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Detection of Urinary *Cannabis* Metabolites: A Preliminary Investigation

Cannabis sativa, in the form of marihuana or hashish, is perhaps the most widely used illicit psychoactive drug in the world. Controversy about its pharmacological effects and potential for harm has prompted a flood of research in the past decade [*1–3*]. In spite of this concentrated research effort the metabolism of the active constituents of *Cannabis* is incompletely elucidated, and analytical methods to supply hard chemical information about marihuana ingestion for both forensic and pharmacological purposes are woefully lacking.

Background

The most active constituent of *Cannabis* is *delta*-9-tetrahydrocannabinol (Δ^9-THC) (see Fig. 1 for formal numbering), although the plant material contains numerous other cannabinoids and natural products [*2*]. *Delta*-9-THC has proved to be a very potent psychoactive agent which requires a dose of only 15 to 25 μg/kg intravenously (iv) in humans to achieve an euphoric effect and marked physiological responses such as tachycardia [*4,5*]. This drug is rapidly redistributed and metabolized so that peak concentrations of 50 to 100 ng/ml in human plasma quickly decrease to levels of no more than a few nanograms per millilitre after 1 h [*6,7*]. Metabolites also appear in the plasma shortly after ingestion or administration of Δ^9-THC and persist at low concentrations for several days in a manner analogous to the parent compound [*8–10*]. Figure 1 indicates the known [*11*] and hypothesized [*12*] metabolic transformations of Δ^9-THC in humans. Several of the monohydroxy metabolites, especially 11-OH-Δ^9-THC, show marked physiological activity and duplicate or exceed the effects of the parent compound [*4,5*]. There is also evidence that the major cannabinoid components of marihuana, cannabidiol (CBD) and cannabinol (CBN), are not as extensively metabolized as Δ^9-THC [*13*].

Studies employing radiolabeled Δ^9-THC in both chronic and naive marihuana users indicated that about 70% of the total radioactivity was excreted in the urine and feces during the first week [*4,8–10*]. Of this amount 22% was excreted in the urine of non-users as compared to 31% in the urine of chronic users. In both groups almost half the total urinary excretion occurred within the first 24 h. Unmetabolized Δ^9-THC accounted for less than 1% of the urinary radioactivity [*10*]; indeed, more rigorous mass spectral methods indicated that only 0.005 to 0.01% unchanged Δ^9-THC is excreted by humans

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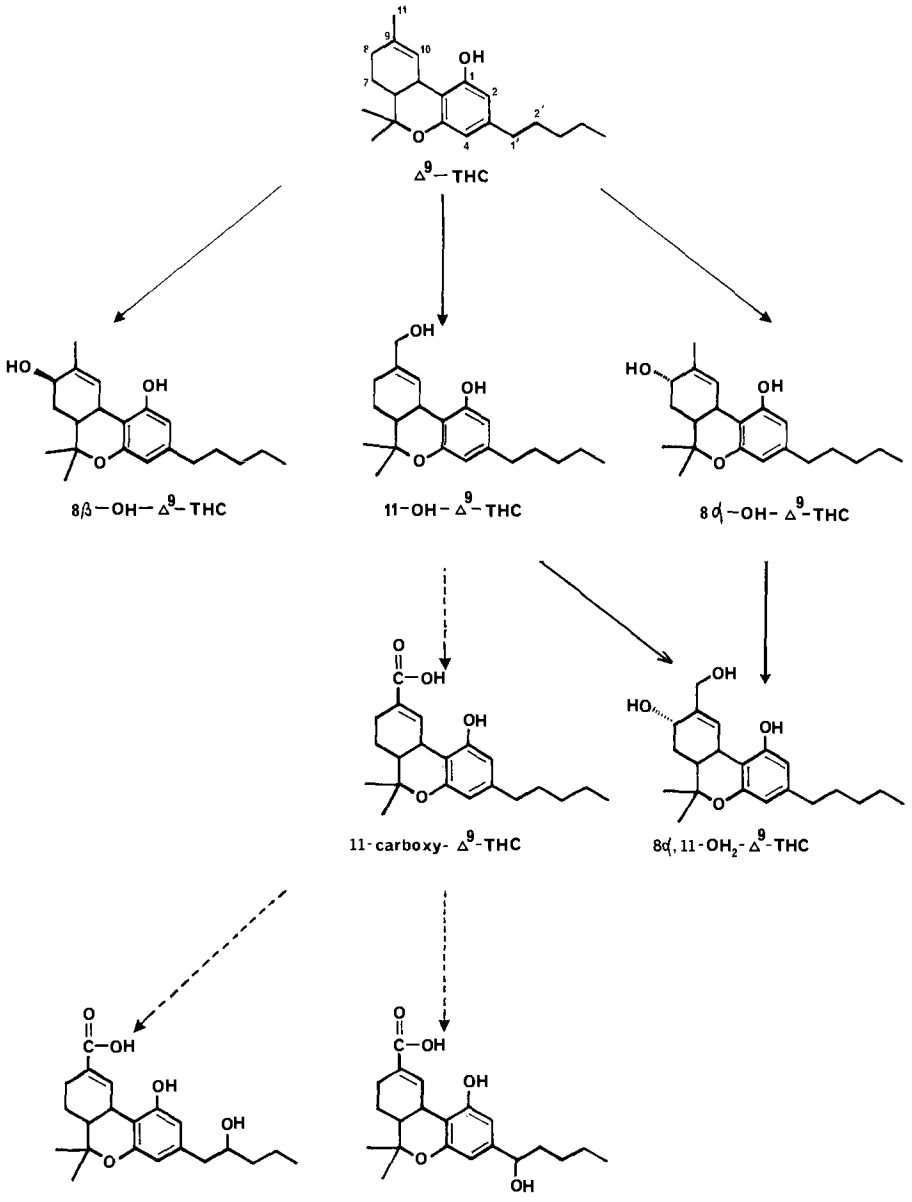


