DOT HS-801 721

THE DEVELOPMENT OF TECHNOLOGY FOR DETECTION OF MARIJUANA INTOXICATION BY ANALYSIS OF BODY FLUIDS

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16. Abstract

A method employing high pressure liquid chromatography plus mass spectrometry was developed for the detection of low concentrations of various marijuana metabolites in body fluids. A new marijuana metabolite was found which could be detected in blood for twenty four hours after smoking.

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SUMMARY

A method employing high pressure liquid chromatography - mass spectrometry (hplc-ms) was developed for detecting trace amounts of the principal biologically active constituent (Δ^9-THC) in marijuana. The method was then successfully applied to detecting and quantitating Δ^9-THC which had been added to human blood plasma. This hplc-ms method was used to quantitate the levels of Δ^9-THC in blood of young male volunteers after they smoked a marijuana cigarette. Levels of Δ^9-THC could be easily detected and quantitated for twenty-four hours following marijuana smoking.

Results from the human marijuana smoking studies also indicated that a marijuana metabolite is present in blood plasma. This metabolite was detectable up to twenty-four hours following smoking and in fact was higher at this time than any other time following smoking. Detection of this metabolite was accomplished using an ultra-violet spectrophotometer attached to the hplc. Of the ten control subjects (non-marijuana smokers) tested by the analysis method, none showed the presence of this marijuana metabolite. Therefore, it appears that numerous blood samples can be quickly and inexpensively screened by the hplc method for presence of this marijuana metabolite which would indicate prior marijuana use.

C O N T E N T S

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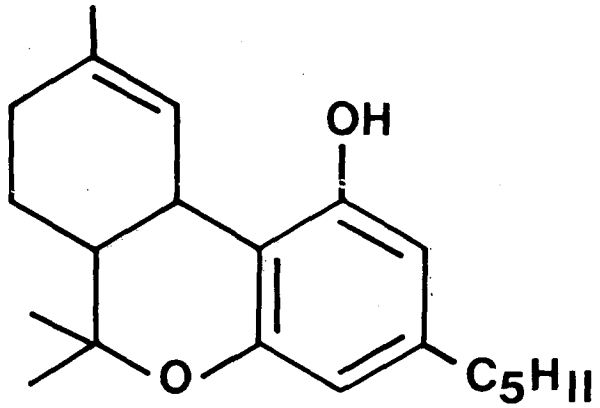
INTRODUCTION

Analyses of blood, blood plasma and/or blood serum for the presence of therapeutic or illicit drugs is rather routine in most clinical and toxicologic laboratories. Most often these analyses are performed using spectrophotometric or gas chromatographic methods. Both of these experimental techniques are adequate for most drugs which are generally present in the microgram (μg) per milliliter (ml) range in human blood. However, certain drugs are given in such low amounts that the resultant blood levels fall in the nanogram (ng) per ml range. One such drug is marijuana which is reportedly being used by 20 million Americans (1).

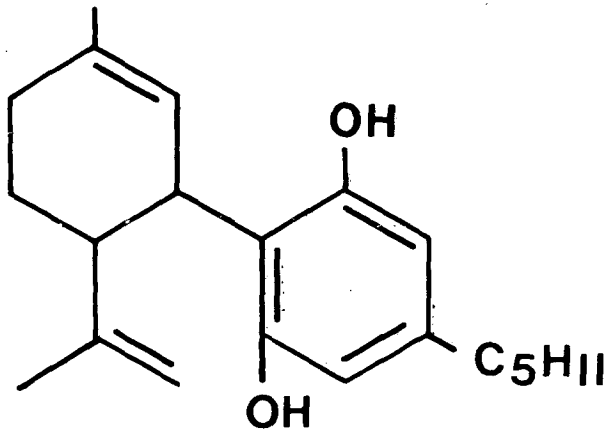
The major emphasis in the present work was to develop and validate in humans a method capable of detecting ng/ml amounts of Δ^9-THC. From prior studies (2-4) it was known that Δ^9-THC accumulated in amounts less than 30 ng/ml in blood plasma after smoking of one marijuana cigarette. Δ^9-THC was chosen for analysis since it is known to be one of the major biologically active agents in marijuana (5). The study by Agurell, et al. (2), had shown that peak blood levels of Δ^9-THC were achieved 15 minutes following smoking and thereafter dissipated very rapidly to less than 5 ng/ml after only 2 hours.

Marijuana commonly has four major components present (6), viz., Δ^9-THC, cannabidiol (CBD), cannabinol (CBN) and cannabichromene (CBC). Inspection of the structural formulas (cf., Figure 1) reveals that Δ^9-THC, CBD and CBC all have the same atomic weight as well as some identical functional groups. Although CBN has a different atomic weight, it is structurally very similar to Δ^9-THC, CBD and CBC. Thus it became important in any method developed for the analysis of Δ^9-THC, to have a method capable of separating Δ^9-THC from CBD, CBN and CBC as well as from blood constituents.

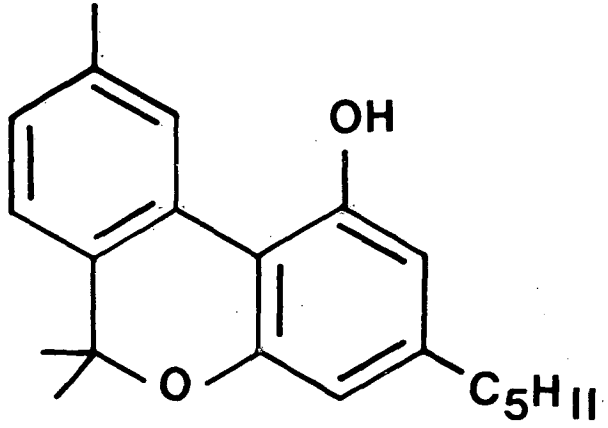
The method developed has utilized the separating powers of high pressure liquid chromatography (hplc) and the quantifying ability of mass spectrometry (ms). This hplc-ms technology is capable, as described later, of separating Δ^9-THC from CBD, CBN and CBC as well as from some of the blood plasma constituents while quantifying 1 ng/ml amounts.



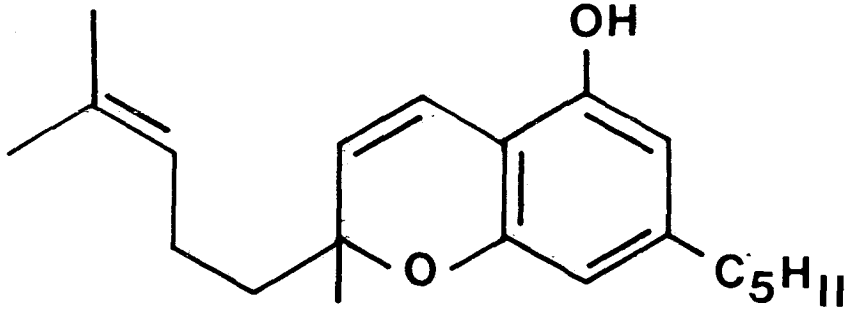
Δ^9-THC
(314)



CBD
(314)



CBN
(312)



CBC
(314)

FIGURE I

EXPERIMENTAL METHODS

A. High Pressure Liquid Chromatography

High pressure liquid chromatography (hplc) analyses were conducted using a Varian 8520 liquid chromatograph utilizing a Varian 635M recording spectrophotometer as a detector. The column chosen for this work was a 10 micron silica gel, 25 cm in length and 2 mm i.d. (Varian Si-10). To adequately separate the major constituents of marijuana, a gradient elution program was developed using heptane and methylene chloride. As work progressed, it became quite obvious that purity of solvents was absolutely essential to obtain reproducible separations of the cannabinoids as well as to assure accurate ms quantitation discussed in the following section. The most direct method to insure solvent purity is to routinely take a uv spectrum of all heptane and methylene chloride purchased. Figures 2 and 3 are typical uv spectra obtained with methylene chloride and heptane, respectively. In each figure the lower curve illustrates the purity desired, whereas the upper curve is typical of unacceptable material. Thus this routine check of solvent purity revealed any impurities which might have interferred with determination of the cannabinoids.

The gradient elution program developed for the separation of the cannabinoids was optimized so as to allow a clear distinction between Δ^9-THC, CBD, CBN and CBC. This gradient elution program requires a total of 18 minutes to complete. Initially the program begins with a 95:5 percent, heptane:methylene chloride mixture and proceeds to 95:5 percent methylene chloride:heptane mixture over a 9 minute period. Once the 95:5 percent methylene chloride:heptane composition has been reached, the program is reversed to the initial 95:5 percent heptane:methylene chloride mixture, thereby regenerating the column. A solvent flow rate of 120 ml per hour was used for all determinations. Figure 4 illustrates the gradient program by the dark line which is superimposed upon the hplc chromatogram of a mixture of

Δ^9-THC, CBN, CBD and CBC. For example, Δ^9-THC appears at a retention time of 4.3 minutes or at a gradient elution mixture (GEM) of 52:48 percent, methylene chloride:heptane. Likewise, the retention time and GEM of the other cannabinoids can be ascertained from this figure.

