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7

CANNABINOID ASSAYS IN HUMANS

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Editor

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CANNABINOID ASSAYS IN HUMANS

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FOREWORD

This monograph describes ways of determining the levels of cannabinoids in the human body after smoking marihuana. Investigations of a number of serious social and health problems have had to wait for the refinements of these techniques.

Chief among these concerns is the effect of marihuana smoking on driving. That investigation along with others can now be undertaken with an expectation of far more precise findings than was formerly possible. We now have the means not only of detecting cannabinoids, but also of determining in what quantity they are present. Thus, we can begin to establish specific correlations between cannabinoid levels and driving impairment. This information is necessary in order to build marihuana into the highway safety campaign now largely restricted to alcohol.

These assay techniques will be useful tools for a range of other problems including simple screening procedures, epidemiological studies, forensic toxicology, as well as for more fundamental pharmacokinetic and pharmacological research. The developments in method and instrumentation described here occur at a crucial juncture for drug abuse research, as we strive to assess the impact of marihuana on our culture.

Robert L. DuPont, M.D.
Director
National Institute on Drug Abuse

PREFACE

For the past several years, there has been an increasing demand for qualitative and quantitative assays for identifying and measuring the constituents of marihuana in the human body. This demand is prompted by the need for such assays in several research investigations that are attempting to improve our understanding of how this complex drug affects the body. In addition to these more fundamental issues, there is a growing feeling that suitable analytical methods will be required for determining the presence of cannabinoids in drivers suspected of being under the influence of the drug.

We have now reached a stage in the search for and development of such methods that many of them can now be employed in a routine manner. As we gain in experience and confidence with these methods, their validity will become increasingly better established and their applicability to critical decisions more acceptable. It will be apparent upon reviewing the procedures described herein that some were designed for or are by their complexity only suited for research purposes or in validating other methods. Others described are more amenable to routine screening or survey applications.

The road to acceptable methods has been long and arduous. Early attempts continued to suffer from lack of adequate sensitivity. It was eventually learned, as the studies on the composition of marihuana and the metabolism of its constituents progressed, that the problem of detecting any specific cannabinoid in the body after use would be an extremely difficult task. The primary active constituent, delta-9-tetrahydrocannabinol (THC) is rapidly distributed and metabolized in the body, making its quantification a major challenge. It is now very gratifying to be able to present a collection of manuscripts that delineate the tremendous progress that has been made over the past few years.

The methods described are grouped together under three major headings. The first are based on the increasingly used immunoassay techniques. In general, immunoassays offer speed and sensitivity and are very amenable to the screening of large numbers of samples. They often suffer from lack of specificity, but this is often acceptable if they cross react only with metabolites of the target drug and no other drug. The four methods described are at various stages of development and refinement, and some are being employed in various research studies.

Methods of the second group are based on the older technology of chromatography, but are applied in rigorous and innovative ways to provide the degree of sensitivity required to measure the low levels of drugs and metabolites. Two different approaches were taken to reduce the background interference. Using a conventional gas chromatograph, Dr. Garrett employed a high pressure liquid chromatograph (HPLC) to "clean-up" the sample. The dual-column instrument designed by Dr. Fenimore reduces the amount of effort required for sample preparation. As the technology in HPLC progresses, this method is becoming extremely popular, offering the ability to separate difficult mixtures at low temperatures. The effort described here is moving forward and parallels similar efforts that are being carried out by Dr. Valentine, in an outgrowth of his project's mass spectroscopic method.

The last group of methods all employ the mass spectrometer as the detector for identifying and quantifying the cannabinoids. Rapid advances in the development of mass spectroscopy have made it the method of choice in terms of sensitivity and specificity. Because of its present size, cost, and complexity, it is not ideal for routine application to routine screening. It is, however, being used for the routine validation of other methods and to confirm the presence of cannabinoids in samples found positive by less specific screening methods, like the immunoassays. The six papers included represent some of the most outstanding work done in the field of mass spectroscopic analysis. Since Dr. Agurell first published his method in 1973, tremendous strides have been made by him and the others included in this monograph.

We now feel very confident in our ability to get on with many of the critical studies that have awaited these methods.

This collection of papers does not signal the end of the road in the development of suitable methods for quantifying cannabinoids in the body. As our understanding of the effects of marihuana progresses, so must the methods used in studying it. We now have methods to study the effects of marihuana on driving, and if the evidence indicates that it poses a significant hazard, then a simple- perhaps roadside- test may be required. Other examples could be cited, but it is sufficient to end with the recognition of a notable achievement in this difficult area of research and a sense of satisfaction that our perseverance is paying off.

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Uivision of Research
National Institute on Drug Abuse

April 1976

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QUANTITATION OF CANNABINOIDS IN BIOLOGICAL FLUIDS BY RADIOIMMUNOASSAY

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INTRODUCTION

There is an expanding need to assay tetrahydrocannabinol (Δ^9THC) and its metabolites in biological fluids, particularly in the area of medical research. Methods used for the quantitation of cannabinoids include gas chromatography coupled with mass spectroscopy (GC/MS) , liquid chromatography and radioimmunoassay. All methods are of value, especially when used to confirm the results of another procedure, so that the method of choice depends on the particular application. Radioimnunoassay is a common clinical method which is highly specific, sensitive and particularly well suited to the routine, simultaneous analysis of multiple samples, often without prior purification. The equipment required is routinely used in university, research, industrial, and clinical laboratories and hospitals. Results can be obtained rapidly and are easy to interpret.

THE PRINCIPLES OF RADIOIMMMUNOASSAY

Radioimmmunoassay depends on the affinity of a biological molecule, the antibody, for the antigen in question, in this case Δ^9THC. Sensitivity is achieved through the use of a radioactive tracer molecule of high specific activity, called the labelled antigen. The extent to which the unlabelled antigen (Δ^9THC) competes with the radioactive antigen for a limited number of receptor sites on the antibody serves as the basis for quantitation in the radioimmunoassay. The assays are simple to perform. Mixtures containing the labelled antigen, the antibody and the sample are incubated, free labelled antigen is separated from antibody-bound labelled antigen and the exent of binding is determined by counting the disintegrations per minute of the radiolabel. Unknown samples are quantitated accurately by comparison with the binding levels achieved with known samples.

Antibodies generally show a remarkable ability to bind selectively the antigen that stimulated their production. This specificity is comparable to that of an enzyme for its substrate. The ability of an antibody to discriminate between the

antigen and the myriad of other compounds of widely diverse structure, which are found in biological fluids, is of fundamental importance in its use as an analytical tool. Macromolecules, such as proteins nucleic acids and polysaccharides, usually elicit an immune response when injected directly into an animal. However, low molecular weight compounds, such as Δ^9THC, cannot elicit an immune response unless they are bound covalently to an antigenic macromolecule such as a protein or polypeptide. The development of a radioimmunoassay for a molecule, such as Δ^9THC, involves (i) the synthesis of suitable covalent conjugates for immunization, (ii) the production of antisera, (iii) the preparation of a radioactive antigen, and (iv) the establishment of the assay based on the antigen-antibody reaction.

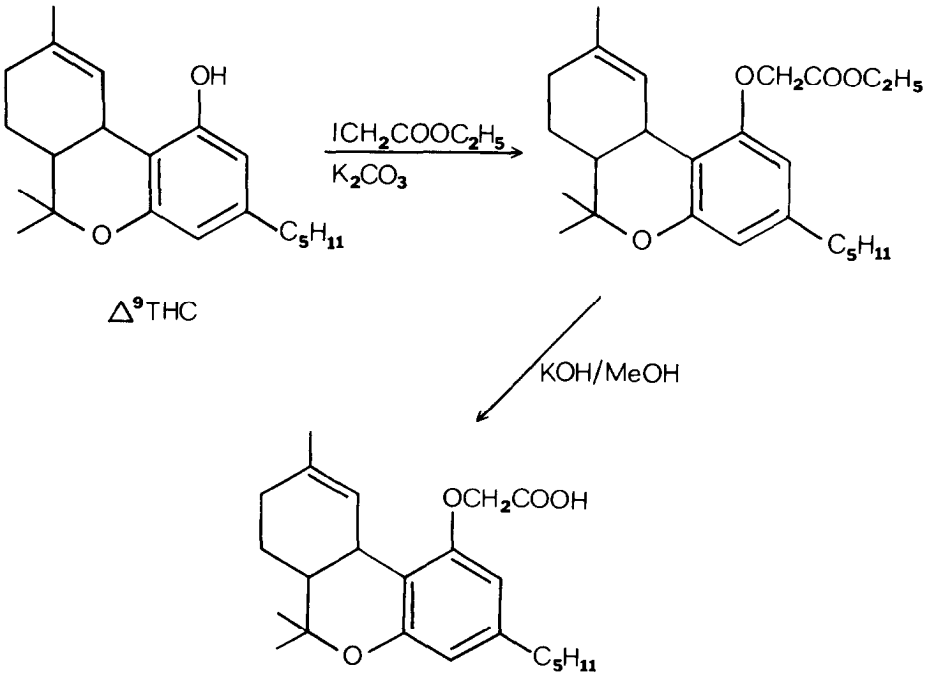
SYNTHESIS OF COVALENT CONJUGATES FOR IMMUNIZATION

Molecules containing an amino or carboxy function can be coupled directly to the amino or carboxy groups of amino acid residues in proteins or polypeptides by formation of amide bonds. Since the specificity of an antibody is usually directed toward those structures on the hapten that are distal to the linkage group, the hapten should be coupled to the carrier so that characteristic functional groups are exposed to the antibody synthesizing cells.

In the case of Δ^9THC several derivatives containing carboxy groups have been synthesized. and coupled to macromolecules. The use of a hemisuccinate ester of Δ^9THC has been reported (Teale et al, 1975) as well as an azobenzoic acid-derivative (Grant et al 1972 and Gross et al, 1974) . We have used O-carboxymethyl-THC prepared by reaction of Δ^9THC with iodoethylacetate followed by basic hydrolysis as shown in Figure 1.

Figure 1

Synthesis of O-Carboxymethyl-THC



O-Carboxymethyl-THC

O-carboxymethyl-THC was coupled to bovine serum albumin (BSA) using two different dehydrating agents. In the first preparation the water soluble carbodiimide, 1-ethyl-3-(3-dimethyl aminopropyl) - carbodiimide (EDC), was used and in the second preparation, coupling was effected by Woodward's reagent, N-ethyl-5-phenyl isooxazolium-3'-sulphonate. These reactions are surmmarized in Figure 2. Uncoupled CBM-THC and other small molecules were removed from the two preparations of O-carboxymethyl-THC-BSA (CBM-THC-BSA) by dialysis and ethanol precipitation. The number of THC residues incorporated was estimated by spiking the preparations with ^{14}C-Δ^9THC.

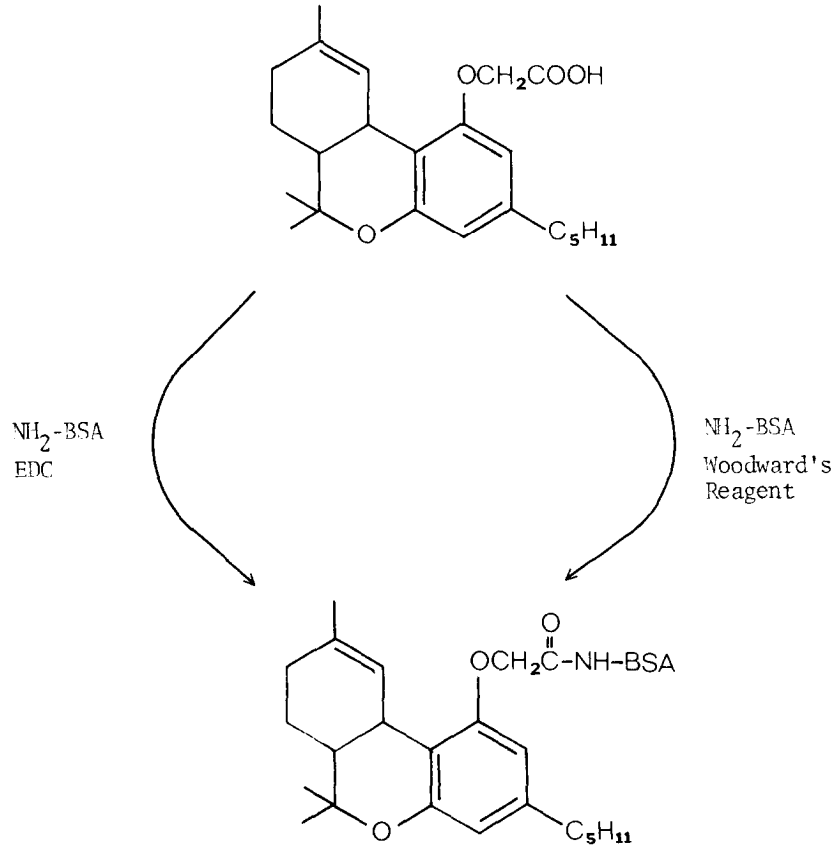
PRODUCTION OF ANTISERUM

The two preparations of the conjugate, CBM-THC-BSA, were both used to immunize rabbits. While the response varied from animal to animal? antisera were generated to both preparations. The presence of antibody was demonstrated by the fact that the antisera would bind radiolabelled Δ^9THC and, moreover, this binding could be inhibited by unlabelled Δ^9THC.

The response of two of the rabbits is summarized in Figure 3. These plots show the titer of antiserum required to give approximately 50% binding of ^3H-Δ^9THC. It can be seen that antisera were generated to both preparations. Most of the data described in the rest of this paper were obtained using bleeding H of rabbit 56.

Figure 2

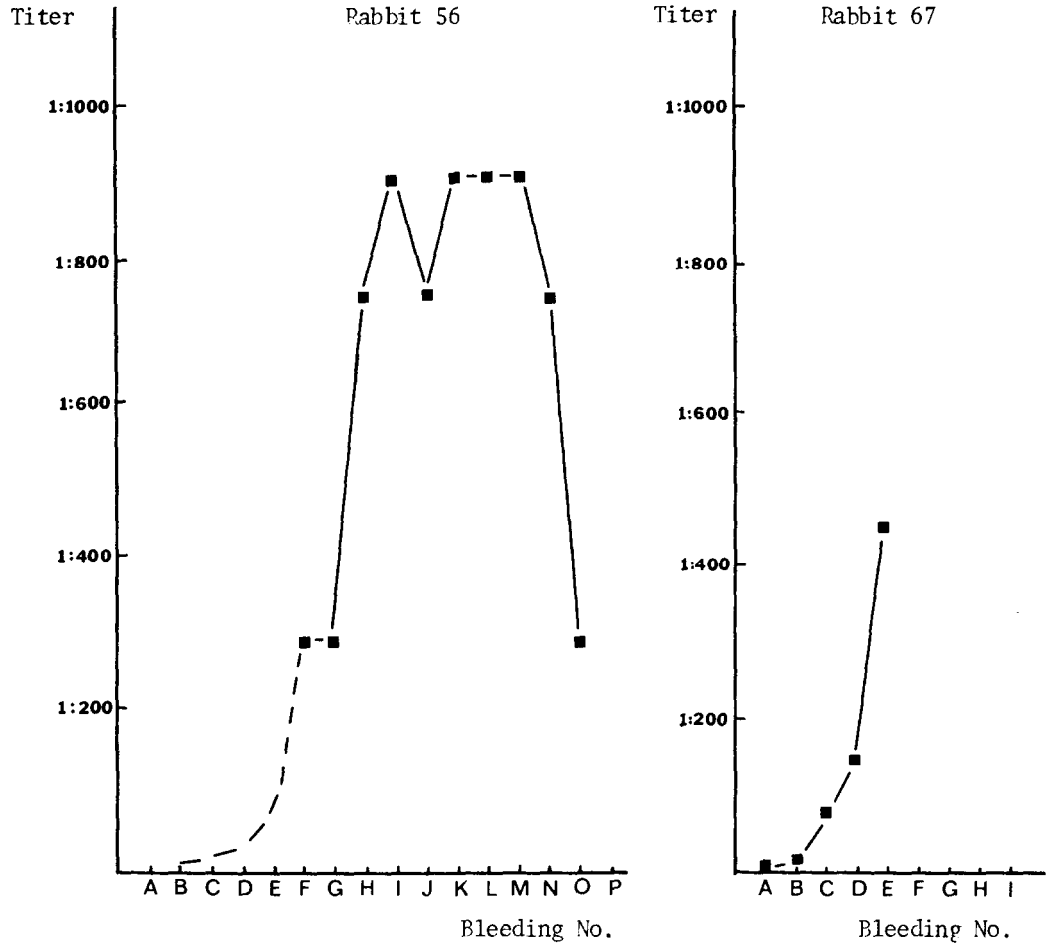
Synthesis of O-Carboxymethyl-THC-Bovine Serum Albumin



O-Carboxymethyl-THC-BSA

Figure 3

Titer of Antiserum Giving Approxomately 50% Binding of ^{3}H-Δ^{9}THC Following Immunization



Note: Rabbit 56 was immunized with CBM-THC-BSA prepared using EDC.

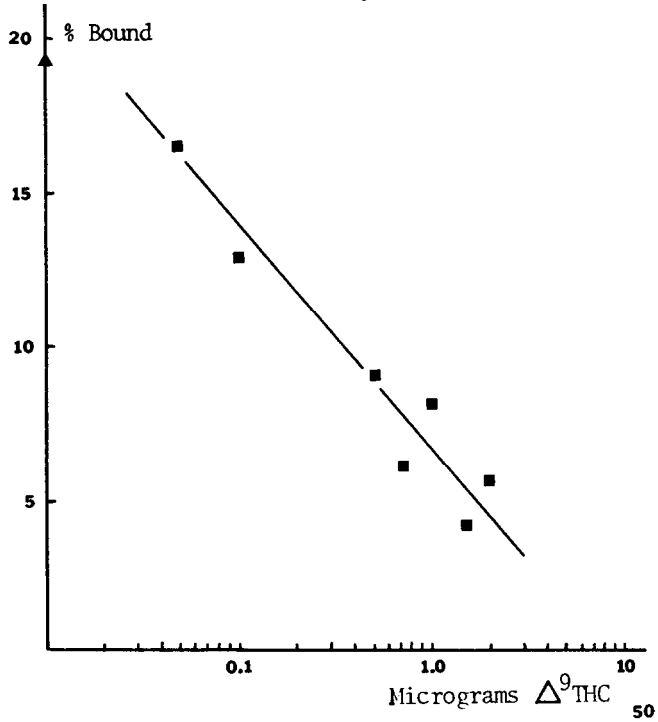
Rabbit 67 was injected with CBM-THC-BSA coupled using Woodward's Reagent.

RADIOLABELLED ANTIGEN, BINDING AND INHIBITION STUDIES

High specific activity, radiolabelled compounds are used to develop sensitive radioimmunoassays. We have used both ^{3}H and ^{14}C-Δ^{9}THC in our work.[1] The ^{14}C-Δ^{9}THC had a specific activity of 0.07 μCi/μg. At a final titer of 1:3 the antibody gave a maximum binding of this ^{14}C-Δ^{9}THC of 19.3%. A standard curve, prepared by inhibiting this binding with unlabelled Δ^{9}THC, is shown in Figure 4. The 50% inhibition point occurred at 330 nanograms of Δ^{9}THC. As expected, this system was too insensitive to be used as a working radioimmunoassay.

Figure 4

Inhibition of the Binding of ^{14}C-Δ^9 *THC and Antibody of* Δ^9 *THC*



Practical sensitivity could be achieved, however, using ^{3}H-labelled Δ^9THC with a specific activity of 41 μCi/μg. Binding levels of 30-50% were routinely achieved at a final titer of 1:750. The binding was inhibited by nanogram levels of unlabelled Δ^9THC as shown in Figure 5. The 50% inhibition point occurred at 0.7 nanograms Δ^9THC. This system was used as the basis of a working radioimmunoassay.

THE TRITIUM BASED RADIOMMUNOASSAY SYSTEM

A working radioimnunoassay has been developed based on the use of ^{3}H-Δ^9THC and antiserum generated to CBM-THC-BSA. The assay system which is described below is available to interested investigators. The assay is carried out in a buffer system of 0.1M phosphate pH 7.0, 0.1% Triton X-405, 0.2% sheep gamma globulin, fraction II. Tubes containing antibody, ^{3}H-Δ^9THC and either the standard or unknown THC sample are incubated for four hours at 4°C. Antibody bound and free labelled THC are separated by dextran coated charcoal. The percent of the radiolabel bound by the antibody is determined by counting samples of the supernatant in a liquid scintillation counter.