FIG. 1—*Metabolic transformations of* Δ⁹*-tetrahydrocannabinol (THC). Solid arrows represent pathways to known human metabolites and dotted arrows, to probable human metabolites.*

[*14*]. In view of this information urinalysis for THC metabolites seems to hold the most promise for determination of recent *Cannabis* use. In addition, urine is quite readily obtained; and since the level of any specific cannabinoid can be expected to be quite low, collecting large volumes is a feasible alternative. With this in mind, we approached the problem of detecting *Cannabis* use.

Experimental

Delta-9-THC, Lot SSC 75518, was obtained from the National Institute of Mental Health (NIMH) as an ethanol solution with a concentration of 10 mg/ml and an assay of 96% Δ^9-THC. Lot SIR-2151 (assay 96%) of 11-OH-Δ^9-THC was obtained from Dr. C. G. Pitt, Chemistry and Life Sciences Division, Research Triangle Institute, Research Triangle Park, N.C., via NIMH. Glusulase®, Lot FJ182A, was purchased from Endo Laboratories, Garden City, N.Y. This preparation is a mixture of β-glucuronidase and sulfatase with an activity of 177 and 99 units per μl, respectively, at optimum pH and 37.5 °C (99.5 °F). All other reagents were of analytical grade and were used without further purification unless noted.

Glassware was thoroughly cleaned by soaking in a concentrated sulfuric acid bath and subsequently rinsing with copious amounts of distilled water before drying at 110 °C (230 °F). All the glassware employed in the extraction procedure was silanized[2] with a 5% v/v solution of dimethyldichlorosilane in toluene. The silanized glassware was then rinsed with toluene and methanol and dried before use.

Blank urines were obtained from normal adult males, aged 22 to 28 years, who indicated no previous use of *Cannabis* or any recent (within 48 h) use of medication or other drugs. These urines were always obtained fresh and used immediately. Sample urines were obtained anonymously from male individuals who professed recent (within 15 h) *Cannabis* use. A 400-ml volume of urine (1.35 mg creatinine/ml) collected 3 to 4 h after initiation of marihuana smoking was available from a 25-year-old male who had smoked marihuana of an unknown strength and composition. Lesser volumes (9.8 to 15.3 ml) of urine were available from four males, aged 19 to 25 years, who had used marihuana or hashish the previous evening. No information was available about the ingestion of other drugs or therapeutic agents for the above sample urines. Because of lack of control over generation and receipt of these samples, the urines were not further characterized in this preliminary study. Sample urines were stored in polyethylene bottles on receipt and frozen until use.

Aliquots of urine (10 to 20 ml) were hydrolyzed in a 50-ml Erlenmeyer flask. The urine was buffered to pH 4.6 with 0.2 ml of 0.1*M* acetate buffer/ml and 60 μl of Glusulase® was added to each sample. Buffered urines were incubated aerobically for 16 h at 37 °C (98.6 °F) with shaking or stirring (see Fig. 2).