Using the above described program and column, it was found that maximum resolution of cannabinoids was achieved if the sample to be injected onto the column was in heptane of the quality used is the GEM. The amount of heptane used to reconstitute the sample was not critical since as much as 500 µl has been injected without loss of resolution. In contrast, if methanol was used for reconstitution of samples, resolution of the cannabinoids was affected as evidenced by broad peaks on the chromatograms.

As discussed earlier, a spectrophotometer was used as the detector for the effluent emanating from the hplc column. Since the spectrophotometer used had scanning capabilities, it was determined by stop-flow techniques that the maximum uv absorbance for Δ^9-THC in the GEM was 273.7 mµ. Thus all chromatograms were recorded at this wavelength, e.g., Figure 4. Using this wavelength it is possible to detect 100 ng of Δ^9-THC, but as pointed out in an earlier section the blood plasma levels were shown to be one-fifth of this amount. Therefore in the present work a small amount of deuterated Δ^9-THC (d_3-Δ^9-THC) was used to aid in uv detection of Δ^9-THC. Also as will be pointed out in the following section, this allowed quantitative measurements to be made with the ms. A 10 cm "zero-dead-volume" stainless steel tube was attached to the flow cell of the spectrophotometer to facilitate collection of the effluent droplets almost instantaneously after they had passed through the flow cell.

B. Mass Spectrometer Quantification

Mass spectrometry (ms) work was accomplished using a Varian MAT SMI-B, high resolution, double-focus mass spectrometer. A new ion-counting technique was developed in conjunction with the peak