The insolubility of Δ^9THC in aqueous systems is well established. Correspondingly, to provide an internal check on each standard solution, ^{14}C-Δ^9THC is used as a standard. The exact number of nanograms of Δ^9THC in each standard solution is then verified by counting an aliquot in a liquid scintillation in the ^{14}C channel. The ^{14}C label does not interfere with the counting of the ^{3}H label in the ^{3}H-channel.

The assay conditions described above were used to obtain the standard curve shown in Figure 5. The system is capable of assaying as little as 0.25 nanograms of Δ^9THC reproducibly.

CROSS REACTIVITY STUDIES

The cross reactivity of various cannabinoids, drugs and other compounds was established for the assay system. The data is summarized in Table 1, and shows that the antiserum apparently reacts exclusively with cannabinoids. None of the non-cannabinoid drugs, hormones or other compounds cross

Figure 5

Inhibition of the Binding of ^{3}*H-*Δ^9 *THC and Antibody by* Δ^9 *THC*

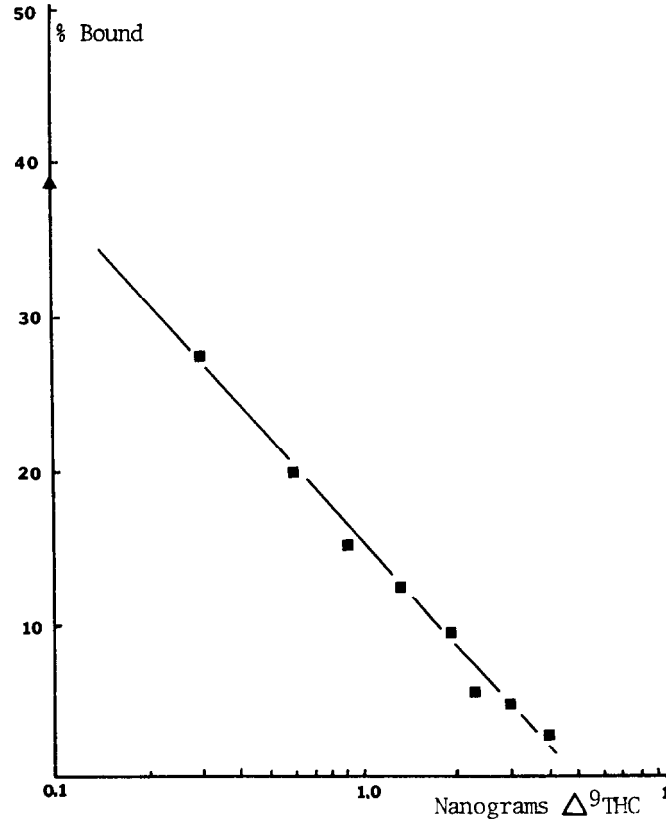


Table 1

Gross Reactivity of Certain Cannabinoids and Other Compounds With

Δ^{9}-THC Antiserum

Cannabinoids	50% Inhibition In Nanograms
Δ^{9}-THC	0.7
Δ^{8}-THC	0.7
11-OH-Δ^{9}-THC	0.4
11-OH-Δ^{8}-THC	0.7
8β-OH-Δ^{9}-THC	2.0
11-Nor-Δ^{9}-THC-9-COOH	0.25
11-Nor CBN-9-COOH	1.5
Cannabinol (CBN)	3.5
Cannabidiol (CBD)	100
Cannabidiolic acid (CBD acid)	40
Cannabicyclol	8
Cannabichromene	40

Other compounds		Level of Detection In Nanograms
Caffeine		>100,000
"Aldactone" or Spironolactone		>5,000
LSD tartrate		>32,000
Secobarbitol (Technam spiked urine)		>100*
Gibberellic acid		>100,000
Nicotine	Drugs of Abuse	>1,000,000
Morphine Sulfate	Drugs of Abuse	>100,000
Cocaine (Technam spiked urine)	Drugs of Abuse	>60*
Methadone HCl	Drugs of Abuse	>100,000
Phenobarbitol, sodium	Drugs of Abuse	>10,000
Tetracycline	Drugs of Abuse	>100,000
D,L-Ephedrine		>10,000
Salicylic Acid		>100,000
Carvone	terpenes	>95,000
(+) Limonene	terpenes	>80,000
$\alpha\beta$- Ihujone	terpenes	>90,000
Aldosterone-21-hemisuccinate	steroids	>10,000
Progesterone	steroids	>10,000
Estradiol	steroids	>10,000
Δ-1 cortisone	steroids	>10,000
Testosterone	steroids	>10,000
Cholesterol hydrogen succinate	steroids	>20,000

* Highest level available

reacted to any significant extent. The results obtained with the various cannabinoids show that the antiserum reacts as well with Δ^{9}THC as with some of its metabolites. The antibody recognizes Δ^{9}THC, Δ^{8}THC and several hydroxy metabolites. It can differentiate to a limited extent between Δ^{9}THC and cannabinol, cannabidiol, cannabidiolic acid and cannabichromene.

The specificity of the antiserum for cannabinoids without absolute specificity for THC is advantageous since native THC is not excreted in the urine and an assay capable of detecting THC only would be of little value. The assay system described here can detect Δ^{9}THC and its hydroxy metabolites, and therefore can be used to detect cannabis use.

APPLICATION TO BIOLOGICAL SAMPLES

Before applying the assay to the detection of cannabinoids in urine samples, normal urines were assayed to determine any non-specific interference. It was found that normal urine was negative in the assay system. A pool of normal urine was spiked with known amounts of Δ^{9}THC and assayed. The results are shown in Table 2, and it can be concluded that (i) urine can be assayed directly with no pre-treatment required, (ii) urine does not interfere with the quantitation of Δ^{9}THC, and (iii) 20 microliter samples can be quantitated accurately.

Table 2

Assay of a Spiked Normal Urine Pool

Sample No.	Nanograms Δ^{9}THC Added to Sample	Nanograms Δ^{9}THC By Radioimmunoassay
1	11.2	11.2
2	5.6	5.2
3	2.2	2.0
4	1.6	2.0
5	0.8	0.7

ASSAY OF BIOLOGICAL SAMPLES

Clinical studies were undertaken to establish the validity of the assay under experimental conditions. All samples were assayed blind, with no knowledge of the key to the sample number code.

The first study involved analysis of 24 hour urine speciments taken from heavy pot users. Prior to receiving oral doses of THC, the subjects were maintained free of any drugs for nine days. They were then maintained for 12 days on a dose of 120 mg/day of hashish oil in ethanol. Urine specimens were assayed for cannabinoids on day 9 and day 21. From an examination of the data, summarized in Table 3, it can be concluded that the assay did detect the presence of large amounts of cannabinoids in the urine samples obtained on day 21.

Table 3

Cannabinoid Levels Found in Urine Samples From Heavy Pot Smokers, Before and After Oral Administration of Hashish Oil

No drug for 9 days	Level Found
Patient A	30 ng/ml
Patient B	40 ng/ml
Hashish Oil for 12 days	
Patient A	7000 ng/ml
Patient B	4000 ng/ml

Note: At the time of these assays, the minimum detectable level of cannabinoids was 12.5 ng/ml.

A second series of 24 hour urine samples from a similar experiment were also assayed. The patients were maintained for seven days without any drugs and were then treated with oral doses of THC. Subject 2 was a fairly heavy cannabis user before admission to the hospital, and the initial urine cannabinoid level was consistent with this fact. At 8:00 a.m. on day 8 treatment was initiated on a schedule of 10 mg of THC in sesame oil every 4 hours, This schedule was maintained until 8:00 a.m. on day 12 when the dose was increased to 30 mg every 4 hours. The treatment was discontinued on day 24 at 4:00 p.m. On days 13 and 22 each subject was also given two cigarettes each of which contained 20 mg of THC.

As can be seen from the results in Table 4, inhaled doses of THC can be detected in the urine for 5-7 days after the last exposure to the drug. The administration of oral doses of THC results in increased urine levels of cannabinoids and the level falls following removal of the drug. The urine levels also reflect an increase in the dosage level. These studies indicate that the radioimmunoassay will give meaningful results.

Table 4

Cannabinoid Levels in 24 Hour Urine Samples

Day	Dose Schedule	Patient 1	Patient 2
1		170 ng/ml	1498 ng/ml
2		76	168
3		47	106
4	No drug	38	81
5		No sample	69
6		17	28
7		240	75
8		1718	992
9		1850	3721
10	10 mg/4 hr.	1582	2994
11		2768	4255
12		3725	5425
13		4175	7819
14		7375	3675
15		3338	6088
16		6992	9950
17		9413	6313
18	30 mg/4 hr.	5600	15767
19		6563	10813
20		4938	13275
21		10254	9556
22		10722	11181
23		5088	1325
24		5513	8775
25		3550	No sample
26	No drug	1600	1075
27		1662	442
28		808	223

*Two cigarettes each containing 20 mg. of Δ^9THC smoked in addition to regular dose.

SUMMARY

A tritium based radioimmunoassay for Δ^9THC and its metabolites has been developed for the use of investigators studying the epidemiological, medical, clinical, and research aspects of cannabis use. The assay is sufficiently sensitive to detect cannabinoids in the urine of marijuana smokers for several days after their last exposure to the drug. The results obtained from a 28 day study indicate that the assay reflects the administration and removal of oral doses of THC. The specificity of the antisera, as determined in cross reactivity studies, allows not only the assay of metabolites in biological samples without interference from other drugs, but also the evaluation of extracts of other kinds of samples which may contain unmetabolized Δ^9THC.

The technique of radioimmunoassay has many advantages over other methods of analysis. It is simple to perform and can be readily applied to the rapid analysis of large numbers of samples, It can be used in the direct analysis of physiological fluids and other biological samples which ordinarily have to be processed before other techniques can be applied. The method is non-destructive and obviates the need to use radiolabelled drugs in man during metabolic and other studies.

This radioimnunoassay has been designed with particular emphasis on ease of use by other investigators. We anticipate that it will prove useful to investigators and scientists for determining the absence, or presence and amount, of THC metabolite in a biological specimen, for epidemiologists in determining the full extent of cannabis use and to the medical/clinical community for establishing the minimum effective dose of Δ^9THC for each patient. The widespread application of a single method of analysis should also remove a great deal of the controversy surrounding marihuana studies performed to date.

[1] ^{14}C-Δ^9THC=(-)Δ^1-Tetrahydro (3',5'-^{14}C) cannabinol, 31 mCi/mmole, was purchased from Amersham Searle. The radiolabel purity was found to be 97-98% by radioscan in two different TLC systems capable of separating Δ^8 and Δ^9 THC. ^{3}HΔ^9THC=Δ^1(G-^{3}H) tetrahydrocannabinol. 13 Ci/mmole was purchased from Amersham Searle. The radiolabel purity was found to be 98% by radioscan in three different TLC systems capable of separating Δ^8 and Δ^9 THC.

ACKNOWLEDGMENTS

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SEPARATE RADIOIMMUNE MEASUREMENTS OF BODY FLUID Δ^9-THC AND 11-NOR-9-CARBOXY Δ^9-THC

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INTRODUCTION

Pharmacologic and metabolic studies with cannabinoids have remained largely unquantitative due to lack of simple assay techniques. Initial gas chromatographic and mass spectroscopic methods were tedious (Fenimore 1973; Garrett 1973; Agurell 1973). Our immune approach led to early development of a simple radioimmune assay for Δ^9-THC in plasma and urine (Gross 1974). This has now been extended to measure separately in body fluids 11-nor-9-carboxy-Δ^9-THC (C-THC) (Soares) a major cannabinoid metabolite (Bernstein 1972).

ASSAY METHODS

^{3}H-Δ^9-THC (50 Ci/mM) and C-THC were obtained from Research Triangle Institute, North Carolina. Δ^9-THC was obtained from the National Institute of Drug Abuse. The generation of antisera for Δ^9-THC and C-THC has been described (Gross and Soares 1974; Soares and Gross 1976). Plasma Δ^9-THC and C-THC were extracted, the extracts reconstituted in 50% ethanol or phosphate buffer and assayed. Urine specimens were assayed directly. Plasma and urine samples were obtained from subjects at various intervals after smoking one to three marihuana cigarettes (Containing 19.8 mg Δ^9-THC) and subsequently assayed for both Δ^9-THC and C-THC. Pooled plasma from non-users of marihuana spiked with various known amounts of Δ^9-THC and C-THC was used to establish the standard inhibition curves. The urine standard curves were similarly determined.

Plasma Δ^9-THC and C-THC: Plasma samples[1] were obtained from five subjects after smoking a single cigarette and from three subjects after smoking three consecutive cigarettes. None of the first group and only one of the second group had measurable preintoxication levels of Δ^9-THC. However, four of the eight subjects had measurable preintoxication levels of C-THC.

In five single smoke subjects definitive increments (10-130 ng/ml) of Δ^9-THC occurred 15 minutes after a single cigarette becoming almost undetectable by 2 hours (Table 1). C-THC peaked at 30-60 minutes; significant but low levels remained at least three hours after exposure.

Table 1

Plasma Δ^9-THC and C-THC levels in occasional THC users 15-240 minutes after smoking a single 900 mg THC cigarette (2.2% Δ^9-THC/cigarette)

Subject		Pre intoxication	Post intoxication (minutes)						
		0	5	15	30	60	120	180	240
S. W.	Δ^9-THC	7	-	31	31	21	11	14	16
	C-THC	50		144	224	116	71	5	9
Z. W.	Δ^9-THC	0		130	92	38	0	0	60
	C-THC	0		---	116	24	59	36	34
B. W.	Δ^9-THC	0		90	9	7	5	5	0
	C-THC	0		---	7	9	25	8	23
P. G.	Δ^9-THC	0		10	7	0	0	0	0
	C-THC	19		---	76	12	75	0	40
T. S.	Δ^9-THC	0	73	32	14	0	0	0	0
	C-THC	0	278	228	216	108	115	0	0

The rapid shifts of plasma Δ^9-THC and C-THC 15 minutes to 48 hours following repetitive exposure are shown in Figure 1 and summarized in Table 2. Expectedly plasma Δ^9-THC peaks (100-260 ng/ml) were much higher after multiple consecutive cigarettes than a single one, permitting detection of Δ^9-THC 2-3 hours after completion of the last cigarette. Significant amounts of this metabolite were measured in plasma 48 hours later.

Table 2

Plasma Δ^9-THC and C-THC equivalent levels in occasional marihuana users 15 min - 2 days after consecutively smoking three 900 mg marihuana cigarettes (2.2% Δ^9-THC/cigarette)

Subject		Pre intoxication	Post intoxication ng/ml							
		0	15'	30'	60'	120'	180'	240'	24hrs	48hrs
W. Z.	Δ^9-THC	8	185	78	123	39	72	12	41	10
	C-THC	7	285	112	292	211	251	296	97	75
T. T.	Δ^9-THC	0	260	- -	37	15	7	0	21	9
	C-THC	40	68	- -	158	43	111	140	94	71
B. W.	Δ^9-THC	0	100	63	87	37	5	7	5	8
	C-THC	46	375	460	401	413	363	428	60	24

Figure 1

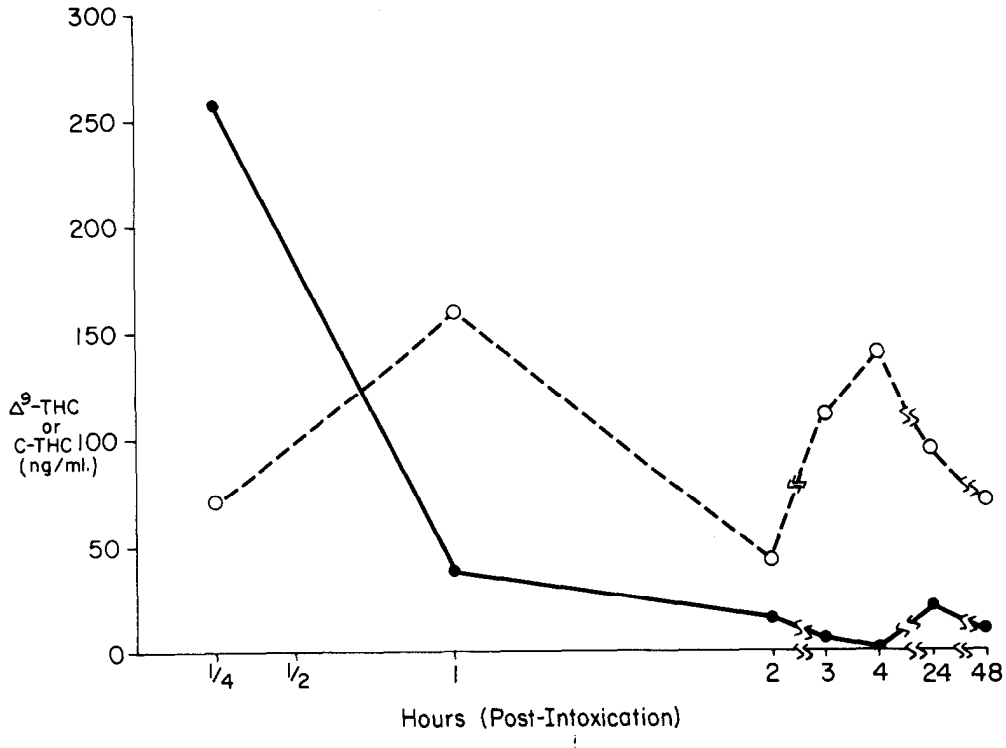

Figure 1: Post-intoxication plasma Δ^9-THC and C-THC (ng/ml) in a representative subject. —— = Δ^9-THC; ---- = C-THC.

Figure 2

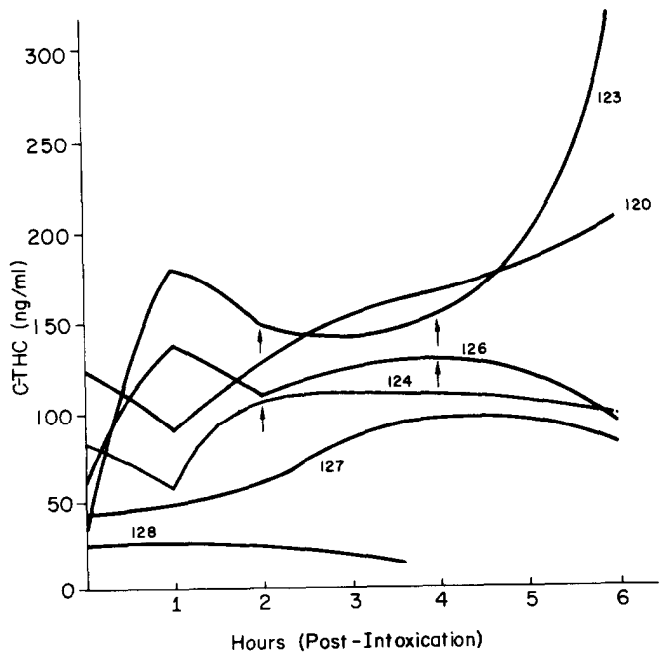

Figure 2: Post-intoxication urine C-THC (ng/ml) in chronic subjects. Arrows indicate an additional smoke.

Table 3

Urine C-THC levels in chronic THC users 1-12 hrs after consecutive smoking 900 mg THC cigarettes (2.2% Δ^9-THC/cigarette)

No.	Subject	Preintoxication	C-THC level (ng/ml) Postintoxication (hrs) 1	2	4	6	8	12
1	119	72			101*		129	164
2	123	28	176	144*	148*	325*	196	
3	124	79	55	106*	105	97*	48	
4	126	61	134	107	126	91*	196*	290
5	127	40		57	93	*	78	
6	120	123	90	125	166*	205	215	
7	121	13	14	21	10	8	5	
8	121	19	4	18	10*	23	23	
9	132	17	31	28	23		31	
10	128	17		17	UD	UD	UD	
11	128	9	11	12	UD	UD	UD	

*Repeat cigarettes on subject demand

UD = undetectable

Table 4

Urine C-THC levels in occasional THC users 1-48 hours after smoking 900 mg THC cigarettes (2.2% Δ^9-THC/cigarette)

Subject	Urine C-THC level (ng/ml) Preintoxication	Postintoxication (hrs) 1	2	3	4	6	8	12	24	48
B. W.	5	33	18	17	33	39	23	15	3	2
K. G.	UD	3	17	2	16	2	3	--	5	3
R. B.	5	9	10	12	21	7	7	4	4	3
W. Z.	UD	7	18	21	9	54	31	--	5	UD
L. G.	2	6	7	4	7	15	3	--	7	5
T. T.	4	20	26	44	59	--	29	14	--	9

UD = undetectable

Urine Δ^9-THC: None of four subjects had measurable urinary Δ^9-THC. This is consistent with previous work (Hollister 1974) describing urine Δ^9-THC levels to be far below present immune assay sensitivity,

Urine C-THC: Assays of urine from 9 chronic users 1, 2, 4, 6 and 8 hours after completion of the first standard cigarette are summarized in Table 3 and Figure 2. Preintoxication levels varied from 28-123 ng/ml. Large C-THC increases occurred (Table 3) peaking at 2-4 hours after initial exposure. Unfortunately the rigid clinical protocol permitted additional cigarettes during the 8 hour study period, C-THC continuing to rise after each additional cigarette. Urine C-THC levels in occasional smokers were considerably lower (Table 4) than in chronic users (Table 3).

