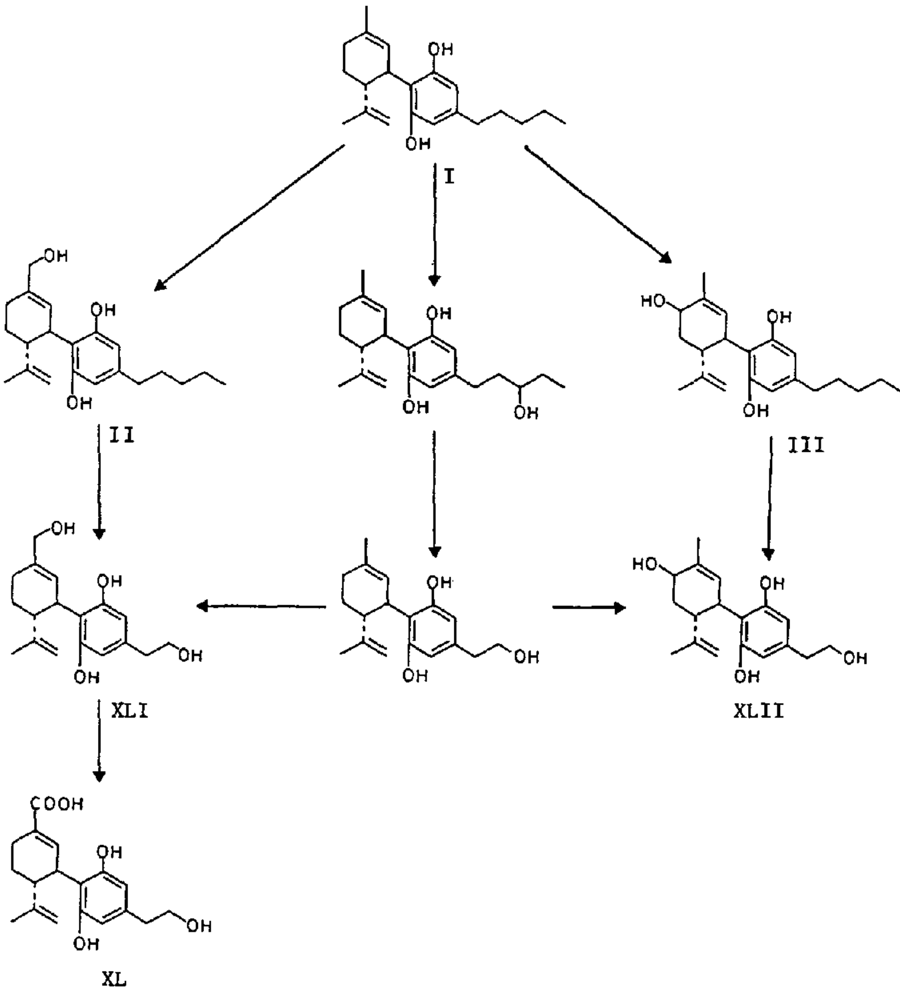


Fig. 8 Metabolism of CBD to give the metabolites with two carbon atoms in the side-chain. The order in which the metabolic steps leading to disubstituted metabolites occurs is speculative.

tion of glucose as its TMS derivative by GC–MS analysis of the aqueous fraction [17] following hydrolysis with β-glucuronidase Type HP-2. The aglycone ions in the spectra of the intact glucosides indicated that they were conjugates of side-chain hydroxylated metabolites and of a keto metabolite. GC–MS analysis of the organic fraction following enzymatic hydrolysis revealed the presence of 4″- and 5″-hydroxy-CBD and 6-oxo-CBD, compounds not produced as metabolites in the unconjugated state, thus confirming the identity of the conjugates as the glucosides of these three cannabinoids. The formation, by TMS migration, of ions in their mass spectra (TMS derivatives), corresponding to the TMS derivatives of the unconjugated aglycones, confirmed the point of attachment of the