Sample urines were then adjusted to pH 4.6, if necessary after hydrolysis, and saturated with ammonium sulfate. The urine was repetitively extracted with 15 ml, 10 ml, and 10 ml of either 2% *iso*-butanol (*i*-BuOH)/$CHCl_3$ (v/v) or 3% *i*-BuOH/heptane (v/v). Each aliquot of solvent was vigorously shaken or vortexed for 3 min, after which the sample was centrifuged to separate the aqueous and organic layers. The organic layers were combined and evaporated to dryness at room temperature under a stream of prepurified nitrogen (N_2). The residue was dissolved in 50 μl of absolute ethanol, and suitable aliquots were analyzed directly or after derivatization to the corresponding O-trimethylsilyl ethers by gas chromatography (GC) or combined gas chromatography-mass spectrometry (GC-MS) for cannabinoids. A recovery of 73 $\pm$ 8% (n = 6) was obtained

[2] Silanization is the process of substituting a trimethylsilyl group, $-Si(CH_3)_3$, for any active hydrogens.

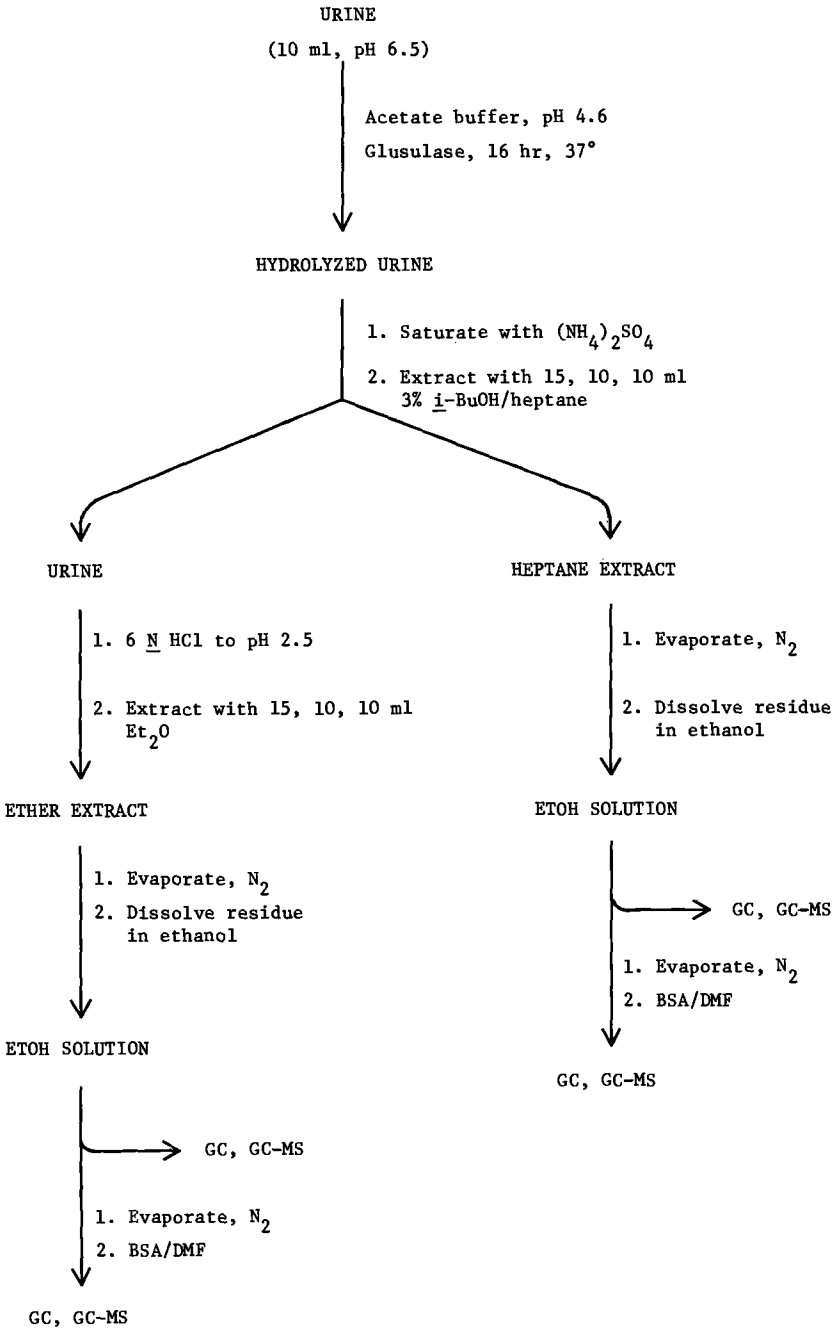


FIG. 2—*Scheme for extraction and analysis of* Cannabis *metabolites in urine.*

for urines to which 100 to 500 ng/ml 11-OH-Δ^9-THC was added before enzymatic hydrolysis. The urine that had been previously extracted at pH 4.6 was adjusted to pH 2.5 by dropwise addition of 6*N* HCl. The sample was then extracted with anhydrous diethyl ether in the same manner as above to isolate the more polar and more acidic metabolites.