Though relatively few subjects were studied a pattern of relative Δ^9-THC and C-THC levels did emerge. In occasional smoker subjects there was a 15-30 minute plasma Δ^9-THC peak and a 30-60 minute C-THC peak after use of a single standardized marihuana cigarette. Importantly, Δ^9-THC became almost undetectable in plasma 1-2 hours after exposure while significant amounts of C-THC persisted in circulation for several hours. This critical divergence of Δ^9-THC and C-THC was even more obvious in subjects who had smoked 3 consecutive marihuana cigarettes. Δ^9-THC was almost undetectable in all plasma samples four hours after the last cigarette had been consumed despite the prolonged (48 hrs) persistence of significant levels of C-THC.

Urine from occasional marihuana subjects was negative or marginal for Δ^9-THC while C-THC (60 ng/ml or less) was detected 1-48 hours after one standard cigarette. The pre-smoke values were zero (Δ^9-THC and C-THC) for occasional smokers. However chronic smokers had significant pre-smoke levels (17-23 ng/ml) in addition to a vastly greater rise of urine C-THC 2-4 hours after consumption of a single cigarette.

Clearly, a single THC metabolite level or use of a significantly crossreacting antiserum (Marks, 1975; Teale, 1975) cannot be used to ascertain "post-intoxication" intervals. Such a result could reflect a single exposure just prior to an examination or multiple exposures several days earlier. The failure to detect plasma Δ^9-THC indicates unambiguously that marihuana was not smoked within the preceeding hour, whereas detection of plasma C-THC in the absence of Δ^9-THC indicates distant exposure. A large controlled experimental population is now essential to correlate Δ^9-THC and C-THC (ratios) with individual metabolic variants for behavior differences.

[1]Samples were provided by the Department of Psychiatry, UCLA School of Medicine.

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RADIOIMMUNOASSAY OF Δ^9-TETRAHYDROCANNABINOL

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INTRODUCTION

Since its introduction by Yalow and Berson (see Berson and Yalow, 1971), radioimmunoassay (RIA) has played an ever-increasing role in the quantitative analysis of drugs and hormones. A number of review articles and books are available (inter alia, Skelley et al., 1973; Spector et al., 1973; Abraham, 1974). In particular a dramatic improvement in assay methodology for many steroid hormones has resulted from the application of radioimmunoassay to these substances.

Due to its extreme sensitivity, RIA can permit the quantitation of substances present in concentrations as low as a few pg/ml of a biological fluid. The inherent selectivity of antibodies introduces a further advantage since it often makes sample preparation requirements minimal and assay methodology simple and thus permits the analysis of large numbers of samples.

These obvious assets of RIA have naturally led to its consideration as a means for measuring blood levels of cannabinoid compounds. A valid RIA procedure for Δ^9-THC would be useful in studying the pharmacokinetics of the drug. Furthermore it is conceivable that RIA methods could be used for the identification of THC and/or its metabolites, thus facilitating forensic investigations. Therefore a number of investigators have been interested in the development of RIA procedures for the cannabinoids.

We believe it is fair to state that development of useful and simple RIA procedures for Δ^9-THC has proven to be a relatively difficult research problem. The difficulties involved in THC RIA development may be summarized as follows:

(1) The highly lipophilic nature of the molecule causes problems in working with the aqueous systems in which RIA is carried out. The compound adheres well to glass and plastic--generally in preference to dissolving in an aqueous medium.

(2) In plasma the compound is tightly bound, principally to a lipoprotein fraction (Klausner, et al., 1975).

(3) It has proven difficult to obtain antibodies highly selective for Δ^9-THC vs its metabolites and various analogs.

(4) The plasma levels of interest (ca. 1-100 ng/ml, although not low by the usual standards of RIA, are sufficiently low that dilution of plasma samples and direct analysis, a technique which has proven highly successful with certain other drugs (Spector et al., 1973; Cook et al., 1973, 1975b; Christensen et al., 1974) is not readily feasible for the lower levels.

(5) Highly concentrated (i.e., high titer) antisera have been difficult to obtain.

(6) High specific activity radioligand for competitive binding studies has been difficult to obtain and often unstable.

It is the purpose of this paper to illustrate these problems and to discuss progress made in overcoming them.

MATERIALS AND METHODS

Definitions

Titer - The dilution of original antiserum which must be added to the assay to obtain X% binding. If one adds 0.1 ml of antiserum which has been diluted 1:500 and obtains 50% binding of radioligand, the 50% titer (initial dilution) is 1:500 If in the above example the total assay volume was 0.5 ml the 50% titer (final dilution) would be 1:2500. In this paper we will quote initial dilution titers unless otherwise noted.

T, B, and N-tubes - Designation for different assay tubes. At end of assay, volumes in all tubes are equal. T tubes measure total radioactivity; N tubes measure nonspecific radioactivity (activity not adsorbed by charcoal) and B tubes measure the amount of labeled drug bound to antibody in the absence (B_o) or presence (Bi) of added unlabeled compound (see Table 1).

Table 1

DEFINITION OF ASSAY TUBES

Tube Designation	Tube Contents				
	Radioligand	Unlabeled Compound	Buffer	Antiserum	Charcoal
T	+	-	+	±	-
N	+	-	+	-	+
B_o	+	-	+	+	+
B_i	+	+	+	+	+

Table 1. Definition of abbreviations for assay tubes

Materials

Chemicals used in the work were reagent grade obtained from commercial sources. Bovine serum albumin was obtained from Sigma Chemical Co., St. Louis, MO (crystallized and lyophilized, No. A4378; or Fraction V, fatty acid free, No. A6003). Norit A (C-176) was obtained from Fisher Scientific, Pittsburgh, PA. Triton X-100 was obtained from Palmetto Chemical Co., Monroe, NC. Antiserum was obtained from Dr. James Soares of the UCLA School of Medicine (Lot No. G2532; Gross et al., 1974) and from Dr. V. Marks thru the National Institute for Drug Abuse (Teale et al., 1974, 1975; S133Y/30/9). Additional antiserum was prepared at Research Triangle Institute (see below).

RIA buffer (0.1 M, pH 6.8) contained 16.35 g $Na_2HPO_4 \cdot 7H_2O$, 5.38 g $NaH_2PO_4 \cdot H_2O$, 9.0 g NaCl, 1.0 g NaN_3, 1.0 g bovine serum albumin (BSA), and 1000 ml double distilled water adjusted to pH 6.8 and was stored in a refrigerator for no more than one month.

Fine particles were removed from charcoal by several decantations from a suspension in distilled water. The charcoal was dried at 200°C and 25 g was suspended in 1000 ml of buffer.

Scintillation fluid contained 2 liters toluene, 1 liter Triton X-100 [purified by stirring for 30 min with 606 Tell-Tale Silica Gel (25 g/ℓ) and filtering] and 18 grams Omnifluor® (New England Nuclear).

Undiluted serum was stored in a freezer (-20°C). Serum diluted in RIA buffer could be stored in a refrigerator for several months.

The radioligand (Δ^9-THC-^{3}H; Pitt et al., 1975) stock solution was kept in benzene/10% ethanol and refrigerated. Solutions for assays were made in 50% ethanol:50% water (double distilled). The radioligand was diluted for assays to 10,000 cpm/10 µl which gives ca. 100 pg Δ^8-THC/10 µl. Dilutions in EtOH/H_2O may be kept refrigerated for no more than 7-10 days. Radioligand decomposition becomes significant after that time. Stock solutions were tested frequently for decomposition by thin layer chromatography. Silica gel plates were developed in 100% benzene and the purity of the radioligand determined by radioscan.

Δ^9-THC for standard curves and metabolites for cross reaction studies were prepared at RTI. For radioimmunoassay, 1 mg/ml stock solutions (prepared from solutions used as glc standards and obtained from K. H. Davis of this laboratory) were stored in 100% ethanol and refrigerated. Dilutions were pre-

pared in 50% ethanol/50% water. These dilutions may be kept refrigerated for approximately one month. Periodic analyses by thin layer chromatography are required to test the purity and stability of the compounds.

RESULTS AND DISCUSSION

Antigen synthesis

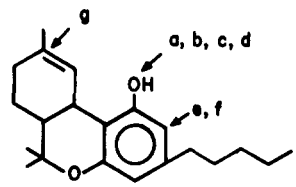
In the first part of this century, Land-Steiner (see Landsteiner, 1962) showed that covalent bonding of a small molecule (haptenic component) to a protein gave an antigenic substance and that antibodies formed to this antigen were capable of selectively binding the small molecule. He further established that the antibody selectivity is influenced primarily by those portions of the hapten which are removed from the linkage to the protein carrier. Thus to achieve an antibody which will be selective for the parent drug in the presence of its metabolites, proper synthetic design of the antigen is essential. Those portions of the molecule which are subject to metabolic alterations must be left free to influence antibody selectivity.

The very fact that the hapten must be attached to the protein via a covalent bond then means that it is impossible to devise a conjugate in which all portions of the hapten will equally influence antibody selectivity. No linkage can be considered completely inert. In addition, in designing the synthesis of the antigen one must consider the chemistry involved and the ease or difficulty in relation to the expected benefit. Depending upon their needs, then, it can be expected that different investigators will synthesize antigens in different ways.

Most of the metabolic alterations of Δ^{9}-THC occur in the cyclohexene moiety with hydroxylation of the Δ^{9} compound occurring at the 11-and 8-positions along with conversion of the 11-carbon to a carboxyl group. More recently it has been reported that hydroxylation can also occur in the amyl side chain of the phenolic ring (Wall, 1975).

A number of previously reported antigens are summarized in Figure 1. Teale et al. (1974, 1975), formed a hemi-ester linkage with the phenolic hydroxyl group and then coupled this compound to bovine serum albumin to form an antigen. Tsui et al. (1974), report formation of a hemisuccinate from the phenolic hydroxyl, and also formed an ether linkage at this position to give a carboxymethyl derivative. The products were coupled to a variety of proteins as shown in Figure 1. The phenolic group apparently undergoes no metabolic

Figure 1



a) CO-NH-BSA (Teale, *et al.*, 1974)

b) $CO\text{-}CH_2CH_2CO\text{-}NH\text{-}BSA$ (Teale, *et al.*, 1975)

c) $CO\text{-}CH_2CH_2CO\text{-}NH\text{-}PGG$ (HSA, SGG, or PLL) (Tsui, *et al.*, 1974)

d) $CH_2CO\text{-}NH\text{-}HSA$ (or SGG) (Tsui, *et al.*, 1974)

e) N=N-  CO- NH-PGG (HSA or SGG) (Tsui, *et al.*, 1974)

f) N=N-  CO-NH-KLH (Gross, *et al.*, 1974)
(+4–isomer)

g) 10-I-9-NH-CO-NH-HSA (or PGG) (Tsui, *et al.*, 1974)

Figure 1. Some positions through which Δ^{9}-THC has been linked to protein

alterations, but spatially the attachment to protein is relatively close to the metabolically important cyclohexene ring. To avoid this problem Tsui et al. also prepared a 2-azophenylcarboxy derivative, a substitution empioyeh by Gross et al. (1974) as well. This substitution frees the hydroxyl group for binding, although at the possible expense of introducing a relatively immunodominant azobenzoyl moiety. Finally, Tsui et al. (1974) also prepared a 10-iodo-9-ureido linked THC.

Our attention was drawn to the amyl side chain of the aromatic ring as a potential position for attachment to the protein. Such a linkage would certainly fulfill the requirement of distance from the metabolically reactive cyclohexene ring (although not from sites of hydroxylation on the amyl side chain). Also it was of interest to examine the effectiveness of the flexible hydrocarbon chain in exposing the tricyclic moiety to influence antibody selectivity. Use of such a long flexible chain had proved quite successful for us in synthesis of antigens for caffeine (Cook et al., 1974) and phenylbutazone (Cook et al., 1975a). For synthetic reasons our initial attempts dealt with the Δ^{8}-THC analog as a substrate.

Synthesis of the Δ^{8}-THC antigen is shown in Figure 2. 5'-Carboxy-Δ^{8}-THC labeled with

Figure 2

SYNTHESIS OF Δ^8 – THC ANTIGEN

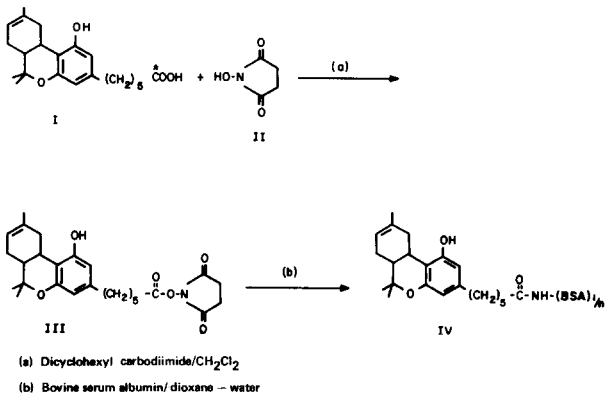


Figure 2. Synthetic route to antigen based on 5'-carboxy-Δ^8-THC

tracer amounts of carbon-14 in the carboxyl group (Pitt et al., 1975) was converted to its N-oxysuccinimidoyl ester by reaction with N-hydrbxysuccinimihe and diclohexylcarbodiimide. This active ester readily coupled with bovine serum albumin in a mixture of dioxane and water to yield the desired antigen. Radioactivity measurements indicated the incorporation of ca. 33 residues of Δ^8-THC/ molecule of bovine serum albumin. We have found this mode of coupling to be a very useful one in certain circumstances. Where it is applicable, the number of molecules of drug moiety incorporated into the bovine serum albumin is relatively easy to control.

Formation of antisera

Rabbits were immunized with the conjugate dissolved in sterile 0.9% NaCl and homogenized with an equal volume of Freund's complete adjuvant. Immunization with 200 µg of antigen was carried out by intradermal technique of Vaitukaitus et al. (1971). This was followed after two weeks by another intradermal immunization (100 µg) and then at four week intervals subcutaneous booster injections (100 µg of antigen) were given. Rabbits were bled on day 52 after the initial dose and every 28 days thereafter. After the third bleeding the immunization program was discontinued for a three-month period. Antigen booster injections were then resumed and the fourth bleeding was taken 10 days after immunization. Reasonable titers (50% binding of ca. 125 pg of labeled Δ^8-THC at an initial dilution of 240-540 or a final dilution of 1200-2700) were achieved in two out of four rabbits at the first bleeding, but no significant increases were observed on subsequent booster administrations. A third rabbit eventually achieved a titer (final dilution) of 1:2250 at the fourth bleeding. Titers generally declined at the fifth and sixth bleedings.

These titers compare quite favorably with those reported by others (Teale et al., 1975; Gross et al., 1974) and demonstrate that with this antigen the rabbit is a reasonable animal to use for antibody production. A further comparison of the azobenzoyl-THC-derived antiserum obtained from UCLA and the antiserum prepared from the amyl-linked antigen is shown in Figure 3. The affinity constants

Figure 3

ANTISERUM CHARACTERISTICS

	UCLA	RTI-RS-81
Affinity Constant (for Δ^8-THC)	1.6 X 10^9	2.5 X 10^9
Std. Curve Characteristics (Logit - log Plot)		
50% Intercept	0.5 ng	1.4ng
Linear Range	0.3 - 4.1 ng	0.3-6 ng

Figure 3. Characteristics of two antisera as determined in this laboratory: UCLA (Gross, et al., 1974); RTI-RS-81-2 (this paper).

are quite close. The slightly higher affinity of the Δ^8-antiserum for Δ^8-THC is understandable. Due to the heterologous nature of the systems employed (Δ^8-radioligand, unlabeled Δ^9 and antiserum generated to either Δ^8- or Δ^9-), the standard curve characteristics are not similar for the two antisera. When standard curves are converted to the logit-log basis (Rodbard et al., 1969), the UCLA antiserum exhibits a steeper slope with a lower 50% intercept and a somewhat narrower linear range than does the RTI antiserum. The lower limit in Figure 3 is a conservative number. If one defines sensitivity as the amount of Δ^9-THC which will reduce binding to a level two standard deviations below the initial value, the limit for the RTI antiserum is ca. 100 pg.

Antibody selectivity

The avidity of the antisera for various metabolites and analogs of Δ^{9}-THC was measured by determining the relative amount of compound required for 50% displacement of the initially bound radioligand. This is a relatively crude measure of cross-reactivity. It is strictly valid only if the displacement curves for the two compounds in question are completely parallel and it does not take into account the possibility of small subpopulations of antibodies with high avidity for one substance in preference to the other. Nevertheless, it offers a useful if rough guide to antibody selectivity if its limitations are realized. Figure 4 shows

Figure 4

STRUCTURE BOUND	% CROSS-REACTION WITH ANTISERUM TO A	B	C
(Δ^9)	100% (100)	100% (100)	100%
(Δ^8)	63 (100)	48	271
(HHC)	94	144	102
CH_2OH	244 (100) (as Δ^8)	49 (47)	13
COOH	157 (<0.4) (as Δ^8-COOMe)	2 (3)	0.6
OAc	–	38	68
OMe	–	21	4
(CBN)	92	26	24
(CBD) HO	2	0.1 (0.5)	0.1

Figure 4.
Structure-binding relationships of various antisera for cannabinoids. Numbers in parentheses are from the references cited: A (Teale, et al., 1975); B (Gross, et al., 1974)

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the cross-reactivity of three types of antisera, all measured in our own laboratory. Selectivity can be significantly influenced by the assay conditions. Therefore the values reported by the producer of the antiserum are also given in the figure in parentheses. The displacement ability of Δ^{9}-THC is taken as a standard at 100%. For reasons discussed later, the radioligand used was Δ^{8}-THC-^{3}H.

A change which alters the tricyclic character of the molecule (see cannabidiol, CBD) essentially destroys binding to all of the antibodies. However, much more subtle changes can also have significant effects. In the case of the antibody to Δ^{8}-THC, shift of the double bond from the Δ^{8}- to the Δ^{9}-position results in a two and one-half fold decrease in cross-reaction. A similar (two-fold) decrease is seen on comparison of the binding of Δ^{9}- and Δ^{8}-THC to antibody obtained from the other two antigens.

Reduction of the cyclohexene double bond has no greater effect with the Δ^{8}-antibody than does the shift to the Δ^{9}-position and actually somewhat increases the cross-reaction with the Δ^{9}-antiserum prepared from the azobenzoyl antigen. Aromatization of the cyclohexene ring (to CBN) results in a four-fold decrease in cross-reaction as compared with Δ^{9}-THC in the case of the azobenzoyl- and amyl-linked antigens. It has little effect on antibody to the O-succinoyl antigen.

Oxygenated C-11 metabolites cross-react relatively little with the antibody from the amyl antigen. Cross-reaction of the 11-hydroxy metabolite with the azobenzoyl antiserum is rather significant (49%) but the cross-reaction drops markedly when the 11-nor-9-carboxy metabolite is considered (2%). Both of these metabolites cross-react strongly with antibody from the O-succinoyl antigen.

Thus it appears that good, but not outstanding, selectivity for Δ^{9}-THC vs a number of metabolites and analogs can be achieved and that modest but usable antibody titers can be obtained in either the goat, sheep, or rabbit. Let us now consider some of the other problems in the development of an assay.

Assay parameters

General procedure.--The general assay procedure finally adopted is shown in Figure 5. Data leading to definition of the various conditions are presented in suceeding paragraphs.