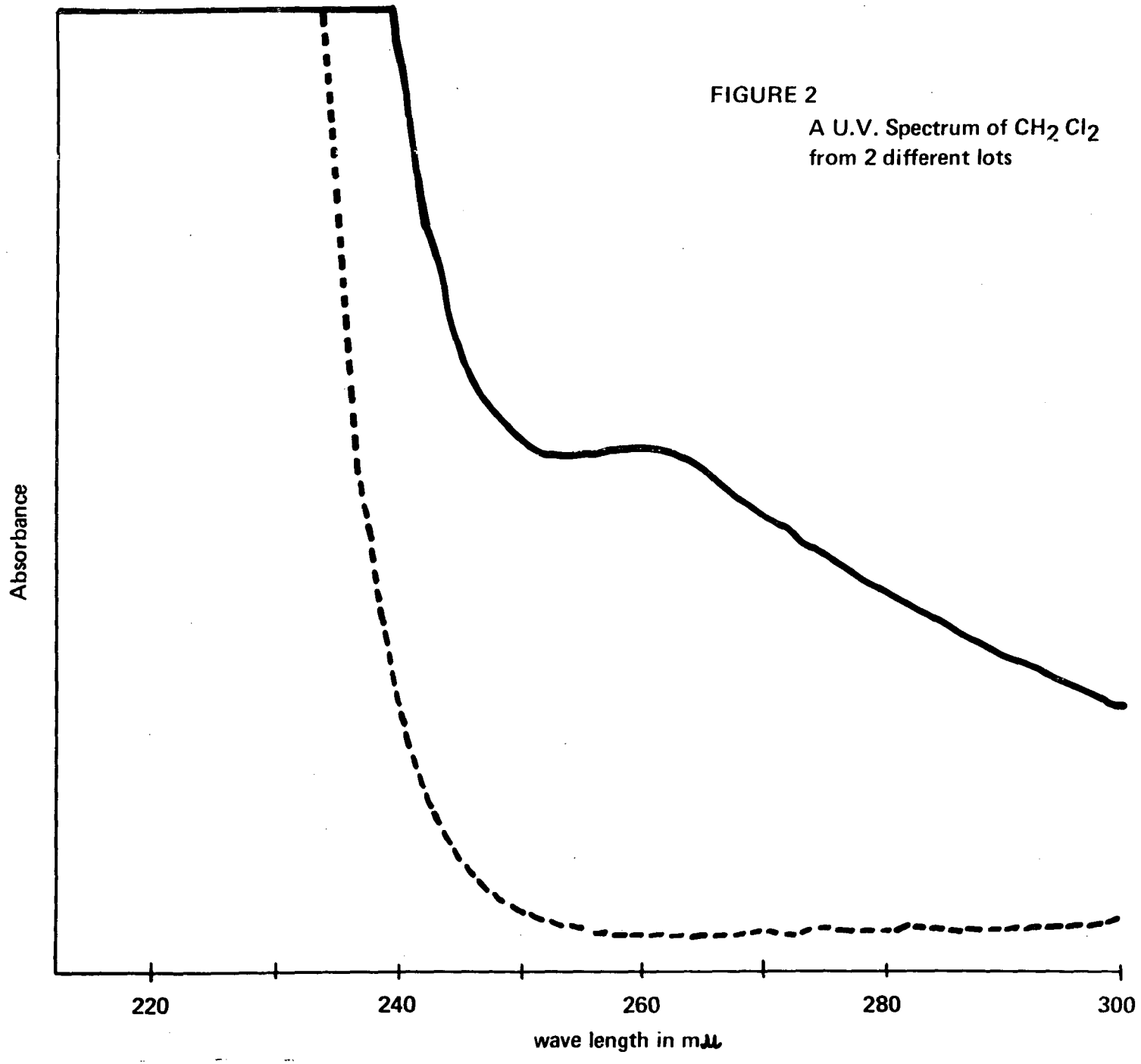


FIGURE 2

A U.V. Spectrum of CH_2Cl_2 from 2 different lots

FIGURE 3

A U.V. Spectrum of Heptane
from 2 different lots

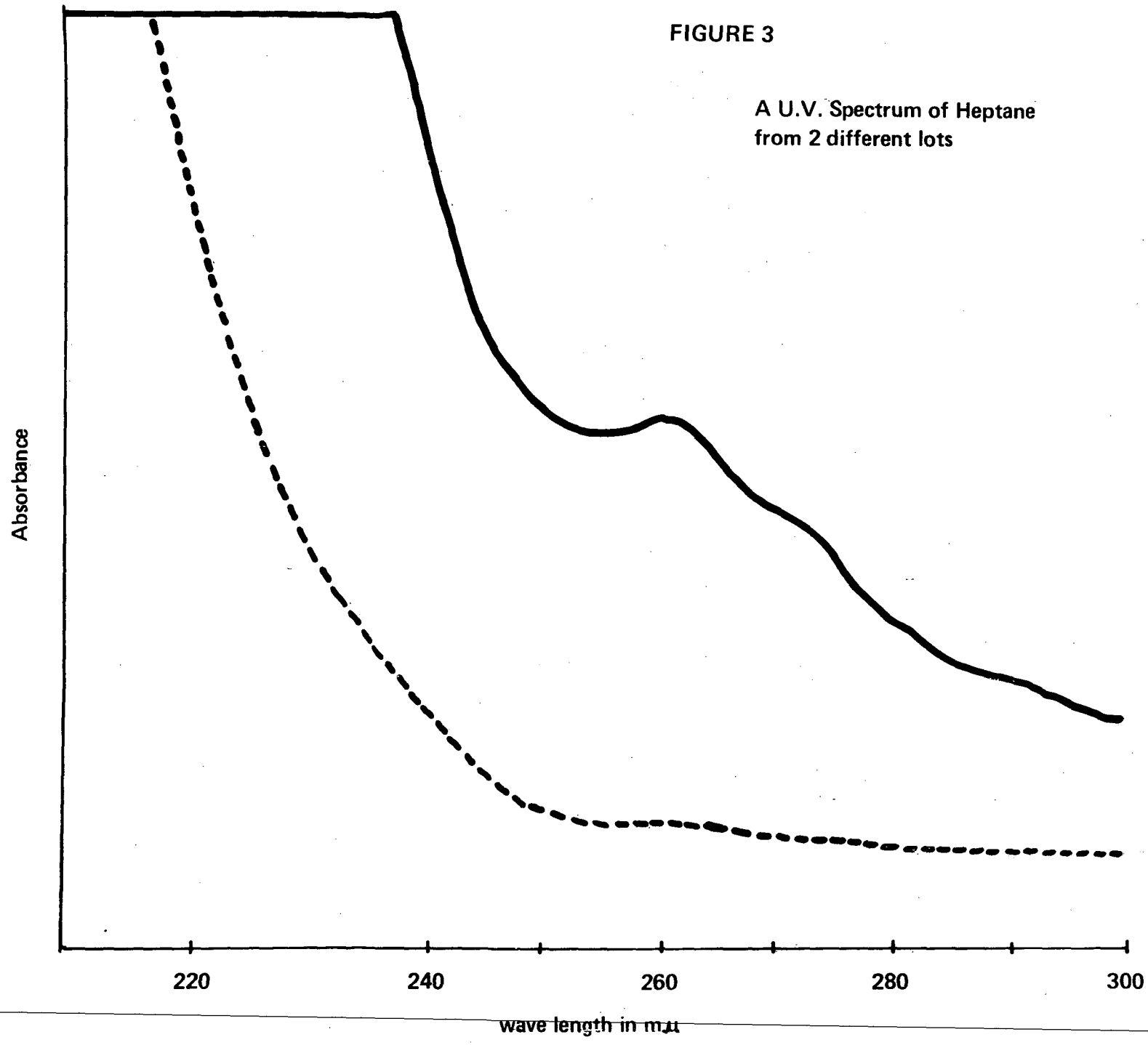
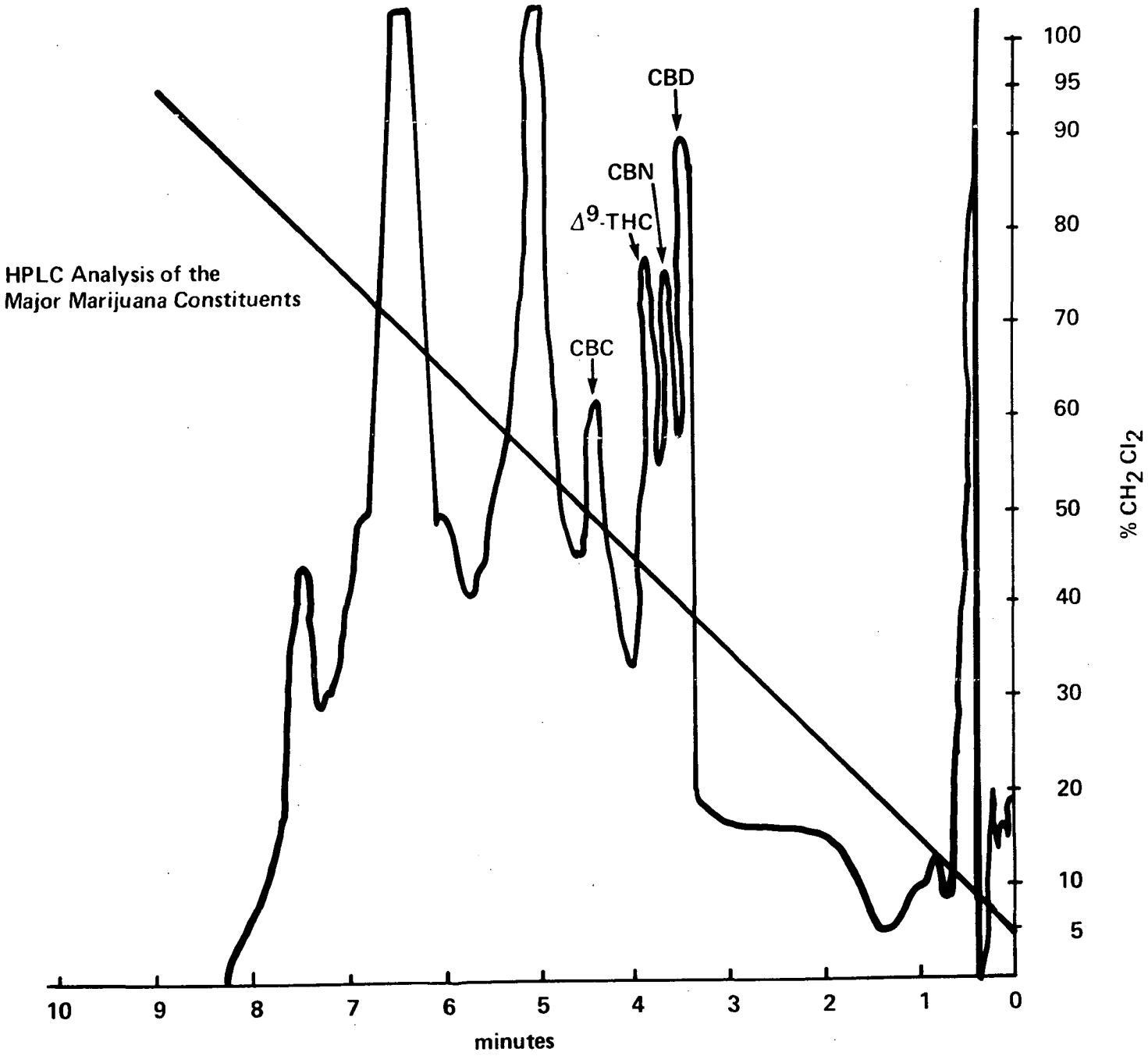


FIGURE 4


HPLC Analysis of the
Major Marijuana Constituents
CBD
CBN
Δ9-THC
CBC
100
95
90
80
70
60
50
40
30
20
10
5
% CH2 Cl2
10
9
8
7
6
5
4
3
2
1
0
minutes

matching accessory which provided for a rapid (essentially continuous) comparison between data from the internal standard (d_3-Δ^9-THC, mass 317) and the assayed compound (Δ^9-THC, mass 314). As was noted earlier, a known amount of d_3-Δ^9-THC is added to each sample to be analyzed for Δ^9-THC content prior to hplc analysis. Each sample coming from the hplc is introduced into the mass spectrometer and the instrument is initially focused exactly on the 317 (d_3-Δ^9-THC) mass signal, then through the action of the peak matching unit, and with the high resolution capability alternately focused to the 314 (Δ^9-THC) mass signal, cf., Figure 5. As this alternation from one signal to another occurs the exact number of ion counts for each compound is recorded and stored in two channels of a dedicated computer. The ion counter-integrator (computer) used was a Princeton Applied Research model -SSR 1110. This unit performs a summation of the number of ion events occurring in both mass peaks (314 and 317) and stores these in two registers. Thus a running total of ions detected from the 317 internal standard and the unknown amount of the 314 mass are stored separately by the counter as shown schematically in Figure 6. The peak matcher accessory is set to dwell for 67 milliseconds on each mass signal before it alternates to the other mass signal. Repeated experimentation has shown that a total counting time of 67 seconds produces good statistical data values. Thus in 67 seconds the alternating cycle is repeated 500 times yielding 1000 bits of data for comparison and quantification.

The temperature at which the Δ^9-THC sample is run proved to be quite critical. After considerable experimentation, it was found that a probe temperature of 65°C combined with the 67 seconds counting time gave optimum results. Figure 7 illustrates how the 314/317 ratio remains constant with time even as the sample is being depleted. Thus the ratio based upon the internal standard remains linear and provides for dependable quantification. The slope of the line drawn through these data points yields the 314/317 ratio or the percent 314 present.

FIGURE 5

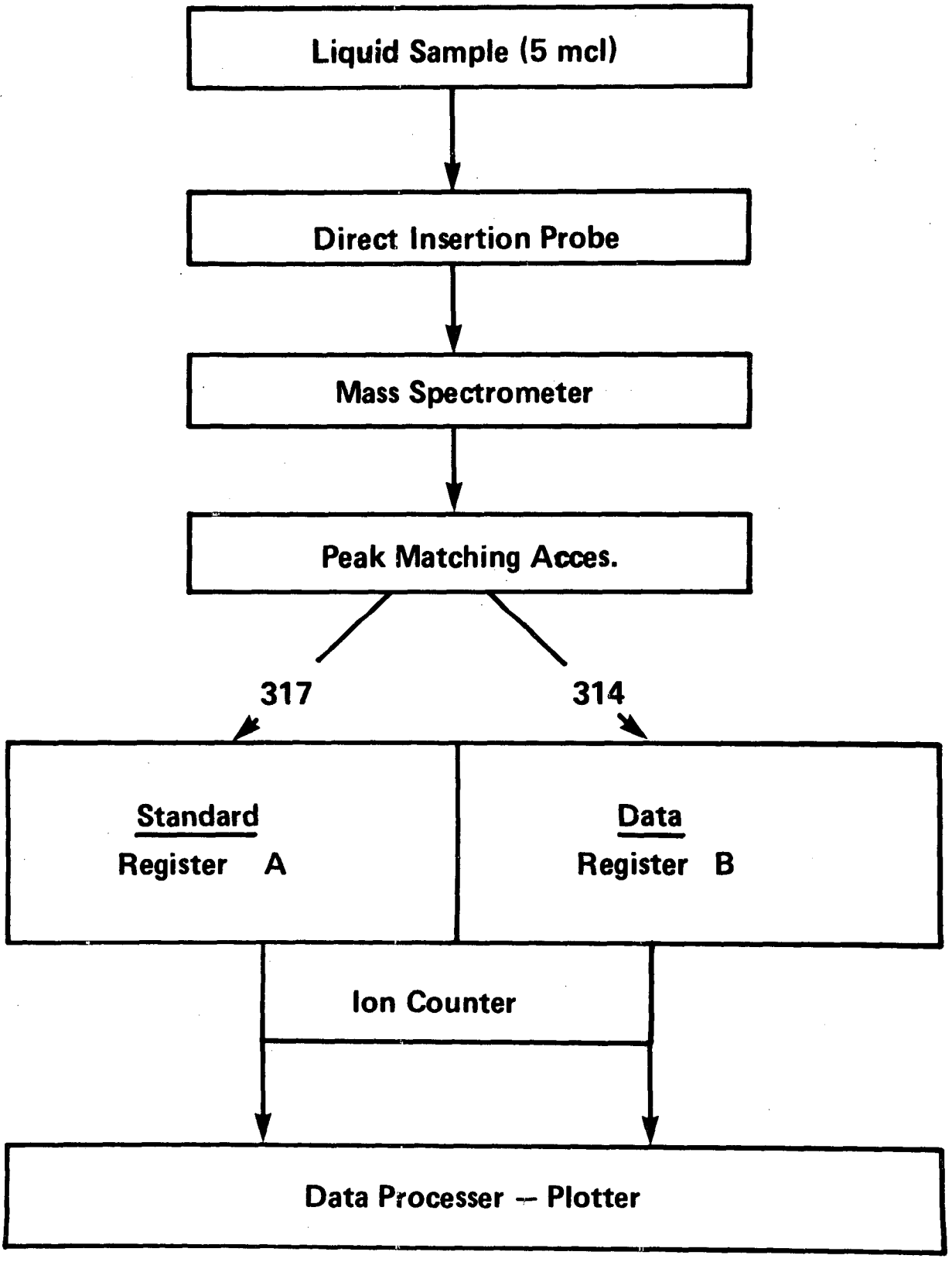

Liquid Sample (5 mcl)
Direct Insertion Probe
Mass Spectrometer
Peak Matching Acces.
317
314
Standard
Register A
Data
Register B
Ion Counter
Data Processer – Plotter