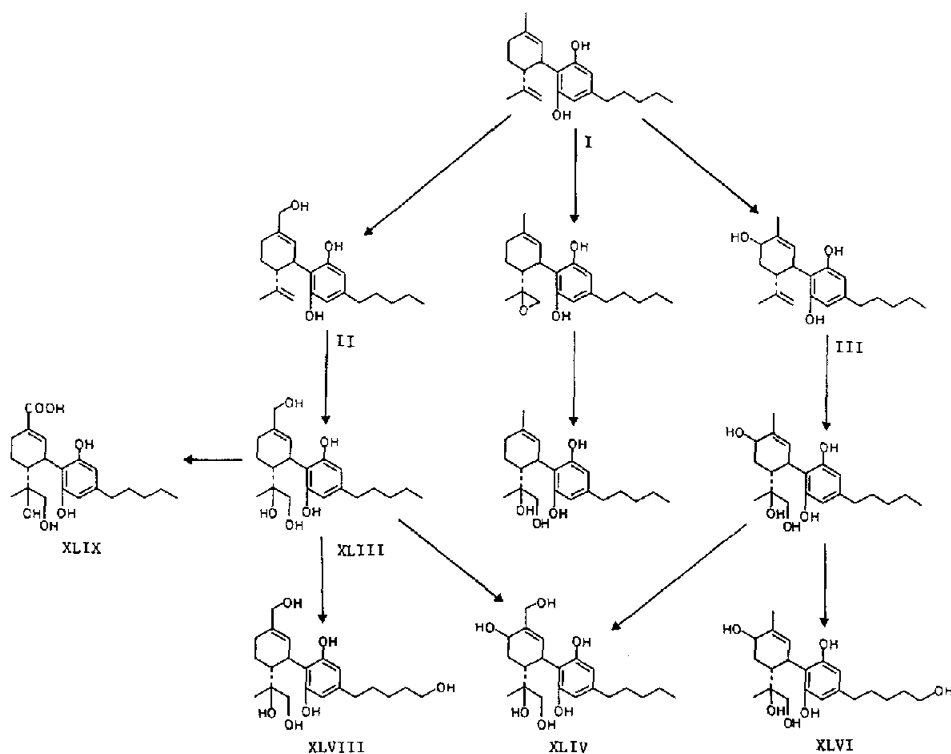


Fig. 9. Summary of the metabolic routes of CBD involving the epoxide-diol pathway. The epoxide itself was not observed. Additional metabolites were found with hydroxylation in the side-chain, but are not shown on this simplified chart. The order in which metabolic transformations occur has not been determined.

sugar as the phenol group of CBD [16]. These conjugates were not seen in samples taken at later times or in samples of urine from the other two species. Full details of the identification of these conjugates have been published [9].

Oxidised metabolites

Fig. 11 shows the reconstructed ion chromatogram of metabolites extracted from a urine sample taken at 10 h from one of the dogs. All three dogs gave metabolic profiles that were very similar. Most of the metabolites were acids as shown by their reaction with diazomethane and most were formed by β-oxidation of the side-chain as shown by the mass of the tropylium ion (*b*) (see Table I and Fig. 7). The very prominent retro-Diels–Alder ion and weak molecular ion in the major metabolites showed that most contained a 6-hydroxy group [14] (both α- and β-isomers). 6-Oxo-CBD-4″,5″-*bis,nor*-CBD-3″-oic acid (XXXVI, Table IV) was also identified and its identity confirmed by preparation of the methyloxime

