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IDENTIFICATION OF (−)-Δ⁹-6a,10a-*TRANS*-TETRAHYDROCANNABINOL AND TWO OF ITS METABOLITES IN RATS BY USE OF COMBINATION GAS CHROMATOGRAPHY-MASS SPECTROMETRY AND MASS FRAGMENTOGRAPHY

FRANTISEK MIKES, ALFRED HOFMANN and PETER G. WASER
Pharmacological Institute of the University of Zürich, Zürich, Switzerland

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Abstract—Two metabolites of (−)-Δ⁹-6a,10a-*trans* tetrahydrocannabinol (Δ⁹THC), one of the psychotomimetic components of *Cannabis sativa* L, were found after this substance was injected intraperitoneally into rats. Extracts of rat urine, blood, bile and feces were analysed by gas chromatography, mass spectrometry and mass fragmentography. In urine and feces unchanged Δ⁹THC was found. After glucuronidase treatment of feces, cannabinol (CBN) and an equal amount of Δ⁹THC was also found. Dihydroxy-Δ⁹-THC was identified in urine and feces. Diacetyl-Δ⁹THC was isolated from bile.

THERE exist today about 2000 publications concerning the phenomenon of marihuana usage.[1] There is also much literature on the composition of marihuana, its structure and the synthesis of its components.* The investigation of psychotomimetic compounds of marihuana and its metabolites, however, is far from complete. The question whether Δ⁹THC, an active substance of marihuana, or its metabolites, appear in blood, urine, etc., in animals and especially in man, has not been unambiguously answered in recent publications (see Table 1).[5–19]

We have attempted to answer two questions: is Δ⁹THC present in urine, blood, bile and feces of rats injected i.p. with this compound; and which metabolites are produced by this animal.

MATERIALS AND METHODS

Female rats (200–250 g, 4–6 months old)[20] were injected i.p. with synthetic Δ⁹THC (77·5 per cent pure) dissolved in 1,2-propylene glycol. Gas chromatography showed the synthetic compound to contain 5 per cent Δ⁸THC, 3 per cent CBN and less than 1 per cent of five other unidentified compounds. Urine, blood and feces from groups of four rats, bile from three rats, and blank samples from a control group were collected over a period of time and prepared for extraction. The rats were kept in metabolic cages, where commercial chew diet and water were offered freely.

* See comprehensive publications by R. Mechoulam *et al.*[2,3] and bibliography on Cannabis by V. Hin Yin Kwan *et al.*[4]

