for nonionic surfactants, substituting Eq. A16 in Eqs. A17 and A18 yields:

$$\Sigma C_{iw} = C_{NaOH} + C_T - VC_m \quad \text{(Eq. A19)}$$

for ionic surfactants and:

$$\Sigma C_{iw} = C_T - VC_m \quad \text{(Eq. A20)}$$

for nonionic surfactants.

By substituting values for Z_s, C_{is}, ΣC_{im}, and ΣC_{iw}, Eq. A13 is reduced to:

$$Z_i F\phi_m = \frac{K_1 K_2 n C_m}{A_m\sqrt{C_T - VC_m}} \quad \text{(Eq. A21)}$$

for nonionic surfactants and:

$$Z_i F\phi_m = -\frac{K_1 n(1 + K_2 C_m)}{A_m\sqrt{C_{NaOH} + C_T - VC_m + C_s}} \quad \text{(Eq. A22)}$$

for the ionic surfactant. Equations A21 and A22 are equivalent to Eqs. 8 and 9.

Evaluation of C_m (Derivation of Eq. 10)—The value for C_m can be obtained as follows. Since the only ion likely to be solubilized is that of indomethacin, then $C_{im} = C_m$ and $C_{iw} = C_w$. When solving Eq. 7 for C_w:

$$C_w = \frac{C_m}{K' \exp(-Z_i F\phi_m/RT)} \quad \text{(Eq. A23)}$$

Equation A23 is equal to Eq. A15. Solving for C_m yields Eq. 10.

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High-Pressure Liquid Chromatographic–Mass Spectrometric Determination of Δ^9-Tetrahydrocannabinol in Human Plasma following Marijuana Smoking

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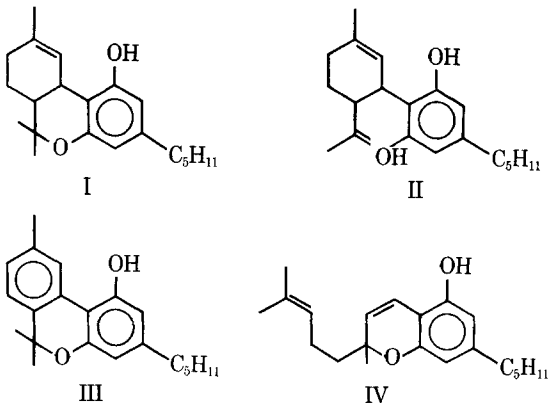
Abstract □ A method was developed for analyzing Δ^9-tetrahydrocannabinol (I), a psychotomimetic constituent found in marijuana smoke. The developed method utilizes a high-pressure liquid chromatographic (HPLC) gradient elution program to separate I from the other major cannabinoids in marijuana smoke. To achieve the sensitivity required to detect I in human plasma following marijuana smoking, a mass spectrometric quantification method was developed to analyze the HPLC eluant. To 1 ml of human plasma was added a known amount of internal standard, trideuterated I. This stable isotope provided a check on extraction efficiency, a marker for UV monitoring of the HPLC effluent and subsequent collection, and a convenient mass for mass spectrometric quantification. An ion-counting techique was used in conjunction with the peak matching accessory of the mass spectrometer to provide for a rapid comparison between molecular ions of I and the internal standard. The method was linear, accurate, and reproducible over the concentration range expected for I in plasma following marijuana smoking; 2.5 ng/ml was the lower practical limit of detection. Plasma from 11 male subjects was analyzed by the method at appropriate intervals up to 24 hr after the smoking of a marijuana cigarette containing 10.8 mg of I. Results demonstrated that levels of I could be determined accurately in the plasma of marijuana smokers in the 1-hr period following smoking.