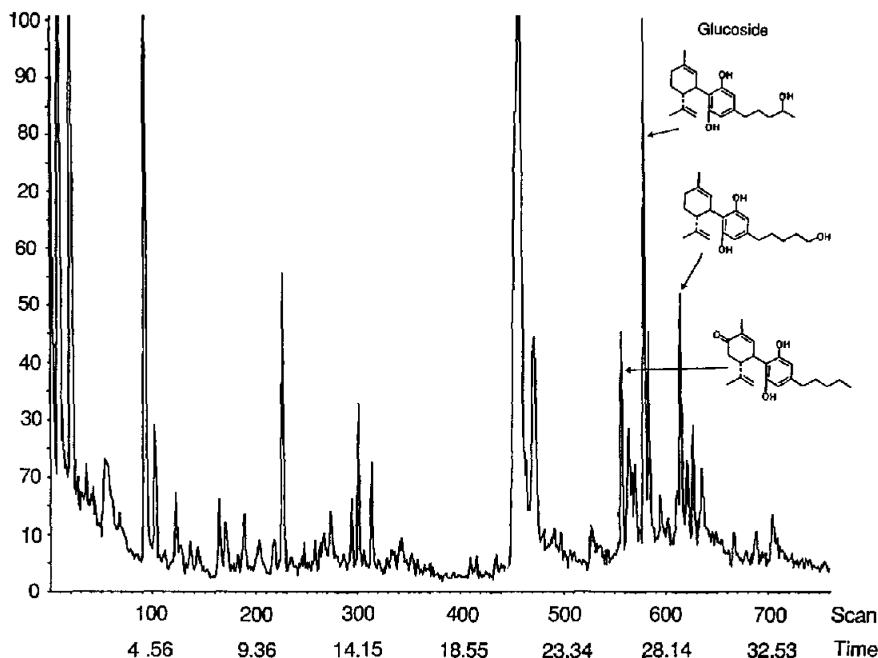


Fig. 10. Reconstructed ion chromatogram (m/z 330–700) of metabolites of CBD (TMS derivatives) from the dog taken 3 h after drug administration showing the glucoside metabolites. Separation was made with a 25 m × 0.2 mm I.D. OV-1 fused-silica capillary column operated as described in the Experimental section. Most peaks were produced by metabolites as identified in Tables III–V and in the text. Major peaks not identified are urinary constituents.

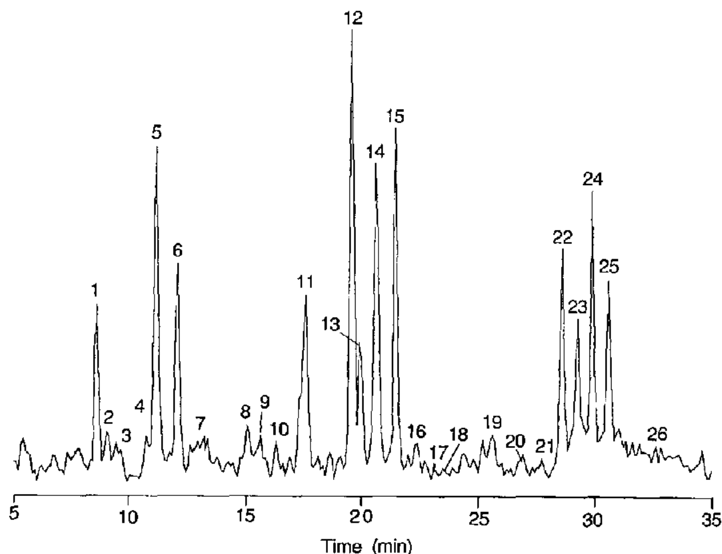


Fig. 11. Reconstructed ion chromatogram (m/z 330–700) of the metabolites (TMS derivatives) extracted from canine urine 10 h after dosing. Separation was made with a 25 m × 0.2 mm I.D. OV-1 fused-silica capillary column operated as described in the Experimental section. Most peaks were produced by metabolites as identified in Tables III–V and in the text. Major peaks not identified are urinary constituents.

derivative. Major identified compounds are listed in Table IV. Peak 15 was produced by 4″,6-dihydroxy-CBD (IV) and was the only compound whose peak intensity rose significantly after hydrolysis of the urine with β-glucuronidase.