TABLE 1. RECENT INVESTIGATIONS OF Δ^9THC METABOLISM

Research group	Exper. animal	Material tested	Administration method	Analysis method	Material analysis	Findings
J. Bonzani da Silva[5]	man	marihuana	inhal.	TLC	saliva blood urine	CBN CBN CBN
H. Aramaki *et al.*[6]	rabbits	Δ^9THC	i.p.	GC	urine	CBN, no Δ^9THC
G. Joachimoglu *et al.*[7]	rats	Δ^9THC-^{14}C	i.p.	RG	urine feces	11·9% ^{14}C–5 days 68·3% ^{14}C–5 days
J. Christiansen *et al.*[8]	man	marihuana resin	oral	TLC	urine	cannabidiol no Δ^9THC
S. Agurell *et al.*[9]	rats	Δ^9THC Δ^9THC-^{3}H	i.p. i.v.	TLC, GC RG	urine feces urine	no Δ^9THC ~10% ^{14}C–8 days ~40% ^{14}C–8 days
S. Agurell *et al.*[10]	rabbits	Δ^9THC-^{3}H	i.v.	TLC RG	urine tissues	no Δ^9THC liver, kidneys highest ^{3}H conc.
S. H. Burstein *et al.*[11]	rabbits	Δ^9THC	i.v.	TLC, GC GC–MS	urine	OH-Δ^9THC
Z. Ben-Zvi *et al.*[12]	rabbits	Δ^9THC	i.v.	TLC, n.m.r. PC, GC–MS	liver homog.	11-OH-Δ^9THC
I. M. Nilsson *et al.*[13]	rabbits	Δ^9THC	*in vitro*	TLC, n.m.r. i.r. GC–MS	liver homog.	11-OH-Δ^9THC
R. L. Foltz *et al.*[14]	rats	Δ^8THC	*in vivo* *in vitro*	TLC, n.m.r. GC–MS	liver homog.	11-OH-Δ^9THC
M. E. Wall *et al.*[15, 16]	rats	Δ^9THC-^{3}H	*in vitro*	TLC, n.m.r. GC–MS, u.v. i.r., RG	liver homog.	11-OH-Δ^9THC 8,11-diOH-Δ^9THC 11-acetyl-Δ^9THC
B. T. Ho *et al.*[17]	rats	Δ^9THC-^{3}H	inhal.	TLC, RG GC	tissues	conc. ^{3}H highest in lungs until 24 hr.
M. Wahlquist *et al.*[18]	man	Δ^9THC-^{3}H	*in vitro*	EP, RG	blood	Δ^9THC bond 80–95% with lipoproteins
L. Lemberger *et al.*[19]	man	Δ^9THC-^{14}C	i.v.	TLC, RG	blood urine feces	Δ^9THC, 11-OH-Δ^9THC 1% Δ^9THC excreted and 85% ^{14}C recovered during 8 days

RG = Radiography.
PC = Paper Chromatography.
EP = Electrophoresis.

Extraction

Fecal material was first crushed and diluted with water. All biological materials were then saturated with NaCl and extracted at pH 7 with ether and *n*-butanol. Chloroform was also used in extraction of bile samples. The materials were further extracted at pH 3 (1·0 N HCl) with ether. Each sample was extracted four times with equal volumes of solvent and dried with Na_2SO_4.

Glucuronidase treatment

The samples of urine, blood and feces were treated with Glucuronidase Type II (Sigma Chemical Co.) in acetate buffer (pH 5·4) during 1 hr at 37°, in order to split any glucuronides of Δ⁹THC or its metabolites, if present.

Silylation

Samples were silanized according to Pierce's method.[21] For each silylation 20–50 μl of a solution of hexamethyldisilazane, trimethylchlorsilane and dimethyldichlorsilane in pyridine, dried with KOH (8:4:1:40) was used.

Thin layer chromatography (TLC)

TLC was carried out on Silikagel plates N-HR/UV_{254} (Macherey–Nagel & Co., Germany). Extracts of urine, blood and feces were developed in hexane containing 12% ether. Extracts from bile were spotted on Silikagel plates which had been impregnated with 20% dimethylformamide in acetone, and then developed in light petroleum (b.p. 40–60°) containing 20% ether. Spots were detected with u.v. (254 mμ) and by spraying with 0·2% Echtblausalz (Merck AG) in 2 N NaOH.

Gas chromatography (GC)

Analyses were made with a gas chromatograph model GI 450 (Carlo Erba, Italy), on a glass spiral column (2·7 × 3 mm) filled with 1% SE-30 on Chromosorb W (silanized). A column temperature of 200–220°, and injector and flame ionization detector (FID) temperatures of 250° were used. Nitrogen at 30 ml/min was used as carrier gas. 1–2 μl of each extract was injected. The GC detection limit for Δ⁹THC using the FID is 15–20 ng.

Gas chromatography—mass spectrometry combination (GC–MS)

A GC–MS model 9000 (LKB, Sweden) was used. Gas chromatographic conditions were as above. Mass spectral conditions were: injector temperature 250°, separator and ion source temperatures 270°, ionization potential 70 eV, multiplier voltage 2·5 kV, trap current 65 μA. Helium at 30 ml/min was used as carrier gas. About 20 per cent of the positively charged ions from the ion source are recorded by a total ion current (TIC) detector and the remaining 80 per cent are recorded in the mass spectra. The TIC detection limit for Δ⁹THC is about 10 ng.

