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DEVELOPMENT OF A LOW COST PORTABLE FLUOROMETRY TECHNOLOGY AND QUANTIFICATION OF CANNABINOIDS IN BODY FLUIDS

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16. Abstract

Technology was developed for determining Δ^9-tetrahydrocannabinol (I) and it's major metabolite 11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid (II) in human blood plasma utilizing high pressure liquid chromatography (hplc)-ultraviolet (uv) detection. The methods were found to be capable of determining 10 ng of I or II in 1 ml of human plasma. A third type of assay method was developed for identifying in human saliva one of the major constituents of marijuana, cannabinol (III). Results indicated that III photolytically converted to a highly fluorescent compound in the absence of air. Using this technique, a limited study of marijuana smoker's saliva and saliva from non-marijuana smokers to which I and III had been added was conducted. This study demonstrated that 1 ng/ml of III can be detected in saliva.

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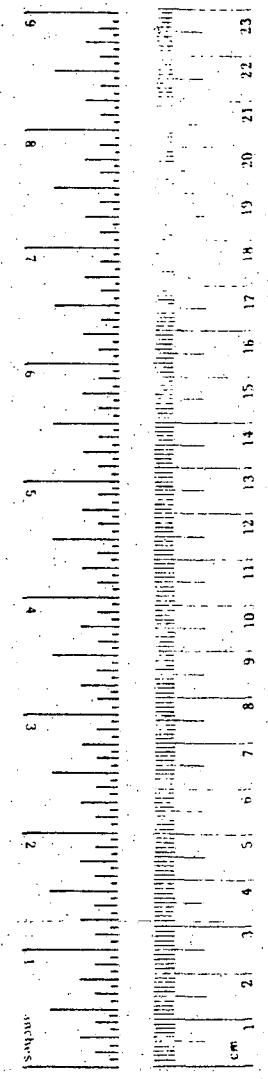
METRIC CONVERSION FACTORS

Approximate Conversions to Metric Measures

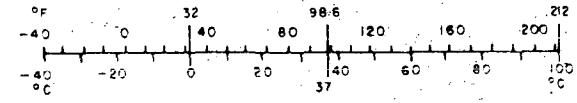
Symbol	When You Know	Multiply by	To Find	Symbol
	LENGTH			
in	inches	*2.5	centimeters	cm
ft	feet	30	centimeters	cm
yd	yards	0.9	meters	m
mi	miles	1.6	kilometers	km
	AREA			
in^2	square inches	6.5	square centimeters	cm^2
ft^2	square feet	0.09	square meters	m^2
yd^2	square yards	0.8	square meters	m^2
mi^2	square miles	2.6	square kilometers	km^2
	acres	0.4	hectares	ha
	MASS (weight)			
oz	ounces	28	grams	g
lb	pounds	0.45	kilograms	kg
	short tons (2000 lb)	0.9	tonnes	t
	VOLUME			
tsp	teaspoons	5	milliliters	ml
Tbsp	tablespoons	15	milliliters	ml
fl oz	fluid ounces	30	milliliters	ml
c	cups	0.24	liters	l
pt	pints	0.47	liters	l
qt	quarts	0.95	liters	l
gal	gallons	3.8	liters	l
ft^3	cubic feet	0.03	cubic meters	m^3
yd^3	cubic yards	0.76	cubic meters	m^3
	TEMPERATURE (exact)			
°F	Fahrenheit temperature	5/9 (after subtracting 32)	Celsius temperature	°C

*1 in = 2.54 (exactly). For other exact conversions and more detailed tables, see NBS Misc. Publ. 286, Units of Weights and Measures, Price $2.25, SD Catalog No. C13.10:286.



Approximate Conversions from Metric Measures

Symbol	When You Know	Multiply by	To Find	Symbol
	LENGTH			
mm	millimeters	0.04	inches	in
cm	centimeters	0.4	inches	in
m	meters	3.3	feet	ft
m	meters	1.1	yards	yd
km	kilometers	0.6	miles	mi
	AREA			
cm^2	square centimeters	0.16	square inches	in^2
m^2	square meters	1.2	square yards	yd^2
km^2	square kilometers	0.4	square miles	mi^2
ha	hectares (10,000 m^2)	2.5	acres	
	MASS (weight)			
g	grams	0.035	ounces	oz
kg	kilograms	2.2	pounds	lb
t	tonnes (1000 kg)	1.1	short tons	
	VOLUME			
ml	milliliters	0.03	fluid ounces	fl oz
l	liters	2.1	pints	pt
l	liters	1.06	quarts	qt
l	liters	0.26	gallons	gal
m^3	cubic meters	35	cubic feet	ft^3
m^3	cubic meters	1.3	cubic yards	yd^3
	TEMPERATURE (exact)			
°C	Celsius temperature	9/5 (then add 32)	Fahrenheit temperature	°F



SUMMARY

Technology was developed for determining Δ^9-tetrahydrocannabinol (Δ^9-THC) and its major metabolite 11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid (9-CO_2H-Δ^9-THC) in human blood plasma utilizing high pressure liquid chromatography (hplc) and ultraviolet (uv) detection. The method developed for analysis of Δ^9-THC was accomplished using an extraction of 1 ml of plasma with petroleum ether followed by normal phase hplc analysis. With the method, the lower practical limit of quantification was found to be 10 ng/ml, but a detection limit of 5 ng/ml was readily achieved. Thus the method would be useful for quantifying Δ^9-THC in the one-hour period following marijuana smoking. The precision and accuracy of the method was comparable to the previously developed hplc-ms methodology.

For the analysis of 9-CO_2H-Δ^9-THC, an hplc-uv method was developed utilizing a reverse phase column. Although the lower practical quantification limit was found to be 10 ng/ml of plasma, the plasma levels determined in several marijuana smokers were found to give a response greater than that observed for a 100 ng/ml standard. Mass spectrometry was used to demonstrate that the quantified peak in the hplc analysis was consistant with the mass spectrum of 9-CO_2H-Δ^9-THC. The developed method was used to determine the plasma levels of 9-CO_2H-Δ^9-THC in both marijuana smokers and patients receiving Δ^9-THC. Results indicated that 9-CO_2H-Δ^9-THC does not give a smooth plasma decay curve as does Δ^9-THC and that levels remain quite high for 24 hours following marijuana smoking. This assay method may be of practical value in identifying a marijuana user.

A third type of assay method was also developed during the study for one of the major constituents of marijuana, viz. cannabinol (CBN). Spectral studies with the various cannabinoids indicated that CBN could be photolytically converted to CBN I if air was excluded and to CBN II and CBN III in the presence of air. Of these photolytic compounds, CBN I proved to be highly fluorescent. An assay

was developed for saliva which allowed CBN present from marijuana smoking to be detected. A limited study was conducted using saliva from both marijuana smokers and nonsmokers as well as the saliva from nonsmokers to which 1 ng/ml of CBN was added. The results indicate that a marijuana smoker can be identified if at least 1 ng/ml of CBN is present in the saliva.

All three of the methods developed during the study represent an advance in reducing the complexity, time and expense in assaying for marijuana use. In the case of Δ^9-THC and 9-CO_2H-Δ^9-THC, the developed method is somewhat more involved since quantification is the major objective. With the CBN assay, the procedure is not lengthy but gives only qualitative information as to marijuana use. All three analysis utilize comparatively simple techniques. Although not all clinical laboratories are equipped with hplc instruments to perform the Δ^9-THC and/or 9-CO_2H-Δ^9-THC assays, there would be the universal ability to perform the CBN assay since a readily available fluorometer is used.

ACKNOWLEDGEMENT

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INTRODUCTION

During a previous contract (HS-4-00968 and TSC-389), technology was developed which permitted determination of nanogram amounts of Δ^9-tetrahydrocannabinol (Δ^9-THC) in human blood, saliva or breath. This technology utilized a high pressure liquid chromatography-mass spectrometry (hplc-ms) method. The hplc step permitted Δ^9-THC to be separated from the other major cannabinoids found in marijuana smoke as well as endogenous body constituents, whereas use of the mass spectrometer permitted detection of trace amounts of Δ^9-THC. Selectivity, sensitivity, reproducibility and accuracy were all well demonstrated for this technique when applied to blood plasma (1). However, the method, although excellent, was not amenable to low sample cost or rapidity of processing. Thus the present contract period was initiated in an attempt to develop alternate technology for the detection of Δ^9-THC.

Prior work in our laboratories had led us to believe that Δ^9-THC had fluorescent properties in various solvents such as 1,4-dioxane. Also this preliminary work had shown that Δ^9-THC had other optical characteristics which should be explored in order to find an optimal detection system for this chemical compound.

Results from our earlier work (2,3) also indicated the consistent appearance of an unknown substance in the blood and breath of marijuana smokers. Since this substance appeared only in the marijuana smokers and not in the nonsmokers, an assumption was made that this compound must be an uncharacterized marijuana metabolite. Therefore, one facet of the present work was devoted to elucidating the structure of this substance.

EXPERIMENTAL

A. Ultraviolet Studies of the Cannabinoids

As a prerequisite to the study of the fluorescence properties of any chemical substance, it is necessary to know the region of maximum uv absorbence to determine the proper wavelength for excitation. Literature values had been reported for

Δ^9-THC (4), CBD (5), CBC (6) and CBN (5). However, since most of these determinations had been reported more than ten years ago, it was felt that a redetermination was appropriate. As shown in Figure 1, the uv spectrum of each cannabinoid was recorded in methyl alcohol using a Cary 118 spectrophotometer. Concentrations of each cannabinoid in methanol was varied to achieve measurable absorbences over the usable uv spectrum. Cells used for all determinations had a 10 mm pathlength. The extinction coefficient of each compound was calculated using the formula:

$$E = \frac{A}{cb}$$

where: A = absorbence

c = concentration in g/100 ml

b = cell path length

B. Fluorescence Studies of the Cannabinoids

The fluorescence of the cannabinoids was studied in two ways. First, using an Aminco-SPF-125, Δ^9-THC, CBD, CBN and CBC as well as the two major metabolites of Δ^9-THC, viz., 11-OH-Δ^9-THC and 11-CO_2H-Δ^9-THC were placed in a methanol solution and their emission spectra obtained. For the excitation wavelength, the uv maxima for each respective compound were used. For example, Δ^9-THC which gives uv maxima at 208, 273.6 and 280 nm had three emission spectra recorded using each of these wavelengths as the excitation wavelength. Figure 2 gives the fluorescent spectra of these compounds.

A second method used to study fluorescences was the hplc-fluorometry method. This method was used to delineate between individual cannabinoids and impurities which were contained in the cannabinoid being studied via the first method. For example, Δ^9-THC appeared to fluoresce quite intensely at higher concentrations, but this was later shown to be due to a slight contamination present in Δ^9-THC. A more detailed discussion of the apparatus used for these studies is given in C below.

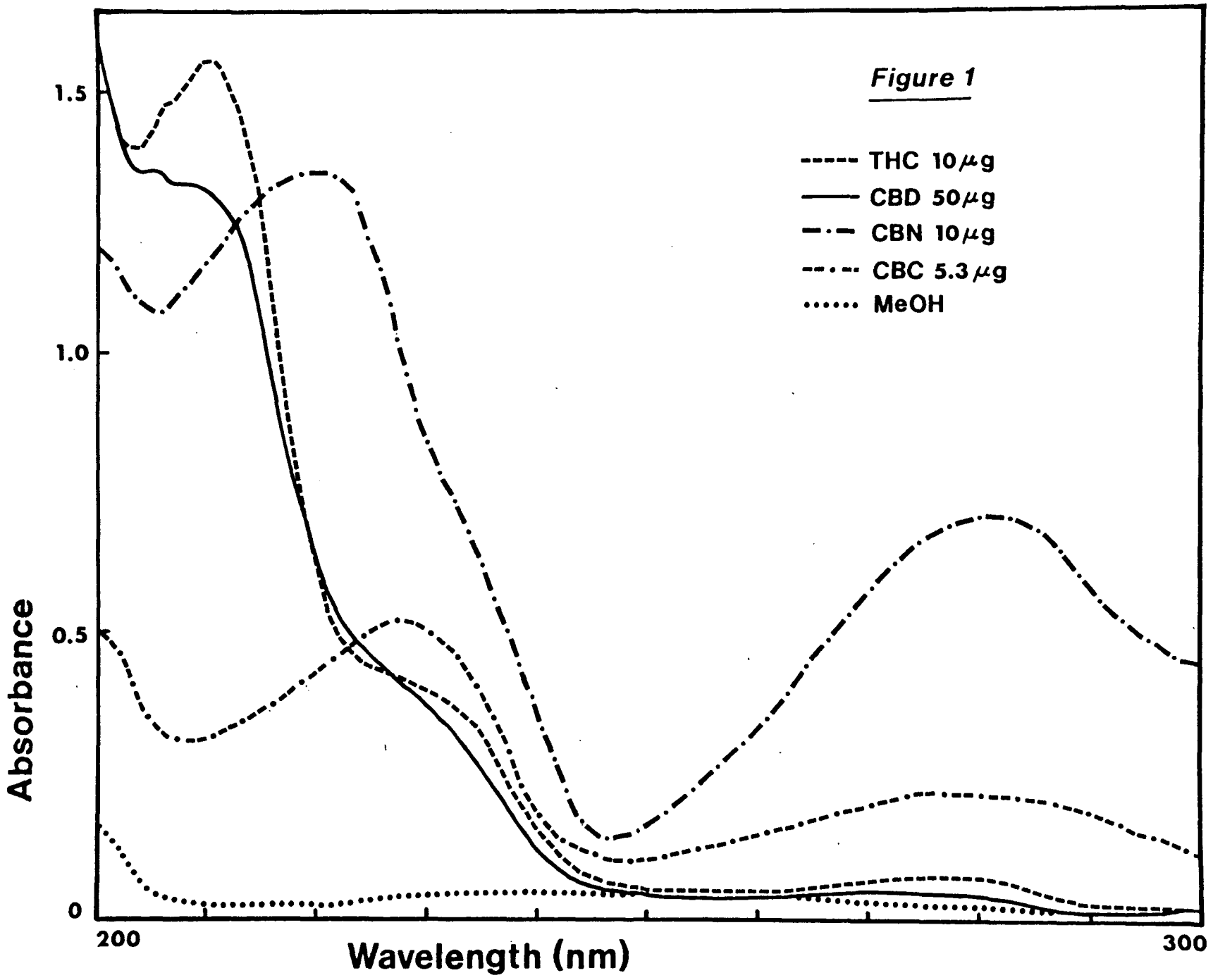


Figure 1
THC 10μg
CBD 50μg
CBN 10μg
CBC 5.3μg
MeOH
Absorbance
1.5
1.0
0.5
0
200
300
Wavelength (nm)