Radioligand.--The sensitivity of a radioimmunoassay is very much a function of the specific activity of the bound radioligand. Thus, in general, one would prefer the highest possible specific activity. Storage of such high specific activity compounds can often lead to radiation-induced decomposition. The general experience of our laboratory in the preparation and storage of such materials is that unlabeled substances which readily decompose through autoxidation, etc. are often particularly unstable when prepared radiolabeled and with high specific activity. This was found to be the case for

Figure 5

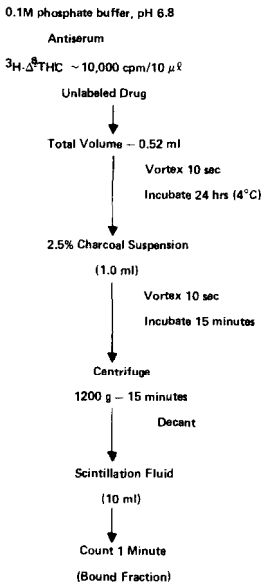

Figure 5. General procedure for RIA of Δ^9-THC

Figure 6

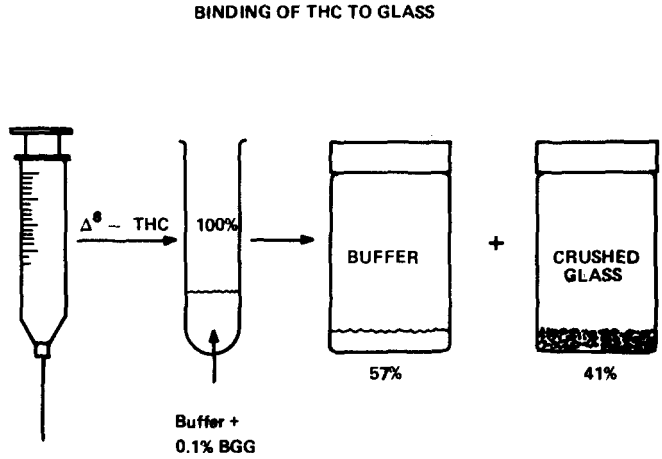

Figure 6. Illustration of the binding to glass observed with THC

Δ^9-THC which was prepared at a specific activity of ca. 50 Ci/mmole (Pitt et al., 1975) but had a very short shelfilife. On the other hand, Δ^8-THC-^{3}H, prepared in similar specific activity, has proven to be a quite stable entity. Its binding characteristics with the antisera raised to either Δ^8- or Δ^9-THC make it a useful radioligand. Hexahydrocannabinol also exhibits good cross-reactivity with the antisera but synthetic problems have discouraged our use of tritiated HHC as a radioligand.

Buffer Protein.--The relatively intractable nature of THC in aqueous systems is by now presumably well-known to all interested in this field of research. Because of the small amounts of material involved, RIA procedures for most compounds call for addition of some type of protein to the buffer in order to enhance solubility of the various components and prevent binding to the incubation tubes. Our experience has been that the nature of this protein is of crucial import in work with Δ^9-THC. This is illustrated in Figure 6. When ca. 5 ng of tritiated Δ^8-THC in a small amount of ethanol was added to a normal RIA buffer (phosphate buffered saline) containing 0.1% bovine γ-globulin as the protein, very significant amounts of the THC were bound to the glass. This was illustrated by decanting the buffer into scintillation liquid. The glass tube was then broken up, and the pieces were placed in a scintillation vial and treated with a little methanol. Scintillation fluid was then added. This experiment indicated that only 57% of the THC remained in the buffer and 41% adhered to the glass tube (percentages are corrected for quenching). Even allowing for incomplete removal of buffer from the glass prior to crushing it, this is still a very significant loss of the THC.

Experiments on the effect of silanizing glass tubes or washing them with nitric acid gave no improvement and in most cases the results were worse. However we found that in our laboratory, the use of bovine serum albumin as the protein component of the buffer resulted in much diminished adherence to glass and a better assay system. This is illustrated in Figure 7. Labeled THC was incubated with buffer containing either bovine serum albumin (BSA) or bovine γ-globulin (BGG) plus varying dilutions of antiserum. The solutions were then decanted into scintillation liquid and radioactivity determined. Results were not corrected for quenching, which had an approximately equal effect on all values.

At high concentrations of antiserum, there is no difference between the two buffer proteins. In effect the antiserum itself is keeping the THC in solution. However, as the antiserum concentration is decreased (dilution increased) less and less of the label can be recovered from the tubes containing the BGG and eventually the recovery

Figure 7

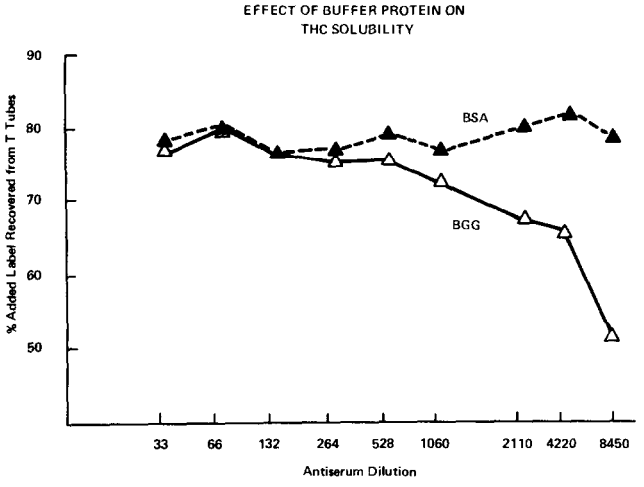

Figure 7.
Variation of THC solubility as a function of antiserum dilution and buffer protein. Antiserum of Gross, et al. (1974); BSA = bovine serum albumin; BGG = bovine gamma globulin

Figure 8

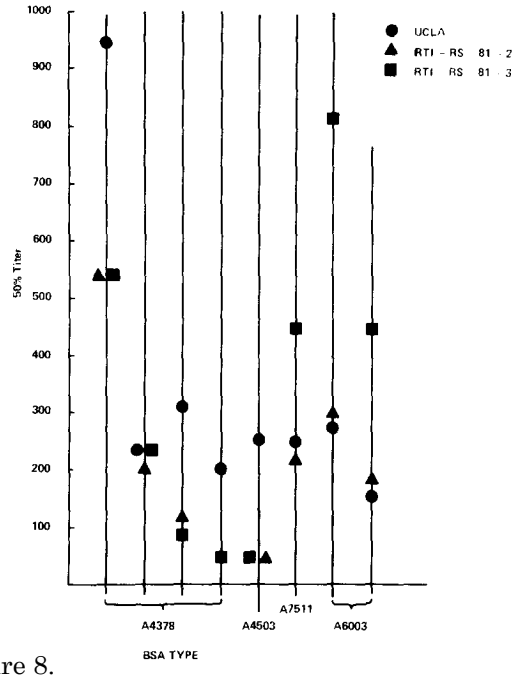

Figure 8.
Influence of different types and lots of bovine serum albumin on apparent antiserum titer (initial dilution). Each vertical line represents a separate lot of BSA: A4378 (crystallized once and lyophilized); A4503 (Fraction V powder); A7511 (crystallized and lyophilized; less than 0.005% fatty acids); A6003 (Fraction V; less than 0.005% fatty acids)

falls to essentially the level found in the previous experiment. The adsorption to glass may be looked upon as competing with the binding to antiserum. Although the problem may be overcome by higher concentrations of antiserum, it was to us preferable to avoid this effect if possible, remove another source of variability from the assay, and increase the practical titer of the antiserum by using bovine serum albumin in the buffer system. At the concentrations employed, this protein did not decrease the amount of radiolabel which was adsorbed to charcoal.

Not only was the type of protein important, but the assay characteristics were also greatly influenced by the type of bovine serum albumin used and even by different lots of the same type of BSA. This is illustrated in Figure 8, where the 50% titers for three different antisera (one obtained from UCLA and two prepared at RTI) are compared in the presence of the same concentration of protein (0.1%) but varying the type or lot of bovine serum albumin used. The first lot of crystallized and lyophilized BSA used gave the best overall binding characteristics. Subsequent lots gave significantly lower titers with one in particular giving extremely poor results. Somewhat more consistent results were obtained with Sigma's fraction V fatty acid-free BSA. Because this material is less expensive than the crystallized type, we have settled on it in practice. A third type (fraction V powder) gave very poor results and a BSA type prepared by Sigma specifically to aid in the RIA of insulin proved worst of all.

We were unable to pinpoint the difference in the various lots which might have influenced the results. The presence of fatty acids as a contaminant was suggested since batches characterized as fatty acid-free gave generally better results. Treatment of one of the less useful batches with charcoal to further remove fatty acids (Chen, 1967) gave some improvement, but did not result in raising the quality to that of some of the better batches. We have, therefore, concluded that (a) each batch of BSA used should be checked out before large amounts of antiserum are diluted in the buffer, (b) large batches should preferably be checked and then used in the assay to attain consistency, (c) the quality of an antiserum in terms of titer may be strongly influenced by the composition of the protein in the buffer (which may account for inter-

Figure 9

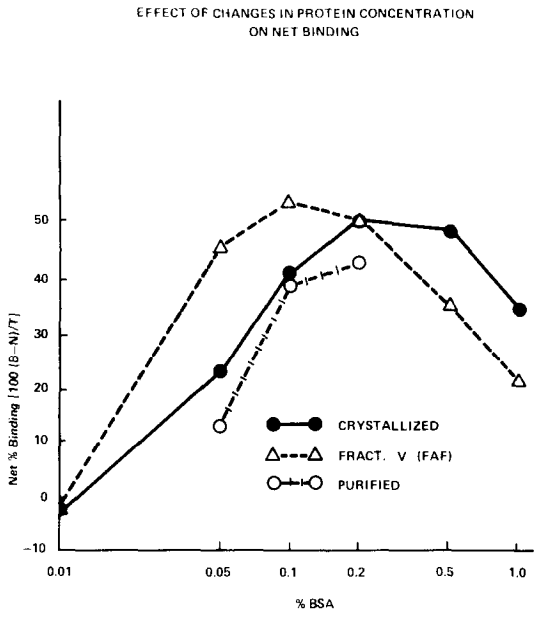

Figure 9.

Net binding as a function of BSA concentration and type: Δ---Δ Sigma fraction V (<0.005% fatty acids); o-|||-o different lot of the above after second removal of fatty acids; ●—● crystallized and lyophilized

Figure 10

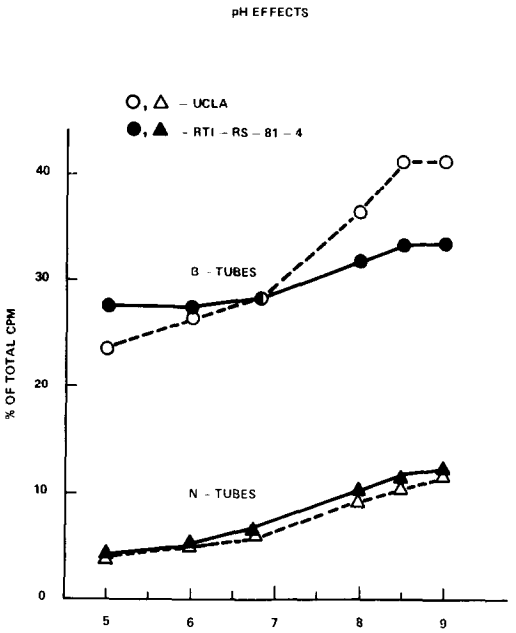

Figure 10.

Effect of pH on binding and non-adsorbed radioactivity. Antisera were obtained from UCLA (Gross, et al., 1974) and as described in this paper (RTI-RS-81-4)

laboratory differences in antiserum titers) and (d) some antisera are more sensitive to changes in the protein than are others. This is particularly true of RTI RS-81-3.

As would be expected, the concentration of protein had an effect on the net percent binding and this concentration dependence was also somewhat a function of the antiserum and the type of bovine serum albumin used. This is illustrated in Figure 9. At low concentrations (<0.05% BSA) the net percent binding [100 (B-N)/T] was low, principally due to the fact that the total binding was very low. Non-specific radioactivity (N tubes, radioactivity not adsorbed by charcoal) was also relatively low up to 0.2% concentration of BSA. At concentrations of 0.5-1% it became a significant factor and resulted in a considerable lowering of the net percent binding.

Best results were generally obtained at 0.1-0.2% protein for the antisera and protein samples examined. Therefore for reasons of both economy and best binding, 0.1% was chosen as the best concentration.

pH Effects.--As shown in Figure 10, pH had a definite effect on both total binding and radioactivity not adsorbed by charcoal. This effect was again somewhat sensitive to the type of antibody used. The UCLA antiserum exhibited considerably better binding at pH values of 8 or above, but the non-specific radioactivity also increased. Although an increase in apparent binding also appeared to occur at higher pH with the RTI antiserum, the net binding showed no change. To minimize nonspecific radioactivity pH 6.8 was accepted as the best compromise. Since relatively little THC would be ionized under the conditions used, it seems likely that the change in pH acts principally by its effect upon the conformation of the binding globulin and/or by protonation effects in the binding region.

Incubation Time.--The effect of the time of incubation at 4°C on the percent binding achieved was also studied (Figure 11). In the case of the RTI antiserum a perceptible rise in percent binding occurred up to about 6 hr, although binding appeared to begin leveling off after about 4 hr. Binding equi-

Figure 11

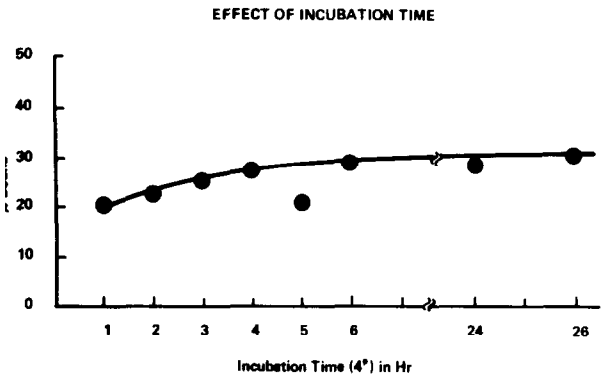

Figure 11.
Percent of radioligand bound as a function of time of incubation at 4°C

Figure 12

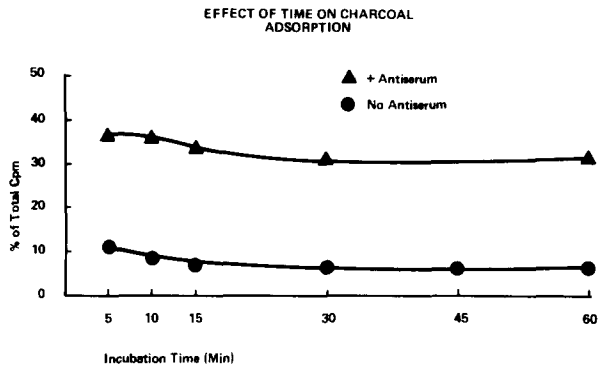

Figure 12.
Effect of time on adsorption of radioligand by charcoal at 4°C. One ml of 2.5% charcoal suspension was added to incubation tubes containing 0.52 ml. Contents were mixed for 10 sec on a vortex mixer and allowed to stand

librium was established more rapidly in the case of the UCLA antiserum and was complete in approximately 2 hr. Incubation times of 4 hr or more would be suitable for either antiserum and there was no deleterious effect if incubation continued for an overnight period.

Charcoal Treatment.--Since the charcoal adsorbant removes free radioligand, one might expect that exposure to charcoal would eventually shift the binding equilibrium so as to remove essentially all of the label from the antibody. The rate at which this would happen would be dependent upon the rate of binding reversal, which in turn should be a function of temperature. A finite time is also required to adsorb the free radiolabel and to effect its removal from weakly binding substances such as protein. The best equilibration time then is often a compromise and must be studied for each antiserum.

Figure 12 shows the effect of letting the charcoal suspension stand with the incubation mixture. Non-adsorbed radioactivity in the absence of antiserum reached the minimum value in approximately 15 min and did not fluctuate for up to 60 min thereafter. In the presence of antiserum, a somewhat similar situation obtained. By 15 min the minimum percent bound was essentially reached. Therefore, in order to reduce the non-specific binding to its minimum, a 15 min time for standing with the charcoal is necessary.

Other experiments on the time of mixing of the charcoal suspension with the incubation mixture showed that this also affected the amount of radioactivity left in solution. It was necessary to compromise in this regard and it was found that a 10 sec mixing on a Vortex mixer gave the best results within a reasonable period of time.

Variation of charcoal concentrations (1 ml of suspension was added in all cases) from 1-10% showed that a 2.5% suspension gave best results. Addition of dextran to the charcoal did not prove useful.

Plasma analysis

Having established assay conditions, one would now like to transfer these to the analysis of samples of biological importance. Ideally, one would like to be able to analyze a diluted sample of plasma directly without carrying out any extraction procedures. Whether this is successful depends upon the amount of plasma which can be added to each assay tube and therefore upon the concentration of the drug in plasma and the sensitivity of the assay itself.

Plasma was added directly to T, B, and N tubes containing both the RTI and UCLA antisera and its effect on each was studied. As expected, T tubes showed no change in going from 0 to 60 µl of plasma in a total volume of 0.52 ml. Addition of 15 or more µl of

Figure 13

ETHANOL – ETHER EXTRACTION PROCEDURE AND RECOVERIES

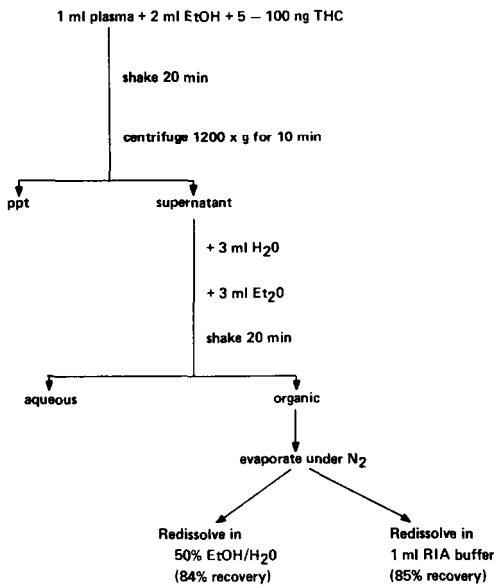

Figure 13.
Procedure used for extraction of Δ^9-THC from plasma

plasma resulted in an increase in the N-tubes and a concomitant decrease in the percent binding so that the net percent bound values at 15 µl of plasma were only 60-70% that of the tubes containing no plasma. Further increases in the amount of plasma resulted in lower levels of net binding. However, if one assumes an assay sensitivity of 0.3 ng and ignores any problems caused by the presence of metabolites, direct assay of plasma down to a level of 20 ng/ml should be feasible. This technique assumes that one has a sample of blank plasma from the subject or is willing to ignore the possibility of intersubject variation in effects on the assay. This latter point will be considered later.

For pharmacokinetic studies, assays at levels below 20 ng/ml are required. Solvent extraction to remove protein and other interferences must apparently be used to analyze low levels of Δ^9-THC. Some care must be used in the choice of solvents since small amounts of certain solvents can have a significant influence on the assay. For example, the addition of only 1 µl of isoamyl alcohol (the solvent commonly used in conjunction with hexane to increase the extractability of THC from plasma) reduces the binding in an assay tube to ca. 60% of that obtained in the absence of the isoamyl alcohol. Therefore traces of this relatively non-volatile substance remaining from an extraction process may have a very significant effect upon assay results.

Ethanol has somewhat less of an effect. Indeed we routinely add up to 10 µl of ethanol to each assay tube in preparing the standard curve. Addition of 20 µl more ethanol reduces binding to about 75% of the initial value. Nevertheless, extraction of Δ^9-THC from plasma by precipitation of proteins with ethanol has been reported to be a useful procedure in RIA of THC (Teale, et al., 1975) and we have therefore studied this particular extraction system with plasma samples. The overall extraction process used is shown in Figure 13. Plasma is treated with twice its volume of ethanol and precipitated material is centrifuged out. Aliquots of the supernatant may be taken for analysis at this point. We tested the possibility of utilizing this supernatant as has been reported by Teale et al. (1975).

Since the plasma/ethanol extract has a definite influence on the standard curve, it is necessary to compensate for this effect by running the standard curve in the presence of the same volume of plasma extract as that to be analyzed. This works reasonably well when it is possible to obtain a blank plasma from the same individual whose plasma THC concentration is being studied. However this would not be easy to do in the case of chronic users of THC and would represent a distinct limitation on the applicability of the method. It was therefore of interest to compare standard curves obtained from different plasmas.

Four human male plasma samples were spiked with five concentrations of THC from 5-100 ng/ml. Each plasma series was then used to generate a standard curve. Each standard curve, in turn, was used as the basis for the "analysis" of the spiked plasma samples. We then looked at the percent of the samples which were analvzed with less than 20% error. Results are shown in Figure 14. As expected, when plasma series A was analyzed by standard curve A (generated from plasma A), all of the samples fell within ±20% of the actual spiked values. Under these-conditions it was possible to add sufficient of the ethanol extract to get down to levels of 5 ng/ml with reasonable accuracy.

With cross plasma comparisons the results are not so good. Plasma samples B and D gave reasonable accuracy when measured against standard curves A and B. However, the accuracy of analysis of samples from plasma C was

Figure 14

COMPARISON OF ASSAY RESULTS FROM SPIKED PLASMA SAMPLES AFTER EtOH PRECIPITATION OF PROTEIN

Percent of Analyses with < 20% Error

Plasma Series Analyzed	Standard Curve Employed A	B	C	D
A	100	80	(20)	80
B	100	100	80	80
C	(20)	60	100	60
D	100	100	80	80

Figure 14.