Trimethylsilylation was conducted with a 1:1 mixture of silylation grade dimethylforamide and N,O-bis-(trimethylsilyl) acetamide (BSA, Pierce Chemical Co., Rockford, Ill.) for 10 min at room temperature. Suitable aliquots of the silylation mixture were analyzed by GC or GC-MS.

Authentic marihuana was obtained from samples submitted to this laboratory for analysis. A 75 to 150 mg portion of the gross sample (leaves, seeds, flowers) was exhaustively extracted with 15 ml 3% *i*-BuOH/heptane for 12 h in a Soxhlet extractor. The Soxhlet thimble was pre-extracted with the same solvent before use. The extract was evaporated to dryness under N_2 and dissolved in 1.00 ml absolute ethanol. Aliquots of this solution were taken directly for analysis or for silylation prior to analysis.

A Hewlett-Packard 7600 gas chromatograph equipped with a flame ionization detector and modified to accept either packed or high capacity capillary columns was used for GC analyses. A 1.9-m, 4-mm inside diameter (ID), glass column packed with either 3% SE-30 or 3% OV-17 on 100/120 mesh Chromosorb Q was operated isothermally at 210°C (410°F) with a helium carrier gas flow of 30 ml/min. High-resolution GC was conducted with a 78-m, 0.61-mm ID glass capillary column of SE-30 on Silanox® 101, which was operated isothermally at either 190 or 210°C (374 or 410°F). A helium carrier gas flow of 3.0 ml/min was used in conjunction with a helium makeup gas flow of 30 to 32 ml/min to ensure optimum flow through the detector. In both instances the injector port was maintained at 265°C (509°F) and the detector block at 275°C (527°F).

When the LKB 9000 GC-MS system was used, GC separations were accomplished with either a 1-m, 4-mm ID, glass column packed with 5% OV-1 on 60/80 mesh Chromosorb Q or a 58-m, 0.61-mm ID, glass capillary column of SE-30 on Silanox® 101. The packed column was operated isothermally at 220°C (428°F) with a helium carrier gas flow of 30 ml/min, while the capillary column was operated isothermally at 210°C (410°F) with a helium carrier gas flow of 3.0 ml/min. Efficient transmission of the sample into the mass spectrometer while using the capillary column required a helium makeup gas flow of 25 ml/min which was admitted at the end of the column but prior to the two-stage Ryhage jet separator. The following mass spectrometric operating conditions were employed: separator, 250°C (482°F); ion source, 190°C (374°F); ionizing current, 3.6 A; electron energy, 70 eV; accelerating voltage, 3200 V. In those analyses where selected ion monitoring was used, the mass spectrometer was focused for the designated ion of lowest mass and the accelerating voltage potentiometers adjusted for the other two masses. A maximum of three masses within 11% of each other could be monitored by this method of periodically alternating the accelerating voltage (AVA). Peak intensities were recorded on light-sensitive oscillograph paper.

Results and Discussion

Metabolic products of Δ^9-THC were expected to be present in only very low concentrations in body fluids. Accordingly, selected ion monitoring [*15,16*] with a GC-MS system was chosen as the means of analysis because of its sensitivity and specificity. Selected ion monitoring enhances the sensitivity of the mass spectrometer over that of its normal scanning mode, since one focuses only for a few specific and abundant ions of interest. Thus the mass spectrometer is adjusted to record a few peaks of high intensity rather than scan the entire mass spectrum. Specificity results from the choice of masses and the signal due to these ions simultaneously maximizing at the expected retention time. An additional check is the ratio of the abundances of the ions being monitored.