Metabolites produced by the epoxide-diol pathway

Fig. 12 shows a chromatogram of metabolites obtained by combining aliquots of the samples taken at each time point. It contained, in addition to the compounds shown in Fig. 11, a series of metabolites (XLIV − XLVIII) formed by the epoxide-diol pathway involving the Δ-9 double bond (Fig. 7). These metabolites were identified by their base peak at m/z 143 and the diagnostic ions listed in Table II. Identified compounds are listed in Table V.

Rat

The reconstructed ion chromatogram of metabolites found in rat urine is shown in Fig. 13. No intact glucuronides or glucosides were identified; other metabolites were identified by the ions listed in Table I. The major metabolites were acids as shown by their reaction with diazomethane. β-Oxidation was again prominent with most identified compounds containing hydroxylation at either

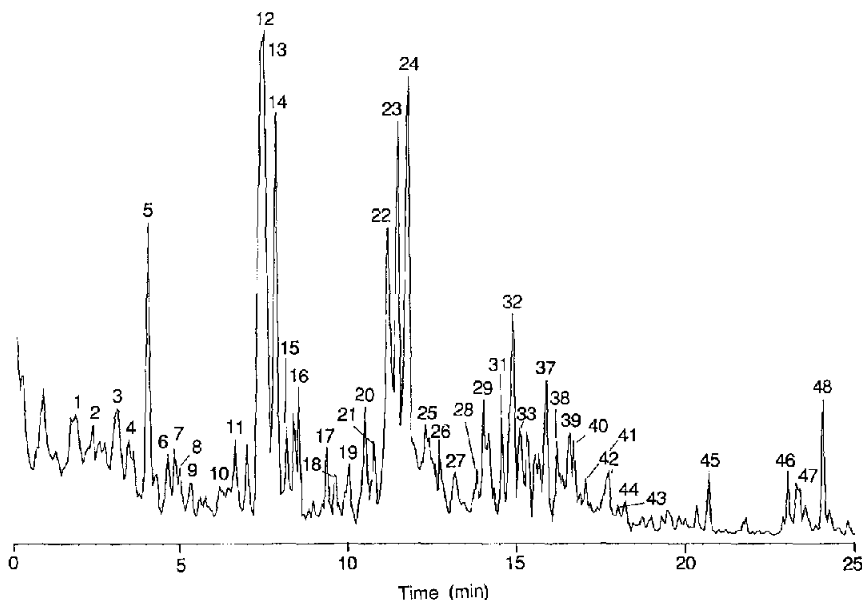


Fig. 12. Reconstructed ion chromatogram (m/z 330–700) of the metabolites (TMS derivatives) extracted from a sample of canine urine obtained by pooling an aliquot from each of the timed samples. Separation was made with a 25 m × 0.2 mm I.D. OV-1 fused-silica capillary column operated as described in the Experimental section. Most peaks were produced by metabolites as identified in Tables III–V and in the text. Major peaks not identified are urinary constituents.