Accuracy of analysis when extracts from one plasma spiked with Δ^9-THC are analyzed by means of standard curves generated from extracts of other plasma samples. Antiserum from UCLA (Gross, et al., 1974)

Figure 15

STD. CURVES FROM SPIKED PLASMA SAMPLES (EtOH PRECIPITATION: Et_2O EXTRACTION; RECONSTITUTED IN BUFFER)

	INITIAL BINDING	SLOPE (LOGIT - LOG)	50% INTERCEPT	CORRELATION COEFFICIENT
Std Curve (Buffer)	35%	-3.54	0.59 ng	0.99
+ 50µl extract A	32%	-2.88	0.81 ng	-0.98
+ 50µl extract A	32%	-2.84	0.77 ng	-0.98
Std. Curve	45%	4.06	0.59 ng	-0.99
+ 50µl extract B	35%	-3.50	0.71 ng	-0.99
+ 50µl extract C	38%	-2.36	0.71 ng	-0.96
+ 50µl extract D	39%	-2.49	0.68 ng	-0.99

Figure 15.

Comparison of standard curves generated in buffer alone with those containing plasma extracts. Results are from two separate experiments. Duplicate extracts A should be compared with the first standard curve; B, C, and D with the second. Antiserum from UCLA (Gross, et al., 1974)

quite poor when the other plasmas were used as the basis of the standard curve. This is particularly true in the case of plasma C analyzed vs standard curve A and plasma A analyzed vs standard curve C, where only one out of five samples fell within the 20% limits. If one requires accuracy within +10%, results are much worse.

We believe this study leads to two conclusions: (1) if one sets modest goals for accuracy, a fair number of plasma samples can be analyzed down to levels of 5 ng/ml of pure Δ^9-THC. However, (2) the influence of plasma extracts on the standard curve is not highly reproducible from one plasma to another and could, on occasion, lead to severe errors in the analysis. This study also does not take into account the potential problems caused by cross-reaction of other cannabinoid materials which may be present in plasma at higher concentrations than the Δ^9-THC itself.

To further purify the plasma material before analysis, we then followed a procedure which was described to us by Soares (private communication, 1975) and which consists of the last part of Figure 13. The ethanol extract is treated with water and ether. After equilibration, ether is removed and evaporated. The residue may be taken up either in 50% aqueous ethanol or in RIA buffer. Since use of the latter eliminates the effects of ethanol on the assay and does not result in lower recoveries, it is the procedure of choice.

Figure 15 shows the results of generating standard curves from plasma samples spiked with 5-100 ng/ml of Δ^9-THC and carried through this procedure. Adding 50 µl (5% of the total extract from one ml) of extract from plasma A did result in a change in the standard curve from one run in buffer alone. The initial binding was lowered, the slope of the logit-log plot was reduced, and the 50% binding intercept was increased. However, duplicate samples of the same plasma gave rather reproducible standard curves. When three different plasma extracts were compared there were found to be some differences. This implies that caution again should be used in analyzing plasma samples from one subject based upon a standard curve generated from the plasma of a second subject. The use of a pooled plasma for generation of standard curves is probably the best compromise at present.

CONCLUSIONS

(1) The work we have done leads us to believe that there are factors which can be extracted from normal plasma under the conditions described which will interfere with the assay. Since these factors have not yet been identified and may vary from subject to subject and perhaps from extraction to extraction, extreme caution should be used in evaluating the data obtained in such a manner. Care must also be taken in obtaining plasma samples and storing them to avoid inadvertent addition of such potentially interfering materials as plasticizers, etc. The effect of freezing and storage on the stability of the THC and on the formation of interfering substances needs to be further evaluated.

(2) The sensitivity of the assay to conditions such as buffer composition and plasma materials makes interlaboratory comparisons difficult. Standardized techniques and materials are therefore a pressing need.

(3) Radioimmunoassay has not yet lived up to its potential in the analysis of cannabinoid substances. The sensitivity of the assay is not as great as one would expect based on other compounds such as the steroid hormones. Nor have the high titer antisera of the type available for the steroid hormones yet been reported for Δ^9-THC. Since factors such as titer and sensitivity can apparently be strongly influenced by the actual assay conditions, this suggests that further manipulation of conditions, the synthesis of even higher specific activity radioligand, and the development of antisera to a variety of antigens and in a variety of animals are all valid approaches to further work.

(4) The reasonable results obtained by immunization of animals with a conjugate prepared from 5'-carboxy-Δ^8-THC have encouraged us to undertake the synthesis of similar antigens based on 5'-carboxy-Δ^9-THC. Work along these lines is in progress and can be reported at future meetings.

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DETERMINATION OF THC AND ITS METABOLITES BY EMIT® HOMOGENEOUS ENZYME IMMUNOASSAY: A SUMMARY REPORT

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INTRODUCTION

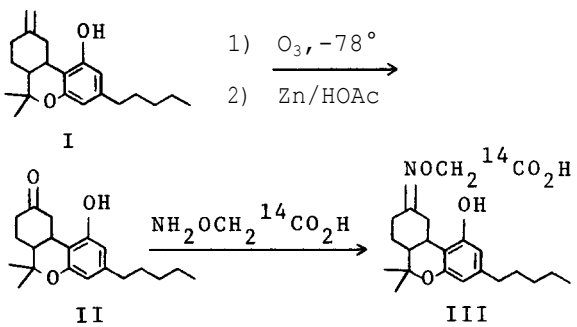
We found that certain enzymes could be inhibited by antihapten antibodies when these enzymes were covalently attached to the corresponding hapten. In 1972 we introduced the first EMIT® homogeneous enzyme immunoassay based on this principle (Rubenstein, Schneider, and Ullman, 1972). Since then we have demonstrated the generality of the technique and have developed immunoassays based on three enzymes, lysozyme (Schneider, Linquist, Wong, Rubenstein, and Ullman, 1973), malate dehydrogenase (Ullman, Blakemore, Leute, Eimstad and Jaklitsch, 1975), and glucose-6-phosphate dehydrogenase (Chang, Crow1 and Schneider, 1975). The change of enzyme activity of hapten-enzyme conjugates produced upon binding antihapten antibodies permits direct measurement of the amount of antibody bound to the conjugates. Enzyme immunoassays based on this principle are classified as "homogeneous" immunochemical techniques since separation of free from bound hapten is not required. Separation is required when the signal of the labeled hapten is not changed by antibody binding as is the case in radioimmunoassay and enzyme-linked immunosorbent assay (ELISA) which are termed "heterogeneous" immunochemical techniques. The observed change of activity in homogeneous enzyme immunoassays is reminescent of inhibition or activation produced when certain antibodies to enzymes bind to their respective enzymes (Arnon, 1973). The mode of action may be similar. Either the antigenic determinant is an integral part of the enzyme surface or it is attached covalently to the surface.

Malate dehydrogenase (MDH) was chosen for development of a THC assay because of its high stability, its availability and its ease of detection by a simple highly sensitive assay. As little as 10^{-11} molar enzyme can be detected in a one minute spectrophotometric measurement. The mechanism of

antibody inhibition of morphine conjugates of MDH had previously been studied and an immunoassay for morphine was developed (Rowley, Rubenstein, Huisjen, and Ullman, 1975) which permitted detection of $\geq 2 \times 10^{-9}$ molar morphine in the assay mixture.

SYNTHESIS OF THE THC HAPTEN

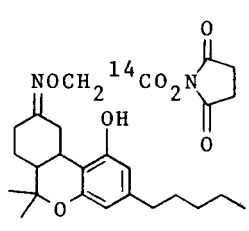
Synthesis of the THC hapten, III, that was used for producing the components of the assay is illustrated below:



$\Delta^{9,11}$-THC, I, was treated with one equivalent of ozone at low temperature to yield an ozonide which was reduced with zinc in acetic acid to yield ketone II in 55% yield. Compound II was treated with 1-C^{14}-O-carboxymethylhydroxylamine in anhydrous refluxing methanol to yield the desired acid, III, in 61% yield.

PREPARATION OF THC ANTIBODY

Acid III was conjugated to bovine γ-globulin via its N-hydroxysuccinimide ester, IV, prepared by condensation with N-hydroxysuccinimide using the condensing reagent, N-dimethylaminopropyl-N'-ethyl carbodiimide hydrochloride in anhydrous dimethylformamide.



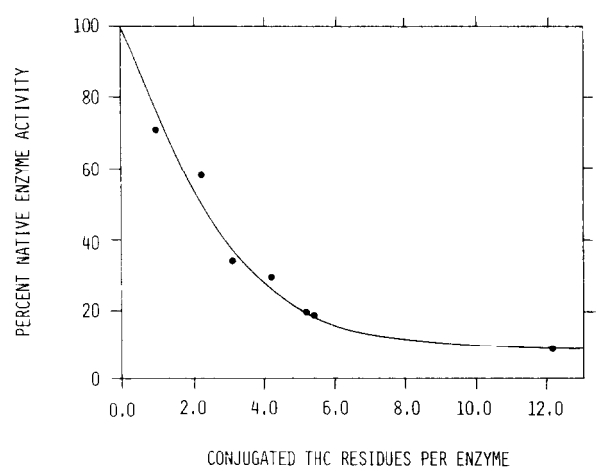
IV

Subsequently, the dimethylformamide solution of "active ester" was added to a buffered solution of the protein containing 30% dimethylformamide cosolvent at pH 8.5. The protein was exhaustively dialyzed to remove non-covalently bound THC residues. The number of bound residues was determined by scintillation counting of the radiolabeled products to yield conjugates containing 21 and 32 THC residues per protein molecule in two separate preparations. Antibodies were obtained from sheep by immunizing with the conjugates.

PREPARATION OF THC-MDH CONJUGATES

Acid III was conjugated to MDH via IV under almost identical conditions. A series of THC-MDH conjugates were thus prepared by adding increasing amounts of IV to constant amounts of MDH in separate reaction vessels. Non-covalently bound THC residues were removed either by exhaustive dialysis or by gel chromatography on Sephadex G-25. The number of bound radiolabeled THC residues and the residual enzyme activity of each conjugate was determined (Figure 1). Enzyme activity of the conjugates decreased sharply with increasing substitution up to about 5 residues bound (20% activity). Substitution beyond 5 residues provided a conjugate (12.2 residues) with only a moderate further loss of activity (8% activity).

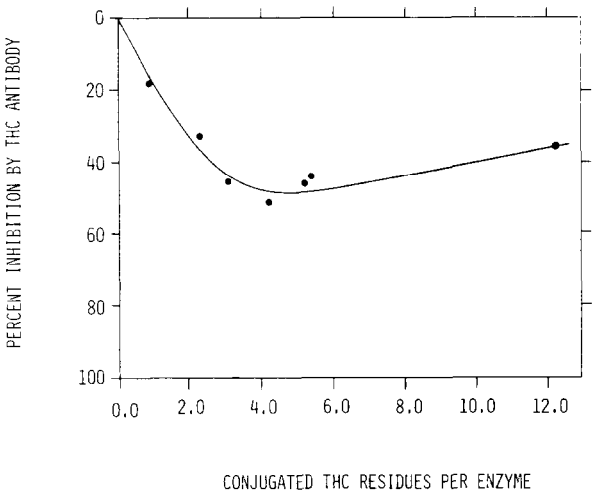
Figure 1



Addition of excess THC antibody to the conjugates reduced their enzymatic activity. The maximum inhibition in the presence of excess antibody was only slightly affected by the animal source. Maximal inhibition was, however, directly dependent on the

number of bound THC residues (Figure 2). It increased sharply with the number of THC groups on the enzyme and reached a maximum when at about 4.2 residues (51% inhibition). Upon substituting the enzyme more heavily (12.2 residues) the antibody induced inhibition decreased to 35%.

Figure 2



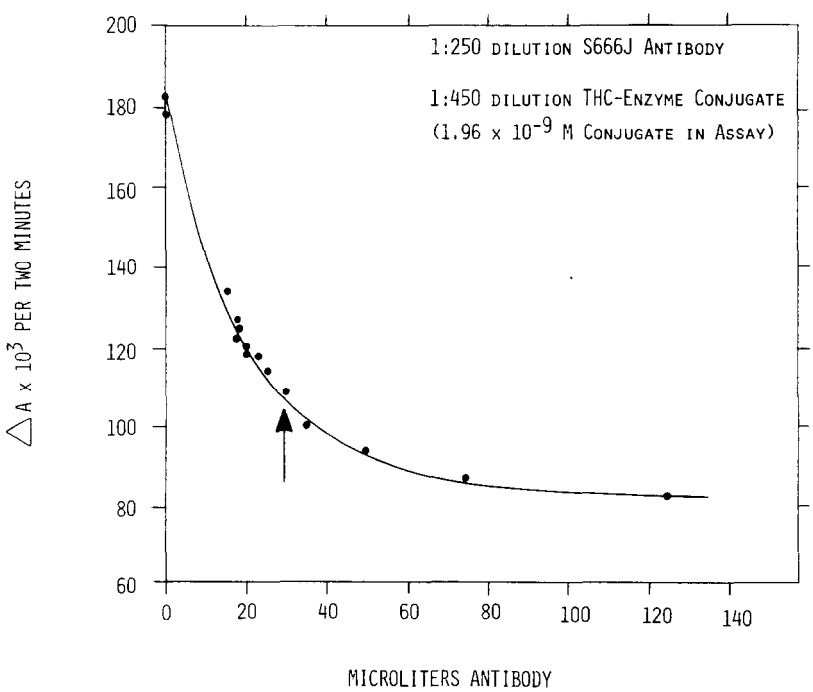
IMMUNOASSAY FOR THC AND 11-NOR-Δ^{9}-THC-9-CARBOXYLIC ACID

THC-MDH conjugate, which was maximally inhibited by THC antibodies (4.2 THC residues), was chosen for the immunoassay. Stability of the conjugate was found to be very sensitive to pH and ionic strength. The conditions selected to store working solutions of enzyme, 0.50 M potassium phosphate, pH 7.4, provide room temperature stability of greater than 34 days.

The conjugate readily adsorbed on glass and plastic measuring devices thus preventing quantitative and reproducible transfer of dilute solutions. Such adsorption is reminiscent of reported adsorption of THC itself to glass and plastics (Garrett and Hunt, 1974). Triton X-405 was found to prevent troublesome adsorption of THC in radioimmunoassays for THC (Teale, Forman, Ring, Piall, and Marks, 1975). We likewise found that adsorption of THC-MDH conjugate was prevented by incorporation of 0.10% Triton X-405 in dilute enzyme solutions.

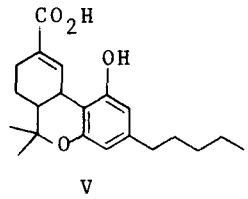
The effect of varying the ratio of antibody to enzyme is given in Figure 3. Upon titration of the conjugate

Figure 3



with antibody, the enzyme activity at first dropped off rapidly and assymptotically approached a limiting value. An antibody to enzyme ratio was chosen for a THC assay where the enzyme was inhibited 39% (arrow, Figure 3). A drug-response curve was obtained at that ratio by adding standard ethanolic THC solutions to the assay mixture (Figure 4). In the assay procedure 1 µl ethanolic THC standard solutions were combined with solutions containing antibody and substrates followed by addition of the THC-MDH conjugate. Enzyme rate measurements were made over 3 minute periods. The lowest detectable level of THC in the assay mixture was 0.5 ng/ml (1.6×10^{-9} M). The total useable range was 0.5 ng - 10 ng/ml.

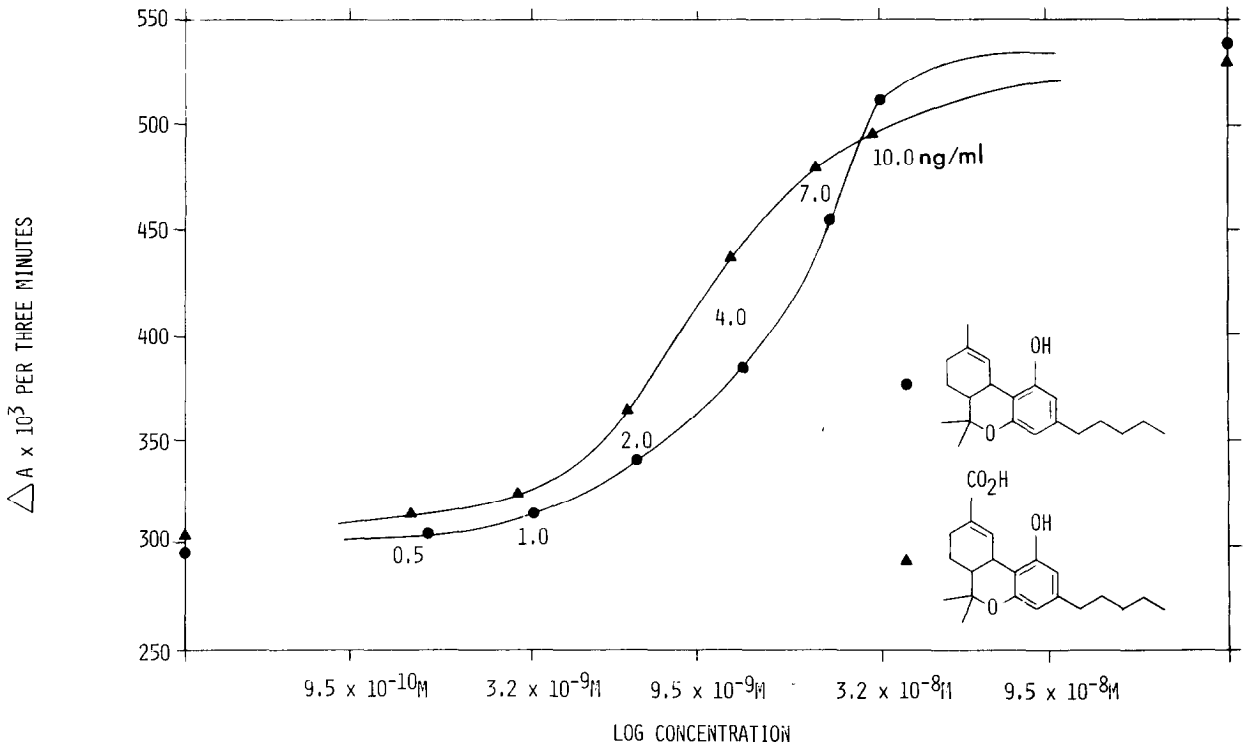
Likewise a drug-response curve for 11-nor-Δ^9-THC-9-carboxylic acid, V was obtained using standard ethanolic solutions in an identical assay with the same antibody (Figure 4).



V

The useable range of detection was the same as that for THC, 0.5 ng - 10 ng/ml. However, response to V in mid-range was about 1.5 times higher than response to THC. The cross-reactive behavior of our antibody both to THC and to V is different from that exhibited by antibodies raised by others against O-carboxymethyl-THC (Van Vunakis and Levine, 1974), 2- or 4- (p-carboxyphenylazo) THC, (Grant, Gross, Lomax, and Wong, 1972), and O-succinyl-THC (Teale, Forman, King, Piall and Marks, 1975) conjugates. All of these produced antibodies that reacted only poorly with V. Our antisera, therefore, appear suited for general screening

Figure 4



for THC and its metabolites, and could also be used to assay either THC or V if these compounds are first separated by an extraction procedure.

Enzyme immunoassays for THC and its metabolites in urine are presently being developed and a serum assay is under investigation.

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SEPARATION AND SENSITIVE ASSAY OF THC IN BIOLOGICAL FLUIDS BY HPLC AND GLC

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ABSTRACT

HPLC systems were developed to permit quantitative separation of Δ^{9}-tetrahydrocannabinol from many of the heptane extractable lipoidal and other endogenous substances in biological fluids. These substances interfered with the quantification by flame ionization GLC of unmodified compound and by electron capture GLC of pentafluorobenzoylated compound. Reverse phase HPLC elution, with 47% acetonitrile in water, and normal phase HPLC with 25% chloroform in heptane, separated tetrahydrocannabinol from 11-hydroxy-Δ^{9}-tetrahydrocannabinol and other monohydroxylated tetrahydrocannabinols. These systems also purified stock solutions of tetrahydrocannabinol from accompanying contaminants. The various monohydroxylated tetrahydrocannabinols were resolved from each other in normal phase, 80% chloroform in heptane. The Δ^{8} and Δ^{9}-tetrahydrocannabinols were separable in normal phase with 5% tetrahydrofuran in hexane. The GLC analysis of pentafluorobenzoylated tetrahydrocannabinol had a sensitivity of 1 ng/ml of plasma with an estimated 5% standard error of an assay with the extraction and GLC procedures given herein. Radiochemical analysis of the HPLC separated fraction had a sensitivity of 0.2 ng/ml of plasma with an estimated 2% standard error of an assay. There was no significant difference between the liquid scintillation and electron capture GLC assays of the HPLC separated Δ^{9}-tetrahydrocannabinol obtained from the plasma of dogs administered the drug. Radiolabelled compounds can be added to pasma samples as internal standards to determine the recovery efficiencies of the several procedures in the analysis of unlabelled tetrahydrocannabinol.