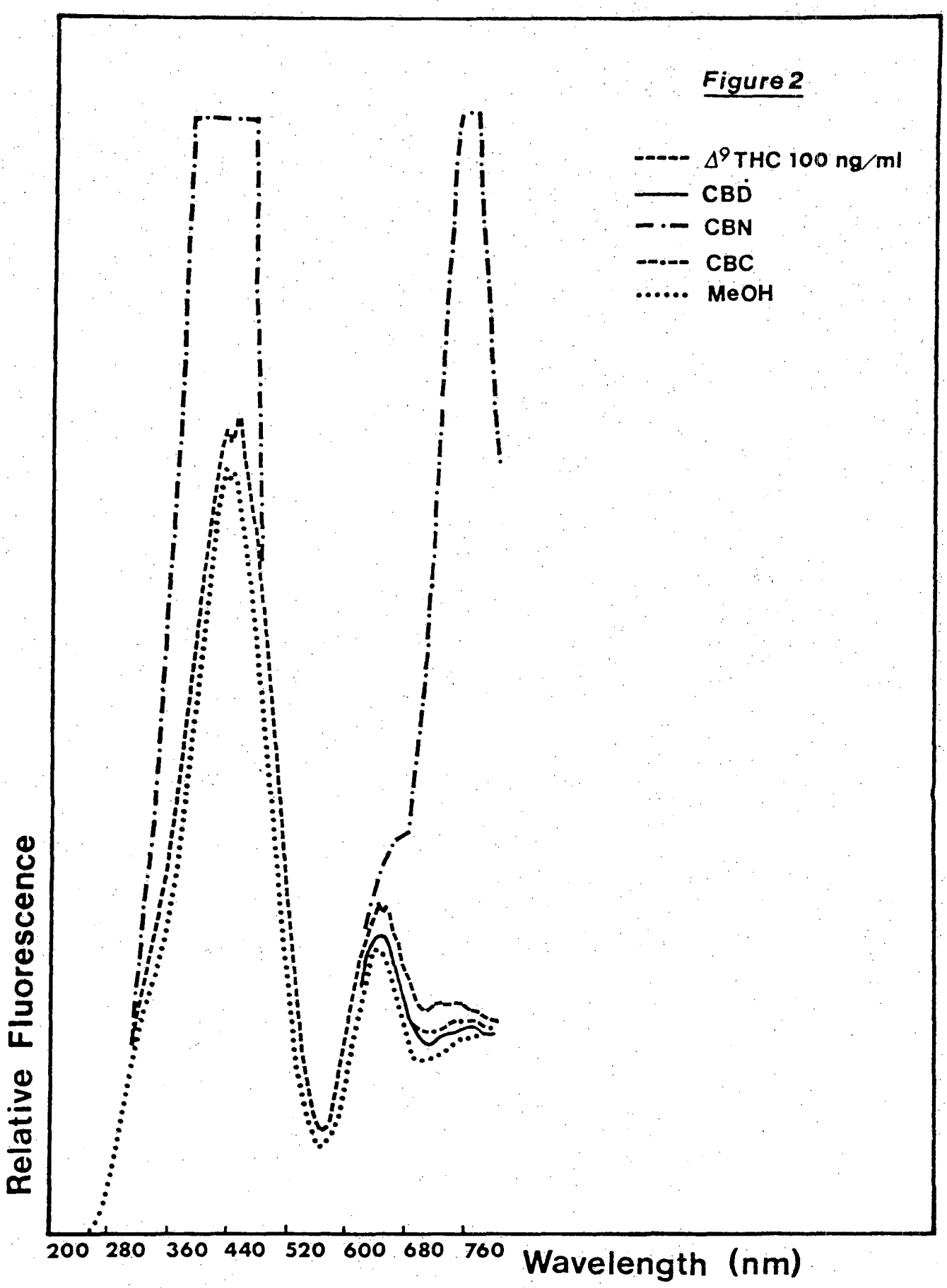


Figure 2
----- Δ9 THC 100 ng/ml
—— CBD
—·— CBN
---- CBC
····· MeOH
Relative Fluorescence
200 280 360 440 520 600 680 760
Wavelength (nm)

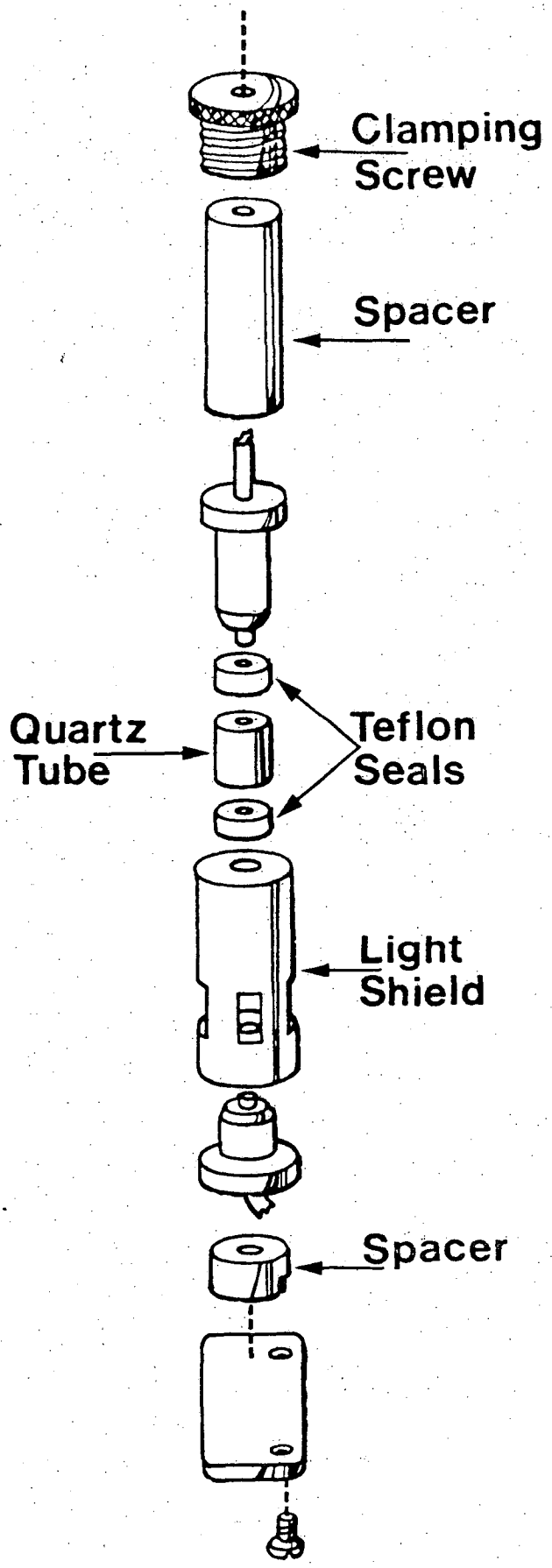
C. Design and Construction of a Fluorometric Flow Cell

At the outset of the present contract, there were no fluorometric flow cells available commercially which were compatible with an hplc. Thus design and construction of such a flow cell was undertaken. A number of different designs were envisioned, but most were rejected because of the difficult task of properly machining the necessary parts. The final design which was reduced to construction, incorporated simplistic characteristics enabling ease of manufacture and flexibility in the size flow cell used. This design is shown in Figure 3. Size of the quartz tube can be varied to give the desired flow volume. The quartz tube used in our work was 5 mm x 2mm i.d. to give a flow volume of 26 µl.

D. Analysis of Δ^9-THC in Human Plasma Using Sephadex-LH-20 and Hplc

A 1 ml sample of human plasma is used for the analysis. To the sample is added 325 ng of DMHP in 3.0 µl of methanol as the internal standard, followed by extraction with 6.0 ml of petroleum ether (3 x 2 ml). The extracts are combined and extracted at room temperature and the resultant residue reconstituted in 0.5 ml of 10:10:1, methylene chloride:heptane:ethanol and the solution placed on a Sephadex LH-20 column. The Sephadex LH-20 was packed into a standard volumetric burette which had been modified in two ways. First the tip was removed to prevent mixing of eluting solvents once separation had been effected. The second modification was to fit each column with a 19/28 ground glass joint in which is placed a 500 ml round bottom flask which has a corresponding 19/28 joint fitted in the bottom. This flask was the reservoir for the eluting solvent. Each column had a small plug of silylated glass wool placed in the end and a slurry of Sephadex LH-20 in the mobile phase poured in until a height of 30 cm was obtained. The flow of each column was set precisely at 0.2 ml/min using a stopwatch and volumetric markings. Using these conditions, DMHP elutes between 14 to 20 ml, and Δ^9-THC at 20 to 26 ml. Both fractions were combined and evaporated prior to hplc analysis.

Figure 3


Clamping Screw
Spacer
Quartz Tube
Teflon Seals
Light Shield
Spacer

This residue is reconstituted in 200 µl of the hplc mobile phase, 0.3% isopropanol in heptane and injected onto a CN-10 bonded phase column. A flow rate of 120 ml/hr and uv detection at 208 mµ gave retention times of 1.35, 1.95, 2.09, 2.25, and 2.95 minutes for DMHP, CBN, Δ^9-THC, CBC and CBD, respectively.

E. Analysis of Δ^9-THC in Human Plasma Using HPLC-UV

To 1 ml of human plasma is added 70 ng (70 µl of a 10 ng/µl solution in methanol) of $\Delta^{9,11}$-THC and the sample mixed for 5 seconds using a Vortex mixer. A silylated 15 ml conical test tube was used. The sample is extracted with a total of 6 ml (4 x 2 ml) of petroleum ether (b.p. 30-60°C) and the extracts combined. For each extraction mixing is continued until a gel is formed, usually requiring about 20 seconds. The gel is dispersed by centrifuging at 2600 rpm for 10 minutes and the top layer removed prior to the next extraction. The combined extracts were evaporated to dryness at room temperature and the resultant residue reconstituted with 100 µl of mobile phase, 0.55% isopropanol in heptane. For hplc analysis, an isocratic program was used at a flow rate of 40 ml/hr with tandem columns consisting of a 10 µ NH and a 10 µ CN bonded phase column. Retention times of 9.2 and 10.3 minutes were noted for $\Delta^{9,11}$-THC and Δ^9-THC, respectively. A total analysis time of 45 minutes was required between samples to permit reequilibration of hplc columns. Detection of the eluting peaks was accomplished using a spectrophotometer (Varichrom) set at 209 nm. Peak height ratio of Δ^9-THC to $\Delta^{9,11}$-THC was determined by measuring peak height from a tangential line to the base of the peaks. Values of unknown samples were determined by substituting the peak height ratio into the least squares equation for standards of 5, 10, 15, 20, 30, 60 and 120 ng/ml.

F. Analysis of 9-CO_2H-Δ^9-THC in Human Plasma Using HPLC-UV

To 1 ml of human plasma is added 1 ml of pH 4.0 buffer and the sample mixed for 5 seconds. The mixture was extracted with a total of 6 ml (2 x 3 ml) of 1.5% isopropanol in benzene, mixing each time until a gel forms. Centrifugation of

the gel at 2500 rpm for 10 minutes produces two layers. Each time 2 ml of the top layer was removed, combined, and evaporated to dryness at room temperature. To the resultant residue was added 50 µl of 50:50, water:acetonitrile. A gradient elution program was used for the hplc analysis which started at 60:40, water: acetonitrile and was held isocratically for 3 minutes, then increased to 40:60, water:acetonitrile over 2 minutes and held for 5 minutes, then reversed to 60:40, water:acetonitrile over a 2 minute period. With this program, the retention time of 9-CO_2H-Δ^9-THC was 2.7 minutes. Detection was accomplished using a spectrophotometer (Varian 635D) set at 210 nm. The column used was a Water's Associates 37-50 µ phenyl bonded phase with a flow rate of 60 ml/hr. The area for 9-CO_2H-Δ^9-THC was determined by triangulation and the ng/ml present calculated by using the least square equation for standards of 50, 100, 300, 500, 700 and 1000 ng/ml.