FIGURE 6

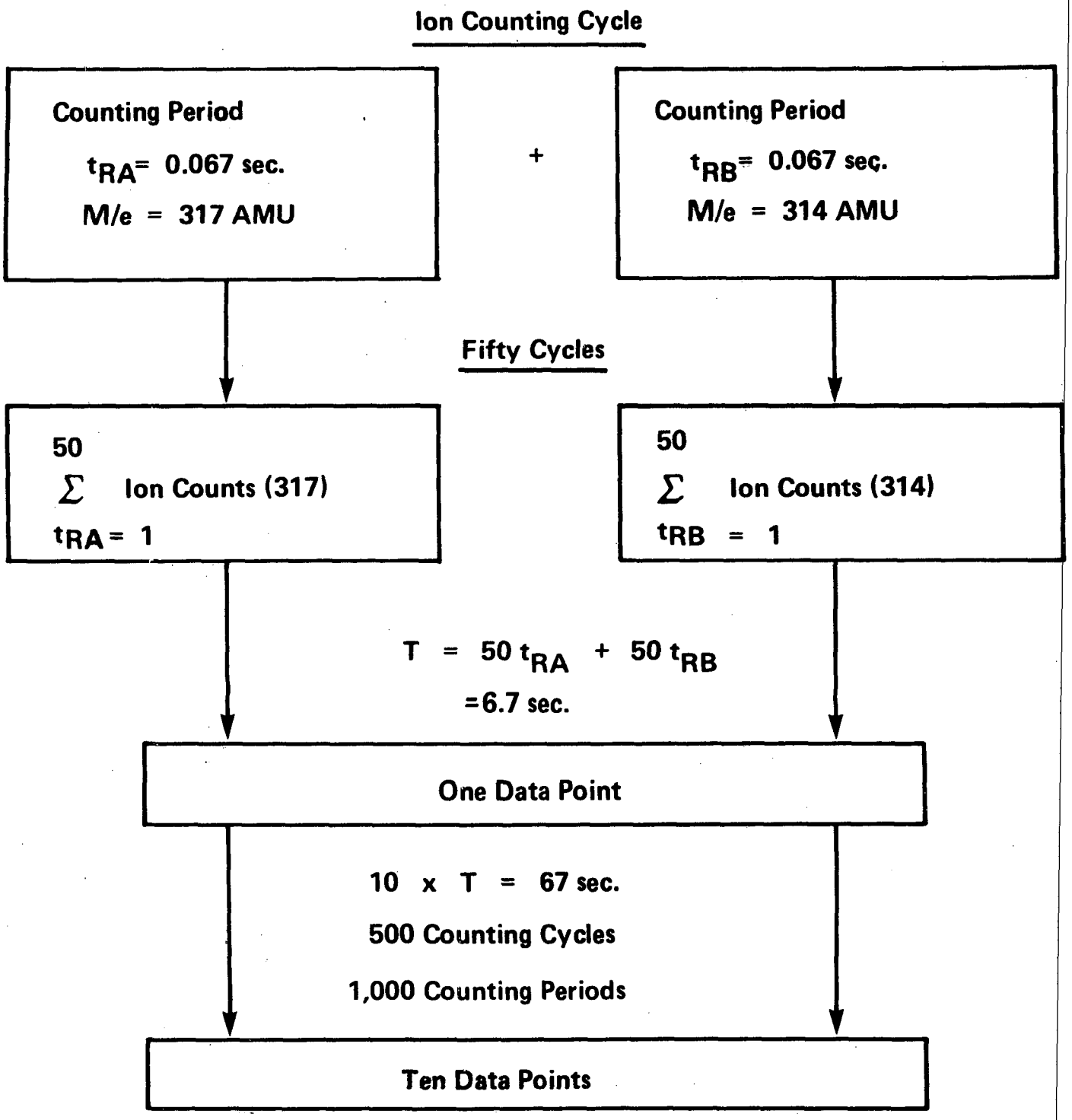
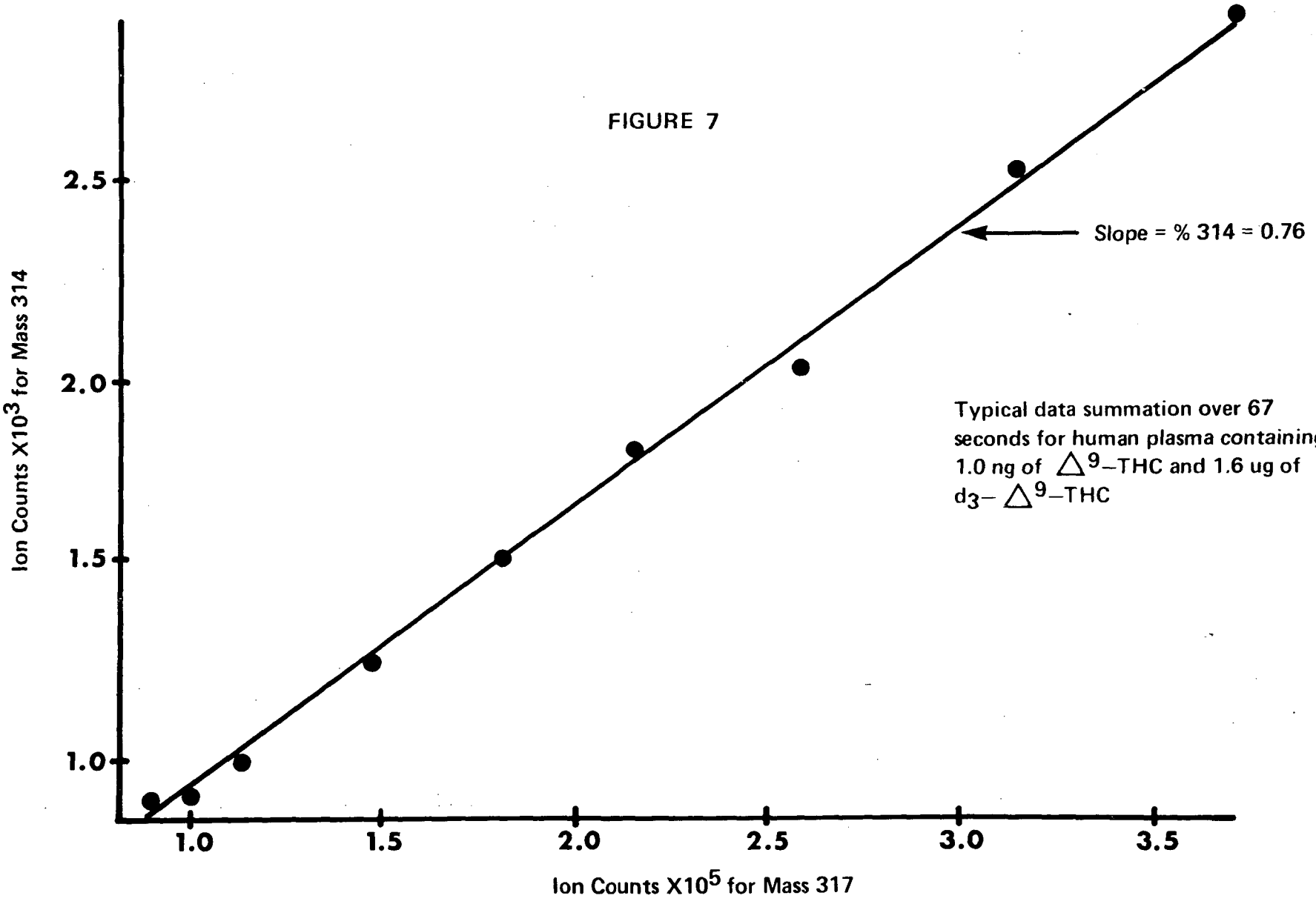

Ion Counting Cycle
Counting Period
t_{RA}= 0.067 sec.
M/e = 317 AMU
+
Counting Period
t_{RB}= 0.067 sec.
M/e = 314 AMU
Fifty Cycles
50
$\sum$ Ion Counts (317)
t_{RA} = 1
50
$\sum$ Ion Counts (314)
t_{RB} = 1
T = 50 t_{RA} + 50 t_{RB}
=6.7 sec.
One Data Point
10 x T = 67 sec.
500 Counting Cycles
1,000 Counting Periods
Ten Data Points

FIGURE 7


2.5
2.0
1.5
1.0
Ion Counts X10^3 for Mass 314
Slope = % 314 = 0.76
Typical data summation over 67 seconds for human plasma containing 1.0 ng of Δ9–THC and 1.6 ug of d3– Δ9–THC
1.0
1.5
2.0
2.5
3.0
3.5
Ion Counts X10^5 for Mass 317

Using the peak matching and ion counting methods just described, several experiments were conducted with a large number of samples to demonstrate validity and reproducibility of the technique. First a known amount of d_3-Δ^9-THC (1.6 mcg) was introduced into the ms via the direct insertion probe and the 314/317 ratio determined. A determination of this ratio was feasible since the d_3-Δ^9-THC contained a small but constant level of undeuterated Δ^9-THC. The data ir Table I summarizes the results of this experiment. As shown, the contribution of Δ^9-THC to the 314/317 ration is 13.8 ± 0.6 ng. A second experiment was conducted to determine what, if any, contribution was made to the 314/317 ratio when a sample of d_3-Δ^9-THC was placed on the hplc instrument followed by fraction collection and ms analysis. Table II is a summary of this data. As shown, very little contribution is attributable to the hplc method over and above that found by the direct analysis shown in Table I. Thus the mobile phase collected from the hplc did not significantly affect the overall results.