Keyphrases □ Δ^9-Tetrahydrocannabinol—high-pressure liquid chromatographic–mass spectrometric analysis, human plasma after smoking marijuana □ High-pressure liquid chromatography–mass spectrometry—analysis, Δ^9-tetrahydrocannabinol, human plasma after smoking marijuana □ Marijuana constituents—Δ^9-tetrahydrocannabinol, high-pressure liquid chromatographic–mass spectrometric analysis, human plasma after smoking marijuana □ Psychotomimetic agents—Δ^9-tetrahydrocannabinol, high-pressure liquid chromatographic–mass spectrometric analysis, human plasma after smoking marijuana

Marijuana smoking is quite prevalent in certain segments of the populace in the United States (1). Most marijuana contains four principal constituents (2): Δ^9-tetrahydrocannabinol (I), cannabidiol (II), cannabinol (III), and cannabichromene (IV). Compound I is believed to be responsible for the psychotomimetic properties of marijuana (3). Some of the physiological responses in humans were shown to change following smoking of cigarettes



containing I, and these changes correlated with plasma levels of I and its metabolites (4). However, such studies were performed using radiolabeled I since no methods were available for plasma assays. More recently, methods such as GLC (5, 6), GLC–mass spectrometry (7, 8), and radioimmunoassay (9, 10) were proposed for the analysis of I in plasma.

The present method utilizes a high-pressure liquid chromatographic (HPLC)–mass spectrometric technique for determining nanogram per milliliter quantities of I in human plasma. Problems associated with direct coupling of the liquid chromatograph to the mass spectrometer (10) are circumvented. Fractions of the mobile phase emanating from the liquid chromatograph are collected and subsequently analyzed *via* the direct insertion probe of the mass spectrometer.

Inherent in this method is the use of a stable deuterated isotope of I for controlling extraction efficiency, as a marker for collection of the HPLC effluent, and as an internal standard for mass spectrometric quantification. Thus, the plasma has enough internal standard added to allow its detection by a UV spectrophotometer connected to the output of the liquid chromatograph. Once the internal standard is detected, fractions of the mobile phase eluant are collected and introduced *via* the direct insertion probe of the mass spectrometer for quantification.

This method was validated for I over a concentration range of 1–100 ng/ml in human plasma. Prior studies (4, 7–9) indicated that this range was expected for plasma levels of I following the smoking of cigarettes containing I. The new assay method was then used to determine plasma I levels in 11 male volunteers during 24 hr following the smoking of a marijuana cigarette.

EXPERIMENTAL

HPLC—All HPLC analyses were conducted on a gradient elution instrument[1] utilizing a recording spectrophotometer[2] set at 273.7 nm and a 10-μm silica gel, 25-cm × 2-mm (i.d.) column[3]. A gradient elution program was developed using heptane and methylene chloride. To separate the cannabinoids satisfactorily as well as to assure accurate mass spectrometric quantitation, it was necessary to record routinely the UV spectrum of each lot of heptane and methylene chloride prior to its use. Only lots that gave minimum absorbance in the 260–280-nm region were used.

A gradient elution program was used starting from 95:5% and proceeding to 5:95% heptane–methylene chloride over 9 min. The program was reversed, *i.e.*, from 5:95% to the initial 95:5% heptane–methylene chloride mixture, thereby regenerating the column. A solvent flow rate of 120 ml/hr was used for all determinations. Using these conditions, both I and its stable isotope, trideuterated I, had a retention time of 4.8 min or appeared at a gradient elution mixture of 53:48% methylene chloride–heptane. The other major constituents of marijuana, II, III, and IV, had retention times of 4.3, 4.5, and 5.6 min, respectively (Fig. 1).

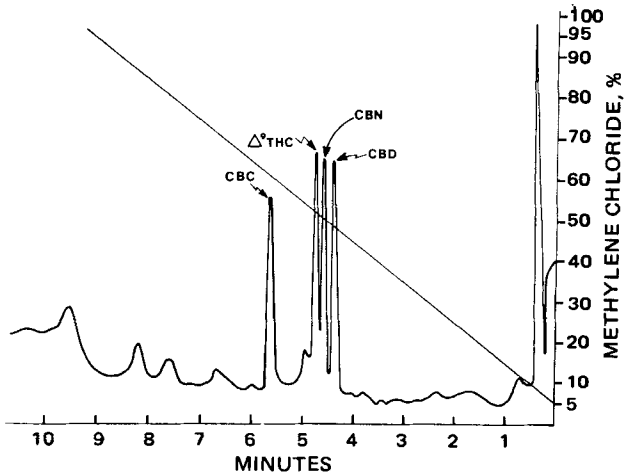


Figure 1—*HPLC analysis of pooled human plasma from nonsmokers to which 1.0 μg of II (CBD), 0.25 μg of III (CBN), 1.0 μg of I (Δ⁹-THC), and 0.25 μg of IV (CBC) were added.*