Results and Discussion

A. Hplc-Fluorescence Studies

Prior to the initiation of the present study, results from our laboratory had shown that Δ^9-THC had some fluorescent characteristics in certain solvents. Thus a concerted effort was made to determine how to optimize these fluorescent properties of the cannabinoids. Foremost in this study was the concurrent development of a fluorometric flow cell which was compatible with the hplc and a mobile phase system which would permit separation of the cannabinoids as well as permit their fluorometric identification. Using the hplc-fluorometric system, it was found that the fluorescences observed originally with Δ^9-THC were attributable to an impurity. A solution of Δ^9-THC in 0.4% dioxane in heptane gave acceptable fluorescences but when analyzed no fluorescence was noted at the retention time for Δ^9-THC. Instead, the fluorescence was noted at the retention time of CBN. Since Δ^9-THC had very little native fluorescence, a study was begun of ways to induce fluorescence into Δ^9-THC. Several reports (7,8) had indicated that Δ^9-THC would react with dansyl chloride to give a reaction product as shown in Figure 4, which was highly fluorescent.

Numerous attempts were made to duplicate the reported procedures both on a semi-macro and micro scale. Using the procedure of Forest, et al (7), no reaction

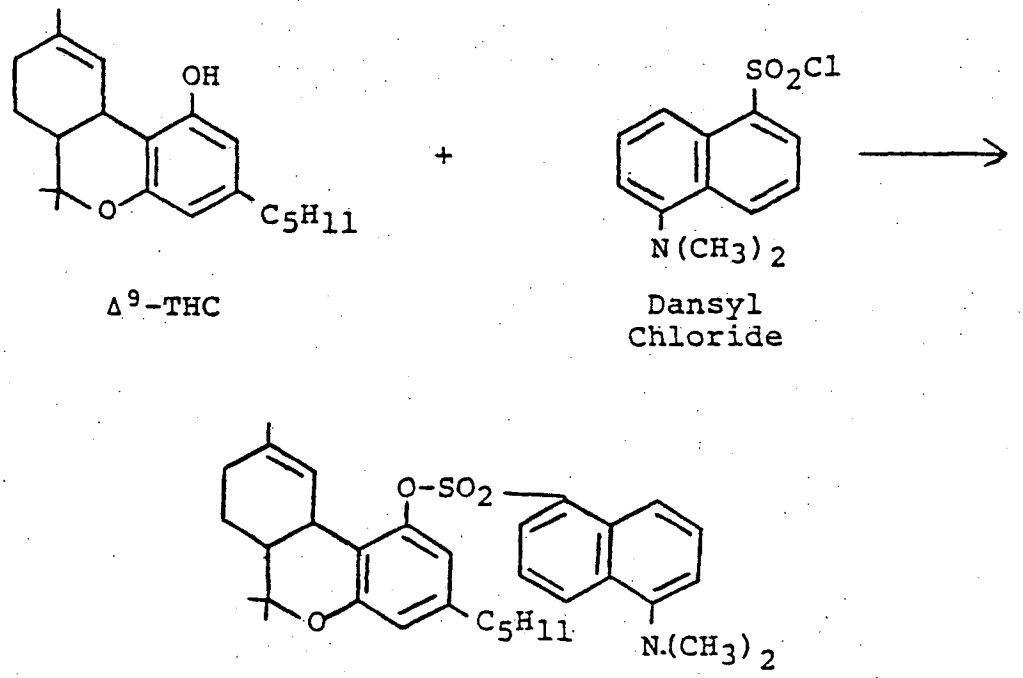


FIGURE 4

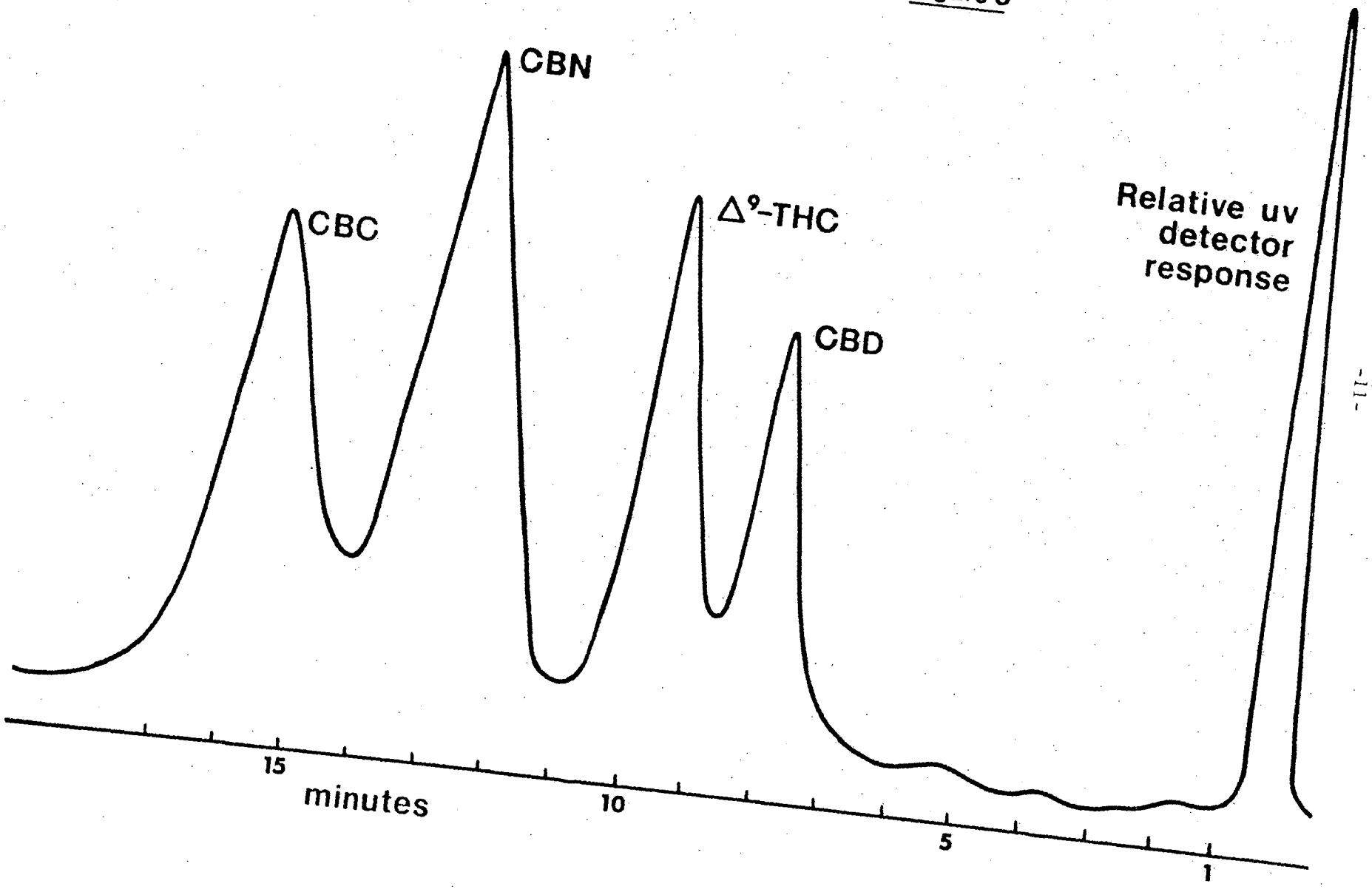
was consistently found to occur. The most logical explanation was that the dansyl chloride was being converted to the unreactive sulfonic acid, although this was not conclusively demonstrated. A number of different modifications in the reaction conditions were made in an effort to get the desired condensation product. However, all these modifications resulted in giving a mixture of reaction product, none of which were the desired product. Although several of the reaction products did show fairly nice fluorescent properties, there was no consistent reproducibility in the resultant intensity. These findings led to the conclusion that the reaction between Δ^9-THC and dansyl chloride is not a simple, straight-forward chemical reaction as had been indicated in the prior reports. Owing to the multiple reaction products and lack of reproducibility, the entire method was abandoned.

B. Hplc-Ultraviolet Studies for Δ^9-Tetrahydrocannabinol

In our prior hplc-ms work (1-3), hplc with uv detection had been routinely used. For this work, 273.6 nm had been used as the wavelength for detecting Δ^9-THC. As a result of the uv studies done as a prelude to the fluorometric studies, it was serendipitously found that Δ^9-THC gave its maximum uv absorbence at 209 nm rather than 273.6 nm (cf., Figure 1). In fact, the absorption was found to be approximately 10 times greater at 209 nm as evidenced by the molar absorbtivity shown in Figure 1. To utilize this newly found wavelength for detection of Δ^9-THC for monitoring the hplc effluent, it was necessary to develop some different hplc mobile phase solvents which did not have a uv cut-off at 209 nm. One such isocratic system was found which utilizes 0.4% dioxane in heptane and a silicic acid column. This system adequately separates Δ^9-THC, CBD, CBN and CBC, while allowing monitoring at 209 nm as shown in Figure 5. Initial work with this system demonstrated that 20 ng of Δ^9-THC could be easily detected. However, to detect amounts of Δ^9-THC lower than 20 ng became a problem due to the signal to noise ratio. That is, if the amplitude of the recorder was increased to detect the signal for Δ^9-THC, the ambient noise became so great that the signal could not be detected. To overcome this limitation, a number of electronic modifications as well as optical modifications were made to the spectrophotometers used as detectors for the hplc. Experimentally, it was found that best results were found by using a dual column-dual beam configuration on the spectrophotometer. This modification allowed 10 ng of Δ^9-THC to be clearly detectable when injected onto the hplc instrument.

The finding that small amounts of Δ^9-THC could be detected by the hplc-uv method prompted an evaluation of the method for Δ^9-THC in blood plasma. Initial results were very discouraging because it was found that there was an endogenous constituent in human blood plasma with an hplc retention time virtually identical to Δ^9-THC. Numerous variations in the hplc program were evaluated in an attempt

Figure 5


CBN
CBC
Δ⁹-THC
CBD
Relative uv detector response
15
minutes
10
5
1

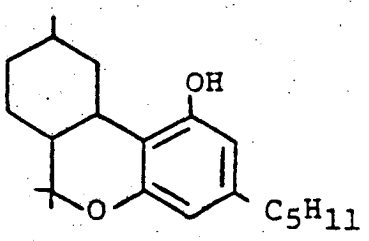
to separate the endogenous plasma constituent from Δ^9-THC. However, no hplc variations were found to be entirely satisfactory. At this juncture, a precolumn clean-up of the blood plasma extract using a Sephadex LH-20 column was investigated. This precolumn clean-up proved to be very satisfactory in removing the interfering blood plasma constituents.

A second hplc method was developed which has proven to be more satisfactory for the cannabinoids. This system uses 0.3% isopropanol in heptane as the mobile phase and a bonded phase nitrile column. Use of the isopropanol as compared to dioxane in the mobile phase accomplished two salient points. First, the signal to noise ratio was dramatically improved, thereby reducing the signal to noise ratio and antiquating the need for dual columns. Second, the detectability of Δ^9-THC was increased since a stronger response for 10 ng was observed. However, to successfully use the isopropanol-heptane mobile phase, it was necessary to switch to the nitrile bonded phase column. The major reason for this is the loss of resolution which occurs on a silicic acid column with isopropanol in the mobile phase.

Once the proper hplc conditions were found, it was necessary to determine the best internal standard for the analysis of Δ^9-THC, CBD, CBN and CBC. The compounds, hexahydrocannabinol (HHCBN, II), $\Delta^{9,11}$-tetrahydrocannabinol (III), dimethylheptylpyran (DMHP, IV) and cyclopentyldimethylheptylpyran (CDMHP, V) were all evaluated as internal standards. Using either the silicic acid or nitrile column system, HHCBN was found to be satisfactory and eluted from the Sephadex precolumn along with Δ^9-THC. However, with the nitrile column, HHCBN overlaps slightly with CBD and would make the analysis of CBD difficult and might possibly interfere in a determination of Δ^9-THC to HHCBN area ratios. Further experimental work clearly demonstrated that DMHP was the best choice for an internal standard when using the nitrile column.