Mass fragmentography (MF) studies were also carried out with GC–MS using the accelerating voltage alternator (AVA).[22,23] About 20 pg is the detection limit for Δ⁹THC by MF.

The results of each experiment were compared with those of blank samples, which had been extracted and treated in the identical manner as those from the biological materials activated with Δ⁹THC.

RESULTS

Urine

Four rats were injected i.p. with 130 mg/kg Δ⁹THC, and their urine collected during 42 hr. The samples were extracted with ether at pH 7 and, following glucuronidase treatment, at pH 3. The extracts were then purified by TLC to remove fatty substances.

GC and GC–MS of the extracts at pH 7 gave no indication of Δ⁹THC. Then the more sensitive mass fragmentography method was used. At the retention time of Δ⁹THC (7·4 min), where no response was detected by the FID or the TIC detector, the AVA was focused on the mass numbers typical for Δ⁹THC (m/e = 314, molecular peak) and two of its fragments (m/e = 231, base peak; 313, M^+—H). These mass numbers were found in the extract at pH 3 and in the same relative intensities as those of the reference compound (see Fig. 1). But in the extract at pH 3 no Δ⁹THC was detected.

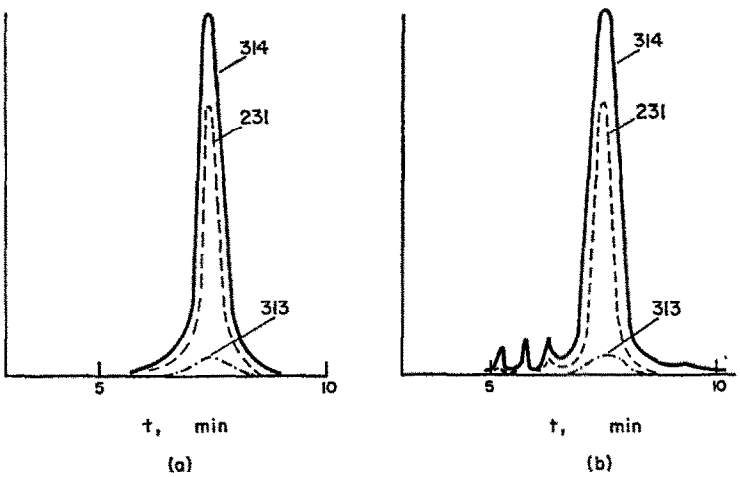


FIG. 1. (a) MF of urine extract from rats injected i.p. with Δ⁹THC. Focusing on the mass numbers, m/e = 314, 231, 313, the AVA recorded relative intensities of 100, 75, 12, respectively. (b) MF of Δ⁹THC. Mass numbers, m/e = 314, 231, 313 were recorded with relative intensities of 100, 72, 15, respectively.

When the urine extract at pH 3 was analyzed by GC–MS, a compound (relative retention time to Δ⁹THC = 0·77) was found, which gave the same fragments (see Table 2) as those reported by Wall *et al.*[16] for dihydroxy-Δ⁹THC (Fig. 2). No dihydroxy-Δ⁹THC had been detected in the extract at pH 7.

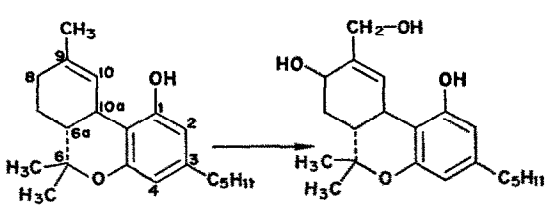
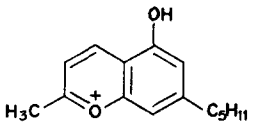


FIG. 2. Dihydroxy-Δ⁹THC observed in urine of rats after i.p. injection of Δ⁹THC.

