II.2.2 Cannabinoids and their metabolites

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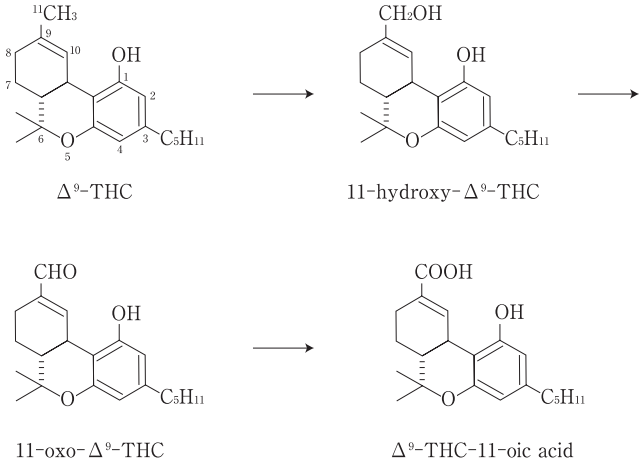
Introduction

The plant *Cannabis sativa* L. has a long history for human being since about BC 2000 for its use as fiber material, food and folk medicine; cannabis (hemp, marijuana) means the whole plant itself and its dried products except for its stem and seeds. The word "hashish" is mainly used for the resin of the cannabis plant. Since the main component of cannabis, Δ^9-tetrahydrocannabinol (Δ^9-THC), has various psychopharmacological effects including hallucination, the cannabis and its components are being controlled under the Cannabis Control Law and the Narcotics Control Law in Japan. For such legal control, analysis of cannabis components and their metabolites is required for plant specimens and human specimens.

The cannabis contains more than 60 analogous components with a C_{21} skeleton; they are comprehensively called "cannabinoids". The main cannabinoids are Δ^9-THC, cannabidiol (CBD) and cannabinol (CBN). The major metabolic pathway of Δ^9-THC is shown in *Fig. 2.1*; the methyl group in the 11-position is oxidized to produce Δ^9-THC-11-oic acid to be excreted into urine [1, 2]. To diagnose the abuse of cannabis or its component, the analysis of Δ^9-THC-11-oic acid in urine is essential. In this chapter, a GC method for analysis of cannabis components in plant specimens and a GC/MS method for Δ^9-THC-11-oic acid in urine are presented.

Figure 2.1



Major metabolic pathway of Δ^9-THC.

GC analysis of cannabinoids in plant specimens[a]

Reagents and their preparation

- Extraction and purification of cannabinoids [3]:
  Cannabis sativa L. is being grown at Hokuriku University, Faculty of Pharmaceutical Sciences with permission from the Government. The dried plant is minced and immersed in 20 volumes of methanol for 2 days for extraction; this procedure is repeated once. The combined methanolic extracts are evaporated to dryness. The residue is decarboxylated by heating at 140 °C for 20 min. The treated residue is applied to a 20 volumes (against the weight of the plant) of florisil and eluted with benzene for partial purification to remove chlorophyl. Finally, column chromatography with 50 volumes of silica gel is performed using benzene/*n*-hexane/diethylamine (25:10:1, v/v) as eluting solvent for getting each cannabinoid standard.
- Δ^9-THC, CBD and CBN are separately dissolved in ethanol to prepare 0.05–0.5 mg/mL standard solution for each compound[b].
- 5α-Cholestane (Sigma, St. Louis, MO, USA) is dissolved in ethanol to prepare a 0.5 mg/mL solution for use as internal standard (IS).

GC conditions

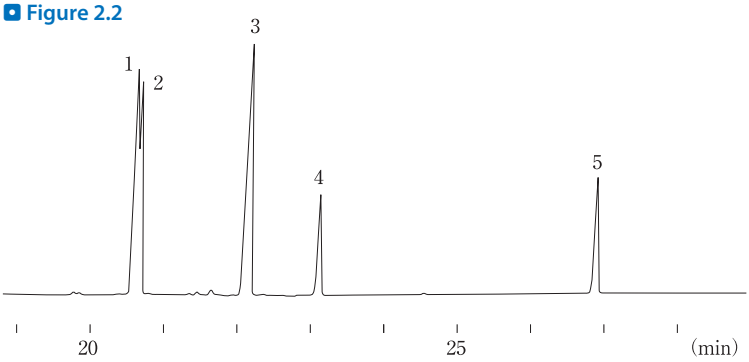
GC column [4]: a fused silica capillary column (slightly polar), HP-5MS (30 m × 0.25 mm i. d., film thickness 0.25 μm, Agilent Technologies, Palo Alto, CA, USA); MDN-5S (30 m× 0.25 mm i. d., film thickness 0.25 μm, Supelco, Bellefonte, PA, USA).