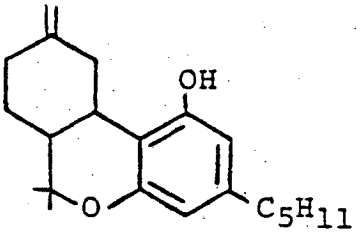
Owing to the overall simplicity of an isocratic hplc system which used only

one column, it was decided that the nitrile column offered the best analysis system for Δ^9-THC. Therefore the scheme shown in Figure 6 was developed for analyzing Δ^9-THC in human blood plasma. Using the Sephadex LH-20 clean-up



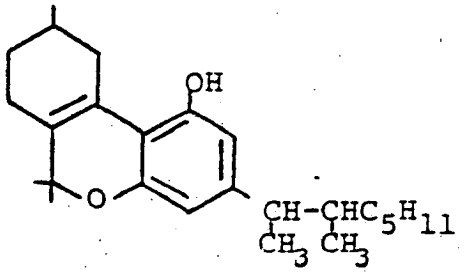
HHCBN

II



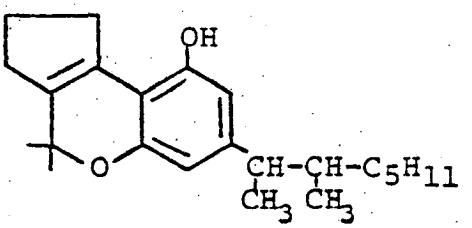
$\Delta^{9,11}$-THC

III



DMHP

IV

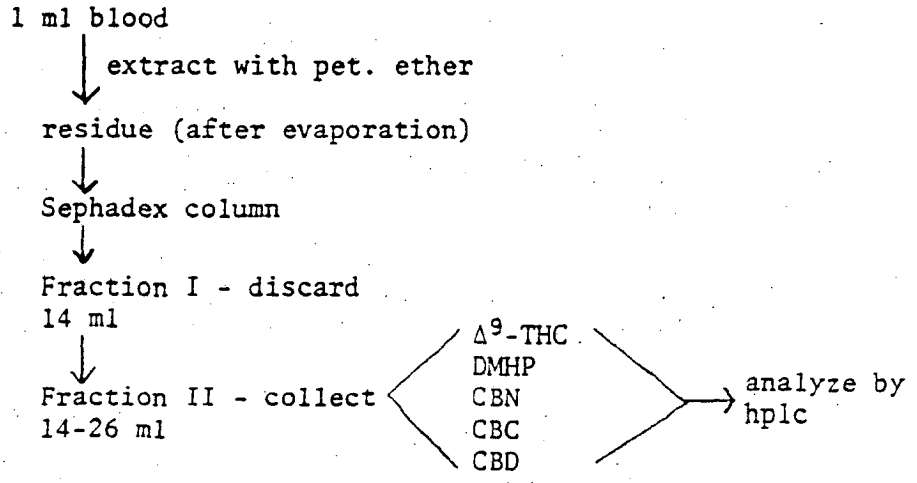


CDMHP

V

FIGURE 6

Analysis of Δ^9-THC in Human Blood Plasma (Sephadex Method)



followed by hplc analysis as depicted in Figure 6, it was possible to develop an analytical technique for Δ^9-THC in blood plasma. The accuracy and precision of the hplc analysis was assessed as shown in Table I. To demonstrate the

TABLE I

Accuracy and Precision of HPLC Method for Analysis of Δ^9-THC

Concentration of Δ^9-THC (ng)	Area Ratio Average (Range)	Number of Determinations	Standard Deviation
5	0.082 (0.070-0.093)	10	± 0.007
10	0.158 (0.148-0.167)	10	± 0.008
20	0.280 (0.257-0.306)	10	± 0.018
30	0.418 (0.403-0.435)	10	± 0.011
40	0.540 (0.504-0.580)	10	± 0.024
50	0.688 (0.653-0.731)	10	± 0.027

reproducibility of the method in blood plasma, samples from two subjects who had smoked marijuana were evaluated and compared to results obtained from the hplc-ms technique. For this evaluation, each subject's plasma was processed on one of two different Sephadex LH-20 columns. That is, the plasma samples from subject T.V. were processed through Sephadex column A, whereas samples from subject R.B. were processed through column B. Four samples from each subject were processed, 0, 0.25, 0.5 and 1 hour post-smoking. A standard curve was obtained for each Sephadex column by processing blood plasma from our laboratory workers to which had been added 2, 5, 10, 20 and 30 ng/ml of Δ^9-THC. The samples were alternated such that a standard was processed followed by a subject's plasma, etc., until all samples had been processed. A total of 4.5 days was required to process the samples on each of the Sephadex columns, since only 2 samples per day can be processed on each of the two columns. However, all samples were processed by hplc on the same day. Results from this study are given in Table II.

As Table II illustrates, the values obtained for subject T.V. are in quite good agreement with the hplc-ms technique. However, the values obtained for subject R.B. using a second Sephadex column were inconsistent at two of the three levels determined. These results point out one of the problems with the Sephadex clean-up, that is, inconsistency of different columns. Such inconsistencies are most likely related to the variability in flow rate from one column to the other. Although the flow rate was carefully adjusted as outlined in the earlier experimental sections, there is some variation during an elution due to the pressure (or lack of pressure) exerted by the reservoir of mobile phase.

Another problem with the Sephadex method is illustrated in Figure 7, which is an hplc chromatogram of blood plasma which is free of Δ^9-THC and has been processed through Sephadex and hplc. Figure 8 is an hplc chromatogram of the same blood plasma to which 30 ng of Δ^9-THC and 375 ng of DMHP have been added. Since both the DMHP and Δ^9-THC appear as shoulders on other peaks, the quantification is complicated. The other major drawback to the Sephadex-hplc method was

TABLE II

Sephadex-Hplc Analysis of Marijuana Smokers' Plasma Compared to Hplc-Ms Analysis

Subject	Sephadex Column	Time (hrs)	ng/ml Δ^9-THC Found HPLC	HPLC-MS
T.V.	A	0	10.0	11.3
		0.25	25.7	34.6
		0.5	19.5	16.5
		1.0	11.7	10.1
R.B.	B	0	4.1	5.7
		0.25	60.9	43.7
		0.5	92.5	18.9

the long analysis time required for an individual sample. Thus as was demonstrated with column A (Table IV), good values could be obtained, but five days actual analysis time was not practical. For these reasons, this method was dropped from

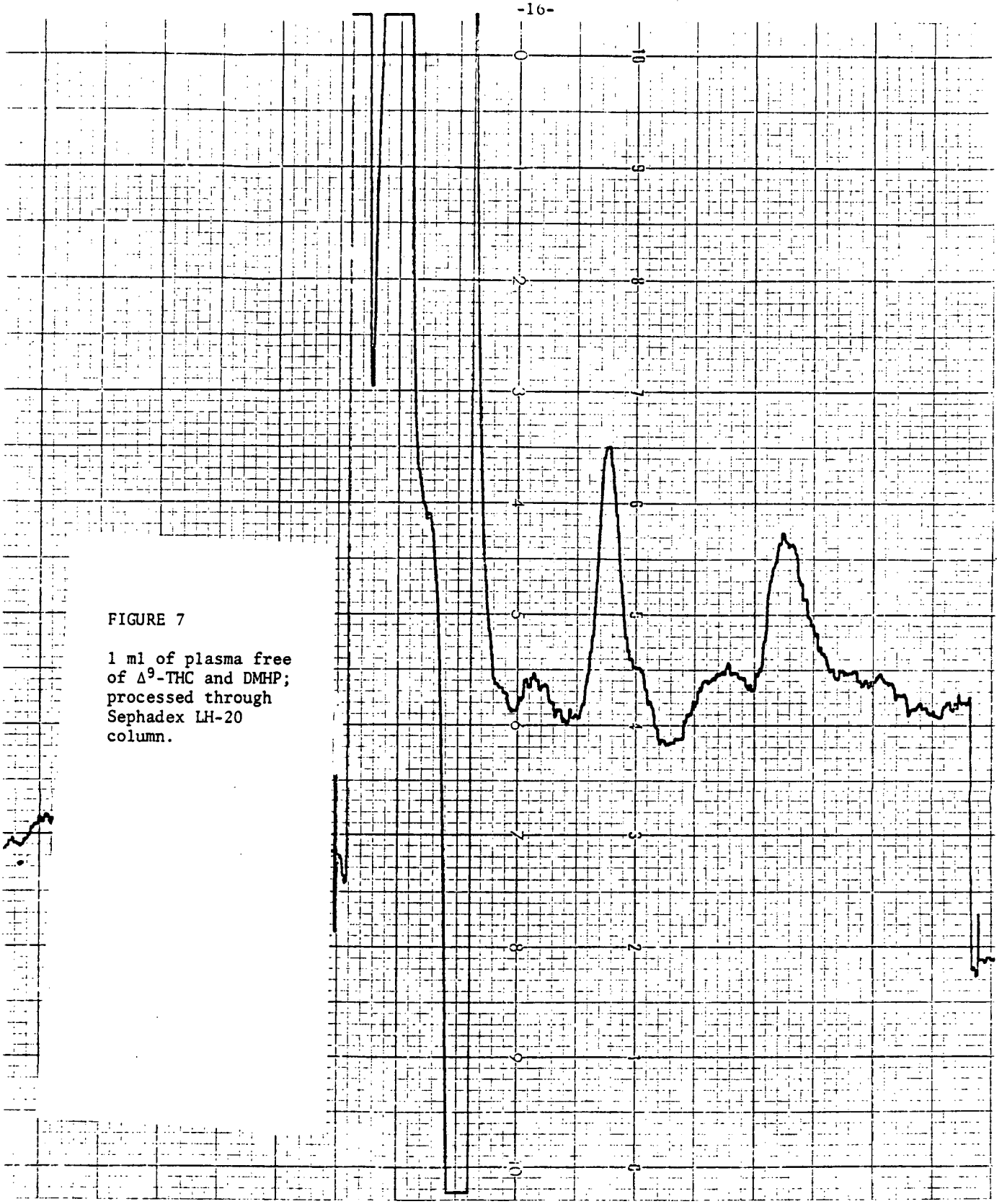


FIGURE 7

1 ml of plasma free of Δ^9-THC and DMHP; processed through Sephadex LH-20 column.

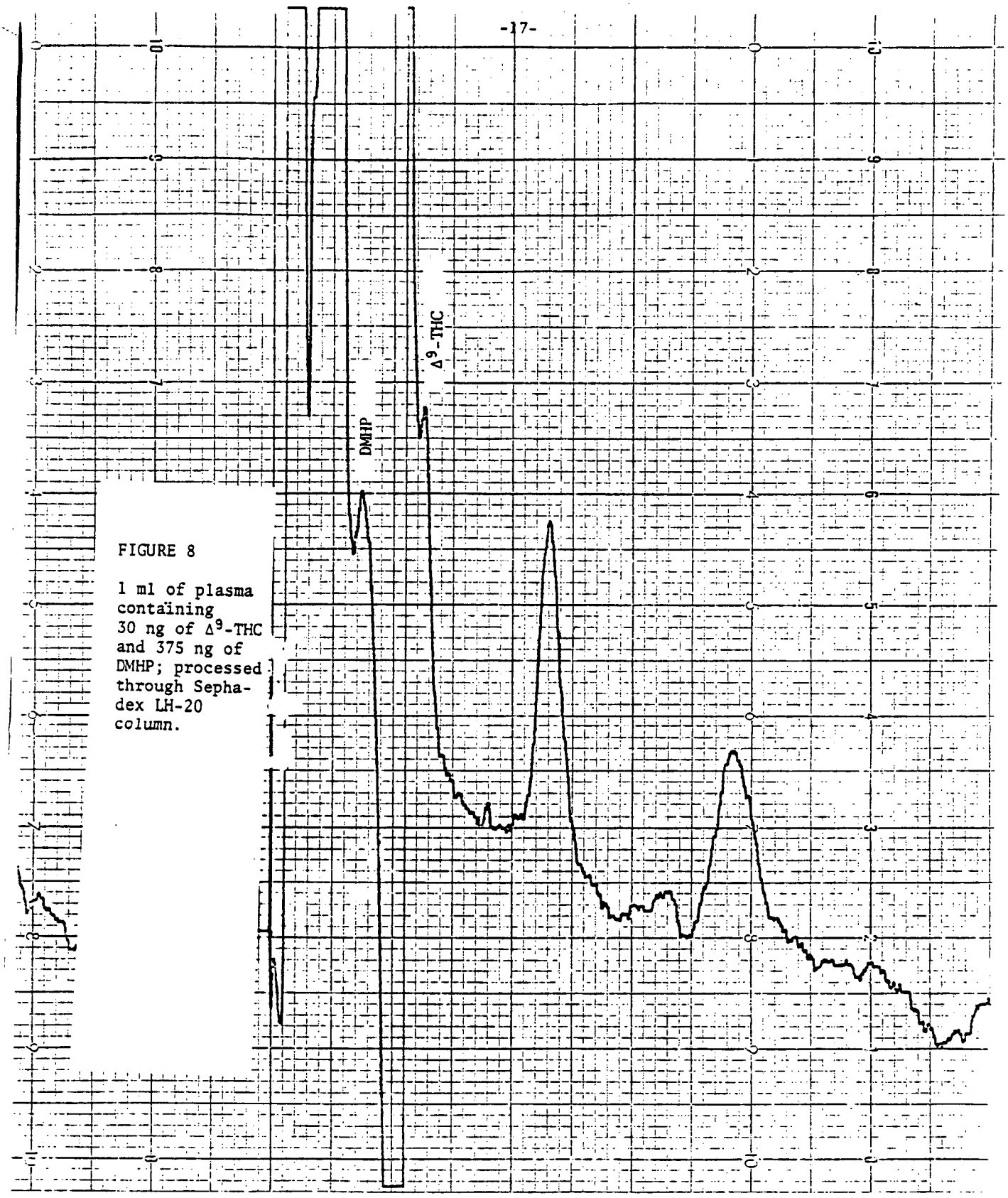


FIGURE 8

1 ml of plasma containing 30 ng of Δ^9-THC and 375 ng of DMHP; processed through Sephadex LH-20 column.

consideration as a replacement for the hplc-ms method.

As was noted in Figure 8, the major problem with hplc analysis was the endogenous blood plasma constituents which caused the peak for Δ^9-THC as well as the proposed internal standards to appear as shoulders and not be clearly resolved. In an attempt to overcome some of these inherent difficulties, a number of experimental variations were tried. One such experimental variation was to attempt to remove these endogenous plasma constituents by using various extraction techniques. For example, instead of extracting the plasma with petroleum ether prior to hplc analysis as was illustrated in Figure 8, other solvents were tried. These were benzene, benzene with varying percentages of isopropanol (0.5-5%), heptane, and heptane with varying percentages of isopropanol (0.5-5%). None of these extracting solvents or solvent combinations gave satisfactory results. That is, the resulting chromatograms were not too dissimilar to that given in Figure 8.