INTRODUCTION

A GLC analytical procedure for tetrahydrocannabinol in biological fluids was developed previously (Garrett and Hunt 1973) and used electron capture detection of the derived pentafluorobenzoylated tetrahydrocannabinol. It could readily detect 0.5 ng of tetrahydrocannabinol added to a 5.0 ml blood sample from a fasting dog. This sensitivity was only obtained when it was realized that tetrahydrocannabinol bound extensively (15%-40%) to glass (Garrett and Hunt 1973; Garrett and Hunt 1974) and rubber stoppers (Garrett and Hunt 1974) and that the time-dependent degree of adsorption could be minimized by prior treatment of all glassware with an organic solution of a silyl reagent. In the case of organic solutions, the tetra-

hydrocannabinol could be reincorporated from the glass into solution on vigorous shaking prior to any sampling.

The method's validity was demonstrated in the fasting dog with a low fat diet. Plasma levels down to 1 ng/ml of blood from 5 ml blood samples were monitored for 12 hours after the administration of 0.1 mg of pure tetrahydrocannabinol per kg (Garrett and Hunt 1973). However when the same method was applied to non-fasting animals a significant increase in GLC background from interfacing plasma constituents was observed, particularly within 4 hours of feeding a previously fasted animal. The resultant minimal detectable quantity unfortunately increased to 5 - 10 ng/ml when a 5 ml blood sample was taken.

Since phannacokinetic studies were contemplated in both dogs and humans over longer periods of time so that fasting would be impractical, such interferences were anticipated that would lower the analytical sensitivity. Thus it was necessary to devise suitable separation and clean-up procedures prior to analysis to improve the sensitivity.

High pressure liquid chromatography (HPLC) provided a powerful method of separation of drugs from their potential metabolites and from endogenous substances in biological fluids. While the classical on-line monitoring devices such as refractive index or UV spectrophotometry were too insensitive for direct assay at the plasma levels anticipated, the separated collected pertinent fractions were analyzed by analytical methods that provided the proper sensitivities. This paper presents HPLC techniques to separate tetrahydrocannabinol from various cannabinoids and endogenous materials of biological fluids with subsequent analysis by various appropriate methods.

RESULTS AND DISCUSSION

Purification and Reproducibility of Collection from Injected Ethanolic Solutions of Δ^9*-Tetrahydrocannabinol* on *HPLC*

The ^{14}C-Δ^9-tetrahydrocannabinol used as supplied by NIDA was reported low in radiolabelled contaminants by TLC. However, reverse phase HPLC (Fig. 1) revealed the presence of a labelled contaminant. The major peak, I, contained Δ^9-tetrahydrocannabinol (> 96%) and Δ^8-tetrahydrocannabino1 (≃ 3%), quantified by flame ionization

Figure 1

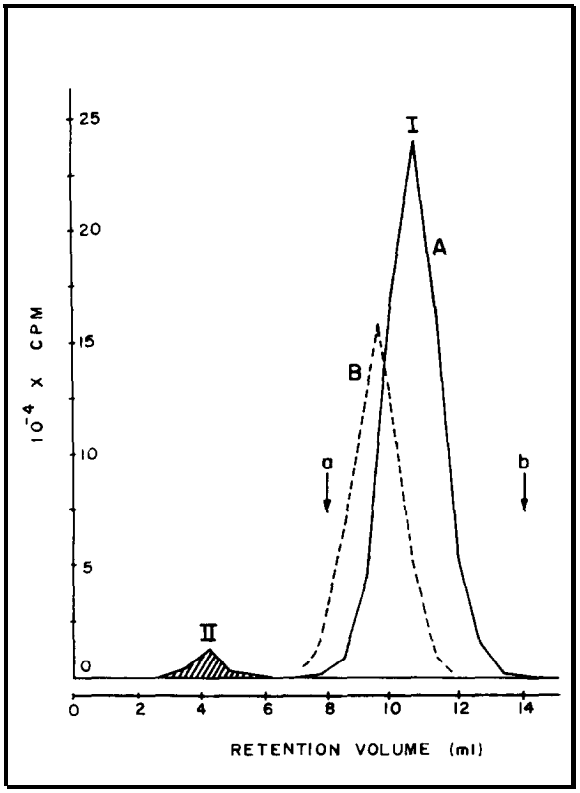

Figure 1. Reverse phase HPLC of stock ^{14}C-Δ^9*-tetrahydrocannabinol. The total radioactivity per eluate fraction is plotted vs retention volume (curve A). Peak I was* ^{14}C-Δ^9*-tetrahydrocannabinol and peak II was an unknown radioactive contaminent. Curve B is a plot of the mean UV detector response (arbitrary units) vs retention volume and is displaced relative to A by the solvent volume between the detector and the collection point. Collection between volumes a and b (peak retention volume ± 30%) for curve B recovered 92.5% of the radioactivity in this case. Column: Bondapack C18; eluent: 45% acetonitrile in water at 1.5 ml/min. Each fraction was corrected for background CPM.*

GLC (Garrett and Hunt 1974; Garrett and Tsau 1974). A labelled contaminant was also found (Fig. 2) with a normal phase column. The contaminant eluted prior to tetrahydrocannabinol in both cases. Since the more polar compounds elute first on reverse phase HPLC whereas the least polar compounds elute first on normal phase HPLC, it suggests that the contaminants observed in the two systems were not the same. The contaminants were not analyzed further.

Figure 2

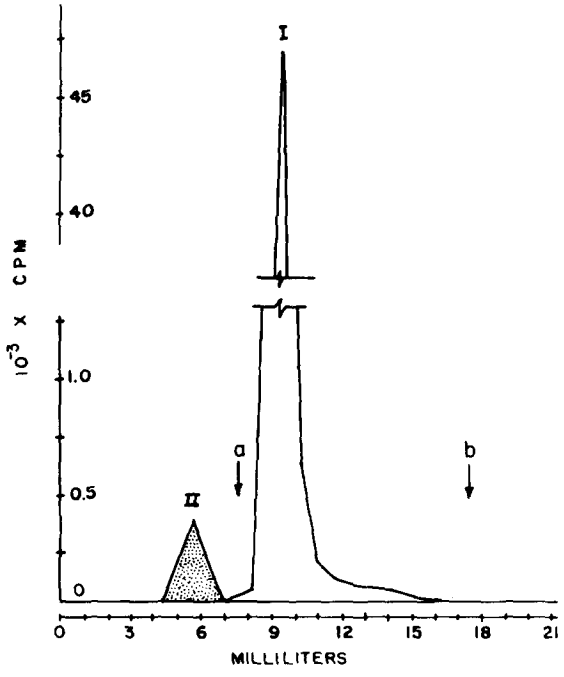

Figure 2. Normal phase HPLC of stock ^{14}C-Δ^9-tetrahydrocannabinol. The total activity per eluate fraction is plotted vs milliliters of eluate. Peak I was ^{14}C-Δ^9- tetrahydrocannabinol and peak II was an unknown radioactive contaminant. Collection between volumes a and b recovered 97.5% of the radioactivity in this case. Column: μ-Porsil; eluent: 20% chloroform in heptane at 2.5 ml/min. Each fraction was corrected for background CPM.

Figure 3

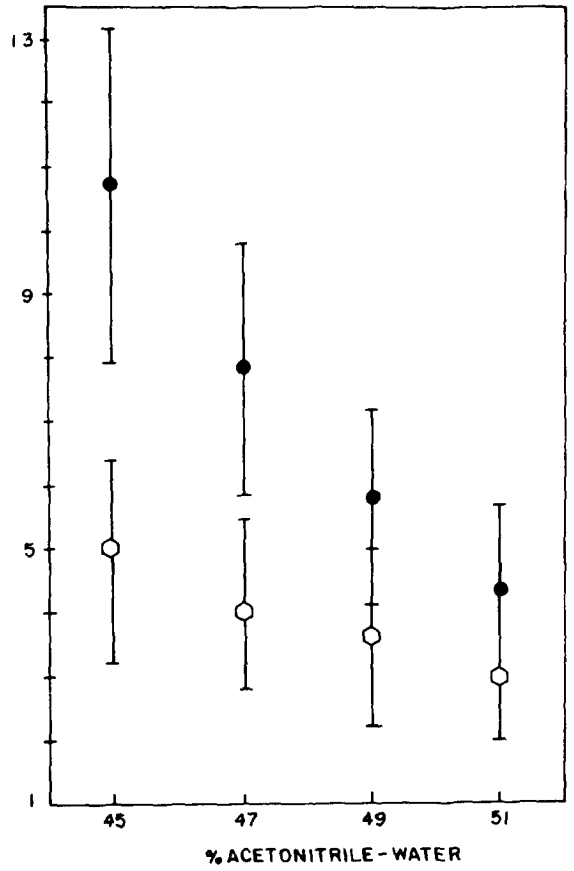

Figure 3. The retention volumes for the peak amounts of Δ^9-tetrahydrocannabinol (●) and 11-hydroxy-Δ^9-tetrahydrocannabinol (⬡) for reverse phase HPLC are plotted vs soluent composition. Each point represents the mean peak retention volume for two determinations. The vertical bars represent the ranges of retention volumes which contained approximately 98% of the area under the plot of recovered radioactivity vs retention volume.

When the tetrahydrocannabinol under peak I (Figs. 1 and 2) was collected, dried, reconstituted in ethanol and re-analyzed on the same HPLC system, only the single peak I was observed. All the stock Δ^9-tetrahydrocannabinol used herein underwent this purification procedure. The percent of the total injected radioactivity under peak I recovered was 97.6 ± 0.7 (standard error of mean) % (Table I) in the reverse phase system for the ranges of volumes collected (Figs. 1 and 3) and was 96.3 ± 1.0 (standard error of mean) % (Table II) for 1 to 1000 ng injected in the normal phase system at a retention volume of 9.7 ml within the collection range of 7.3 - 16.2 ml (Fig. 2).

The fact that the retention volume of peak I and the appropriate collection ranges for 98% recovery of injected labelled tetrahydrocannabinol were sensitive to solvent composition (Fig. 3) necessitated the prior establishment of an appropriate collection range for each newly prepared batch of solvent by radiochemical analysis of the collected HPLC fractions of previously purified ^{14}C-Δ^9-tetrabydrocannabinol. The retention volume and the appropriate collection range increased with decreasing solvent polarity (less % acetonitrile) due to peak spreading (Fig. 3).

Table 1

TABLE I - Recovery of Radioactivity of ^{14}C-Δ^9-Terahydrocannabinol[a] from Reverse Phase HPLC

Eluting Solvent (% acetonitrile in water)	n[b]	10^{-4} CPM Injected[c]	Average 10^{-4} CPM Collected[d]	Average Percent Recovered
51%	2	4.006	3.813	95.18
	2	8.012	7.688	95.95
	2	12.018	11.86	98.7
	2	16.024	15.824	98.75
49%	4	16.024	16.760	98.33
47%	4	16.024	15.804	98.63
45%	4	16.024	15.441	96.36
Overall Avg.	20	———	———	97.57 ± 0.69[e]

[a]The stock solutions of the material had been purified previously by HPLC. [b]Numbers of replicates. [c]Injections of 2, 4, 6 or 8 µl were taken from the same ethanol solution of ^{14}C-Δ^9-tetrahydrocannabinol: 4 x 10^3 CPM/µg and 2.003 x 10^4 CPM/µl. [d]For each solvent the collection range (Fig.3) was constant. All samples were corrected for background counts. [e]Standard error of the mean where 3.07 is the standard deviation.

Table 2

TABLE II - Recovery of Radioactivity of ^{14}C-Δ^9-Terahydrocannabinol[a] from Normal Phase HPLC[b]

n[c]	10^{-3} CPM Injected[c]	Average 10^{-3} CPM Collected[d]	Percent Recovered[e]	
5	0.1420	0.1344	94.7	(6.58)
3	0.7110	0.689	97.04	(3.0)
3	1.423	1.377	96.82	(4.9)
2	14.29	13.615	95.88	(4.13)
2	71.02	69.77	98.27	(1.13)
3	142.06	137.81	97.05	(1.65)
	Total (n = 18)		96.32 ± 1.023[f]	

[a]The stock solutions of the material had been purified previously by HPLC. [b]Eluting solvent: 25% chloroform in heptane. [c]15 µl of n replicates of each solution were injected. The specific activity of ^{14}C-Δ^9-tetrahydrocannabinol was 142 CPM/ng. [d]Each sample was corrected for background counts. [e]The parentheses contain standard deviations as percent of mean. [f]Standard error of the mean where 4.23 is the standard deviation.

The quality of the water used in the eluting solvent acetonitrile-water in reverse phase HPLC was an important factor in maintaining the reproducibility of the percent radioactivity recovered for a given collection range. The UV detector clearly indicated that adsorbed contaminants in impure water could be eluted from a previously used column by 100% acetonitrile, a less polar solvent than the used mixed eluent. Additional evidence of these contaminants was demonstrated when the GLC background significantly varied when distilled water from various sources was collected after reverse phase chromatography, extracted as if Δ^9-tetrahydrocannabinol were present, then treated by the derivatization procedure and analyzed by electron capture GLC.

HPLC Separation of Δ^9-Tetrahydrocannabinol from Selected Cannabinoids and Metabolites

Tetrahydrocannabinol, cannabinol and cannabidiol were resolved by the normal phase HPLC system (Fig. 4). If 98% of the tetrahydrocannabinol were to be collected after normal phase HPLC in this system, it is apparent that the chosen range (Fig. 2) would also collect cannabinol and cannabidiol. The retention volumes of cannabinol and cannabidiol relative to tetrahydrocannabinol increased as the percent of chloroform in heptane increased.

The monohydroxylated metabolites had large retention volumes (> 15 ml) on the normal phase column when 20 - 25% chloroform in heptane was the solvent (Fig. 2) and could be completely separated from tetrahydrocannabinol on this system. They were resolved from each other with a more polar solvent, 80% chloroform in heptane (Fig. 5).

Δ^9-Tetrahydrocannabinol and 11-hydroxy-Δ^9-tetrahydrocannabinol were quantitatively separable on the reverse phase HPLC system at 47% (or less) acetonitrile in water (Fig. 3). The collection efficiencies in the ranges given were 98% of the recoverable radioactivities of ^{3}H-11-hydroxy-Δ^9-tetrahydrocannabinol and ^{14}C-Δ^9-tetrahydrocannabinol.

Δ^8 and Δ^9 -Tetrahydrocannabinols were not readily resolvable in any of these systems. However an HPLC system that readily resolved and separated these two compounds was 5% THF in hexane on the normal phase column at 0.5 ml/min with retention volumes of 8.15 and 8.45 ml, respectively. The tetrahydrocannabinols collected under peak I (Figs. 1 and 2) could be further separated by this system.

Figure 4

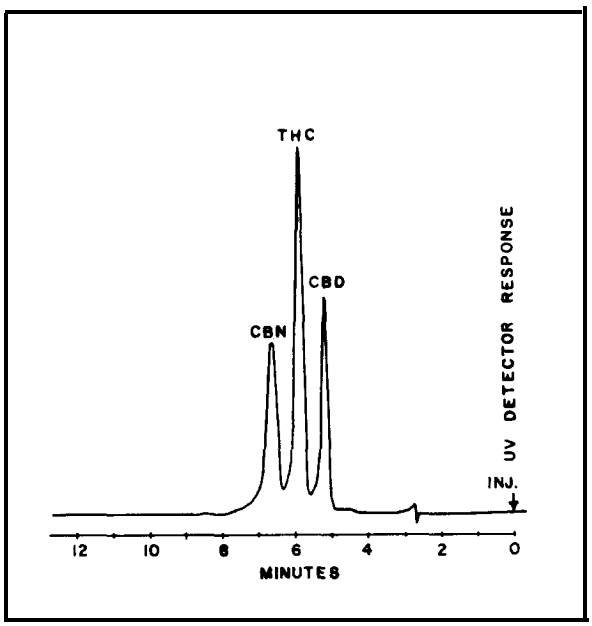

Figure 4. Normal phase HPLC separation of cannabinoids. The UV detector response (arbitrary units) is plotted vs retention time after injection (inj.) of a mixture of cannabidiol (CBD), Δ⁹-tetrahydrocannabinol (THC) and cannabinol (CBN). The amounts of Δ⁹-tetrahydrocannabinol and cannabidiol injected were approximately twice that of the cannabinol. Column: μ-Porsil; eluent: 20% chloroform in heptane at 1.5 ml/min.

Figure 5

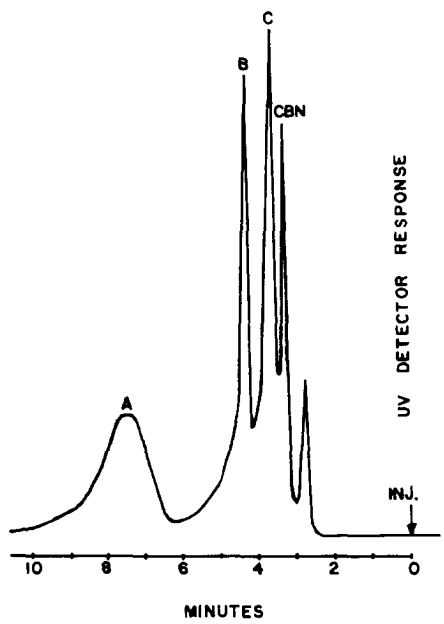

Figure 5. Normal phase HPLC separation of Δ⁹-tetrahydrocannabinol metabolites. The UV detector response (arbitrary units) is plotted vs retention time after injection (inj.) of a mixture of cannabinol (CBN), 11-hydroxy-Δ⁹-tetrahydrocannabinol (C), 8α- and 8β-hydroxy-Δ⁹-tetrahydrocannabinol (B and A, respectively). Column: μ-Porsil; eluent: 80% chloroform in heptane at 1.5 ml/min.

Table 3

TABLE III Percent Recoveries[a] of ^{14}C-Δ⁹-Tetrahydrocannabinol in the Heptane Extract of Plasma, in the Collection of the Proper Fraction of the Heptane Extract Separated by HPLC and the Overall Recovery after Both Extraction and HPLC Collection as Monitored by Both Electron Capture-GLC and Scintillation Analysis

^{14}C- Δ⁹-Tetrahydrocannabinol[b], ng/2 ml plasma	Extracted by Heptane, %	Extract HPLC Collected[c], %		Overall Recovery[d], %	
	Scintillation	Scintillation	GLC	Scintillation	GLC
2.25	90.2 ± 4.7	93	92	84 ± 8	83 ± 32
22.5	90.8 ± 2.6	88	97	80 ± 6	88 ± 12
225	90.9 ± 8.5	97	89	8.8 ± 4	81± 7
Overall Average ± std. error	90.6 ± 0.7	92	93	84 ± 2	84 ± 5

[a]Given as the mean from 4 separate plasma samples ± standard deviation. The scintillation analysis of recovered radioactivity was performed on a different set of four studies and on a different day than the electron capture GLC analysis of the derivatized HPLC collection. [b]142.47 CPM/ng. Two ml of plasma was extracted with 15 ml heptane and 14 ml was analyzed for total ^{14}C. [c]Quotient divided by the extraction efficiency (0.91). Additional studies were conducted for 26 ng ^{14}C-Δ⁹-tetrahydrocannabinol per 2 ml plasma on two other day for 4 samples each and the % recovered on HPLC from the extract were 94 ± 4 and 92 ± 3% (standard error) respectively. [d]Quotient of amount recovered corrected for volumes of extract used and amount added, which is the product of the extraction efficiency and the collection efficiency.

Effect of *HPLC Separation on GLC Analysis* of Δ^9 *Tetrahydrocannabinol in Plasma*

An equal amount of Δ^9 tetrahydrocannabinol and 11-hydroxy-Δ^9-tetrahydrocannabinol in 2 ml of dog plasma was extracted and separated from a majority of the extracted components by reverse phase HPLC. The reduction in potential contaminants from plasma observable on GLC was demonstrated by flame ionization GLC analysis (Garrett and Hunt 1974; Garrett and Tsau 1974) both before and after HPLC treatment (Fig. 6).