The amount of I-d_3 added to the plasma was sufficient to allow UV detection of total I (labeled plus unlabeled) as it eluted from the column. A 10-cm "zero dead volume" stainless steel tube was attached to the flowcell of the spectrophotometer to facilitate collection of the effluent droplets almost instantaneously after detection in the flowcell.

Mass Spectrometric Quantification—All mass spectrometric analyses were accomplished using a high-resolution, double-focusing mass spectrometer[4]. A new ion-counting technique was developed in conjunction with the peak matching accessory of the mass spectrometer, which provided for a rapid comparison between data from the molecular ion of the internal standard (*m/e* 317) and the assayed compound (*m/e* 314). Each sample coming from the liquid chromatograph was introduced into the mass spectrometer *via* the direct insertion probe, and the instrument initially was focused on *m/e* 317. Then, through the action of the peak matching unit and with a resolution of 2500, it was alternately focused to *m/e* 314.

As this alternation from *m/e* 317 to 314 occurred, the number of ion counts for each compound was recorded and stored in two registers of a dedicated computer[5]. This unit summed the number of ion events occurring in both *m/e* 314 and 317 and stored them in two registers (A and B). Thus, a running total of ions detected from the *m/e* 317 internal standard and the unknown amount of *m/e* 314 were stored separately by the counter. The peak matching accessory was set to dwell for 67 msec on each mass before alternating to the other mass. A time of 67 msec was required to sweep each mass peak across the electron multiplier.

A total counting time of 67 sec combined with a probe temperature of 65° gave optimum results. The probe temperature was programmed from ambient to 65° within 2 sec. Thus, in 67 sec, the alternating cycle was repeated 500 times, yielding 1000 bits of data for comparison and quantification. With time, the sample was depleted, but the ratio based on the internal standard remained linear and provided dependable quantification.

Linear plots were obtained from a computer-plotter[6] by using the data from Register B (*m/e* 314) for the ordinate and from Register A (*m/e* 317) for the abscissa and determining the least-squares best straight line. The slope of the line gives the *m/e* 314–317 ratio, which can be multiplied by the amount of internal standard added to get the concentration of I. In actual practice, the stable isotope compound used[7] contained a small amount of I. A mass spectrometric determination of the amount of *m/e* 314 in the internal standard was made by analyzing 10 samples of 1.6 μg of I-d_3 that had been added to 1 ml of plasma (Table I). This value for

[1] Varian 8520 liquid chromatograph.
[2] Varian 635M UV-visible spectrophotometer.
[3] Varian Si-10.
[4] Varian MAT model SM1-B.
[5] Princeton Applied Research, model SSR 1110.
[6] Hewlett-Packard model 9820A.
[7] Obtained from Research Triangle Institute through the National Institute on Drug Abuse. The trideuterated label was in the 11-position.

Table I—Precision and Accuracy in Recovery of I Added to Human Plasma

Added, ng/ml	Found[a], ng/ml	*n*	±*RSD*	*RE*
0	11.3 (9.35–13.6)[b]	10	11.15	—
1	1.2 (0.6–1.5)	3	43.3	20.0
5	4.5 (4.0–4.7)	3	9.0	−11.12
10	10.5 (9.5–12.4)	10	10.0	5.0
20	20.0 (19.1–21.1)	3	5.14	0.0
40	41.2 (39.6–42.5)	3	3.58	3.0
60	62.1 (61.3–62.8)	3	1.26	3.5
80	82.8 (74.9–90.7)	3	9.55	3.5
100	103.7 (99.3–106.0)	3	3.65	3.7
	Average percent recovered = 103.8			

[a] Average (range) of *n* determinations. [b] This value represents the amount of I found in 1.6 μg of the internal standard. Other values are corrected for this amount.