Cannabinol (CBN), a *Cannabis* constituent with an activity several times less than Δ^9-THC [*17*], illustrates the use of selected ion monitoring. This technique, of course, requires a knowledge of the fragmentation pattern of the compounds in question. The mass spectrum of CBN (Fig. 3a) shows that a large portion of the ion current is carried by the ion at a mass to charge ratio (*m/e*) 295 resulting from loss of a methyl group. Furthermore, the molecular ion (*m/e* 310) is also present with the ratio of *m/e* 295 to *m/e* 310 being 6.5:1.0. Thus a highly specific and very sensitive test for CBN would be to monitor these two masses during the course of a GC separation and note whether their signals maximize simultaneously with the correct ratio at the expected retention time. In the case of 11-OH-Δ^9-THC (Fig. 3b) the masses one would monitor are 299, 312, and 330. When trimethylsilyl (TMS) derivatives of these cannabinoids are used, masses 367 (M-15, 100%) and 382 (M+, 11.9%) are monitored for CBN-TMS and mass 371 (M-CH_2OTMS, 100%) is observed for 11-OH-Δ^9-THC-TMS_2. It should be pointed out that some of these masses are also indicative of other cannabinoids; for example, *m/e* 299 represents the M-15 peak for Δ^8-THC, Δ^9-THC, and CBD while *m/e* 371 represents the corresponding M-15 peak for the TMS ethers of these same compounds. Therefore, a judicious choice of two or three masses can allow one to determine the probable presence of several closely related compounds such as the parent drug and several of its metabolites.

An aliquot of marihuana extract containing CBN and a standard solution of 11-OH-Δ^9-THC were used to focus the mass spectrometer for the ions of interest. Figure 4a demonstrates how monitoring masses 295, 299, and 310 determines CBN and 11-OH-Δ^9-THC. Aliquots of the $CHCl_3$ and ether extracts of the hydrolyzed urine from a professed marihuana smoker taken 3 to 4 h after smoking were then subjected to analysis in the above manner (Fig. 4b). The presence of CBN is clearly indicated since both 295 and 310 maximize at the required retention time and possess an intensity ratio that corresponds to the standard. The presence of 11-OH-Δ^9-THC is also suggested, although the evidence is not conclusive from this one experiment. An additional analysis was performed to more conclusively identify this monohydroxylated metabolite of Δ^9-THC. Masses 299, 312, and 330 (see Fig. 3b) were monitored. Figure 5a shows how these masses locate the 11-OH-Δ^9-THC in the 2% *i*-BuOH/$CHCl_3$ extract of a spiked and enzymatically hydrolyzed blank urine, while Fig. 5b shows the corresponding results for a 2% *i*-BuOH/$CHCl_3$ extract of the same sample shown in Fig. 4b. Corresponding results were obtained for the trimethylsilylated extracts; and mass spectra, taken at the times in question, verified the presence of these two compounds even though background contamination was present.

Selected ion monitoring analysis of both the underivatized and trimethysilylated extracts of other urines also revealed the presence of both cannabinol and 11-OH-Δ^9-THC in each of the four cases where *Cannabis* use was indicated. These two cannabinoids have been previously identified, but never reported in combination, in the urine of human marihuana users by employing detection methods whose specificity and sensitivity has varied from thin-layer chromatography (TLC) of radiolabeled [*8–10,18*] and unlabeled [*13,19*] components to MS of positive spots eluted from a TLC plate [*20*]. To our knowledge this is the first instance where these two cannabinoids have been detected in urine in a single analysis or where GC-MS in a selected ion monitoring mode has been successfully employed for this purpose.

The limit of detection of both cannabinoids in this study was approximately 1 ng/ml urine, so smaller volumes of urine could also be used in contrast to previous studies [*19,20*]. Neither cannabinol nor 11-OH-Δ^9-THC was present in detectable amounts when unhydrolyzed sample urine was subjected to the above extraction and analysis procedure. This indicates, as has been previously noted [*19,20*], that both cannabinoids were excreted as urinary conjugates. No attempt was made to quantify either CBN or 11-OH-Δ^9-THC