Figure 6

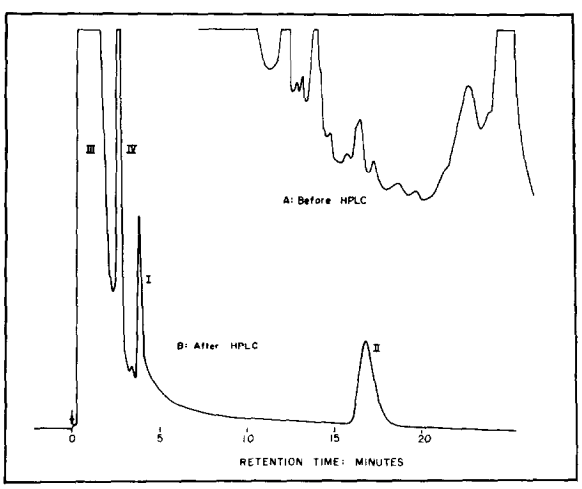

Figure 6. GLC chromatograms (flame ionization *detection) of an extract of 2 ml of plasma containing* Δ^9*-tetrahydrocannabinol, I (200* ng/ml), *before (A) and after (B) reverse phase HPLC separation of both cannabinoids over a range of predetermined collection volumes (Fig. 1). One* µl *of 18* µl *of extract was injected into the GLC prior to HPLC (A). Ten* µl *of 18* µl *of the extract was injected into the HPLC, the collected fraction was reconstituted in 10* µl *of chloroform and 1* µl *was injected into the GLC (B). Peak III is the solvent; peak IV is an unknown from plasma. HPLC: column, Corasil C18; eluent, 52% acetonitrile in water at 1.5 ml/min. GLC: column, 5' x 2 mm 1.9% OV-225 at 245° with a* N_2 *flow of 24 ml/min. and an attenuation of 8 x* 12^{-12} for *both chromatograms. The initial base lines and injection times for both chromatograms are superimposed for comparison.*

The normal phase HPLC with 20% chloroform in heptane could separate Δ^9-tetrahydrocannabinol from monohydroxylated metabolites and from 11-hydroxy-Δ^9-tetrahydrocannabinol. However, a minor overlap could be avoided by collecting the tetrahydrocannabinol in a slightly narrower volume range. The prior heptane extraction of alkalinized plasma had separated these non-polar constituents from any acidic metabolite. This separation of plasma extracts and normal phase HPLC collection of volumes in the appropriate range resulted in a substantial, reduction in GLC background from plasma components for derivatized tetrahydrocannabinol analyzed with electron capture detection as shown by the comparison of curves A and B or A and C in Fig. 7.

Figure 7

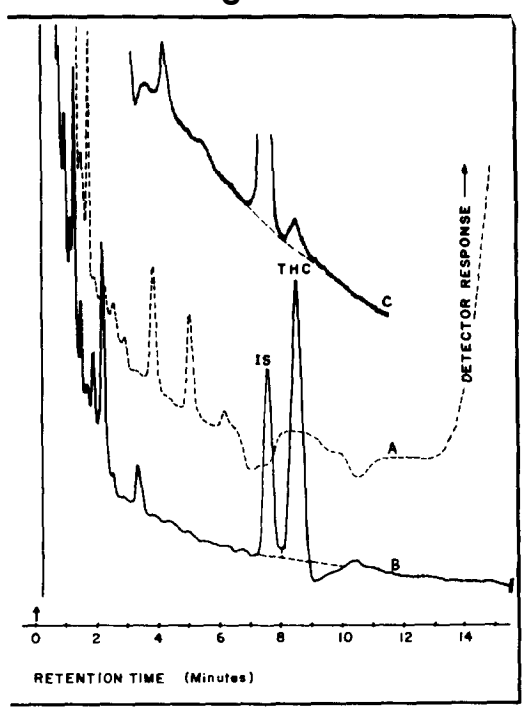

Figure 7. GLC (electron capture) analysis of derivatized samples. The chromatograms represent: A) the injection of 1 of 300 µl *from a derivatized extract of 2 ml of blank plasma without HPLC purification: B) the injection of 1 of 200* µl *of the final solution of derivatized compound which was 70% of the total amount from 2 ml of dog plasma containing 200 ng/ml* Δ^9 *tetrahydrocannibinol pentafluorobenzoate (THC) are appropriately labelled. Typical estimated base lines are shown. GLC conditions: 6' OV 17 column, 225°; detector, 280°; injector, 255°;* N_2 *flow, 45 ml/min; attenuations 4 x* 10^{-9} *(A and B) and 8 x* 10^{-10} *(C).*

Plasma samples obtained from dogs administered Δ^9-tetrahydrocannabinol solutions intravenously were analyzed by the electron capture GLC in accordance with the modified procedures described herein that included extraction, normal phase HPLC separation and derivatization except that no internal standard was added. These procedures would have included any cannabinol or cannabidiol in the HPLC collection volume range (Fig. 2 and 4) used. However, no peaks were seen at the retention times of cannabinol or cannabidiol pentafluorbenzoate and no significant amounts of cannabinol or cannabidiol could be detected as metabolites of Δ^9-tetrahydrocannabinol in the dog. Thus, either compound, when purified, should serve as an appropriate internal standard in pharmacokinetic studies. However, since cannabinol has been reported as a minor metabolite (McCallum 1973; McCallum et al. 1975; Widman et al. 1974), cannabidiol pentaflurobenzoate was chosen as the internal standard. It must be realized that cannabinol is known to be a contaminent of degraded Δ^9 tetrahydrocannabinol (Garrett and Tsau 1974).

The GLC methodology presented herein differed from the prior studies (Garrett and Hunt 1973) in that the short 30 cm column of 3% OV 225 was supplanted by a longer OV 17 packed column to be consistent with the data in the literature accumulated for the reso-Lution of the cannabinols (Fetter-man and Turner 1972; Turner and Hadley 1973; Turner et al. 1974).

Efficiency, Reproducibility and Sensitivity of the Steps in the Radiochemical and Electron Capture Assay of ^{14}C-Δ^9-Tetrahydrocannabinol in Dog Plasma

The heptane extraction efficiency from plasma was highly reproducible (Table III) over a wide range of plasma concentrations, 90.6 ± 0.7% standard error of mean.

The recovery of Δ^9-tetrahydrocannabinol from the heptane extract of dog plasma by the normal phase HPLC was reproducible (Table III) over the range of plasma concentrations studied. Equivalent overall recoveries (Table II) were obtained by both radiochemical analysis (83.7 ± 1.8% standard error) and by electron capture GLC analysis (84.0 ± 4.9% standard error) of the derivatized tetrahydrocannabinol (Fig. 7). Both methods permitted estimation of a 92.5% recovery of the amount in the heptane extract injected on the normal phase HPLC and collected in the chosen range. The normal HPLC collection range was chosen to be slightly smaller than in the studies on ethanolic solutions of tetrahydrocannabinol (Fig. 2, Table II) since the procedure had to be modified to separate tetrahydrocannabinol from possible monohydroxylated or 11-hydroxy-Δ^9-tetrahydrocannabino1 metabolites in plasma. A slight overlap of their HPLC peak areas with tetrahydrocannabinol would have occurred if the larger collection ranges had been used.

A similar study of the reproducibility of collection of ^{14}C-Δ^9-tetrahydrocannabinol in plasma assayed by liquid scintillation after extraction and reverse phase HPLC was also conducted. The amounts recovered were proportional to the amounts injected (Fig. 8) and the HPLC recovery efficiency of the drug in the heptane extract in this case was 95.7%.

Figure 8

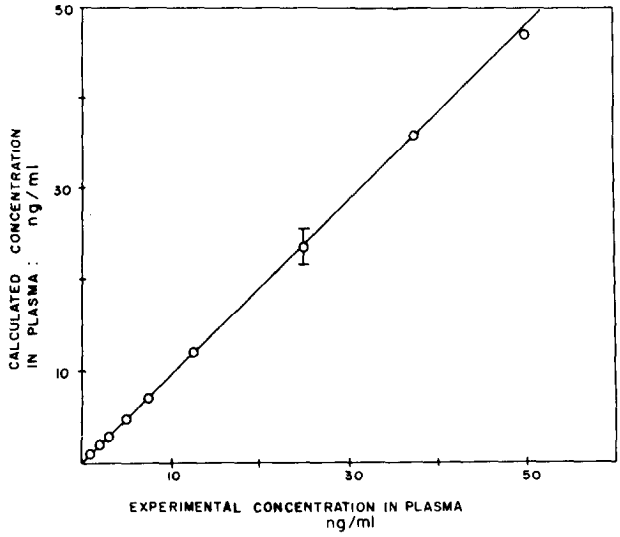

Figure 8. Recovery of ^{14}C-Δ^9-tetrahydrocannabinol calculated as concentration in plasma corrected for an extraction efficiency of 91% against the experimentally prepared concentrations. The drug extracted from plasma and collected over the proper volume range (Table I and Fig. 1) after reverse phase HPLC with a 45% acetonitrile in water eluent. The collection was analyzed by liquid scintillation. The slope, 0.957, is the HPLC collection efficiency for Δ^9-tetrahydrocannabinol and was typical of the values obtained. The vertical bar is the range for ± one standard deviation (n=4).

Equivalency of Radiochemical Analyses of ^{14}C-Δ^9 *-Tetrahydrocannabinol and GLC Electron Capture Detection of Derivatized Material after Normal Phase HPLC in Dog Plasma during Pharmacokinetic Studies*

The plasma of a do intravenously administered solutions of ^{14}C-Δ^9 - tetrahydrocannabinol was monitored with time after heptane extraction by both radiochemical analysis and electron capture GLC of the derivative of the appropriately collected eluate fraction from normal phase HPLC. Typical plots of the time course of the results from both methods are given in Figs. 9 and 10.

Figure 9

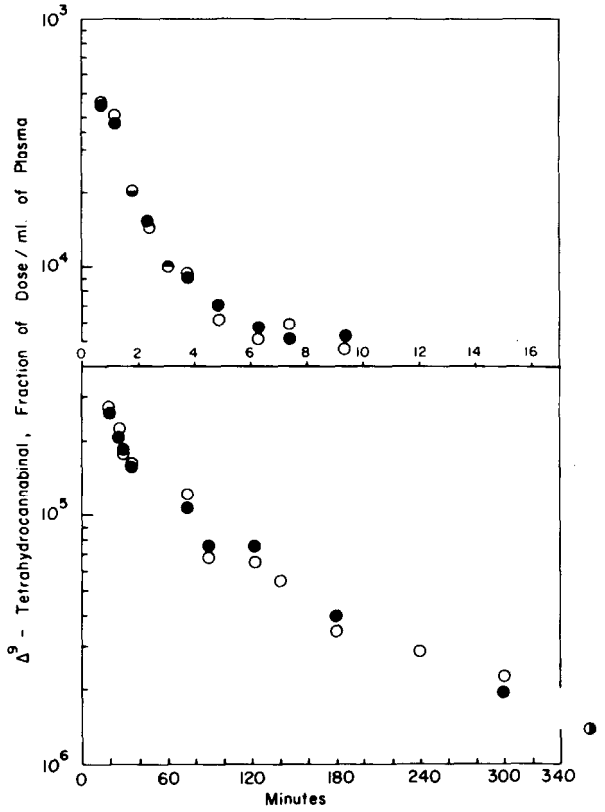

Figure 9. Semilogarithmic plots of fraction of the Δ^9 *-tetrahydrocannabinol 0.1 mg/kg dose per ml of plasma against time for dog A plotted from the liquid scintillation analysis* of *the total* ^{14}C *collected as* Δ^9 *-tetrahydrocannabinol on normal phase HPLC (O) and from the electron capture GLC of the derivatized HPLC collected fraction (●). The values were corrected for the fractions of extracts and total collection range used.*

Figure 10

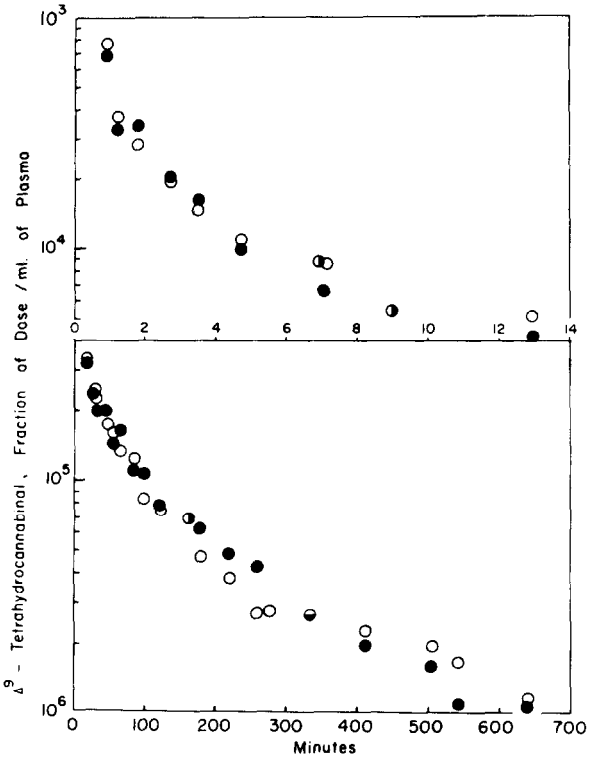

Figure 10. Semilogarithmic plots of fraction of the Δ^9 *-tetrahydrocannabinol 2.0 mg/kg dose per ml of plasma against time* for *dog A plotted from the liquid scintillation analysis* of *the total* ^{14}C *collected as* Δ^9 *-tetrahydrocannabinol on* normal *phase HPLC (O) and from the elctron capture GLC of the derivatized HPLC collected fraction (●).. The values were corrected for the fractions of extracts and total* collection *range used.*

The procedures for GLC analysis reported herein gave a lower limit for quantitative analysis of tetrahydrocannabinol in plasma of approximately 1 ng/ml from twice the standard deviation (0.32 ng) obtained for the amount of tetrahydrocannabinol recovered from 2.25 ng in 2 ml plasma (Table II). Similarly, the procedure for radiochemical analysis reported herein gave a lower limit of approximately 0.2 ng/ml from twice the standard deviation (0.084 ng). A statistical analysis of the apparent differences between the tetrahydrocannabinol assays at a given time from both analytical methods (Figs. 9 and 10) showed no significance.

This demonstrated that all of the recovered radioactivity from the HPLC separation procedure could be assigned to the Δ^9-tetrahydrocannabinol assayed specifically by electron capture GLC and thus no significant amounts of radiolabelled metabolites were in the collected HRLC fractions.

It can be concluded that Δ^9 -tetrahydrocannabinol can be extracted from plasma and other biological fluids, that it can be separated on HPLC from the simultaneously extracted biologically endogenous materials and metabolites that would interfere with a chosen highly sensitive analytical method such as GLC. It is not necessary to collect all of the material to be analyzed; assurance that a reproducible or known fraction of the total material injected on HPLC is recovered is all that is necessary since it is directly proportional to the total drug concentration. If unlabelled tetrahydrocannabinol in a solution of plasma were analyzed, the calculated recovery of known amounts of labelled tetrahydrocannabinol added either to plasma prior to extraction or to heptane extract subsequent to extraction would permit calculation of the extraction and/or HPLC collection efficiencies for that particular biological sample. These known efficiencies would permit the calculation of the original plasma concentrations. If a labelled ^{14}C-Δ^9 -tetrahydrocannabinol were used in pharmacokinetic studies, extracted and separated on the HPLC, a tritium labelled ^{3}H-tetrahydrocannabinol could be used as the appropriate internal standard to monitor the recovery efficiencies.

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DETERMINATION OF Δ⁹-TETRAHYDROCANNABINOL IN HUMAN BLOOD SERUM BY ELECTRON CAPTURE GAS CHROMATOGRAPHY

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The application of electron capture gas chromatography (ECGC) to the determination of Δ^9-tetrahydrocannabinol in blood samples has been reported by Garrett and Hunt (1973) and Fenimore, Freeman, and Loy (1973). In these studies laboratory animals were used as experimental subjects which, as later experience demonstrated to the present authors, was a fortunate choice. When attempts were made to extend the technique to samples of blood from human subjects with their more varied diet and environment, the presence of interfering components proved to be a serious problem. Had this level of difficulty occurred during the initial development of the methodology, other less sensitive but more tractable alternatives would surely have been pursued. The initial success with animal subjects, however, encouraged modifications of the method which now permit quantitation of Δ^9-THC in human blood at concentrations approaching that attainable with the animal subjects.

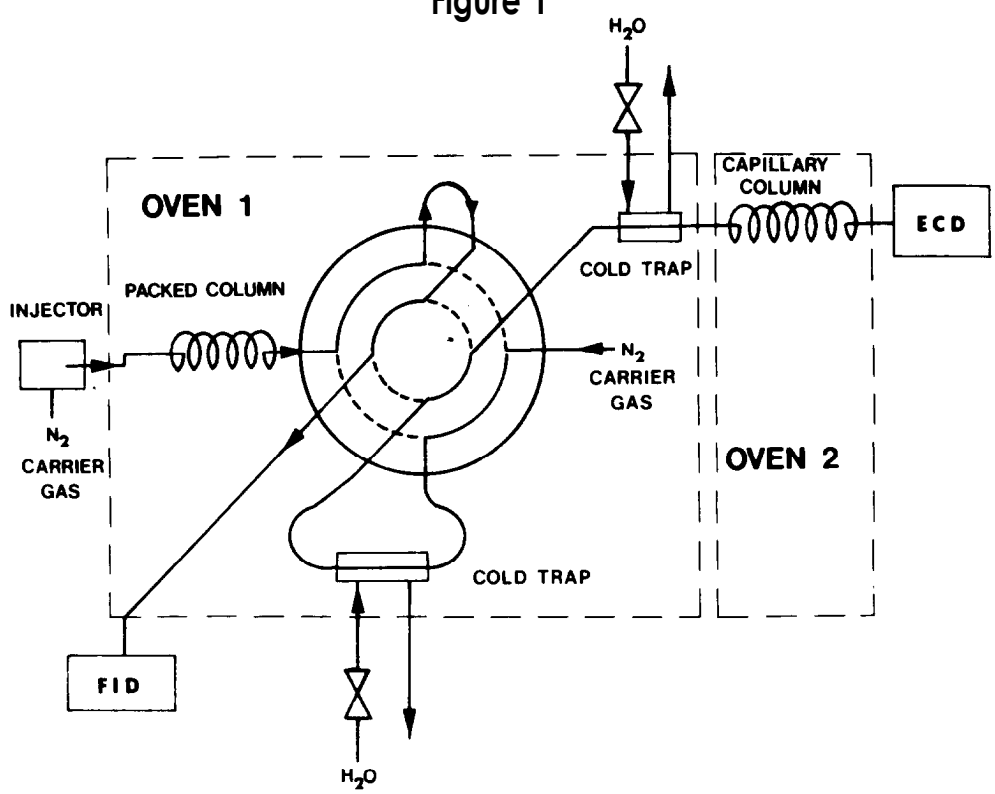
Although the cannabinoids do not possess high affinities for thermal electrons, it is a relatively simple matter to form polyhalogenated derivatives of these compounds in quantitative yield to which the electron capture detector responds with excellent sensitivity. The limit of detection for the heptafluorobutyrate or pentafluoropropionate esters of Δ^9-THC is approximately one picogram (Fenimore, 1973). Because the levels of Δ^9-THC in the blood are about 1000 times this amount per milliliter for a considerable period after a nominally active dose of cannabis preparation (Lemberger 1971, Agurell 1973, Garrett 1973), it is obvious that sensitivity of detection is not the limiting factor. The problems arise from the fact that the derivatizing reactions are relatively non-specific. Not only are there a large number of components present in biological samples at parts per billion concentrations capable of forming derivatives of this type, but also many similarly reactive species may be carried through the initial sample preparation in amounts orders of magnitude greater than the compound or compounds of interest. It is apparent, then, that selectivity becomes all important if the sensitivity of the determination is to be maintained, and if this selectivity cannot be achieved at the detector, it must be restored through the chromatographic process, the sample preparation, or both.

One approach toward eliminating the interfering components is to achieve a relatively pure isolate prior to gas chromatographic analysis through sample preparation involving other chromatographic separations or through extensive partitioning processes. Experience has shown, however, that sample recovery is not favored by manipulations of this kind. Consequently we have relied primarily on improved gas chromatographic technique to alleviate the problem of interference. A dual column-dual oven instrument described previously (Fenimore, 1973) and discussed in some detail in the following section has provided the necessary resolution to enable the determinations of Δ^9-THC in human blood serum.