Another experimental technique which was investigated to remove the interfering endogenous plasma peaks was the classical procedure of protein precipitation. Two different precipitation procedures were investigated, viz. methanol and trichloroacetic acid. With methanol, a 1:1 ratio of methanol to plasma was used; whereas, a 1:1 ratio of 5% trichloroacetic acid to plasma was required. In both methods, the precipitate was removed by centrifugation and the supernatant liquid extracted with petroleum ether. With both methods, this extract was shown by hplc analysis to contain neither Δ^9-THC nor the internal standard. Thus it appeared that Δ^9-THC was bound to the precipitatedproteins. When the precipitate was resuspended in distilled water and extracted with petroleum ether followed by hplc analysis, a chromatogram very similar to Figure 8 was obtained. Therefore, the interfering endogenous plasma constituent was not removed by this protein precipitation technique.

Yet another experimental technique which was tried was using an hplc Sephadex LH-20 precolumn. Since there are no such columns available commercially,

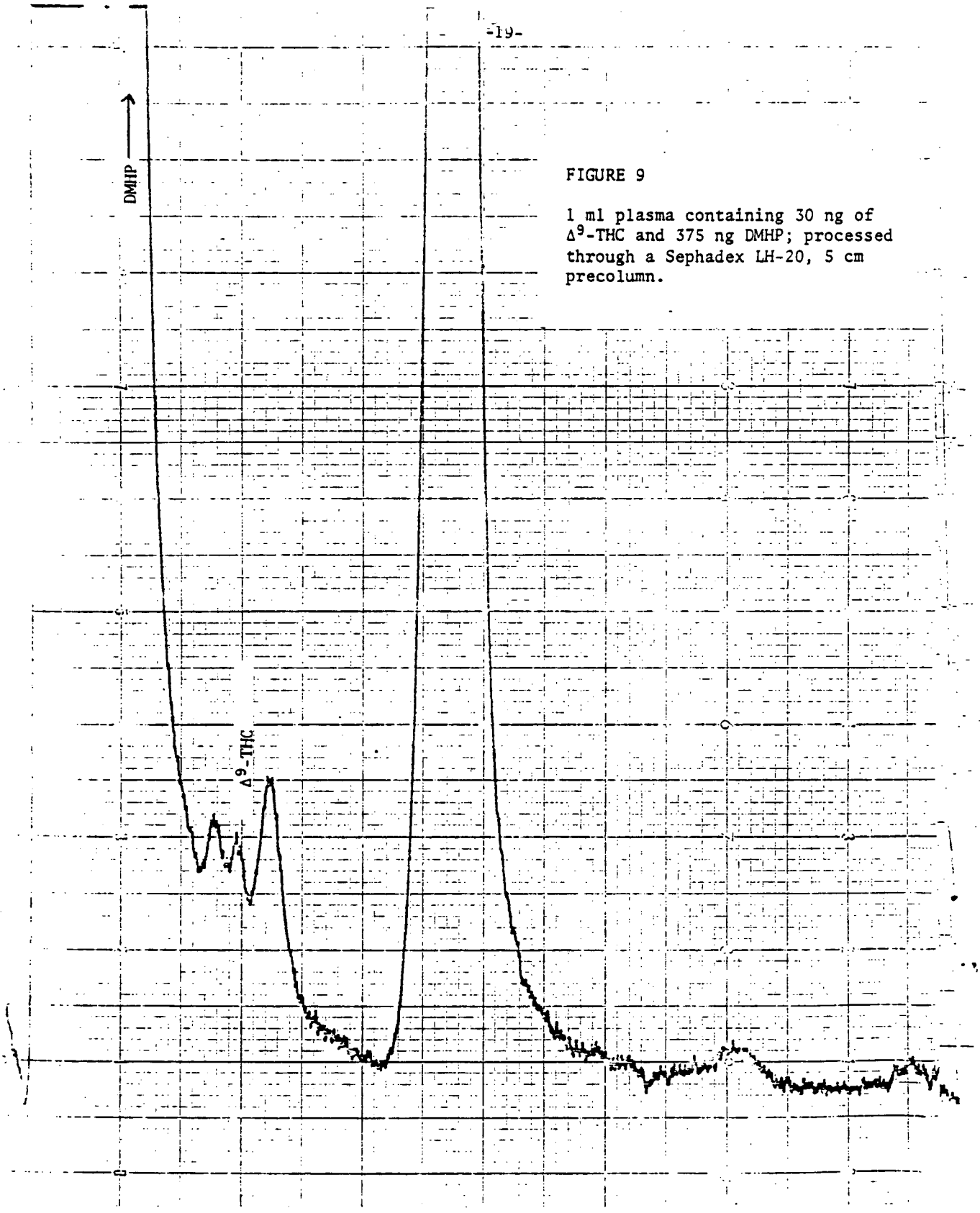


FIGURE 9

1 ml plasma containing 30 ng of Δ^9-THC and 375 ng DMHP; processed through a Sephadex LH-20, 5 cm precolumn.

one was constructed by slurrying Sephadex LH-20 with 100 micron silylated glass beads in heptane and pouring the mixture into an hplc column. This column was placed prior to the CN column on the hplc instrument. As shown in Figure 9, there was still imcomplete resolution of the Δ^9-THC from the endogenous plasma peaks. However, there did appear to be some improvement in the resolution, giving hope that some type of precolumn might be an effective way to resolve Δ^9-THC from the endogenous plasma constituents. Such an effective precolumn was found in a bonded phase NH column. The resolution initially obtained as shown in Figure 10 was better but not completely satisfactory. However, it should be noted that $\Delta^{9,11}$-THC, the internal standard was adequately resolved and Δ^9-THC still needed to be more completely resolved from one endogenous plasma constituent. To accomplish a satisfactory separation of Δ^9-THC, an intensive investigation was undertaken to determine the optimum mobile phase conditions to effect maximum resolution. Table III summarizes some of the conditions tried. Thus it was found that 0.6% isopropanol in heptane at a flow rate of 60 ml/hr gave the optimum resolution of Δ^9-THC from the two endogenous plasma constituents as well as permitting satisfactory separation of the internal standard $\Delta^{9,11}$-THC. However, sometimes

TABLE III

Experimental Conditions for HPLC Analysis With NH-CN Columns

% Isopropanol in Heptane	Flow Rate ml/hr	Resolution
0.1	60	very poor
0.1	120	very poor
0.2	60	fair
0.2	120	fair
0.3	60	good
0.3	120	good
0.3	150	good
0.5	120	good
0.6	60	very good

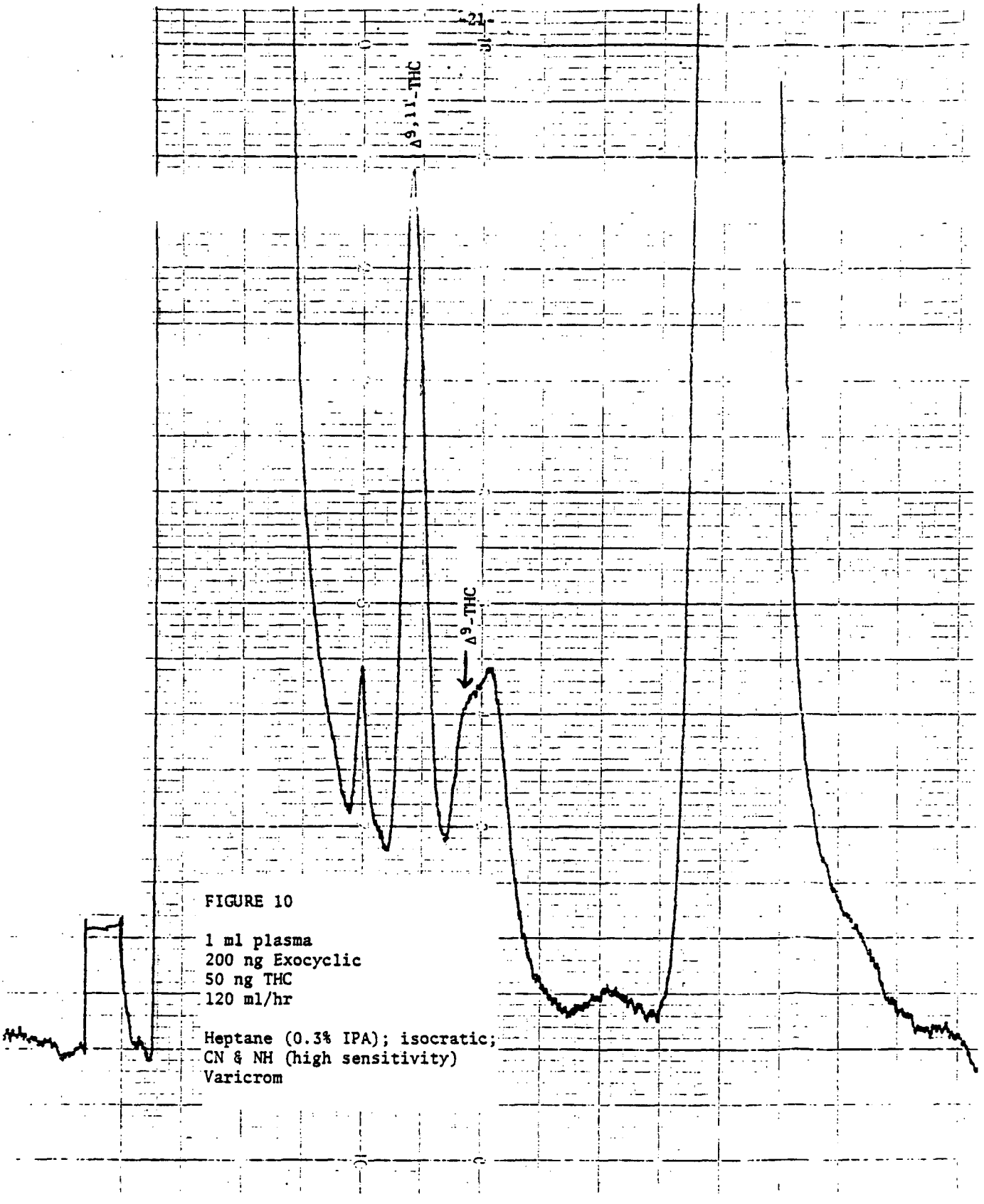


FIGURE 10

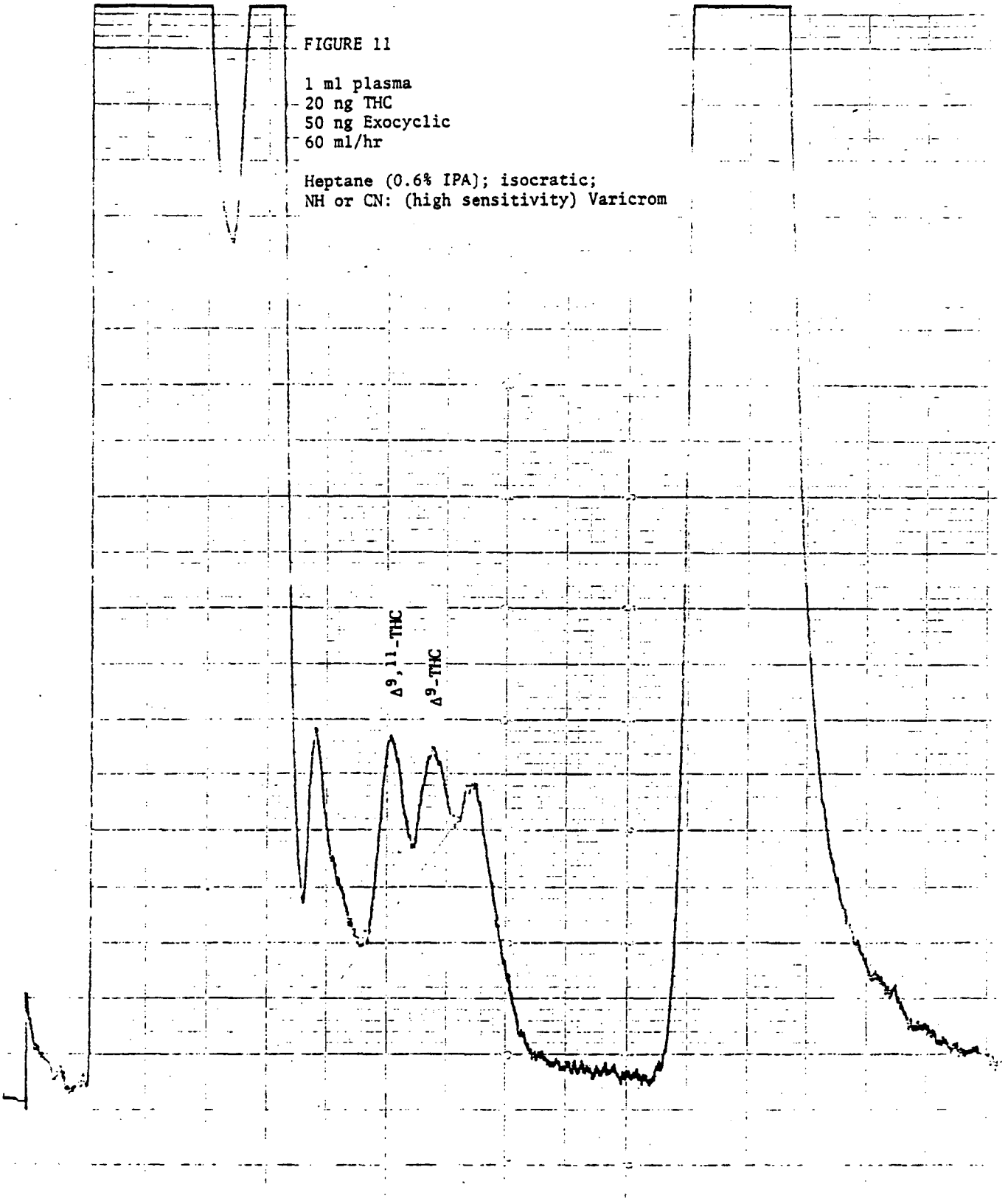
1 ml plasma
200 ng Exocyclic
50 ng THC
120 ml/hr

Heptane (0.3% IPA); isocratic;
CN & NH (high sensitivity)
Varicrom

FIGURE 11

1 ml plasma
20 ng THC
50 ng Exocyclic
60 ml/hr

Heptane (0.6% IPA); isocratic;
NH or CN: (high sensitivity) Varicrom



compromises must be made, and this was the case for the optimum resolution obtained with 0.6% isopropanol in heptane. To keep the overall analysis time short while maintaining an acceptable resolution, 0.55% isopropanol in heptane was chosen as the mobile phase, at a flow rate of 40 ml/hr. These conditions permit the analysis of a blood plasma sample for Δ^9-THC in 35 minutes with a lower limit of sensitivity of 10 ng/ml. Figure 12 illustrates these conditions.