I in the internal standard was subtracted from the amount of I found in the assayed samples to give the actual found value.

Methods of Plasma Analysis—All glassware was silylated using a reported method (11). Whole blood was centrifuged at 2600 rpm for 20 min to obtain plasma. To 1 ml of plasma was added 1.6 μg of I-d_3 (0.5 ml of a 3.2-μg/ml solution in methanol) followed by extraction three times, using 2 ml of petroleum ether for each extraction. The extracts were combined and evaporated to dryness under nitrogen at room temperature. The resultant residue was reconstituted in 300 μl of heptane, and the entire solution was injected into the liquid chromatograph.

A 100-μl wash of heptane was used on the vessel containing the extracts; it was also injected into the chromatograph, and the gradient elution program previously described was used for separation. When the peak for combined I and I-d_3 was observed on the recorder, the eluant was collected in a silanized screw-capped vial[8]. Collection of the eluant was correlated with the recorder tracing so that equal amounts of the top one-half were obtained from both sides of the symmetrical peak. In general, approximately 0.5 ml of eluant was collected for each I-I-d_3 peak.

Samples thus collected from the chromatograph were stored at −5° until mass spectrometric analysis. Prior to analysis, each sample was evaporated under nitrogen at room temperature to approximately 25 μl. A microsyringe was used to transfer this solution in successive portions to a 5-μl gold cup. The solution was allowed to air evaporate, and the gold cup was placed into the direct insertion probe of the mass spectrometer. Quantification was accomplished as described.

Validity of Plasma Assay—Control blood samples from 10 laboratory workers known to be nonusers of marijuana were drawn and analyzed as described. Each sample contained some I, since the 1.6 μg of I-d_3 added to each plasma sample contained an average of 11.3 ng of I (Table I). To demonstrate the reproducibility and accuracy of the developed method, pooled human plasma samples were analyzed to which known amounts of I had been added (Table I). A plot of these data gave a linear curve whose intercept passed not through the origin but rather through a point corresponding to the 11.3 ng of I contained in the internal standard.

Marijuana Smoking Studies[9]—Eleven healthy male volunteers, 21–26 years old, were used. Each subject was within 10% of ideal body weight and received both medical and psychological examinations prior to the experiment. Values for the following tests were determined prior to the study, and each subject was required to be within the normal range: ECG, chest X-ray, creatinine, blood urea nitrogen, lactic dehydrogenase, serum glutamic–oxaloacetic transaminase, alkaline phosphatase, blood sugar, calcium, phosphorus, bilirubin, total protein, albumin, cholesterol, uric acid, hematocrit, hemoglobin, platelet count, and prothrombin time.

All subjects were moderate marijuana smokers. Each was requested not to smoke marijuana for 2 days prior to reporting for the study. The subjects were brought into the hospital ward 12 hr prior to smoking, and they were not allowed food or drink after 12:00 am of the study day. At approximately 8:00 am of the test day, each subject had a heparin-lock placed in a forearm vein and 5 ml of blood was withdrawn and placed in a silanized heparinized vacuum tube. Each blood sample was handled so that the blood did not come in contact with the rubber stopper.

All blood samples were immediately centrifuged at 2600 rpm, and the plasma was removed, placed in another silanized tube, and frozen for later analysis. This initial 5 ml of blood drawn prior to smoking constituted the 0-hr sample. Each subject smoked one marijuana cigarette[10], which contained 10.8 mg of I, 2.16 mg of III, 0.9 mg of IV, and 0.63 mg of II. Upon completion of smoking, timing was begun. Blood samples (5 ml) were withdrawn at 0.25, 0.5, 1, 2, 3, 4, 12, and 24 hr. Each blood sample was handled as described. Table II gives the blood I levels found for each subject.