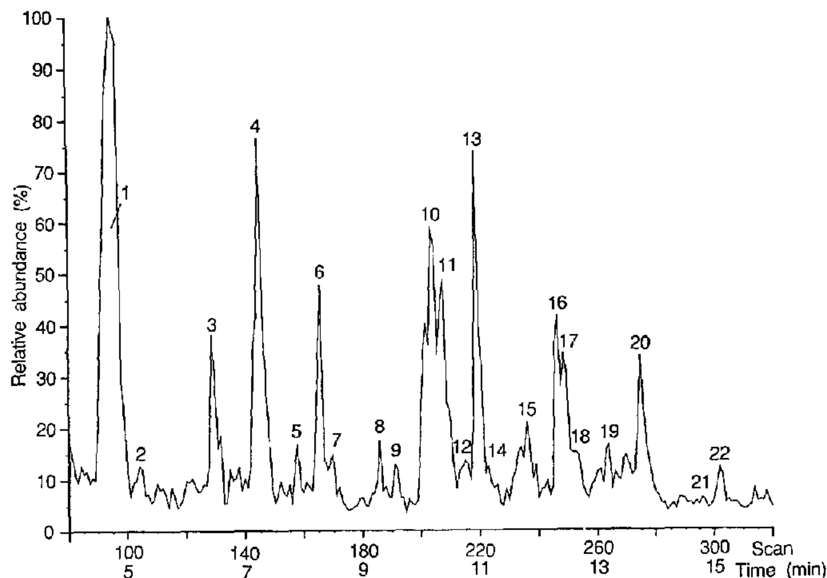


Fig. 13. Reconstructed ion chromatogram (m/z 330–700) of the metabolites (TMS derivatives) extracted from rat urine. Separation was made with a 25 m × 0.2 mm I.D. OV-1 fused-silica capillary column operated as described in the Experimental section. Most peaks were produced by metabolites as identified in Tables III–V and in the text. Major peaks not identified are urinary constituents.

C-6 or C-7. A larger proportion of metabolites was hydroxylated at C-7 than was found in the dog. Identified compounds are listed in Tables III and IV. Full details of the mass spectra will be published elsewhere.

DISCUSSION

GC–MS identification of the metabolites from CBD was straightforward as the molecules fragmented into structurally informative ions. In particular, the retro-Diels–Alder (*a*) and tropylium (*b*) ions were abundant and their masses localised the site of metabolic attack to specific parts of the molecule, Single-ion plots could, thus, be used to identify groups of metabolites with specific structural features. In addition, most metabolites gave diagnostic fragment ions allowing specific sites of metabolic attack to be identified.

Metabolism of CBD showed biotransformation routes reasonably typical for cannabinoids in general [7] with multiple hydroxylations, oxidations to carboxylic acids, β-oxidation and conjugation reactions dominating the profile. These are summarised in Figs. 5 and 7–9.

Fifty-three metabolites were identified in the three species, but the range of compounds found in each individual species varied considerably. Conjugates were not found in the rat urine, but a substantial proportion of the dose appeared

as CBD glucuronide in human urine. In the dog, glucoside conjugates dominated the profile at early times; these were identified as conjugates of 4″- and 5″-hydroxy-CBD and 6-oxo-CBD, three compounds that were not excreted unconjugated. Glucose conjugates are unusual in mammalian systems and have only been identified on a few occasions [18–27]. Most of these reports were to N- rather than to O-linked conjugates, the latter compounds only apparently having been reported on one earlier occasion [26]. It is significant that in several of these earlier reports [18,19,26], the dog appears to favour glucose conjugation.

Most of the other metabolites were acids. These were of three types: hydroxy derivatives of CBD-7-oic and (XII–XX); products of side-chain degradation in which the acid was in the side-chain; and related compounds with a degraded side-chain in which the carboxylic acid group was at C-7. The hydroxylated derivatives of CBD-7-oic acid were most numerous and abundant in human urine. The hydroxy group was found at each position of the side-chain and resolution on the fused-silica capillary column was sufficient to show that the 1″-, 2″- and 3″-hydroxy derivatives of CBD-7-oic acid were produced as *R*- and *S*-isomers. Resolution of such isomers has only been reported on one previous occasion [6] and then only with 1″-hydroxy substitution. The abundance of 2″-hydroxy-CBD-7-oic acid (XIV and XV) was unusually high; in most species except the mouse, hydroxylation at C-2″ is usually a very minor biotransformation route for cannabinoids [7]. In rat and dog urine, the major acids were products of β-oxidation with metabolites formed by one stage of oxidation being the most common. In the dog, these were further hydroxylated mainly at C-6 whereas in rat and human, C-7 was favoured. Products of two stages of β-oxidation increased with time and there was also a greater tendency for oxidation of the 7-hydroxy group to a carboxylic acid with time in the rat. Thus the *bis*-acid, 4″,5″-*bis,nor*-CBD-3″,7-dioic acid (XXXVII) was reasonably abundant after 24 h.