Table I. Mass Spectral Analysis of 1600 ng of d_3-Δ^9-THC.

Sample No.	314/317%	ng of Δ^9-THC Found
1	0.865	13.8
2	0.914	14.6
3	0.815	13.0
4	0.848	13.6
5	0.895	14.3
6	0.817	13.1
7	0.865	13.8
Average	0.860	13.8
Standard Deviation	±0.037	±0.6
% Variation	±4.3	±4.3

Table II. Mass Spectral Analysis of 1600 ng of d_3-Δ^9-THC Injected Onto and Collected From the HPLC.

Sample No.	314/317%	ng of Δ^9-THC Found
1	0.90	14.40
2	1.02	16.32
3	0.93	14.88
4	0.81	12.96
5	0.89	14.24
6	0.80	12.80
7	0.86	13.76
8	0.91	14.56
Average	0.89	14.24
Standard Deviation	±0.07	±0.06
% Variation	±7.8	±1.06

C. Methods of Blood Plasma Analysis.

Whole blood was centrifuged at 2600 rpm for 20 minutes to obtain blood plasma. To 1 ml of the blood plasma is added 1.6 µg of d_3-Δ^9-THC. This amount of deuterated material was added to plasma to ensure that d_3-Δ^9-THC could easily be detected by both the uv spectrophotometer and the oscilloscope of the ms. The blood plasma is extracted 3 times using 2 ml of petroleum ether for each extraction and the extracts combined, followed by evaporation to dryness under nitrogen at room temperature. The resultant residue was reconstituted in 300 µl of heptane and the entire solution injected into the hplc. A 100 µl wash of heptane was used on the vessel containing the extracts and it was also injected into the hplc. Fractions of eluent were collected at the proper retention time for Δ^9-THC and analyzed by ms as described previously under B. Figure 8 is a flow chart of the blood analysis scheme used.

FIGURE 8

ANALYSIS FOR Δ^9 – THC IN PLASMA

1. Add 1.6 mcg of $d_3 - \Delta^9$ – THC to 1 ml of plasma
2. Extract 3x with petroleum ether (2 ml)
3. Concentrate to dryness under N_2
4. Reconstitute with 350 mcl heptane
5. Inject 350 mcl into hplc
6. Collect Fractions
7. Introduce into ms
8. Quantitate by determining 314/317

D. Validation of the Assay Method in Blood Plasma.

Blood from 10 laboratory workers known to be non-users of marijuana was drawn and analyzed as discussed in C. These blood plasmas constituted the control samples. Each sample showed some Δ^9-THC content since the 1.6 µg of d_3-Δ^9-THC added to each contained 11.3 ng of the undeuterated material. Therefore the final quantitation figure given for each blood plasma analyzed has been adjusted to reflect this 11.3 ng of undeuterated material.

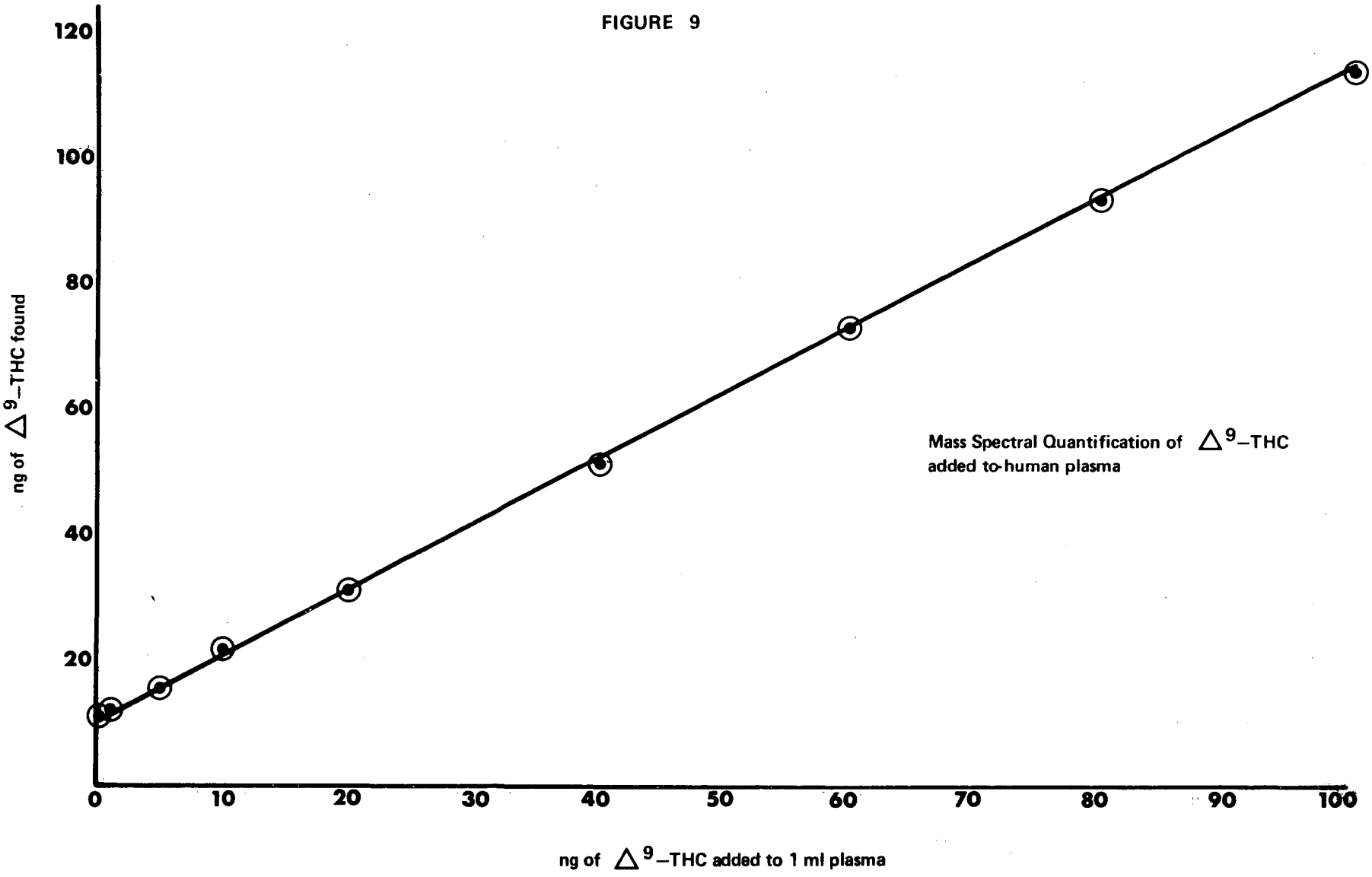
To demonstrate the reproducibility and accuracy of the developed method, pooled human plasma samples were analyzed to which known amounts of Δ^9-THC had been added. Table III is a summary of this study. Likewise Figure 9 is a plot of the data which appears in Table III. As discussed above, the intercept in Figure 9 does not pass through the orgin since some undeuterated Δ^9-THC is found in the d_3-Δ^9-THC used as an internal standard. Also as seen in Figure 9, the ms demonstrated linearity over the range studied.

Table III. Precision and Accuracy In Recovery of Δ^9-THC Added To Human Plasma

Added ng/ml	Found[a], ng/ml	n	RSD ±	RE, %
0	11.3	10	---	---
1	1.2 (0.6-1.5)	3	0.52	20
5	4.5 (4.0-4.7)	3	0.41	10
10	10.5 (9.5-12.4)	10	---	5
20	20.0 (19.1-21.1)	3	1.03	0
40	41.2 (39.6-42.5)	3	1.47	3
60	62.1 (61.3-62.8)	3	0.56	3.5
80	82.8 (74.9-90.7)	3	7.9	3.5
100	103.7 (99.3-106.0)	3	3.78	3.7

[a] Average of n different samples at each added concentration level (range). Values other than 0 ng/ml have been corrected for Δ^9-THC content in the deutrated material.