Table II—Amount of I (Nanograms per Milliliter) Found in Human Plasma following Marijuana Smoking

	Hours								
Subject	0	0.25	0.5	1	2	3	4	12	24
D.L.	3.6	23.0	10.1	12.2	7.1	0.9	3.5	2.7	0
B.N.	1.2	39.0	11.7	3.8	0.9	0	0.7	0	1.7
B.B.	1.6	34.1	23.3	16.8	1.4	1.1	0	2.0	0.9
D.J.	0	66.7	19.7	11.8	3.0	3.8	4.3	0	3.1
F.R.	4.2	38.4	20.1	6.8	8.4	2.8	6.3	0.6	0.9
B.W.	0.4	57.9	24.8	14.8	10.7	2.4	6.2	5.8	2.5
W.Z.	1.2	39.7	19.7	6.8	5.3	4.9	2.0	0	0
R.B.	5.7	43.7	18.9	27.6	6.7	4.4	2.5	0.1	3.4
L.G.	7.6	15.3	39.9	8.1	2.1	11.1	7.5	9.7	2.5
T.V.	11.3	34.6	16.5	10.1	5.1	4.2	2.3	1.6	2.8
K.C.	4.7	20.6	22.4	14.5	8.4	7.7	4.7	5.0	2.5
Mean	3.8	37.5	20.7	12.1	5.2	3.9	3.6	2.5	1.9

RESULTS AND DISCUSSION

Analysis of trace substances in a biological system is often limited by the sensitivity and accuracy of the method employed. This was precisely the case with I, since prior literature reports documented the rather low levels of I occurring in blood following marijuana smoking. Therefore, in the present work, a technique was developed that took advantage of the resolving ability of HPLC and the sensitivity of mass spectrometry to quantitate very low levels of I in human plasma.

To achieve the needed accuracy and reproducibility, a stable isotopic form of I was added to the plasma to control extraction efficiency, to prevent losses during the chromatographic elution, as a marker for eluant collection, and as a convenient mass for mass spectrometric quantification. The HPLC program used permits a selective quantification of I, since the other cannabinoids with *m/e* 314 (II and IV) in marijuana are adequately resolved from I. In addition, the moderate resolution used in the mass spectrometric quantification prevents analysis of other satellite masses present due to constituents in the plasma also having an *m/e* close to 314.

Plasma was extracted three times to introduce to the mass spectrometer sufficient stable isotope for accurate quantification while not using excessive amounts of the isotope as the internal standard. The other important use of I-d_3 was to permit the total amount of I (both labeled and unlabeled) to be detectable by the UV spectrophotometer attached to the output of the liquid chromatograph. When using the described HPLC program and UV wavelength monitoring, a minimum of 100 ng/ml of I in plasma could be detected. This finding was in agreement with the reported (12) low absorptivity of I. However, as already discussed, the levels of I following marijuana smoking were known to be significantly lower than the 100-ng/ml levels detectable by HPLC–UV spectrophotometry. Thus, by using 1.6 μg of I-d_3 as the internal standard, a clearly detectable peak was observed on the resultant chromatogram.

The observed range for the determinations on control plasma with added I-d_3 was 9.35–13.6 ng/ml, with an average value of 11.3 ng/ml (Table I). Applying the Student distribution or *t* values to these data gave ±2.29 ng/ml for a 99% confidence limit. The other data given in Table I were obtained by adding a known amount of I as well as 1.6 μg of I-d_3 as the internal standard to pooled human plasma from nonsmokers of marijuana. Each value obtained from the known I addition experiments was corrected for the contribution from unlabeled material present in the internal standard—*viz.*, 11.3 ng/ml.

The reproducibility and accuracy were good for all levels of I added to plasma except the 1-ng/ml level. The lack of accuracy at this level was due to the overlap of confidence limits between the internal standard and this low level of added I. Although a precise determination of the lower limits of sensitivity was not made, 2.5 ng/ml apparently would be the lower limit of detection for the assay. Such a value would be above the

[8] Reacti-Vial, Pierce Chemical Co.

[9] All smoking studies were performed at the Marijuana Research Institute, School of Medicine, University of California–Los Angeles.