GC conditions; an Autosystem XL (Perkin Elmer, Wellesley, MA, USA) and an FID were used. Column (oven) temperature: 50°C (2 min) →20 °C/min→200 °C(0.5 min)→5 °C/min→ 300 °C(5 min); injection temperature: 250 °C[c]; carrier gas (flow rate): He (1 mL/min); FID gas (flow rate): air (400 mL/min) and H_2 (40 mL/min); make-up gas (flow rate): N_2 (30 mL/min); injection volume: 1 μL (split ratio 1/50).

Procedure

i. A dried specimen is minced and extracted with 10 volumes of methanol with stirring for 10 min at room temperature.
ii. The above methanol extract is condensed or diluted to an appropriate amount and mixed with a fixed amount of 5α-cholestane (IS).
iii. A 1-μL aliquot of the above extract is injected into GC for qualitative analysis and for quantitation using the below calibration curve.
iv. Construction of a calibration curve: the standard solutions at 0.05–0.5 mg/mL mixed with a fixed amount of IS each are prepared for each cannabinoid, and a 1-μL aliquot of each solution is injected into GC to construct a calibration curve with cannabinoid concentration on the horizontal axis and with peak area ratio of a cannabinoid to IS on the vertical axis.

Figure 2.2



Gas chromatogram for cannabinoids. 1: CBD; 2: CBC; 3: Δ^9-THC; 4: CBN; 5: 5α-cholestane (IS).

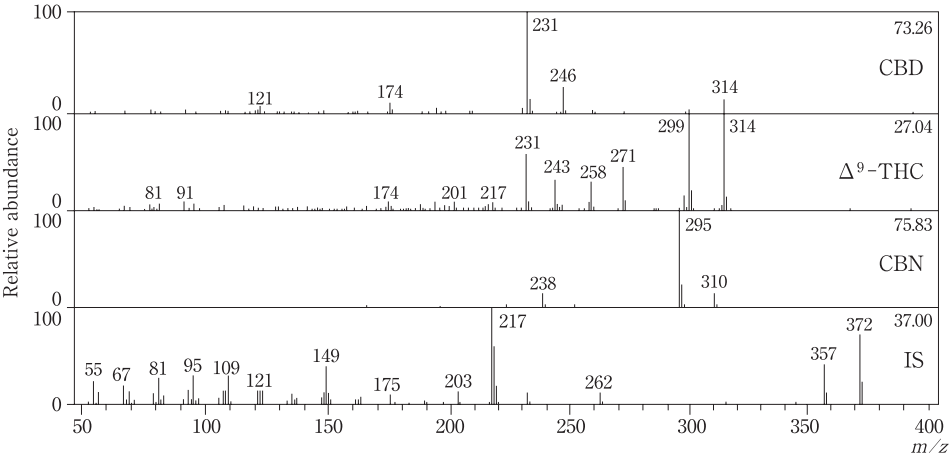
Assessment and some comments on the method

Figure 2.2 shows a gas chromatogram for Δ^9-THC, CBD, CBN and cannabichromene (CBC) obtained under the GC conditions. The peaks of Δ^9-THC (22.2 min), CBD (20.6 min) and CBN (23.2 min) are separated well; but the peak of CBC may overlap that of CBD.

When the dried cannabis is analyzed, the peaks of cannabinoids are not interfered with by impurity peaks in background, because the total concentration of cannabinoids in the dry specimen is as high as 0.5 %. For the specimen which had been stored for a long time, Δ^9-THC and CBD are converted into CBN by oxidation reaction, resulting in relatively high concentrations of CBN [5]. For discrimination of seeds, a GC method for cannabinoids with benzene extraction was reported [6].