Feces

The feces from the same series of experiments were collected during 5 days. The samples were extracted with ether at pH 7, and the extracts purified by TLC. A spot at the same R_f as Δ⁹THC (R_f = 0·55) was eluated with ether and analysed by GC–MS. The mass spectrum was identical to that of Δ⁹THC.

TABLE 2. MS FRAGMENTS OF A COMPOUND SEPARATED BY GC FROM A URINE EXTRACT OF RATS INJECTED WITH Δ⁹THC, SHOWING FRAGMENTS TYPICAL FOR DIHYDROXY-Δ⁹THC

Fragment	*m/e*	Relative abundance (%)
M^+ ($C_{21}H_{30}O_4$)	346	40
$M^+—H_2O$	328	100
$M^+—CH_2OH$	315	39
$M^+—H_2O—CH_3$	313	38
$M^+—CH_2OH—H_2O$	297	83
$M^+—H_2O—C_3H_7$	285	25
 *	231	30

* This fragment was published.[24,25]

The samples were then glucuronized, acidified to pH 3 and extracted with ether. The extracts were directly analysed by GC–MS. Mass spectra of the components in the extracts were compared with those of the reference samples and with those in the literature[24,25] for Δ⁹THC and CBN. CBN (relative retention time to Δ⁹THC = 1·26) and an equal amount of Δ⁹THC were found.

Feces from another group of rats injected i.p. with 75 mg/kg Δ⁹THC were collected during 24 hr. The samples were extracted with *n*-butanol at pH 7, and the dried extracts silanized and analysed by GC–MS. A mass spectrum of one of the GC peaks (relative retention time to Δ⁹THC = 0·32) suggests a monosilanized dihydroxy-Δ⁹THC ($C_{21}H_{29}O_4—Si(CH_3)_3$) (see Table 3).

TABLE 3. MS FRAGMENTS FROM A COMPOUND SEPARATED BY GC FROM A SILANIZED FECES EXTRACT OF RATS INJECTED i.p. WITH Δ⁹THC

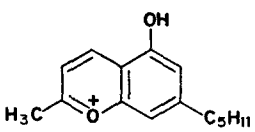
Fragment	*m/e*	Relative abundance (%)
M^+ ($C_{24}H_{38}O_4Si$)	418	10
$M^+—Si(CH_3)_3$	345	19
$M^+—Si(CH_3)_3OH$	328	20
$M^+—Si(CH_3)_3H_2O$	327	22
$M^+—Si(CH_3)_3—CH_2OH$	314	58
$M^+—Si(CH_3)_3OH—CH_3$	313	100
$M^+—Si(CH_3)_3—CH_2OH—CH_3$	299	24
$M^+—Si(CH_3)_3OH—C_3H_7$	285	8
$M^+—Si(CH_3)_3—CH_3OH—C_3H_7$	271	10
	231	50

Bile

Three rats were injected i.p. with 50 mg/kg Δ^9THC and the bile (1 ml) collected from 4 to 12 hr. The samples were then extracted with ether and chloroform at pH 7 and the combined extracts purified by TLC. Analysis by TC–MS showed a compound (relative retention time to Δ^9THC = 3·2) whose mass spectrum is similar to that of diacetyl-Δ^9THC. (See Fig. 3, Table 4.)

No Δ^9THC was found in the bile extract by mass fragmentography.

TABLE 4. MS FRAGMENTS OF A SUBSTANCE SEPARATED BY GC FROM A BILE EXTRACT OF A RAT INJECTED i.p. WITH Δ^9THC

Fragment	*m/e*	Relative abundance (%)
M^+ ($C_{25}H_{34}O_5$)	414	5
M^+—CH_2CO	372	22
M^+—CH_2CO—H_2O	354	23
M^+—CH_2CO—H_2O—CH_2CO	312	5
M^+—CH_2CO—H_2O—CH_2CO—CH_3	297	11
	231	14
	73	70
$(CH_3COOCH_2)^+$	43	100
$(CH_3CO)^+$		

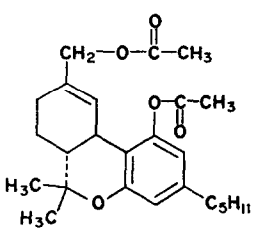


FIG. 3. The supposed structure of diacetyl-Δ^9THC ($C_{25}H_{34}O_5$). The structure has not been confirmed by n.m.r.