The third type of acid, containing a carboxylic acid at C-7 and a hydroxylated side-chain with a reduced number of carbon atoms, was particularly abundant in human urine with 2″-hydroxy-3″,4″,5″-*tris,nor*-CBD-7-oic acid (XL) being a major metabolite. These compounds did not appear to be products of β-oxidation as demonstrated earlier with Δ-9-THC [28] when it was shown that metabolism of 5″-hydroxy-Δ-9-THC and Δ-9-THC-5″-oic acid, the normal intermediates in β-oxidation, did not yield the metabolites with a hydroxy side-chain. On the other hand, metabolism of 3″-hydroxy-Δ-9-THC [29], but not 2″- [29] or 4″-Δ-9-THC [30] did not. This strongly suggests that formation of 2″-hydroxy-3″,4″,5″-*tris,nor*-CBD-7-oic acid from CBD involves initial hydroxylation at C-3″, but the subsequent steps leading to loss of three carbon atoms from the side-chain has yet to be clarified. However, the presence of this metabolite in high concentration in human, but not dog urine, correlated well with the observation that human urine contained much higher concentrations of 3″-hydroxylated metabolites.

Another potentially interesting metabolic route produced the metabolites hav-

ing a side-chain that had lost four mass units and for which a loss of four hydrogen atoms with the introduction of two double bonds appears to be the most likely mechanism. However, this has not yet been confirmed. Metabolites of a similar nature have previously been observed for valproic acid [31], an acidic drug which shows many of the same types of biotransformation pathways as CBD.

Other metabolites were formed by epoxidation and subsequent hydroxylation of the Δ-8-double bond. No metabolites were found in which epoxidation had occurred at the Δ-1 bond (it should be noted that recent work has suggested that the Δ-1-epoxide of CBD is unstable and decomposes by cyclisation to cannabielsoin [32,33]). 8,9-Dihydro-8,9-dihydroxy-containing metabolites were only found in human and dog urine. In human urine, they were further biotransformed, particularly by acid formation at C-7, whereas in the dog, hydroxylation and particularly dihydroxylation was preferred. Hydroxy groups were located at C-6 or C-7 and in the side-chain.

CONCLUSIONS

The cannabinoids in general, and CBD in particular, appear to be very interesting compounds metabolically as they are metabolised along multiple pathways which show considerable species variation. These result from initial hydroxylation at different sites [34]. Thus, although not discussed here, they can be used as probes of the cytochrome P-450 enzyme system. Their metabolism by enzymes usually involved in metabolism of carboxylic acids also enables them to be used as probes of this system. Because they are not ideal substrates, reactions are slower and intermediates of the type not usually seen with endogenous substrates are sufficiently stable to be excreted into the urine. This has already been used to show that the product of one stage of β-oxidation in cannabinoids can occur, not only from the ω (5″-hydroxy) intermediate, but also from ω-1 (4″-hydroxy) and ω-2 (3″-hydroxy) intermediates. In the present study, metabolism of CBD has also shown a new product of a related oxidation mechanism, 2″-hydroxy-*tris,nor*-CBD-7-oic acid. Conjugate formation is also complex with the production of the comparatively rare glucose conjugates. However, as pointed out in our original communication [9], the failure to observe these for some other compounds may be related to methodology; enzymatic hydrolysis by β-glucuronidase from *Helix pomatia*, and comparison of metabolic profiles with those without hydrolysis, is the usual method of inferring the presence of glucuronides. However, as shown here, glucosides are also hydrolysed by this enzyme and it is only with the more powerful technique of GC–MS and the examination of intact conjugates that the presence of these compounds can be conclusively demonstrated.

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