The confirmatory test for cannabinoids should be made by GC/MS. For this purpose, the mass spectra of Δ^9-THC, CBD, CBN and 5α-cholestane (IS) are shown in *Fig. 2.3*.

Figure 2.3



EI mass spectra of Δ^9-THC, CBD, CBN and 5α-cholestane.

GC/MS analysis of Δ^9-THC-11-oic acid in urine [d]

Reagents and their preparation

- Δ^9-THC-11-oic acid can be synthesized by the method of Pitt et al. [7]. The author et al. are using the compound supplied by the National Institute on Drug Abuse in USA. The authentic compound is dissolved in ethanol to prepare 0.1 mg/mL solution. IR, γ^{CHCl_3} 1,680 cm^{-1}; NMR (CD_3COCD_3) δ: 1.08, 1.39 (s, 3H × 2, gem-CH_3), 3.38 (d, C_{10a}-H), 6.15, 6.30 (s, 1H × 2, aromatic-H), 8.10 (m, 1H, C_{10}-H); MS, *m/z* 344 (M^+).
- 5′-Nor-Δ^8–THC-4′-oic acid was synthesized by the method of Ohlsson et al. [8]. The compound is dissolved in ethanol to prepare 0.1 mg/mL solution. IR, γ^{CHCl_3} 1,710 cm^{-1}; NMR ($CDCl_3$) δ: 1.10, 1.38 (s, 3H × 2, gem-CH_3), 1.70 (s, 3H, C_9-CH_3), 3.22 (dd, 1H C_{10a}-H), 5.42 (m, 1H, C_8-H), 6.07, 6.24 (s, 1H × 2, aromatic-H); MS, *m/z* 330 (M^+).
- The solution of 5α-cholestane (IS) is also prepared with the same procedure as those of the above two compounds.

GC/MS conditions

Analysis with a packed column [9]; column: 5 % SE–30 (2 m × 2 mm i. d.); GC/MS: a JEOL GCG-06 gas chromatograph connected with a JEOL JMS-DX300 mass spectrometer (JEOL, Tokyo, Japan); column (oven) temperature: 250 °C; injection temperature: 270 °C; carrier gas: He; its flow rate: 40 mL/min; electron energy: 70 eV.

Analysis with a capillary column [10]; column: DB-5 or DB-1 (30 m × 0.25 mm i. d., J & W Scientific, Folsom, CA, USA); GC/MS: a Varian Model 3500 gas chromatograph (Varian, Harbor City, CA, USA) connected with a Finnigan MAT ITS 40 GC/MS system (Thermo-Finnigan, San Jose, CA, USA); column temperature: 180 °C→20 °C/min→280 °C; injection temperature: 300 °C; carrier gas: He; its flow rate: 1 mL/min; electron energy: 70 eV.

Procedures

i. Liquid-liquid extraction

i. A 10-mL volume of urine and 10 mL of 10 M NaOH solution are placed in a glass centrifuge tube with a ground-in stopper [e] and heated at 50 °C for 15 min in a water bath for hydrolysis [f].
ii. After cooling to room temperature, 2 mL of 1 M potassium dihydrogen- phosphate solution is added to the mixture and its pH is adjusted to 2–3 by adding hydrochloric acid.
iii. A 20-mL volume of *n*-hexane/ethyl acetate (7:1) is added to the mixture, shaken for 10 min and centrifuged [9].
iv. The organic phase is carefully transferred to another centrifuge tube of the same type, mixed with 5 mL of 0.5 M NaOH solution, shaken for 10 min and centrifuged at 3,000 rpm for 5 min.
v. The organic phase is discarded by aspiration; 2 mL of 1 M potassium dihydrogenphosphate solution is added to the aqueous phase and its pH is adjusted to 2–3 with hydrochloric acid.