Blood

Four groups of four rats each were injected i.p. with 50, 75, 80 and 135 mg Δ^9THC/kg. Blood samples (2–8 ml) were taken from heart (group 1), tail (group 2), decapitated rats (groups 3 and 4) after 0·5, 1, 2, 4, 12, 24 and 48 hr. The whole blood samples, to which 3·5% sodium citrate as anticoagulant had been added, were saturated with NaCl and extracted at pH 7 with ether and *n*-butanol, and at pH 3 with ether. A portion of each extract was directly gas chromatographed. Another portion of the extract was silanized and GC analysed. The blood samples were then incubated with glucuronidase, extracted with ether at pH 3, silanized, and GC analysed. The GC spectra of these samples were identical to those of the blanks.

All samples were further analysed by GC–MS, but no Δ⁹THC or its metabolites were found. The extracts were then analysed by MF focusing on mass numbers characteristic for Δ⁹THC (m/e = 314, 313, 231), for a monohydroxylated Δ⁹THC (m/e = 330, 313, 231) (26), and for an *O*-methylated metabolite (m/e = 328), but none of these fragments were found, although the detection limit for Δ⁹THC by MF is about 20 pg.

DISCUSSION

Following glucuronidase treatment and extraction of urine at pH 3, an amount of dihydroxy-Δ⁹THC detectable by the TIC was found and a mass spectrum taken, whereas none of this metabolite was detected by GC–MS in the extract before treatment. The mass spectrum of this metabolite showed the same mass fragments reported for 8, 11-dihydroxy-Δ⁹THC found by Wall *et al.*[15,16] in *in vitro* rat liver homogenate. Wall *et al.*[16] and Ben-Zvi *et al.*[12] also determined the position of hydroxy groups in this compound by n.m.r. The yield of Δ⁹THC from urine was not significantly changed by glucuronidase treatment.

Glucuronidase treatment and extraction at pH 3 of feces resulted in CBN and an equal amount of Δ⁹THC being released. Δ⁹THC, but no CBN, had been detected by GC–MS previous to this treatment.

The substance isolated from bile, showing a mass spectrum similar to diacetyl-Δ⁹ THC, probably has one acetyl group substituted for the hydroxy group at position 1. The appearance of m/e = 43 as the base peak at 70 eV may represent the acetylium ion ($CH_3C{\equiv}O+$) characteristic of phenolic acetates.[27] Lack of NMR data excludes definite position assignment of the other acetyl group. The absence of unchanged Δ⁹THC in bile could be due to the formation of a conjugate polymer, a form which could not be detected by GC. King *et al.*[28] mention that 44% of i.p. administered Δ⁹THC in rats is excreted in the bile as such a conjugate of Δ⁹THC. Whether such conjugated polymers, if present, could be cleaved by glucuronidase was not determined, because of the small amount of samples.

That we were unable to detect any Δ⁹THC or its metabolites in the whole blood extracts, even after glucuronidase treatment, may be due to the fact that these substances are bound to blood lipoproteins as demonstrated by Wahlquist *et al.*[18] By electrophoresis they found 80–95 per cent of the labeled Δ⁹THC-^{3}H associated with lipoproteins. Until now we have not found a method for splitting such complexes.

The next step in our work will be the elucidation of the metabolic processes reported here, in man. The main difficulty in identification of metabolites in man will be the much lower doses of Δ⁹THC permitted to produce psychotomimetic effects.

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