In order to test the adequacy of the developed method for determining the Δ^9-THC levels in marijuana smokers, a study was conducted on plasma from actual marijuana smokers. The samples analyzed were concurrently analyzed by the proven hplc-ms technique. Results from this study are presented in Table IV and clearly demonstrate that the method gives results similar to the hplc-ms method.

C. HPLC-Ultraviolet Studies on 11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic Acid

The major metabolite of Δ^9-THC is 11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid (9-CO_2H-Δ^9-THC). This metabolite has physical properties which are quite dissimilar from Δ^9-THC. One important difference is a lesser degree of binding properties. Other differences include greater polarity, water solubility and acidic properties. Therefore, as a result of these physical property differences, it was necessary to develop an analytical procedure for 9-CO_2H-Δ^9-THC analysis which was somewhat different.

In developing the assay procedure for 9-CO_2H-Δ^9-THC, it was necessary to consider the following factors:

1) optimum pH for extraction from physiological fluids
2) optimum solvent for extraction from physiological fluids
3) proper hplc conditions for analysis
4) internal standard which was compatible with the above conditions.

As was previously discussed, the uv absorption properties of 9-CO_2H-Δ^9-THC were very good at 210 mμ (cf., Figure 13), and therefore uv detection of the compound following hplc analysis was feasible.

FIGURE 12

1 ml of plasma containing 30 ng of Δ^9-THC and 70 ng of $\Delta^{9,11}$-THC; 0.55% isopropanol in heptane at a flow of 40 ml/hr.

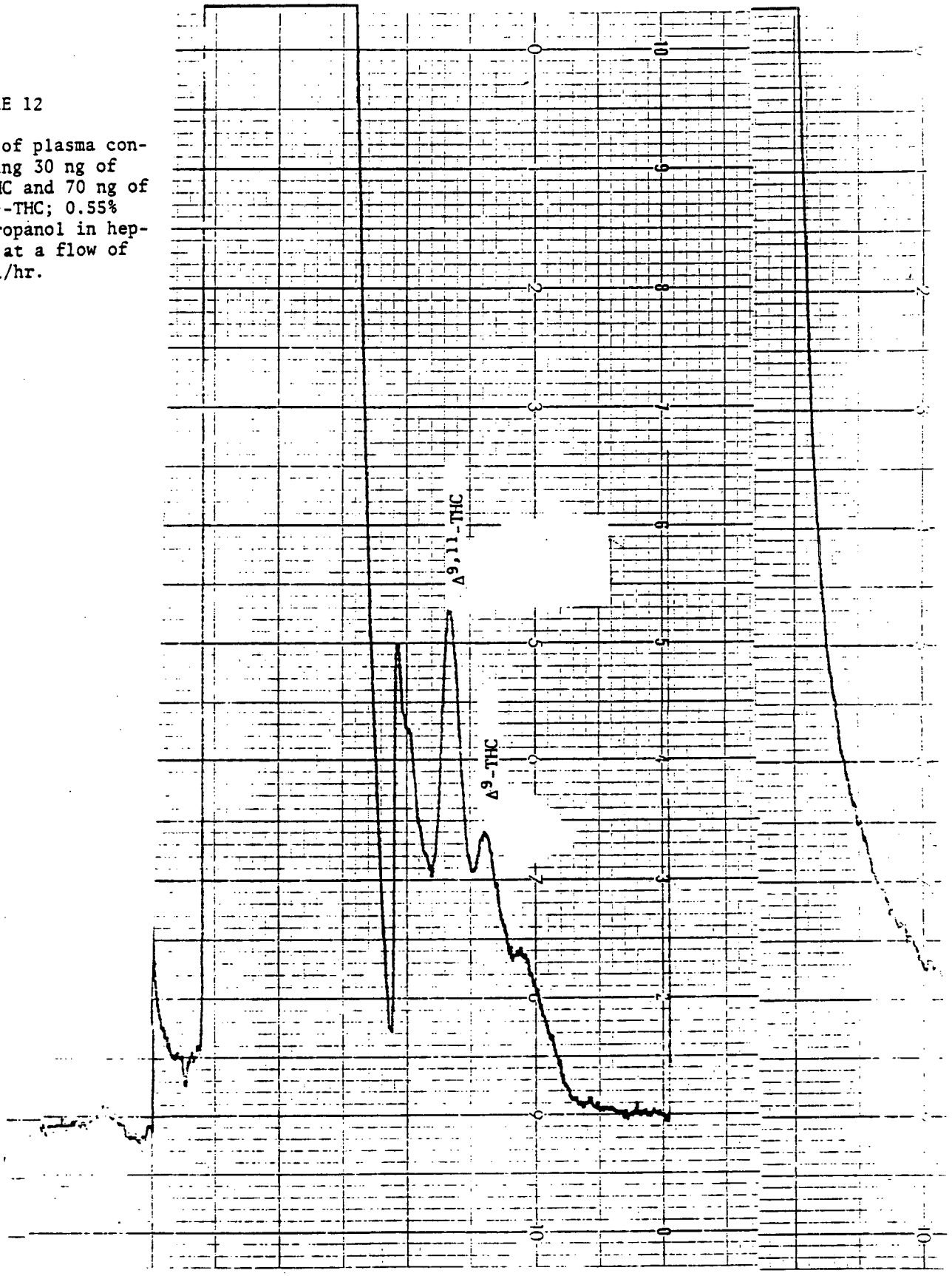


TABLE IV

Comparison of Hplc-uv And Hplc-ms Techniques

Patient Number	Time Post-dose (hrs)	ng/ml Found hplc-uv	hplc-ms
04	0.0[a,b]	12	0
	0.0833	87	85
	0.1666	57	55
	0.25[b]	42	35
	0.50	27	21
	0.75	19	18
	1.00	17	14
	1.50[b]	15	13
	2.00[b]	22	9
	4.00	0	2

[a]Sample taken prior to dosing

[b]Sample broken in transit

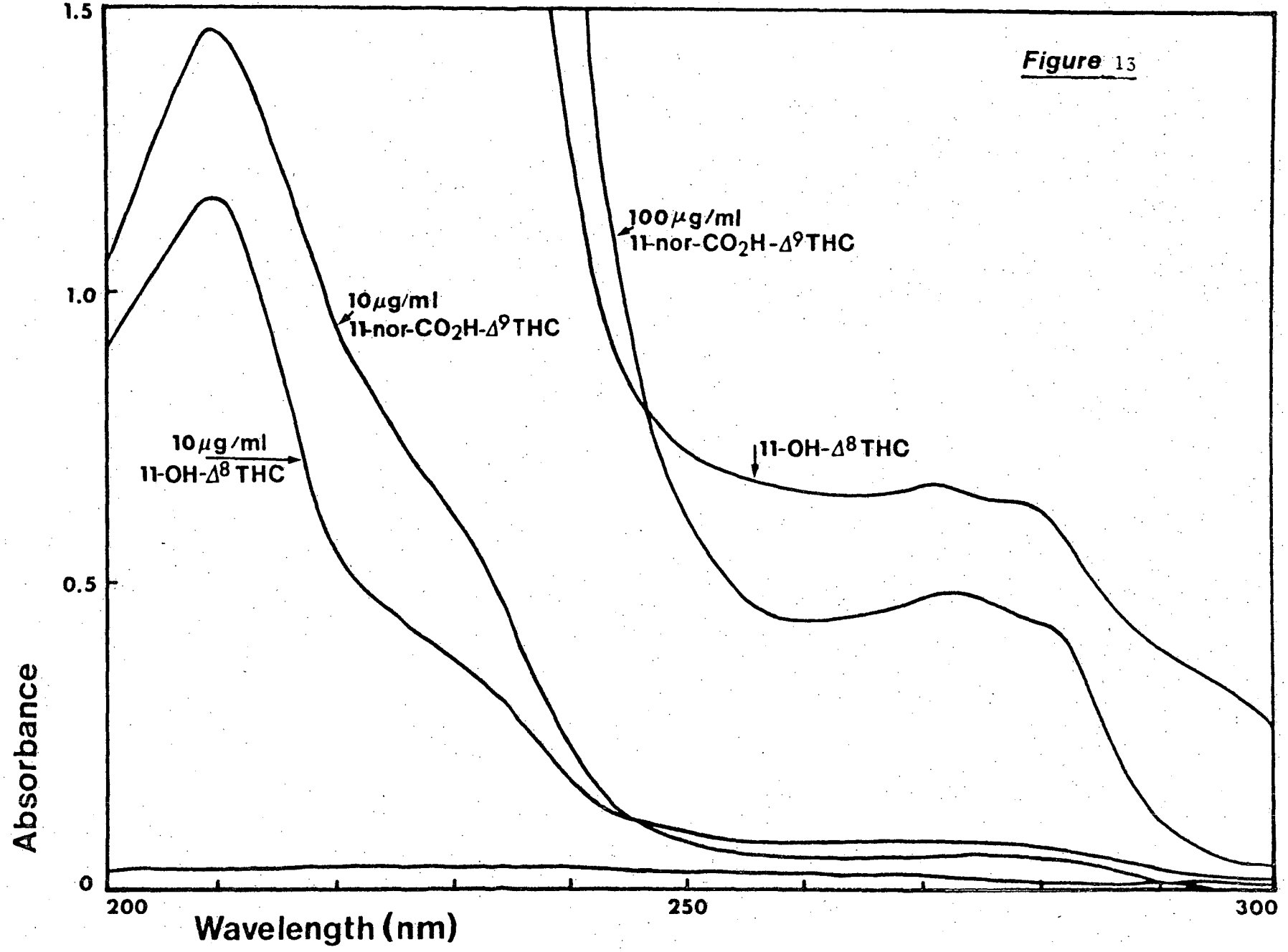


Figure 13
1.5
1.0
0.5
0
Absorbance
200
250
300
Wavelength (nm)
100μg/ml
11-nor-CO2H-Δ9THC
10μg/ml
11-nor-CO2H-Δ9THC
10μg/ml
11-OH-Δ8 THC
11-OH-Δ8 THC

Initial work on the assay for this metabolite using normal phase hplc as was developed for Δ^9-THC was impractical due to the polar nature of 9-CO_2H-Δ^9-THC. Using such a system resulted in long analysis time, broad eluting bands as well as poor sensitivity for the polar metabolite. These findings led then to an investigation of reverse-phase hplc for analysis of this metabolite. The first type of hplc column investigated was a C-18 reverse-phase column. A number of different types of mobile phase combinations were used as are illustrated in Table V. The major problem with the C-18 reverse phase column proved to be the very short retention time for 9-CO_2H-Δ^9-THC. This short retention time placed it upon the trailing edge of the peaks generally found in blood plasma, as illustrated in Figure 14, and made accurate quantification difficult.

The most satisfactory hplc conditions for the analysis of 9-CO_2H-Δ^9-THC was ultimately achieved by using a phenyl-bonded phase column in the reverse mode. Many different solvent combinations were again tried as mobile phase for this type of column, but based upon the previous experience with the C-18 column, water:acetonitrile was used exclusively in the developmental work. To achieve adequate resolution and sensitivity for 9-CO_2H-Δ^9-THC from endogenous blood plasma constituents, a gradient hplc program was employed. The program starts at 60:40, water:acetonitrile for 3 minutes and then is increased to 40:60, water:acetonitrile over a 2 minute period, held at that ratio for 5 minutes, then returned to 60:40, water:acetonitrile over a 2 minute period. Using this hplc gradient program, 9-CO_2H-Δ^9-THC is eluted at 2.7 minutes as shown in Figure 15. As noted in Figure 15, the 9-CO_2H-Δ^9-THC is eluted during the isocratic portion of the hplc program. The major purpose of the gradient program is to clear the endogenous blood plasma compounds so that the overall analysis time is reduced.