A 20-mL volume of *n*-hexane/ethyl acetate (7:1) is added to the above solution to extract Δ^9-THC-11-oic acid again[g].

vi. The organic phase is dehydrated with anhydrous sodium sulfate, passed through filter paper and evaporated to dryness.

ii. Solid-phase extraction [10]

i. A 2-mL volume of the hydrolyzed urine obtained after step i) of the above procedure is mixed with a fixed amount of IS[h], and poured into a Bond Elut Certify II column (Varian, Harbor City, CA, USA), which has been activated by passing 2 mL methanol and 2 mL water, at a flow rate of about 1 mL/min [10].

ii. The column is washed with 9 mL of 50 mM phosphoric acid solution and 3 mL of 50 mM phosphoric acid solution/methanol (4:1).

iii. After drying the column under reduced pressure, the target compound and IS are eluted with 2 mL of *n*-hexane/ethyl acetate (4:1) containing 1 % acetic acid and evaporated to dryness under a stream of nitrogen.

iii. Derivatization-1

One of the above dry residues is dissolved in 10 µL acetonitrile, 15 µL of *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and 5 µL trimethylchlorosilane (TMCS), and heated at 60 °C for 20 min. After cooling, a 1-µL aliquot of it is injected into GC/MS.

iv. Derivatization-2

One of the above dry residues is dissolved in 2 mL of solution of diazomethane in ethyl ether[i], left at room temperature for 30 min and evaporated to dryness. The residue is then subjected to the above Derivatization-1 for trimethylsilylation to obtain methyl ester plus TMS derivatives. A 1-µL of the solution is subjected to the GC/MS analysis.

v. Quantitation

By using various amounts of Δ^9-THC-11-oic acid and an fixed amount each of IS, which had both been spiked into blank urine, a calibration curve is constructed for quantitation of the acid in a test urine specimen.

Assessment and some comments on the method

Jones et al. [11] reported that Δ^9-THC-11-oic acid was stable during storage at –18 °C for 2 months. The author et al. confirmed that the compound did not change at –20 °C for at least 2 months.

Δ^9-THC-11-oic acid is known to be decarboxylated at high temperatures; it, therefore, is necessary to be derivatized for GC/MS analysis. Baker et al. [12] examined various derivatizations and reported that the methyl ester plus TMS derivative of the acid gave the highest sensitivity, although the two-step derivatization procedure is required.

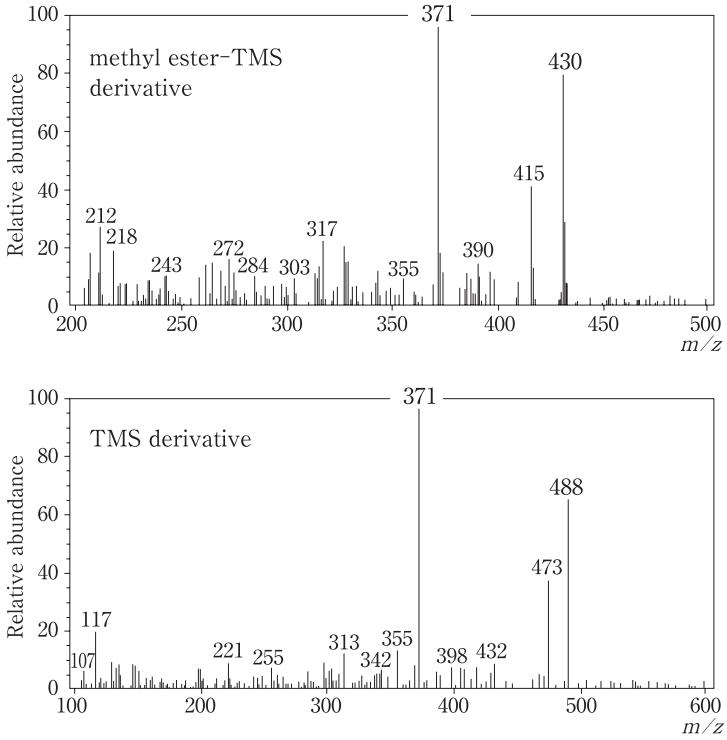
Table 2.1 shows major ions of mass spectra for Δ^9-THC-11-oic acid, 5α-cholestane and 5'-nor-Δ^8-THC-4'-oic acid obtained by both derivatization methods. For the Δ^9-THC-11-oic acid, the base peak appeared at *m/z* 371 and is most suitable for quantitation by SIM. *Figure 2.4* shows mass spectra of Δ^9-THC-11-oic acid after the TMS and methyl ester-TMS derivatizations.