FIGURE 9


120
100
80
60
40
20
0
10
20
30
40
50
60
70
80
90
100
ng of Δ9–THC found
ng of Δ9–THC added to 1 ml plasma
Mass Spectral Quantification of Δ9–THC added to human plasma

E. Human Marijuana Smoking Studies.

All marijuana smoking studies were conducted at the Marijuana Research Institute, UCLA School of Medicine. Twice, our laboratory received samples from studies being done at UCLA. On January 23 and April 3, 1975 smoking experiments were performed using six male volunteers for each study. The subjects were brought into the hospital ward 12 hours prior to smoking. The subjects were not allowed any food or drink after 12:00 a.m. prior to smoking. At approximately 3:00 a.m. of the test day, each subject had a heparin-lock "butterfly" placed in a forearm vein. At this time a 5 ml blood sample was drawn and placed in a silinized green top vacutainer. Each blood sample was handled in such a manner that the blood did not contact the rubber stopper. All blood samples were immediately centrifuged at 2600 rpm and the plasma removed and placed in a second silinized tube and frozen for later analysis. Each subject was then allowed to smoke one marijuana cigarette which contained 10.8 mg Δ^9-THC, 2.16 mg CBN, 0.9 mg CBC and 0.63 mg CBD. Upon completion of smoking, timing was begun. Blood samples were withdrawn at 0, 0.25, 0.5, 1, 2, 3, 4, 12 and 24 hours. After blood samples were withdrawn, breath and saliva samples were also obtained. Collection and analysis of the breath samples was reported earlier (7) while analysis of saliva samples will be a future task.

RESULTS

The blood plasma concentration (C_p) of Δ^9-THC was determined in the marijuana smokers using the newly developed hplc-ms technique. Figure 10 is a plot of C_p *versus* time (t) for the subjects studied. Vertical lines at each data point represent the range of values, i.e. the high and low levels, observed between individuals. From this plot the average maximum plasma concentration ($\overline{C_p}^{max}$) and the time required (t_{max}) to reach the $\overline{C_p}^{max}$ is determined directly as 30.8 ng/ml and 15 minutes, respectively.

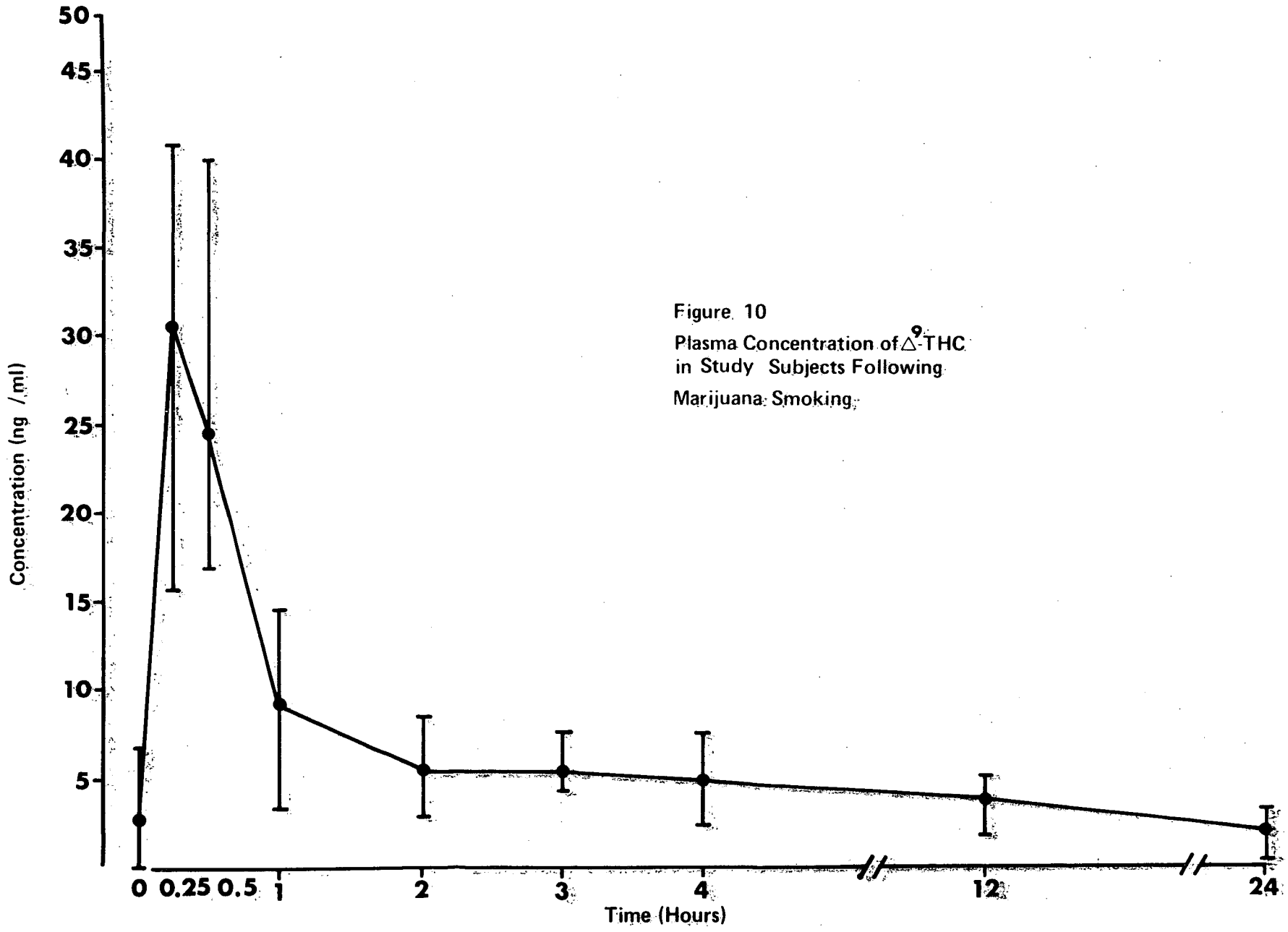


Figure 10

Plasma Concentration of Δ^9-THC in Study Subjects Following Marijuana Smoking

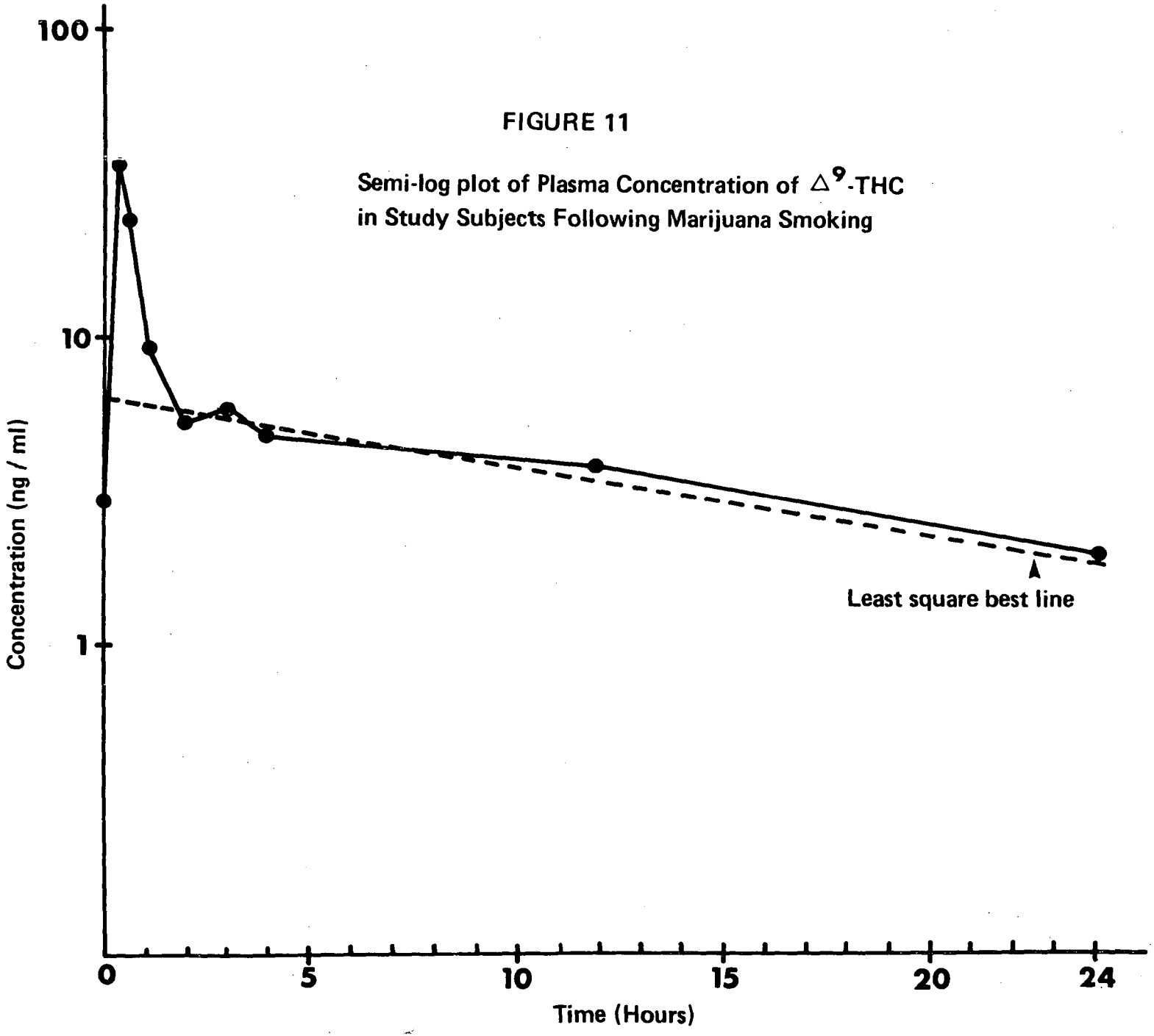


FIGURE 11

Semi-log plot of Plasma Concentration of Δ^9-THC
in Study Subjects Following Marijuana Smoking