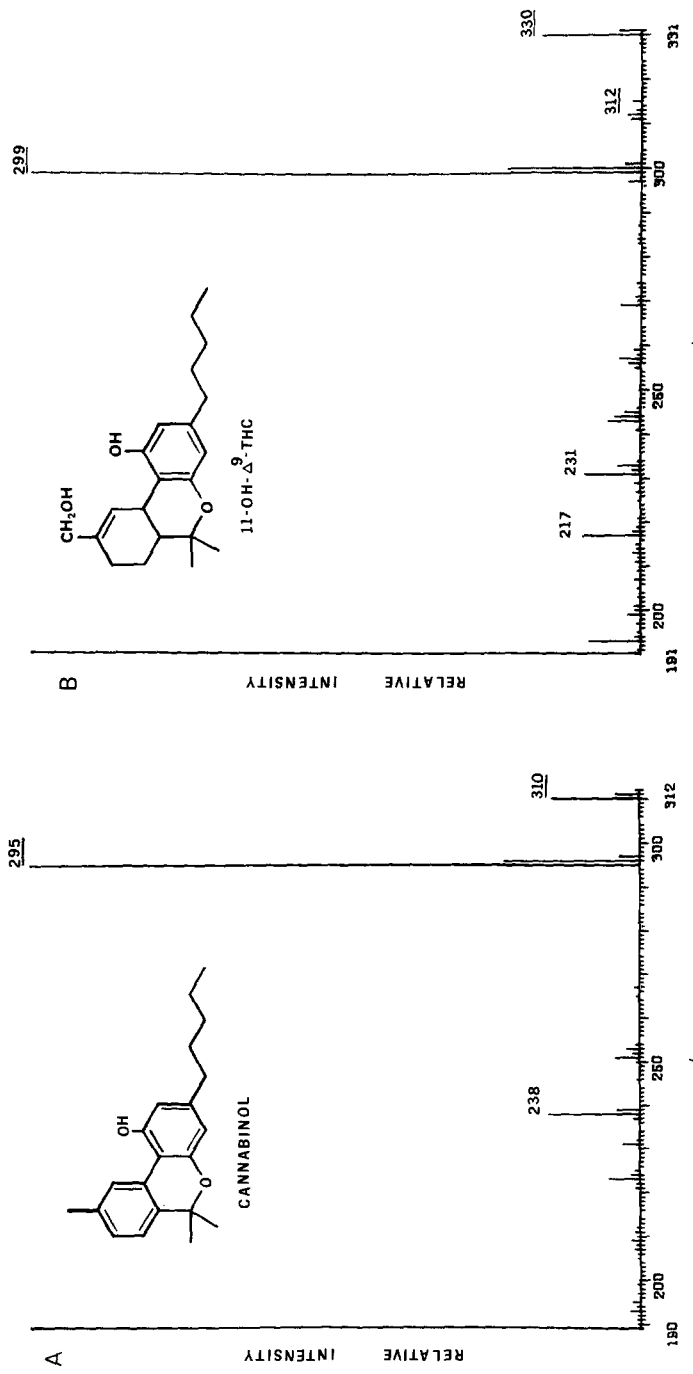


FIG. 3—*Mass spectrum of* (a) *cannabinol and* (b) *11-hydroxy-Δ⁹-tetrahydrocannabinol. No major peaks (relative intensity greater than 10%) occur below* m/e *190. The mass spectrometer is focussed for the underlined masses.*