Once the proper hplc conditions were achieved for 9-CO_2H-Δ^9-THC, a study was conducted to determine which pH and extraction solvent would be optimum. Table VI summarizes the major conditions which were evaluated. As a result of these

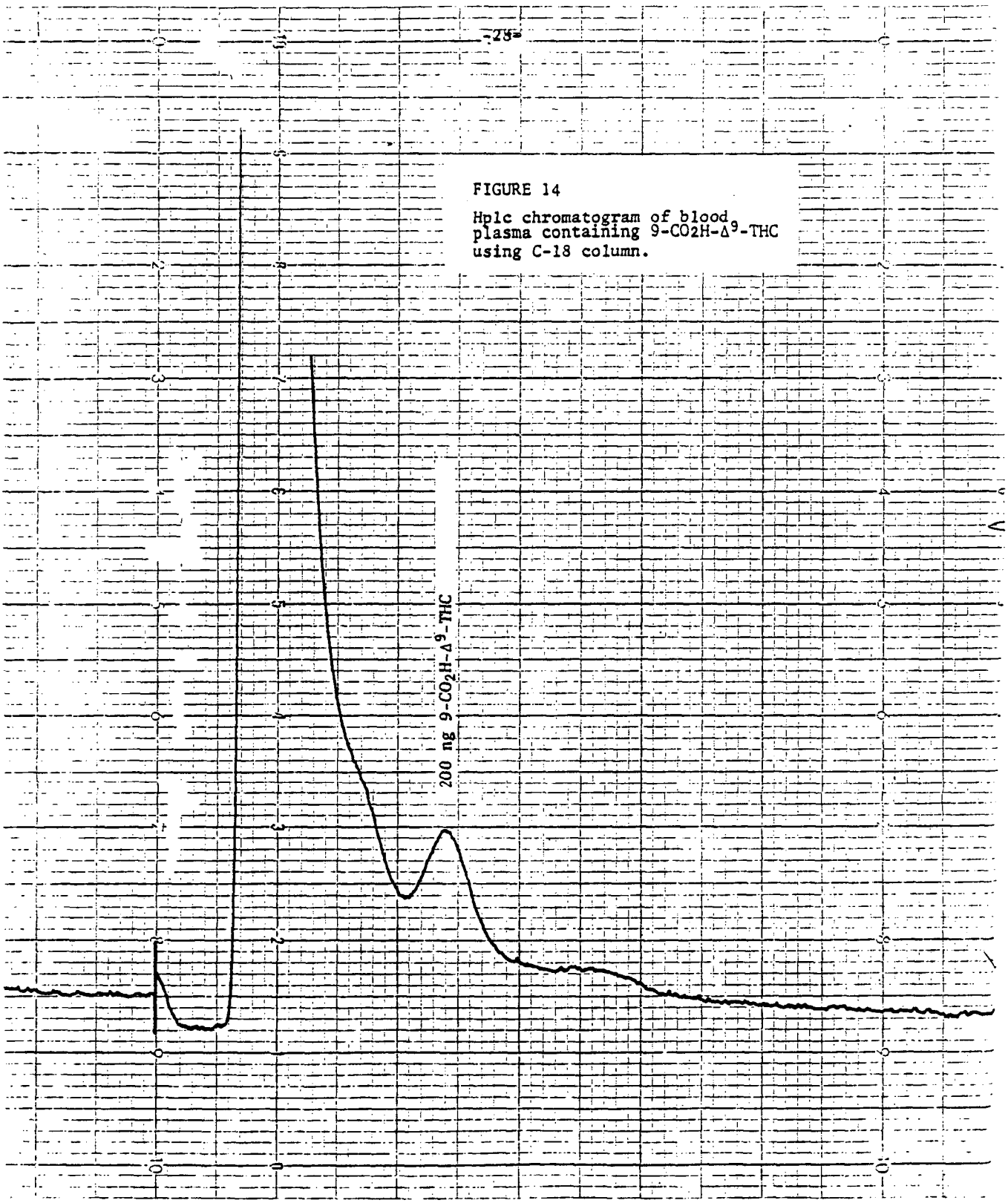


FIGURE 14

Hplc chromatogram of blood plasma containing 9-CO_2H-Δ^9-THC using C-18 column.

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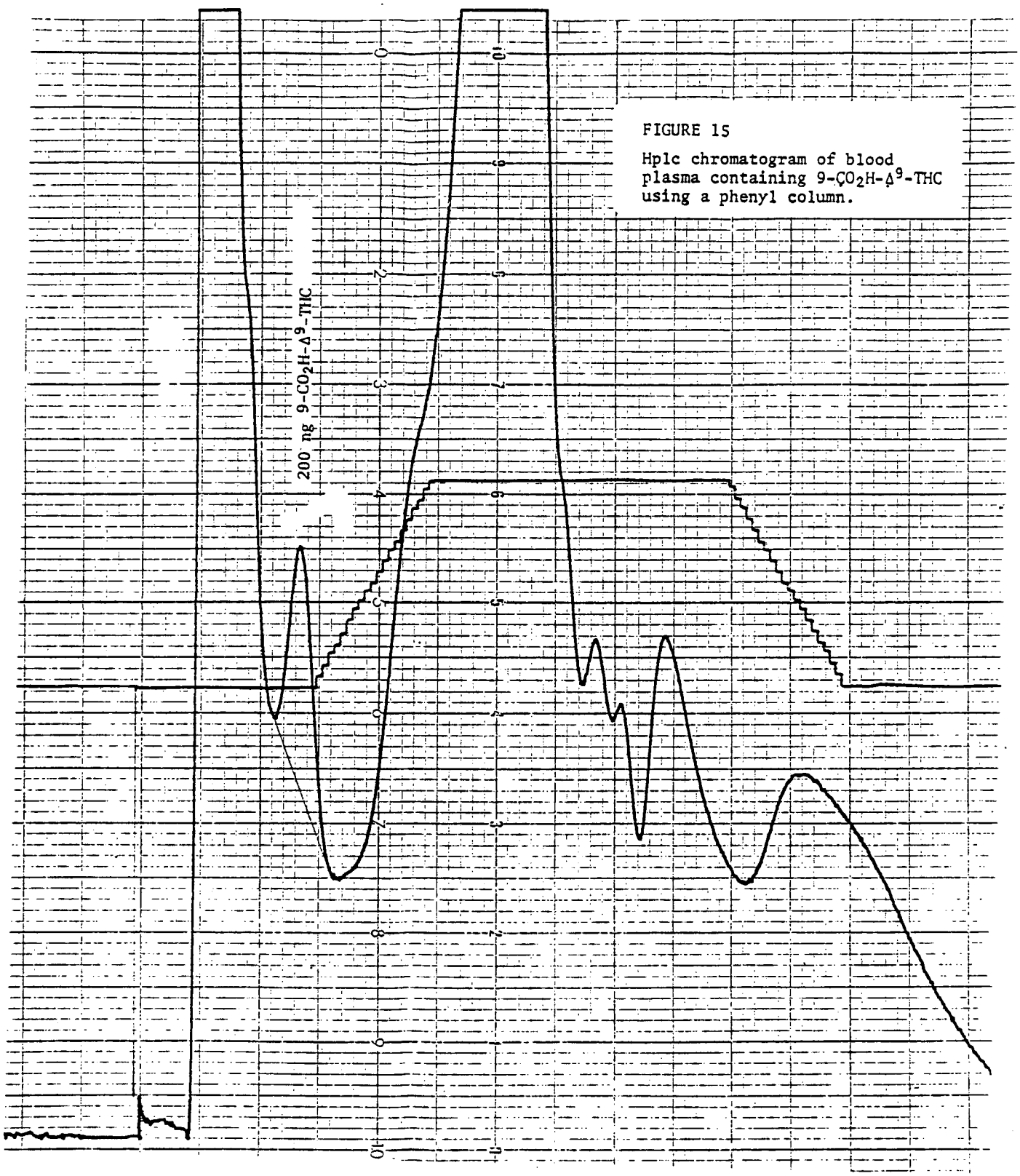


FIGURE 15

Hplc chromatogram of blood plasma containing 9-CO_2H-Δ^9-THC using a phenyl column.

TABLE V

MOBILE PHASE STUDIES FOR REVERSE PHASE CHROMATOGRAPHY (C-18)

Using 100 ng of 11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic Acid

Solvents	Composition	Retention Time (min)	Peak Height (in.)	Half Width (min.)
Water:acetonitrile	30:70	0.8	10	0.15
	50:50	0.9	10	0.15
	55:45	0.9	8	0.15
	60:40	0.95	7	0.15
	65:35	1.45	5	0.25
	70:30	6.0	1	3.00
Water:methanol	35:65	1.1	6.5	0.2
	40:60	1.25	5	0.25
	45:55	1.45	3.5	0.35
Water:acetonitrile: ethylene glycol	50:48:2	1.15	7	0.18
	50:45:5	1.15	7	0.18
	50:40:10	1.15	7	0.18
	55:43:2	1.25	6	0.2
	55:40:5	1.35	5.5	0.22
	60:39:1	1.7	4	0.35
	60:38:2	1.6	5	0.3
Water:acetonitrile: methanol	20:50:30	0.85	6	0.15
	45:45:10	1.15	6	0.2
	50:45:5	1.25	6	0.2
	50:30:20	1.6	4	0.3
	55:40:5	1.5	4.5	0.3
	55:35:10	1.9	2.5	0.5
	58:37:5	1.7	4	0.4
	60:38:2	1.75	4.5	0.3
	60:35:5	2.2	2.5	0.5

TABLE VI

Evaluation of Extracting pH and Solvents for 9-CO_2H-Δ^9-THC From Blood

pH	Extracting Solvent	Relative Amount of 9-CO_2H-Δ^9-THC Recovered
ambient	petroleum ether	none
2.5	petroleum ether	none
2.5	benzene (1.5% isopropanol)	70%
2.5	benzene (3% isopropanol)	60%
4	benzene (1.5% isopropanol)	70%

studies, a pH of 4 and benzene containing 1.5% isopropanol was used as the extracting conditions for analysis of 9-CO_2H-Δ^9-THC. To evaluate the 9-CO_2H-Δ^9-THC level in blood plasma of marijuana smokers standard curves were prepared using combined plasma from our laboratory workers to which known amounts of 9-CO_2H-Δ^9-THC had been added. These standard curves were prepared on the same day as the samples from the marijuana smokers and analyzed among the actual samples. All of the standard curves proved to be linear over the concentration ranges of 50-2000 ng/ml.

Table VII gives the results from a subject who received 10 mg of Δ^9-THC as an aerosol spray. Certain of these samples were analyzed again and as noted, the intra-sample agreement is quite good. Also it was noted that the levels of 9-CO_2H-Δ^9-THC were unexpectedly high, that is, much greater than those of Δ^9-THC.

Next the assay method was used to evaluate the 9-CO_2H-Δ^9-THC level of a marijuana smoker who had received 10.8 mg of Δ^9-THC. Results from this assay are given in Table VIII. Again, it was noted that the levels of 9-CO_2H-Δ^9-THC were quite high and that they were quite random when compared to Δ^9-THC levels; that is, there is no smooth decay with time.

The precision and reproducibility of the assay method was demonstrated by the analysis of some blind samples supplied by Dr. Richard Stillman of NIDA. These

TABLE VII

Blood Plasma Level of 9-CO_2H-Δ^9-THC From a Subject Receiving 10 mg of Δ^9-THC via Aerosol Inhalation

Time Post-dose (hrs.)	9-CO_2H-Δ^9-THC Found (ng/ml)
0	590
0.083	770
0.25	2050, 2100
0.5	800, 780
1.0	820, 740
1.5	930
2.0	1000
3.0	1480, 1500
4.0	680, 700
5.0	380
6.0	250

TABLE VIII

Plasma Level of 9 -CO_2H-Δ^9-THC From a Marijuana Smoker Receiving 10.8 mg of Δ^9-THC

	ng/ml Found	
Time (hrs)	9 -CO_2H-Δ^9-THC	Δ^9-THC[a]
0	300	0.4
0.25	1540	57.9
0.5	1840	24.8
1.0	3000	14.8
2.0	1480	10.7
3.0	2040	2.4
4.0	1900	6.2
12.0	2650	5.8
24.0	1920	2.5

[a]Determined by the hplc-ms method

TABLE IX

Plasma Level of 9 -CO_2H-Δ^9-THC From NIDA Samples

Subject	Time	ng/ml of 11-CO_2H-Δ^9-THC Found in Split Samples	
		A	B
A	pre	180	180
	2 hrs.	180	a
B	pre	0	0
	2 hrs.	30	30

[a]Sample lost through laboratory accident

samples were obtained from two marijuana smokers at the time intervals shown in Table IX. Each sample drawn was divided into two portions, coded and sent to us for analysis. Once the analysis was complete, the results were supplied to Dr. Stillman and the code broken. The results shown in Table IX indicate excellent intra-sample precision.

Since all the samples analyzed by the developed method gave such unexpectedly high levels of 9-CO_2H-Δ^9-THC, there appeared to be some question as to the specificity of the method. That is, was it possible that another metabolite of Δ^9-THC could be eluting at the same retention time as was 9-CO_2H-Δ^9-THC, causing erroneous values for the assay? To answer this question, the eluent from the hplc was collected at the place where 9-CO_2H-Δ^9-THC elutes after 100 µl of mobile containing 500 ng of 9-CO_2H-Δ^9-THC, or 100 µl of mobile phase containing plasma known to be free of 9-CO_2H-Δ^9-THC, or 100 µl of mobile phase containing plasma from a marijuana smoker was injected onto the hplc. Each eluent was analyzed by mass spectrometry. The collected eluent from the mobile phase and the mobile phase containing plasma known to be free of 9-CO_2H-Δ^9-THC were devoid of m/e in the region to be monitored

for 9-CO_2H-Δ^9-THC. In a similar manner, the eluent from the plasma to which 9-CO_2H-Δ^9-THC had been added and that from a marijuana smoker gave identical fragments at m/e 344, 329 and 299.