Figure 11 is a semilog plot of ln C_p *versus* t for Δ^9-THC found in the plasma of the marijuana smokers. Using data points at 2, 3, 4, 12 and 24 hours from Figure 10, it is possible to obtain the least squares value of the slope (β). The average biological half-life ($\bar{t}_{\frac{1}{2}}$) of Δ^9-THC was calculated using the following equation:

$$\bar{t}_{\frac{1}{2}}(\Delta^9\text{-THC}) = \frac{0.693}{\beta} = \frac{0.693}{0.18} = 3.85$$

Data was given in an earlier report (7) on the relationship which exists between levels of Δ^9-THC on breath and in blood plasma. Figure 12 demonstrates the relationship between saliva levels of Δ^9-THC and plasma levels for one subject following marijuana smoking. The saliva values shown have been normalized to 1 ml of volume. The actual values found per unit volume of saliva are given in Table IV. Due to the limited objectives of the present study only one individual's saliva was analyzed for Δ^9-THC content.

Table IV. Δ^9-THC Content In Saliva.

Time (hrs) Post-Smoking	ml of Saliva Analyzed	ng of Δ^9-THC Found	Normalized (ng/ml) of Δ^9-THC
0.25	0.2	29.0	145.0
0.5	0.2	35.1	175.5
1.0	0.2	38.8	194.0
2.0	0.4	26.6	66.5
3.0	0.3	6.6	22.0
4.0	0.4	8.4	21.0
8.0	0.5	24.9	49.8
12.0	0.3	4.4	14.7

As with the breath samples reported on earlier, a new marijuana metabolite was found in the blood plasma of the subjects used in the studies at UCLA. This metabolite appears at a retention time of 3.1 minutes on the hplc chromatogram and is clearly present at all times

Figure 12 Δ^9-THC Content in Saliva and Plasma of One Marijuana Smoker

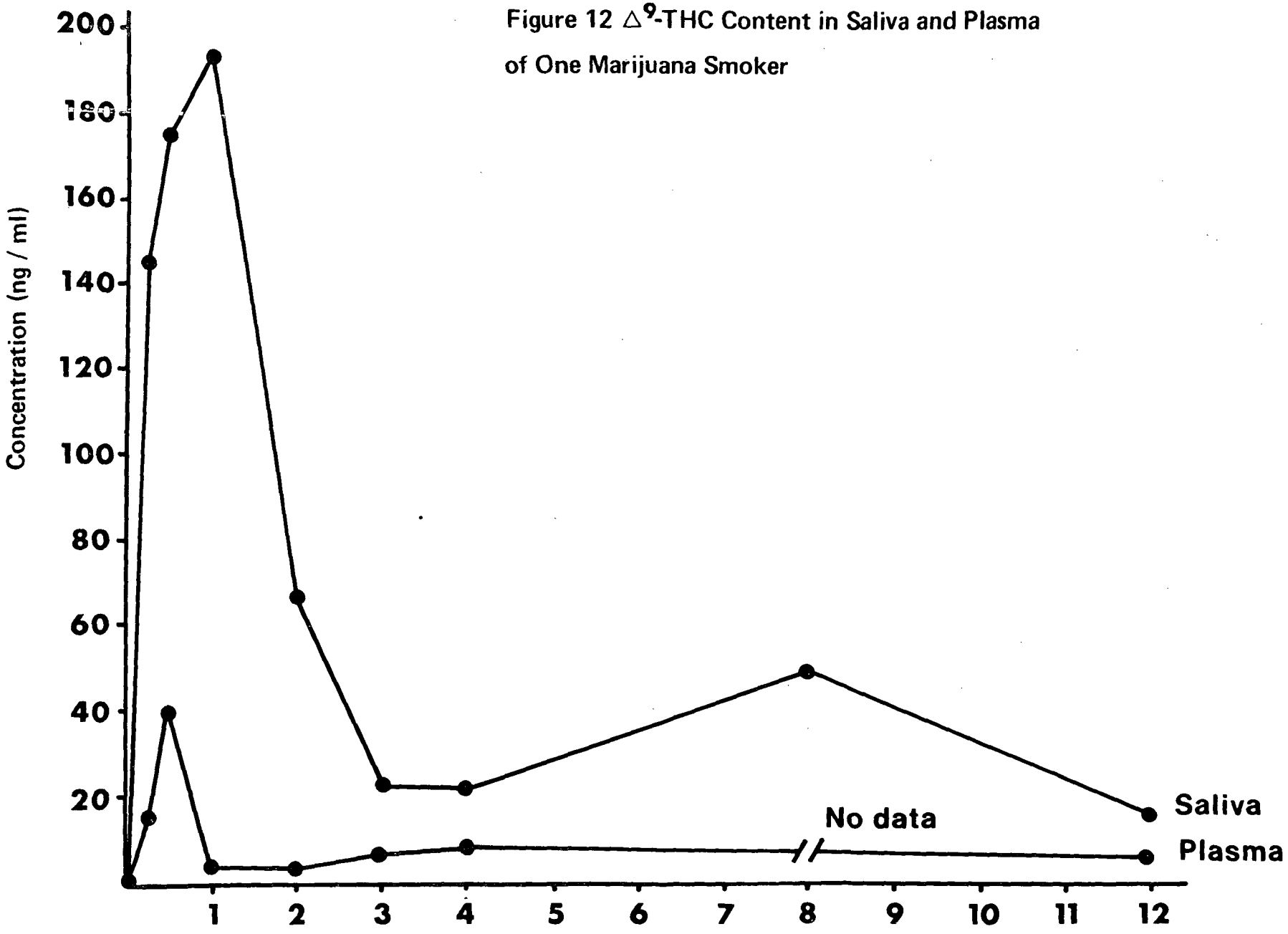


Figure 13

Plasma Concentration of t_R 3.1 Marijuana Metabolite in Study Subject Following Marijuana Smoking