[10] Supplied by National Institute on Drug Abuse.

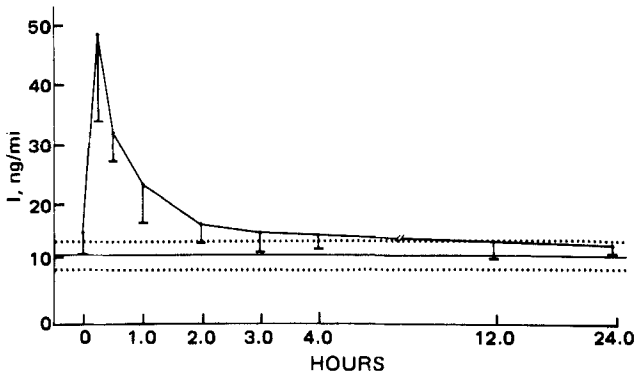


Figure 2—*Average plasma levels of I (Δ^9-THC) for 11 marijuana smokers (not corrected for contribution of I-d_3) and 10 nonsmokers. The solid horizontal line represents the average plasma level of I found in nonusers and is due to the impurity present in the internal standard, whereas the 99% confidence limits for this value are represented by the horizontal dotted lines. The vertical lines at each data point represent the lower half of the confidence limits for those 11 determinations made in the marijuana smokers at the appropriate time interval.*

99% confidence limits of I found as a contaminant in the internal standard.

Figure 2 is a plot of the average uncorrected plasma I levels of 11 male volunteers who smoked one marijuana cigarette of known I content. The maximum blood I level occurred at 0.25 hr. Also the I level in the control or 0-hr sample was positive. This fact can be explained on the basis of the type of subject used in the marijuana smoking studies. All subjects were moderate marijuana users. Each was confined 12 hr prior to smoking and was asked not to smoke marijuana for 2 days prior to the study. The analytical data obtained on the plasma of each subject indicated that only five individuals conformed to the established protocol.

The 99% confidence levels, as determined by the Student distribution (t-value) method, are shown by the vertical bars at each data point. The horizontal lines represent the average value (dark line) for 10 nonsmokers of marijuana as well as the 99% confidence limits (dotted lines). Wherever the confidence limits for data from both marijuana smokers and nonsmokers coalesce, a time period exists where marijuana use could not be assigned with 99% confidence. Thus, for up to 1 hr after marijuana smoking, the levels of I are clearly determinable and marijuana use can be assigned with 99% confidence using the present assay method.

The HPLC–mass spectrometric methodology described would be applicable to the study of other drug entities in biological fluids where very low detection levels are required along with precise specificity. However, for such determinations, the stable isotopic form of the drug is needed.

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Radioimmunoassay of Minoxidil in Human Serum

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Abstract □ A simple, sensitive, and specific radioimmunoassay for determining minoxidil was developed. Antiserums to two minoxidil haptens were compared for cross-reactivity and assay levels on human serums. One antiserum had little cross-reactivity with minoxidil metabolites. The radioimmunoassay is specific for determining minoxidil directly in serum without extraction. Human serum minoxidil levels were determined from a single oral dose.

Keyphrases □ Minoxidil—radioimmunoassay, human serum □ Radioimmunoassay—minoxidil in human serum □ Antihypertensives—minoxidil, radioimmunoassay in human serum

Minoxidil (2,4-diamino-6-piperidinopyrimidine 3-oxide) (I) is a potent hypotensive agent useful in the treatment of severe hypertension (1, 2). A sensitive assay was needed for determining serum I levels. Attempts with a GLC procedure indicated that sizable amounts of the several derivatives tried were lost on the column. High-pressure liquid chromatography (HPLC) would have adequate sensitivity for only the higher serum concentrations encountered at normal dosage levels of I. Chromatographic methods for I in serum also require extraction and a preliminary chromatographic cleanup step. Such procedures are tedious and time consuming. Radioimmunoassay, however, permits analysis of many samples without extraction and with adequate sensitivity.

Antiserums were developed in rabbits against two bovine serum albumin conjugates of the N-4-glutaryl (II) and