The other major problem with developing an assay for 9-CO_2H-Δ^9-THC was the selection of a proper internal standard. A number of different internal standards which had similar physical properties as 9-CO_2H-Δ^9-THC but would not appear in human plasma, were evaluated. These compounds and their retention times are given in Table X. When compared to the retention time of 9-CO_2H-Δ^9-THC, viz., 2.7 minutes, it is obvious that most of the compounds were unsuitable as internal standards. Only one compound, benzylbiphenyl, appeared to have possibilities as an internal standard. However, it was not possible during the present study to fully investigate its use as an internal standard.

TABLE X

Compounds Investigated as Internal Standards for the 9-CO_2H-Δ^9-THC Assay

Compound	Retention Time (min)	Comments
biphenol	1.2	unretained
olivetol	1.2	unretained
catechol	1.2	unretained
2-biphenylcarboxylic acid	1.2	unretained
4-biphenylcarboxylic acid	1.2	unretained
4-phenylphenol	1.4	not resolved from an endogenous plasma peak
4-biphenylphenol	2.9	not resolved from 9-CO_2H-Δ^9-THC
2-biphenylphenol	3.1	not resolved from 9-CO_2H-Δ^9-THC
biphenyl	4.0	not water soluble
4-(p-biphenyl)-2-methylthiazole	5.0	not resolved from an endogenous plasma peak
benzylbiphenyl	11.0	resolved from endogenous plasma peaks

D. Fluorescent Studies With Cannabinol

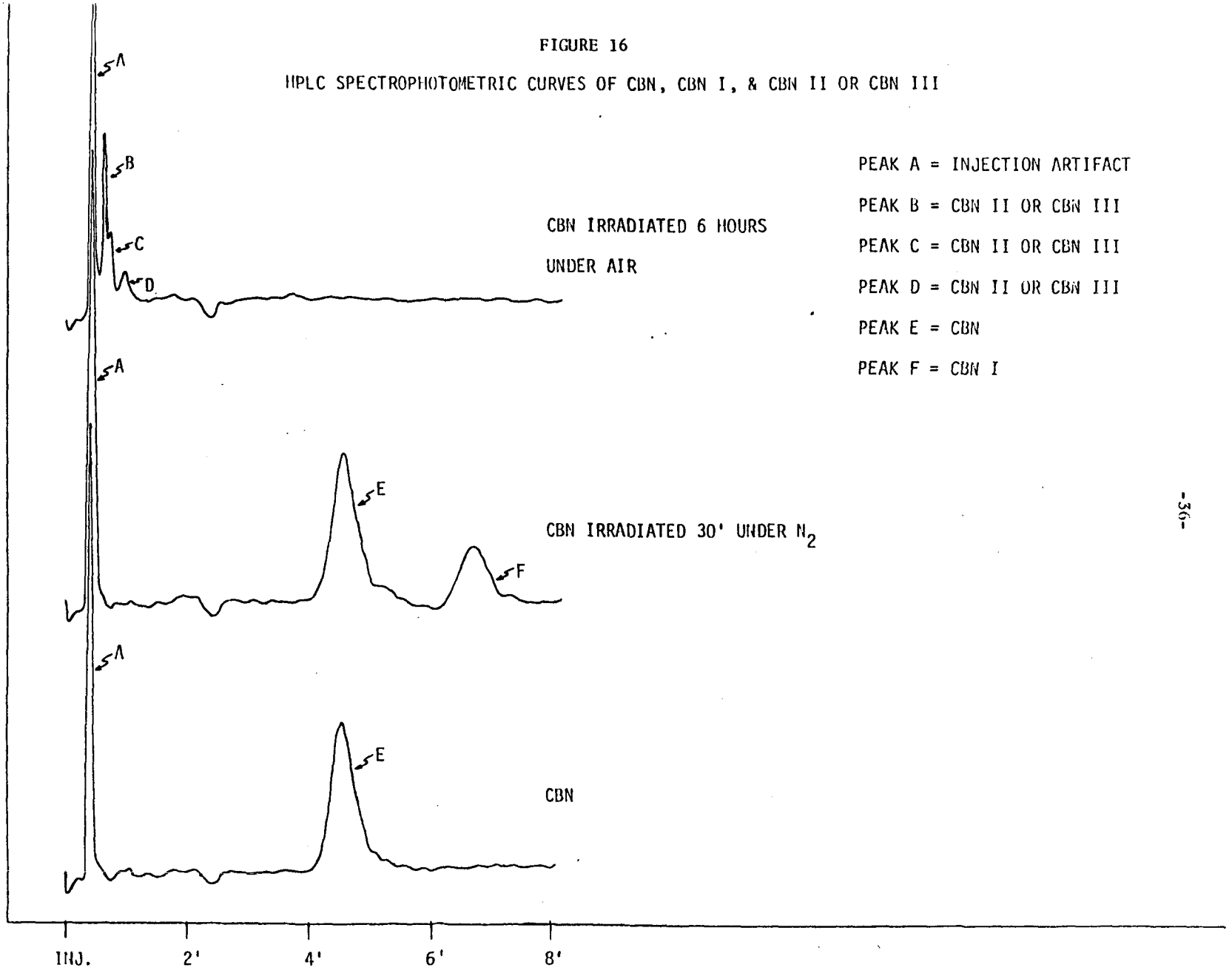
Prior to initiation of the present contract, it was believed that Δ^9-THC had some fluorescent properties. The reason for this belief was based upon our earlier studies with Δ^9-THC in methanol. As was shown in Figure 2, when Δ^9-THC was excited at 279 nm and the emission spectrum recorded, there was some fluorescence noted with Δ^9-THC. However, once the fluorometric flow cell described in B above was attached to the hplc, it became obvious that Δ^9-THC was not the fluorescent entity, but rather cannabinol (CBN). This finding was later confirmed when the hplc system described in C above was used to analyze a 1 µg sample of Δ^9-THC. A trace amount of CBN was found to be present in the Δ^9-THC which had been used in the original fluorescence studies. Thus a small study was conducted to examine the nature and possible uses of this highly fluorescent characteristic of CBN.

One of the first things noted about CBN was that if it was excited at 279 nm and the emission observed at 381 nm, the intensity increased with the time excited, up to a certain point, whereupon the intensity markedly decreased. Some preliminary hplc analyses of these irradiated samples clearly demonstrated the presence of other products. Thus it appeared that CBN was being photolytically decomposed into other compounds. Figure 16 illustrates the types of hplc chromatograms obtained when CBN was irradiated in the presence of air and in the presence of nitrogen. As shown in this figure, if CBN is analyzed by hplc, only one compound is present. However, upon irradiation under nitrogen, another compound is produced and in the presence of air two other compounds are produced.

Using hplc analysis of the irradiated CBN, it was possible to collect the eluent and obtain the mass spectrum of each compound. Figure 17 illustrates the mass spectral findings. From Figure 17 it will be noted that CBN II is obviously obtained from incorporation of an oxygen atom into CBN I, whereas CBN III is a dimeric material. Another use for the collected hplc eluent of CBN I, II and III

FIGURE 16

HPLC SPECTROPHOTOMETRIC CURVES OF CBN, CBN I, & CBN II OR CBN III



PEAK A = INJECTION ARTIFACT

PEAK B = CBN II OR CBN III

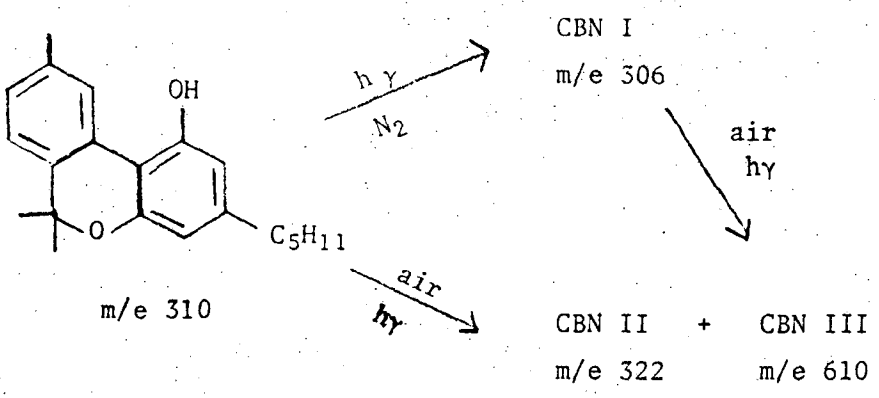
PEAK C = CBN II OR CBN III

PEAK D = CBN II OR CBN III

PEAK E = CBN

PEAK F = CBN I

FIGURE 17



was to determine the fluorescent intensity at 279 nm excitation and 381 nm emission. Only CBN I was highly fluorescent at these wavelengths.

In an effort to utilize the highly fluorescent properties of CBN I, it appeared that a biological sample would have to be irradiated under nitrogen. Using control samples where CBN was added to methanol, it was very easy to detect femtogram (10^{-15} g) amounts of CBN I. Thus it seemed feasible to take a small amount of a physiological fluid such as plasma, urine or saliva, irradiate it under nitrogen and observe any fluorescence produced. However, it was found that both urine and plasma contain endogenous substances which will fluoresce at the same wavelength as CBN I. The only physiological fluid found which appeared to be acceptable was saliva.

The procedure developed for assaying for CBN I in saliva is shown in Table XI. This method was used to assay 14 saliva samples from marijuana smokers as shown in Table XII. The data presented clearly demonstrates that the developed assay method can detect CBN I in a saliva sample of a marijuana smoker.

Following completion of the present work, an article appeared (9) which utilized the same photolytic principle to assay mouthwashes from marijuana smokers. These

TABLE XI

Detection of CBN I in Human Saliva

1. Extract 0.5 ml of saliva with 4 ml of petroleum ether
2. Evaporate under N_2
3. Add 2 ml of methanol
4. Take fluorometer reading
5. Irradiate 5 minutes under N_2
6. Take fluorometer reading

TABLE XII

Saliva Fluorometric Analysis for Cannabinol

Time Sampled After Smoking	n	Average Initial Reading	Average Final Reading	Average Change
control	6	0.03 (0.01-0.06)	0.45 (0.10-0.95)	0.42 (0.07-0.94)
presmoking	2	0.82 (0.69-0.95)	1.45 (1.20-1.70)	0.63 (0.51-0.75)
15 minutes	6	2.17 (1.46-3.05)	7.89 (1.86-35.00)	5.72 (-0.26-32.8)
30 minutes	6	1.71 (0.29-4.00)	8.06 (0.49-38.60)	6.35 (-0.38-36.69)
1 ng standard	6	0.27 (0.12-0.33)	0.61 (0.30-0.85)	0.34 (0.18-0.57)

authors washed the mouth with four 15 ml portions of 9% ethanol in water followed by extraction with petroleum ether. The petroleum ether extract was concentrated and placed on an alumina column. Elution was performed with 100% ethanol and the ethanol eluent was irradiated at 285 nm and the emission read at 365 nm. The authors claim a sensitivity of 15 ng/ml of mouthwash.

Based upon our work, it would appear that Bowd and his coworkers have produced CBN II and III, which are not as highly fluorescent as CBN I. To conclusively demonstrate this fact would require a duplication of their procedure and hplc-ms identification. However, it is worthy to note that both methods can detect CBN in the mouth of a marijuana smoker using somewhat analogous procedures.

CONCLUSIONS

During the present contract period, technology has been developed which will permit the determination of Δ^9-THC and its major metabolite, 9-CO_2H-Δ^9-THC, using hplc with uv detection. Since the developed technology is one which uses chromatography, the specificity for the individual cannabinoids is retained. The usable limit of sensitivity for Δ^9-THC appears to be 10 ng/ml, although this was not vigorously demonstrated in this work. However, the agreement between the proven hplc-ms method and the newly developed hplc-uv method is very good. Therefore, this technology would be adequate for monitoring the one-hour post-marijuana smoking, where the physiologic effect appears to be greatest. Thus for most applications other than pharmacokinetic studies, this methodology should be useful. Additionally, the equipment and level of expertise required to perform the analysis is amenable to a number of laboratories.

Since no other assay method has been developed for 9-CO_2H-Δ^9-THC, it was impossible to perform a comparative validation assay for this metabolite. Yet this work did clearly demonstrate that intra-sample precision was excellent. In addition, it was possible to verify that the hplc results were consistent with 9-CO_2H-Δ^9-THC eluting at the quantitated retention time since the collected substance gave a mass spectrum similar to that of 9-CO_2H-Δ^9-THC using high resolution mass spectrometry. The very high plasma levels of 9-CO_2H-Δ^9-THC would be consistent with the "first pass effect" noted for Δ^9-THC in dogs. (10) That is, Δ^9-THC is rapidly metabolized during the first pass through the liver and the blood levels of metabolites could be much higher than the parent drug. Unfortunately, it appears that the 9-CO_2H-Δ^9-THC levels do not follow a smooth decay curve, as was the case with Δ^9-THC. Thus, at present, it does not appear that measurement of this metabolite would be of any predicative value in determining marijuana intoxication, although an individual could readily be identified as a marijuana user since the levels of this metabolite are so high in plasma at all the time intervals studied.

Another interesting result of the present work was the finding that CBN could be photolytically converted to a highly fluorescent compound. Since CBN is present in marijuana smoke, a study was conducted on the detection of this compound in saliva of both marijuana smokers and nonsmokers. The limited results indicated that photolysis of an extract from marijuana smokers' saliva gave fluorescent characteristics indicative of the photolytic congener of CBN, whereas saliva from a nonsmoker of marijuana did not. These preliminary results suggest that a simple saliva test for marijuana use might be based on this assay.

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