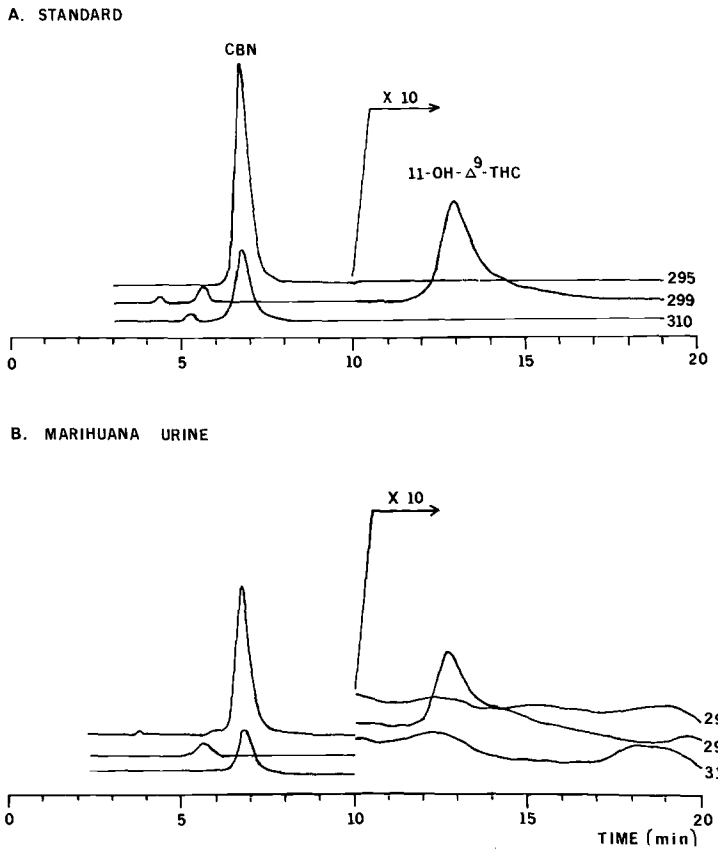


FIG. 4—*Selected ion current profile* [16] *of* (a) *cannabinol and 11-hydroxy-Δ⁹-tetrahydrocannabinol standards and* (b) *diethyl ether extract of hydrolyzed urine from a marihuana user. See text for chromatographic and mass spectrometric conditions.*

since suitable standards were not available. Both compounds were estimated to be present in low nanogram amounts (2 to 20 ng/ml) with the concentration of CBN much greater than that of 11-OH-Δ⁹-THC in all urines. Whether cannabinol is formed during the metabolism of Δ⁹-THC, as has been suggested [*21,22*], is open to speculation since it is a known major constituent of marihuana and may simply be excreted unchanged. Furthermore, either CBD or Δ⁹-THC may be partially dehydrogenated during the smoking process to produce additional amounts of CBN [*23*].

The only other analytical technique with the requisite sensitivity that has been applied to nonlabeled cannabinoid determination in physiological media is electron-capture GC [*21,24,25*]. In the case of cannabinoids a derivative amenable to electron-capture must be formed. This usually requires a chemical reaction of high yield and little specificity on a biological extract. Unless the extract has been subjected to an elaborate cleanup procedure, such as isolation of just a specific fraction via column or liquid chromatography [*6,25*], a high background caused by extraneous material is usually a problem. Since we expected little material and considerable variation in the chemical properties of the metabolites, the extraction and cleanup procedures were kept as simple as possible. The electron-capture approach also requires a knowledge of the gas chromatographic behavior of the compounds in question, and the certainty of identification is much less than selected ion monitoring since only one parameter—retention time—is available.

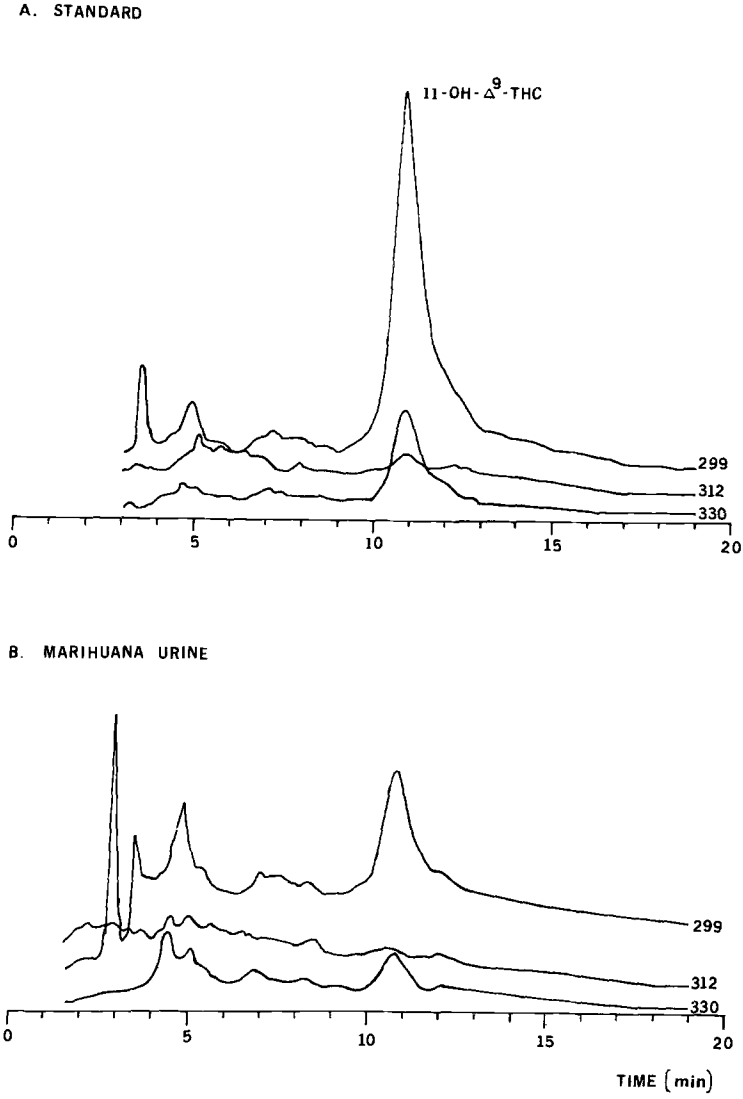
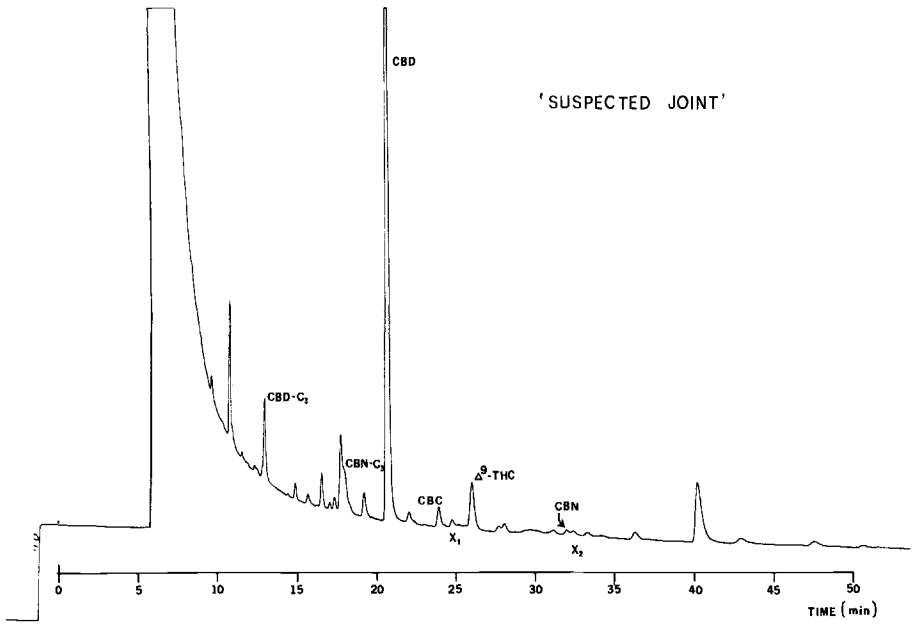