Table 2.1

Diagnostic ions in mass spectra of Δ^9-THC-11-ioc acid, 5α-cholestane and 5′-nor-Δ^8-THC-4′-ioc acid with different derivatizations

	Diagnostic ion (*m/z*)		
	Δ^9-THC-11-ioc acid	5α-cholestane	5′-nor-Δ^8-THC-4′-ioc acid
Derivatization-1	488, 473, 371	372	474
Derivatization-2	430, 415, 371	372	416, 333

Figure 2.4



Mass spectra of the methyl ester plus TMS and TMS derivatives of Δ^9-THC-11-oic acid.

The Substance Abuse and Mental Service Administration (SAMSA) in USA set the cutoff value of Δ^9-THC-11-oic acid in urine at 50 ng/mL by immunoassay and the identifiable value of the acid by mass spectral measurements at 15 ng/mL.

Cases of analysis [13]

Case 1: A 22-year-old male was killed by a mysterious accident during driving a truck. Because of the high severity of injuries, blood specimens could not be obtained; the analysis was made

with urine and the liver tissue. Urine showed a positive result for the cannabinoid metabolite by screening with EMIT. The concentrations of Δ^9-THC-11-oic acid measured by GC/MS were 22 ng/mL in urine and 0.6 ng/g in the liver.

Case 2: A paper factory worker was killed by being caught in a machine during working with it. The concentrations of Δ^9-THC-11-oic acid measured by GC/MS were 78 and 12 ng/mL in urine and blood, respectively; 0.7 ng/mL of Δ^9-THC was also detected from blood.

Case 3: A male died of gunshot injury. His death was estimated to be suicidal or accidental under the influence of a drug. Δ^9-THC at 0.4 ng/mL and Δ^9-THC-11-oic acid at 40 ng/mL were detected from his blood.

Case 4: The author et al. [9] also analyzed a urine specimen of a subject, who had been suspected of cannabis smoking, and found 60 ng/mL of Δ^9-THC-11-oic acid in it.

Notes

a) For the cannabis plant and its resin, established methods are available for their chemical diagnosis [14].
b) The cannabinoids dissolved in ethanol are very stable at –20 °C, and a long storage is possible under the conditions.
c) In a fresh cannabis plant, cannabinoids exist in the carboxylated form. They are easily decarboxylated at 250 °C.
d) There are reviews dealing with GC/MS analysis of Δ^9-THC-11-oic acid in human urine [15, 16].
e) A method of silylation of glassware to be done before analysis was reported [17]; such treatment suppresses the adsorption of cannabinoids to the surface and enhances reproducibility of analysis.
f) Since Δ^9-THC-11-oic acid is excreted into human urine in the form of its glucuronide, it should be hydrolyzed before extraction. A hydrolysis procedure using β-glucuronidase can be used; but the hydrolysis with NaOH is simple and sufficient to be used.
g) By the repeated extraction, the background levels are lowered and interfering impurity peaks become much less; clean and distinct mass spectra can be obtained after the treatments. Since 5α-cholestane cannot be recovered with this extraction procedure; it cannot be used as IS; the external calibration method should be used for quantitation.
h) As ISs for quantitation by SIM, stable isotopic compounds of Δ^9-THC-11-oic acid are most preferable; usually d_3- and d_{10}- substitution products of the acid are used. When Δ^9-THC-11-oic acid-d_3 is used, the mass spectrum shows ions at *m/z* 433, 418 and 374 with the Derivatization-2, and at *m/z* 491, 476 and 374 with the Derivatization-1. Since the stable isotopic IS interferes with the non-isotopic Δ^9-THC-11-oic acid in mass spectral measurements, the ISs such as 5α-cholestane and 5'-nor-Δ^8-THC-4′-oic acid become useful.
i) The solution of diazomethane in ethyl ether can be prepared from 1-methyl-3-nitro-1-nitrosoguanidine using the Diazald kit (Aldrich, Milwaukee, WI, USA).

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