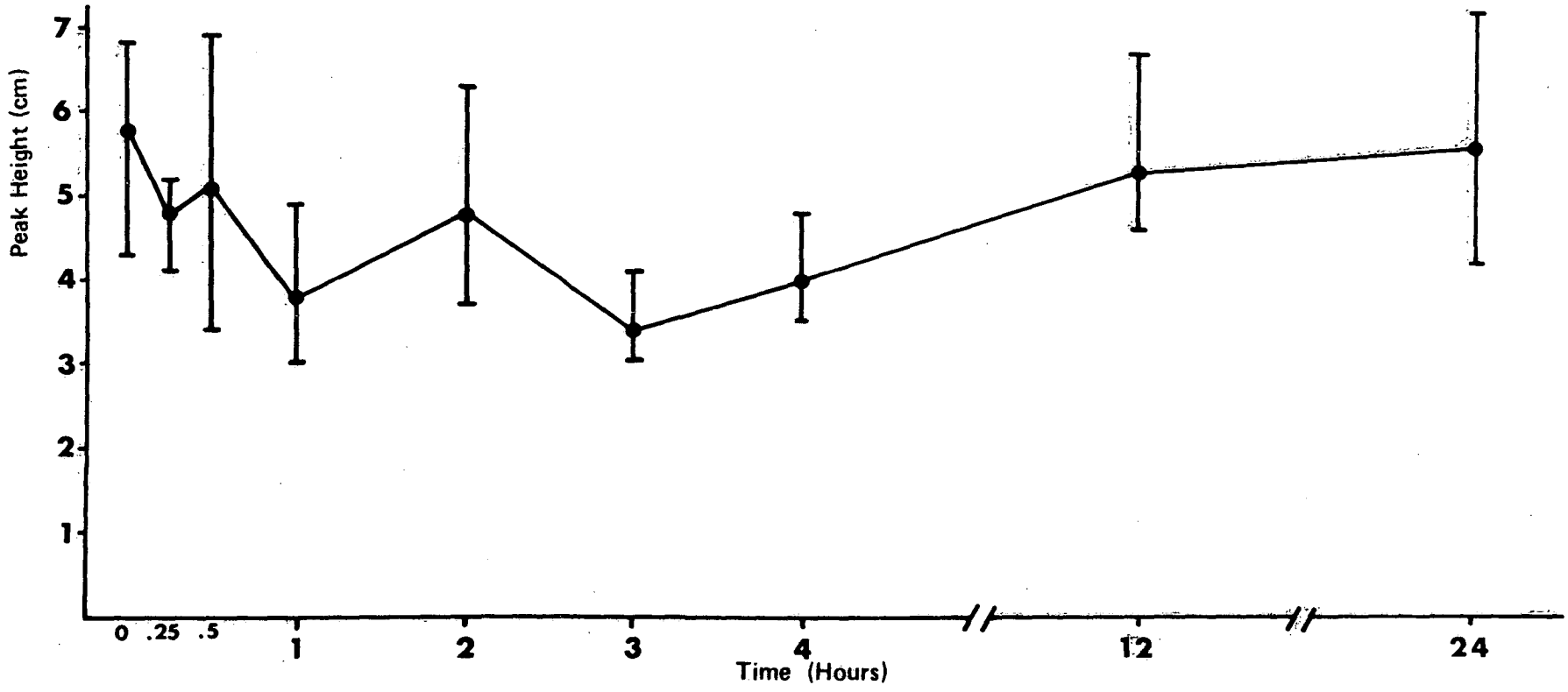
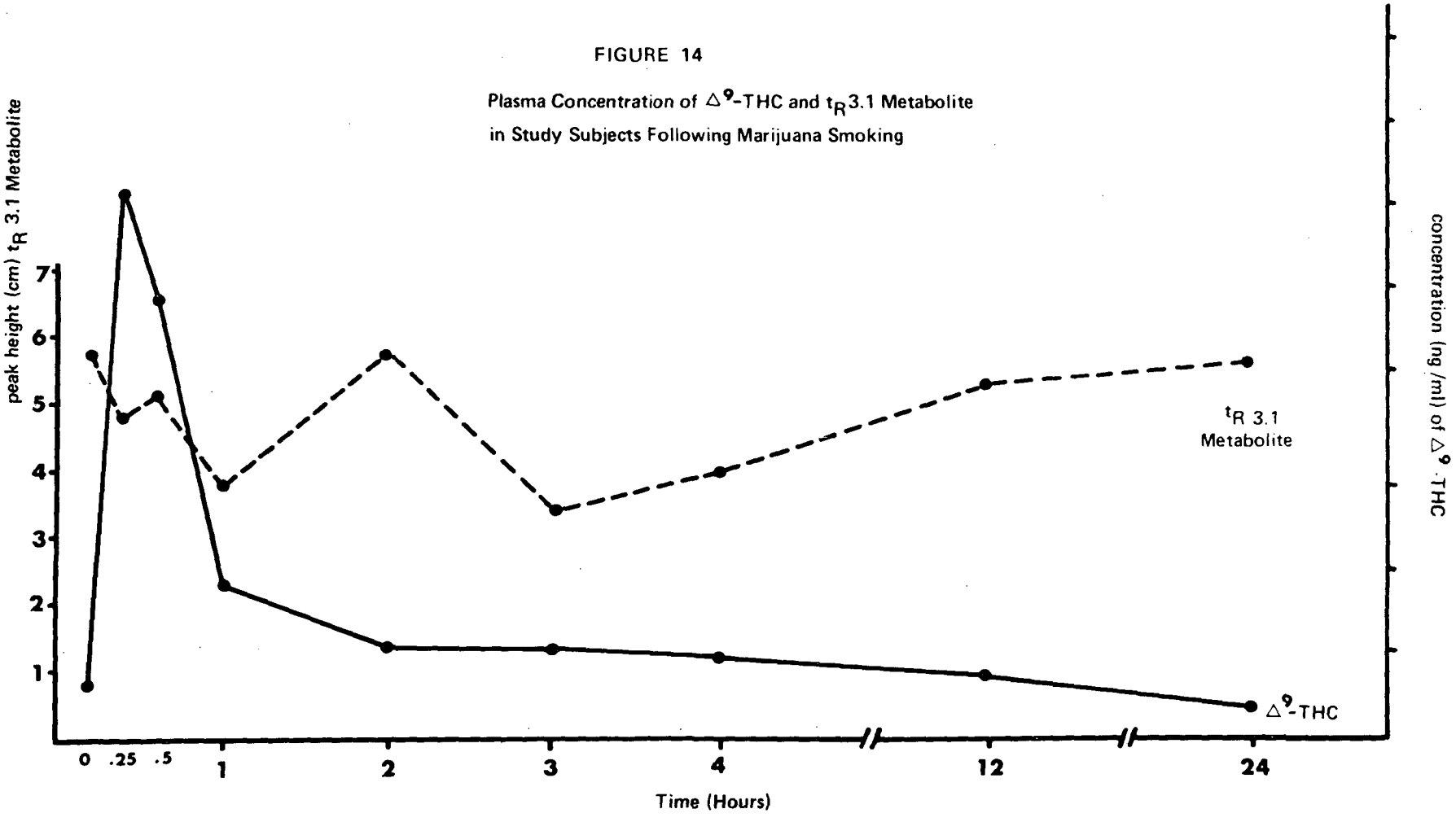


FIGURE 14

Plasma Concentration of Δ^9-THC and t_R 3.1 Metabolite
in Study Subjects Following Marijuana Smoking



studied up to 24 hours after smoking. The earlier report on breath analysis (7) indicated that an additional marijuana metabolite might be present in plasma at 0.6 minutes on the chromatogram. Although this peak is always noted, its peak height seems to depend somewhat on the extent of blood hemolysis. Thus at this juncture the t_R 0.6 peak will be omitted as a metabolite of marijuana until a firm characterization can be made. Figure 13 illustrates the plasma concentration of the t_R 3.1 metabolite in the study subjects following marijuana smoking. The range of values (high and low) is denoted by the vertical line through each datum point. Figure 14 shows the relationship between the Δ^9-THC and t_R 3.1 metabolite in blood plasma with time.

CONCLUSIONS

The major objective of the present study was to develop and validate a method for determining Δ^9-THC amounts in human plasma. A technology employing hplc-ms was developed and validated in human blood plasma to which Δ^9-THC had been added. These studies demonstrated that 1 ng/ml of Δ^9-THC could be accurately determined in blood plasma. Also these studies proved that the ms gave reproducible (linear) results over the concentration range of 1-100 ng/ml.

Human marijuana smoking studies were conducted in which blood, breath and saliva samples were obtained. The results from the breath study were reported earlier. Blood samples were analyzed as plasma using the hplc-ms technology which had been developed and validated in known samples. As was shown in the data presented in Figure 10, the Δ^9-THC levels in plasma could be followed easily to 24 hours after marijuana smoking. Also Figure 10 illustrated that the maximum plasma level of Δ^9-THC occurs at 15 minutes following smoking. All marijuana smokers showed some levels of Δ^9-THC in their plasma even on the pre-smoking samples. This is readily explainable since all subjects in the study were previous users of marijuana and had obviously smoked prior to reporting for the study.

Saliva was examined for Δ^9-THC in one study subject as shown in Figure 12. As was pointed out in the earlier results section (cf., Table IV) the saliva values were normalized to 1 ml volume. Nevertheless the general shapes of the two curves are very similar indicating that a general relationship may exist between blood and saliva levels. That is, saliva may be acting as an excretory pathway for Δ^9-THC. Further work will be needed to elucidate this point.

As was the case with the earlier reported breath work, this study demonstrated that plasma contained a marijuana metabolite. The relationship which exists between this metabolite and Δ^9-THC content in blood plasma was given in Figure 14. This metabolite was found in the plasma up to 24 hours following smoking and was not found in the plasma of any non-marijuana smoker. Again as with the breath, there appears to be no doubt that a marijuana user can be detected simply by the presence of this metabolite. A very convenient feature of this metabolite is that it can be detected with the uv spectrophotometer attached to the hplc, whereas Δ^9-THC cannot. Thus it would be possible to screen a large number of human blood samples for the presence of this marijuana metabolite using the less expensive hplc method. However, it would also be possible on the same sample to make a determination of the Δ^9-THC level by the combined hplc-ms technique as a cross check to eliminate any false positive assays.

The level of Δ^9-THC and metabolite can also be correlated in an individual marijuana smoker. Figure 14 gave this average relationship for the marijuana smokers studied. As noted in this figure, the levels of the metabolite fluctuate in the early time intervals, but after three hours continued to steadily rise. The fluctuation in levels of the metabolite can be most readily explained by enterohepatic circulation and/or suppression of its biosynthesis by Δ^9-THC. Once the structure of this compound and its metabolic precursor are known, a more definitive explanation will be available. Also additional human smoking studies in which a larger number of samples were collected in the earlier time intervals, viz., 0-1 hour, would produce a more conclusive correlation between Δ^9-THC and the metabolite.

With completion of the present study all of the major objectives set forth in the original goals have been accomplished. Basically these were development of a method for determining Δ^9-THC levels in human blood and applying the method to blood from actual marijuana smokers. An additional finding which gives great promise for identifying a marijuana user was discovery of the marijuana metabolite in the human blood plasma for up to 24 hours following marijuana smoking. Therefore, the finding of this metabolite in the blood plasma of a driver identifies him or her as a user. Additional analysis of the same sample would permit assignment of the Δ^9-THC level. Such technology will permit many driver samples to be screened to determine the extent of marijuana use. Also this technology should be useful for future studies in which correlations are made between driver impairment and blood levels of the marijuana constituents.

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