FIG. 5—*Selected ion current profile of* (a) *2% i-BuOH/CHCl₃ extract of blank urine spiked with 11-hydroxy-Δ⁹-tetrahydrocannabinol before enzymatic hydrolysis and* (b) *2% i-BuOH/CHCl₃ extract of hydrolyzed urine from a marihuana smoker.*

Prognosis

Preparations derived from *Cannabis sativa* are extremely complex mixtures of both active and inactive natural products whose effects may be highly variable depending on the method of administration and the composition of the original material. Figure 6, a high-resolution capillary column gas chromatogram of a trimethylsilylated marihuana extract, illustrates a case where cannabidiol is by far the major cannabinoid constituent. It would probably take a good deal of this marihuana to produce a noticeable effect since CBD is inactive [*17*], although it may be partially cyclized to Δ⁹-THC by smoking [*23*]. Thus a highly reliable indication of *Cannabis* use and intoxication could require an analysis for several cannabinoids or metabolites. Numerous researchers have also demon-



$CBD\text{-}C_3$ = cannabidivarin
$CBN\text{-}C_3$ = cannabivarin
CBD = cannabidiol
CBC = cannabichromene
X_1 = unidentified trimethylisilylated cannabinoid, MW = 386
Δ^9-THC = *delta*-9-tetrahydrocannabinol
CBN = cannabinol
X_2 = unidentified trimethylsilylated cannabinoid, MW = 386

FIG. 6—*High resolution capillary column gas chromatogram of a trimethylsilylated Soxhlet extract of a "suspected joint." Identifications were confirmed by GC-MS.*

strated that the bulk of urinary *Cannabis* metabolites may be very polar acidic compounds [*9,12,26-28*]. Elucidating the role these compounds play in marihuana metabolism and further determining a reliable indicator of *Cannabis* use are thus the goals of a more comprehensive, ongoing study.

Summary

Cannabinol and 11-OH-Δ^9-THC have been detected in the individual urines of five professed marihuana or hashish smokers. Both compounds exist primarily as urinary conjugates with the concentration of cannabinol being substantially greater than 11-OH-Δ^9-THC in all urines. These findings are discussed in light of present knowledge of Δ^9-THC metabolism and in view of current analytical procedures for the determination of Δ^9-THC and its metabolites in physiological fluids.

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