DESCRIPTION OF THE GAS CHROMATOGRAPHIC APPARATUS

A schematic diagram of the dual column-dual oven gas chronatograph is shown in Figure 1. Oven N° 1, containing a conventional packed column, is temperature programmed while oven N° 2, equipped with a capillary column, remains isothermal. A switching valve and cold traps are arranged to permit transfer of only that volume of effluent containing the compound or compounds being measured from the packed column to the capillary column. The exact retention volume necessary for accurate transfer is determined previously by detection of relatively large amounts of compound (approx.

Figure 1



1 μg) by flame ionization. The transferred effluent is further separated by the capillary column and detected by a microvolume electron capture detector (Fenimore, 1971).

This system provides the means to eliminate three major problems encountered in high resolution ECGC. First, temperature programming, which is usually not feasible with EC procedures because of exaggerated baseline drift, is accomplished by raising the temperature of the first column while holding the second column at a constant temperature. Second, the packed column tolerates large injection volumes which would rapidly degrade a small bore capillary column. Third, the two columns may contain stationary phases of widely different polarity which increases the probability of separation of compounds having similar partition coefficients on any single chromatographic column.

The problem of transferring the solute from the high volume mobile phase eluate of the packed column to the much smaller volume of the capillary column is solved by interposing two water-cooled traps between the columns. The first trap consists of 1/8" ID nickel tubing containing a 3 cm plug of material identical to the packing of the fore-column, and the second is short length of 1/16" ID nickel capillary coated with the stationary phase used in the second column. Water is allowed to flow through an annular space surrounding the traps immediately before and during the transfer interval. When the temperature of the first trap is restored to oven temperature by interrupting the flow of coolant, the solute is released at the reduced carrier gas flow rate of the capillary column. The band broadening introduced by the volume of the first trap is reduced by re-trapping the solute in the capillary tubing and then releasing it to the capillary column in the second oven. An eight-port switching valve (Valco Instruments, Houston, Texas) is used to transfer the carrier gas flow between columns and traps. All lines connecting the various elements of the system are nickel capillary tubing.

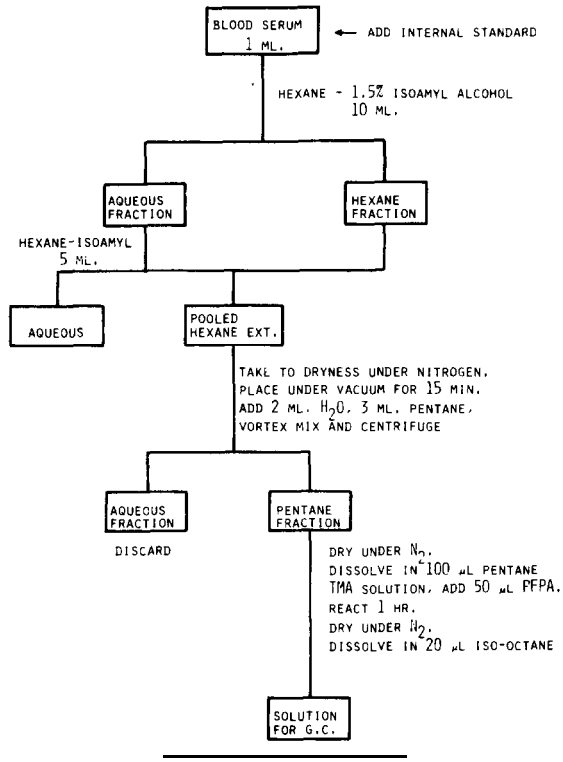
The columns and column conditions used in the present study are as follows:
Packed column: 6 ft x 3 mm ID coiled glass containing 10% OV-101 on 100 to 120 mesh Gas Chrom Q (Applied Science Lab, State College, Pennsylvania). Nitrogen carrier gas flow rate is 40 cc per minute. Column is temperature programmed from 180°C to 240°C at 4°C/min.
Capillary column: 100 ft x 0.02" ID nickel-200 tubing (Handy and Harmon Tube Co., Norristown, Pennsylvania) coated with Poly I-110 (Applied Science Lab, State College, Pennsylvania). Nitrogen carrier gas flow rate is 4 cc/min. Column temperature is 190°C. Additional gas flow is added prior to the electron capture detector to provide a total flow of 10 cc/min.

The electron capture detector contains a 1 Curie scandium tritide foil (U.S. Radium Corp. , Panorama City, California) and is operated at a 1000 μsec. collection pulse. The signal from the detector is converted by an analog linearizer (Antek Instruments, Houston, Texas) prior to the recorder.

SAMPLE PREPARATION

A flow diagram of the blood serum extraction and derivatization procedure is shown in Figure 2. All solvents are

Figure 2



reagent quality or better and are redistilled and stored in glass before use. All glassware is silylated using vapor phase silylation. Derivatization is a standard esterification of the phenolic hydroxyl group of Δ^9-THC using pentafluoropropionic anhydride (PFPA) in the presence of trimethylamine. The final volume of sample prior to gas chromatographic analysis is 20 μl of which 5 μl is taken for each sample injection.

The internal standard is hexahydrocannabinol (HHC) prepared by hydrogenation of Δ^8-THC and purified by preparative scale thin layer chromatography. For most determinations 4 ng of the HHC are added in solution to the blood serum sample before proceeding with the extraction procedure.

RESULTS AND DISCUSSION

As stated previously the amounts of Δ^9-THC which would normally be determined in a convenient volume of blood serum are some three orders of magnitude greater than the limit of detectability attained by electron capture of the pure, derivatized compound. The primary factor which prevents the realization of this detection limit is the presence of inter-

fering substances originating in the blood serum or introduced to the sample during preparation. This latter source of contamination can, at least, be controlled by exercising the usual precautions generally recommended for electron capture determinations, i.e. use of glass-distilled solvents, use of small total solvent volume where evaporative concentration is required, re-distillation of derivatizing reagents, scrupulous cleanliness of glassware, and avoidance of all plastic materials with the exception of polytetrafluorethylene type polymers.

Interference from endogenous compounds in the sample can only be reduced to a practical minimum through selective solvent extraction of the cannabinoid and utilization of appropriate chromatographic separation. The limit to which the extractive isolation of the compound can be pursued is very much dependent on how much loss of that compound can be tolerated. The multiplicative nature of sample loss thus dictates against all but the very minimal number of partitioning manipulations prior to gas chromatographic separation. On the other hand, loss of sample during gas chromatography can be all but be eliminated by careful choice and preparation of the various components of the instrument. In the present study the individual elements of the dual oven chromatograph were examined for effect on peak area of the detected Δ^9-THC-PFP and were found to have no significant contribution to solute loss even at the limit of detectability. It is for these reasons that reliance was placed on gas chromatographic separation rather than extractive techniques for reduction of interference.

In even the most meticulous extraction procedures involving recovery of compounds at part per billion concentration, some loss must be anticipated. Inclusion of an internal standard prior to the extraction and derivatization procedures is therefore necessary to monitor compound recovery, and the chemical and physical properties of the standard should approximate those of the compounds in question as closely as possible. The deuterated Δ^9-THC employed by Agurell in mass fragmentographic assays (Agurell, 1973) certainly approaches the ideal, but, of course. cannot be differentiated from unlabeled Δ^9-THC by electron capture procedures. Hexahydrocannabinol was therefore selected as having similar properties to Δ^9-THC while retaining good chromatographic separation.

There are obstacles to relying almost entirely on gas chromatography to isolate a component of a mixture for measurement, because complete separation may require excessive analysis time, even with highly efficient columns. Thus the columns used and the operating conditions utilized are usually a compromise between resolution and speed of analysis. The variability of blood constituents among human subjects complicates the selection of operating conditions. Therefore in this study numerous blood samples from individuals believed to be abstinant from cannabis use were examined for the purpose of varying the chromatographic conditions until extraneous peaks were absent in the region of the chromatogram where the Δ^9-THC and HHC internal standard were known to appear at the appropriate level of sensitivity. Once the conditions had been achieved which produced a reliable baseline, known amounts of Δ^9-THC were added to random sera samples, and the ratio of detected peak heights. of Δ^9-lHC to the internal standard were computed. The regression was linear from 30 ng per ml to a limit of sensitivity of 500 pg per ml without dilution of the more concentrated trial samples.

Figures 3 and 4 show electron capture chromatograms of human blood serum extracts obtained using this procedure. The blood samples were taken before and one hour after a volunteer had smoked a single marihuana cigarette estimated to have contained 6 mg of Δ^9-THC. Blood serum levels determined over a four hour period are shown in Figure 5, and are in close agreement with those reported by other investigators (Agurell, 1973).

The major disadvantage in applying ECGC to determinations of this type is the amount of instrument time involved in performing a single analysis. For the chromatograms shown in the figures the total time from injection to the point at which the capillary column is clear for a subsequent determination is approximately forty-five minutes, and this time period would undoubtedly be excessive in studies where large numbers of samples were processed daily. On the other hand, this reported time is by no means an irreducible minimum, and improvements in column technology, operating parameters, and sample preparation could well lower this figure substantially. In addition, gas chromatography lends itself readily to instrument modification. A multiple column system utilizing the principles demonstrated successfully in the operation of the dual-oven instrument could be assembled capable of performing analyses

Figure 3

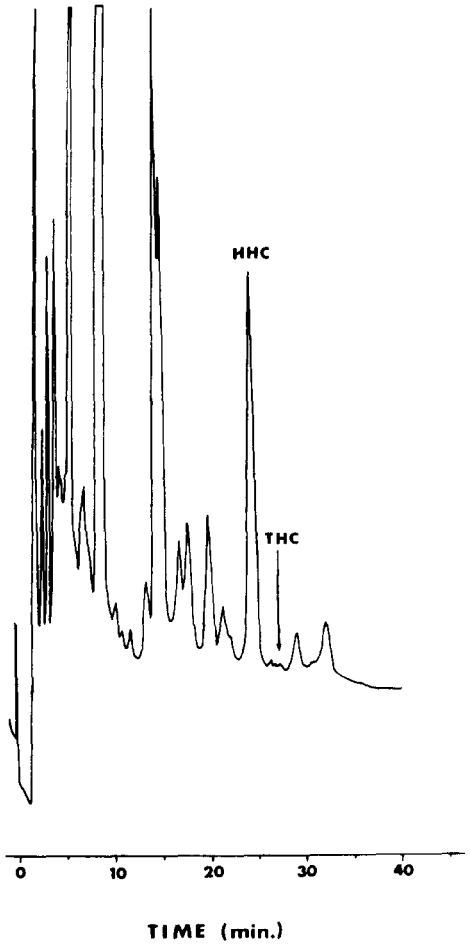


Figure 4

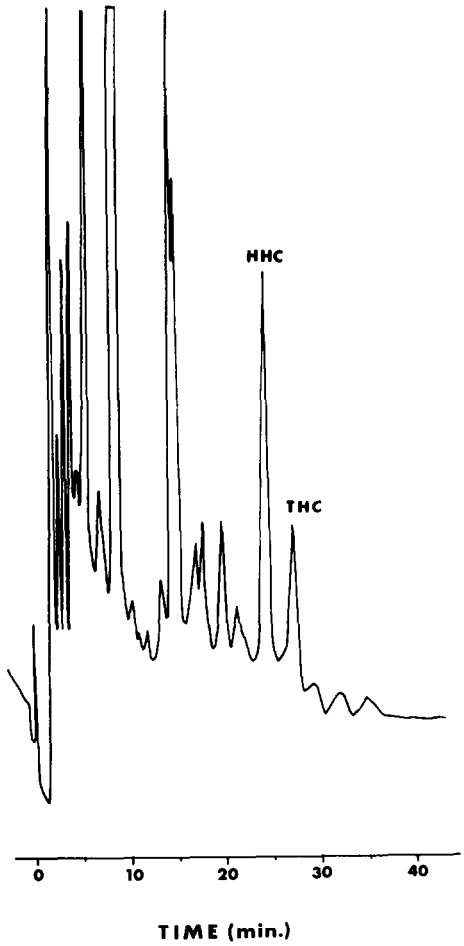
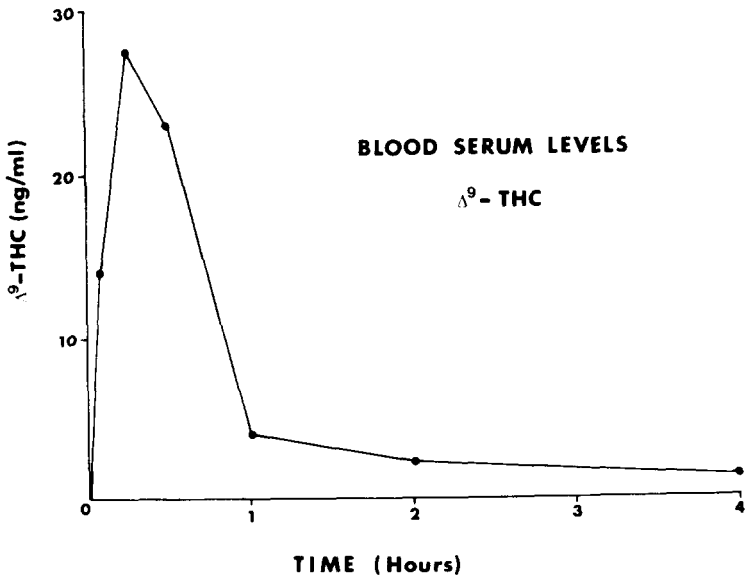


Figure 5



limited only by the rapidity with which the primary temperature-programmed column can be serially operated. With automatic sampling and computer control such an instrument could run continuously with very little technician supervision.

Although this report concerns the determination of Δ^9-THC onlv. the techniques and instrumentation'should be applicable with minor modification to quantitation of other cannabinoids and metabolites of these compounds in biological samples.

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DETECTION AND QUANTIFICATION OF TETRAHYDROCANNABINOL IN BLOOD PLASMA

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INTRODUCTION

During the last few years much effort has been spent on developing methods for the identification of Cannabis users by analysing different body fluids. Also, techniques for the quantitative determination of Δ^1-tetrahydrocannabinol (Δ^1-THC) and related compounds have long been desired. Recent developments in this area have been summarized in a review by Grlič (1974) including some eighty references. This field of detection of cannabinoids in biological fluids is in a state of rapid expansion and for the positive identification and quantification of cannabinoids in body fluids several highly sensitive methods are available.

It must be emphasized that the definition of what a "satisfactory method" is will vary with the goal of the users. For epidemiological studies of Cannabis use, simple screening procedures - where a high reliability may be traded for rapidity and expense - will probably be satisfactory. In forensic toxicology, the requirement for unequivocal identification hardly allows the use of procedures yielding ambigious results. Finally, in pharmacokinetic and pharmacological studies it may be necessary to have a specific as well as accurate quantitative procedure. These considerations would suggest that eventually different

procedures will be used for the determination of cannabinoids in body fluids (blood, urine) depending upon the aim of the investigator.

What a "complex and sophisticated" technique is will also depend upon the trends in analytical chemistry in a specific country. In Sweden, where over seventy gas chromatography - mass spectrometry (GC-MS) instruments are available in a limited scientific community, a mass spectrometric method is obviously less "sophisticated" than in a country where such instruments are scarce.

Radioimmuno assay procedures for cannabinoids in blood and urine have been extensively investigated *(cf.* Gross *et al.,* 1974; Teale *et al.,* 1975). Both authors utilized goat antiserum obtained after immunization with conjugates of Δ^1-THC. After reduction of non-specific binding a sensitivity of ca. 7.5 ng/ml THC in plasma or 1 ng/ml in urine could be achieved The antibodies were specific for the three ringed cannabinoid nucleus in THC and cross-reacted with *e.g.* 7-hydroxy-Δ^1-THC and cannabinol (CBN) but not with cannabidiol (CBD). As pointed out by Teale *et al.* (1975) this cross-reactivity has disadvantages but also advantages in e.g. epidemiological studies. Another immunological approach - free radical immunoassay - has been tested by Cais *et al.* (1975).

Gas chromatography in combination with sensitive detection systems has also been widely investigated. In general, the problems have been to remove lipid materials interfering with the gas chromatographic separation of *e.g.* THC and increase the sensitivity in the detection step. Fenimore *et al.* (1973) used a dual column separation, utilizing a capillary column for the final resolution, and the electron capture (EC) sensitive heptafluorobutyrate derivative. McCallum (1973) utilized a flame photometric detection of phosphate esters of Δ^1-THC and related compounds.

Garrett and Hunt (1973) described another EC-based procedure using the pentafluorobenzoate ester. Later, the same authors (Garrett and Hunt, 1976 a) have discussed the use of high pressure liquid (HPLC) and gas chromatography (GLC) for separation and analysis of THC in biological fluids.

We have published methods for the quantitative determinations of Δ^1-THC and Δ^6-THC in the blood of Cannabis smokers (Agurell *et al.,* 1973, 1974). These methods are based upon the purification of THC extracted from blood plasma by liquid chromatography on Sephadex LH-20 followed by mass fragmentographic assay using the deuterated THC-analogue as internal standard. Rosenfeld and co-workers (1974) also used mass fragmentography for the detection of Δ^1-THC in humans after smoking. The latter authors used trimethylaniliniur hydroxide for on column methylation of the phenolic group and relied on the trideuteromethyl ether as internal standard in the last step.

Many other techniques have been tried as reviewed by Grlic (1974) but a later result utilizing HPLC of dansyl derivatives should be mentioned (Loeffler *et al.,* 1975). Wall and co-workers in this volume present in detail a mass spectrometric procedure for the determination of THC in humans after Cannabis smoking.

Of course, the crucial test for any method is its performance (sensitivity, specificity, capability) when applied to the actual problem in man - this is unfortunately often lacking in the papers published.

The purpose of the present paper is to summarize previously published results and to present additional information on the mass fragmentographic procedures for THC and other cannabinoids developed in our laboratory.

METHODS

Cannabinoids

Synthesis of olivetol-d_7. The synthetic procedure of Pitt *et al.* (1975) was used with the following modifications in order to increase the amount of deuterium incorporated into the molecule (Fig. 1). The ylide was prepared from methyl 4-bromocro-. tonoate and triphenylphosphine, was reacted with 3,5-dimethoxybenzaldehyde to yield methyl 5-(3,5-dimethoxyphenyl)penta-2,4-dienoate (1) (Buchta & Andree, 1959, 1960). 1 was then reduced with LiAld_4 in ether at room temp. for 2 h. The complex was degraded with $d_2$0 to insert one more d in the side chain. The isolated compound, with the hydrogen atom of the alcohol group exchanged for deuterium, and the catalyst tris(triphenylphosphine)rhodium chloride was stirred in benzene under d_2 atmosphere for 16 h at room temp. The 5-(3,5-dimethoxyphenyl)pentan-1,1,2,3,4,5- d_6-l-ol was then reacted with PBr_3, LiAl d_4 and BBr_3 according to Pitt et al. (1975) to yield olivetol-d_7 (over-all yield from 3,5-dimethoxybenzaldehyde 24%). Deuterium content according to mass spectrometry, m/e (180-187): d_0 2%, d_1 2%, d_2 2%, d_3 3%, d_4 3%, d_5 9%, d_6 30%, d_7 100%.

CBD-d_7. Olivetol-d_7 (160 mg) was condensed with (+)-*trans*-*p*-mentha-2,8-dien-l-ol in benzene using traces of oxalic acid as described by Petrzilka *et al.* (1969). After final purification by TLC (Agurell *et al.*, 1974) on pre-washed silica gel G plates with methylene chloride-methanol (95:5) as solvent, 33 mg of 97% pure (GLC) CBD-d_7 was obtained. Deuterium content according to mass spectrometry (m/e 314-321): d_0 2%, d_1 2%, d_2 2%, d_3 3%, d_4 3%, d_5 9%, d_6 30%, d_7 100%.

Δ^{11}-THC-d_7. *Olivetol-d_7* was condensed with (+)-*trans*-p-mentha-2,8-dien-l-ol using *p*-toluensulfonic acid (Petrzilka *et al,* 1969), The preparative purification was carried out on silica gel G plates eluted three times with 5% ether in light petroleum to separate the Δ^6 and Δ^1-isomers. The purified (94% by GLC, 37 mg) Δ^{11}-THC-d_7 showed the following deuterium content (m/e 314-321): d_0 2%, d_1 2%, d_2 2%, d_3 3%, d_4 3%, d_5 9%, d_6 30%, d_7 100%.

CBN-d_7. Δ^{6}-THC-d_7 was treated with two equivalents of chloranil and traces of *p*-toluenesulfonic acid in refluxing dry toluene for 5 h (Mechoulam *et al.,* 1968). The solution was chromatographed on a silica gel column with toluene eluent and further purification by TLC yielded 96% pure CBN-d_7. The isotope distribution was the same as in Δ^1-THC-d_7; this in contrast to dehydrogenation methods using sulphur which causes extensive deuterium scrambling.

Figure 1

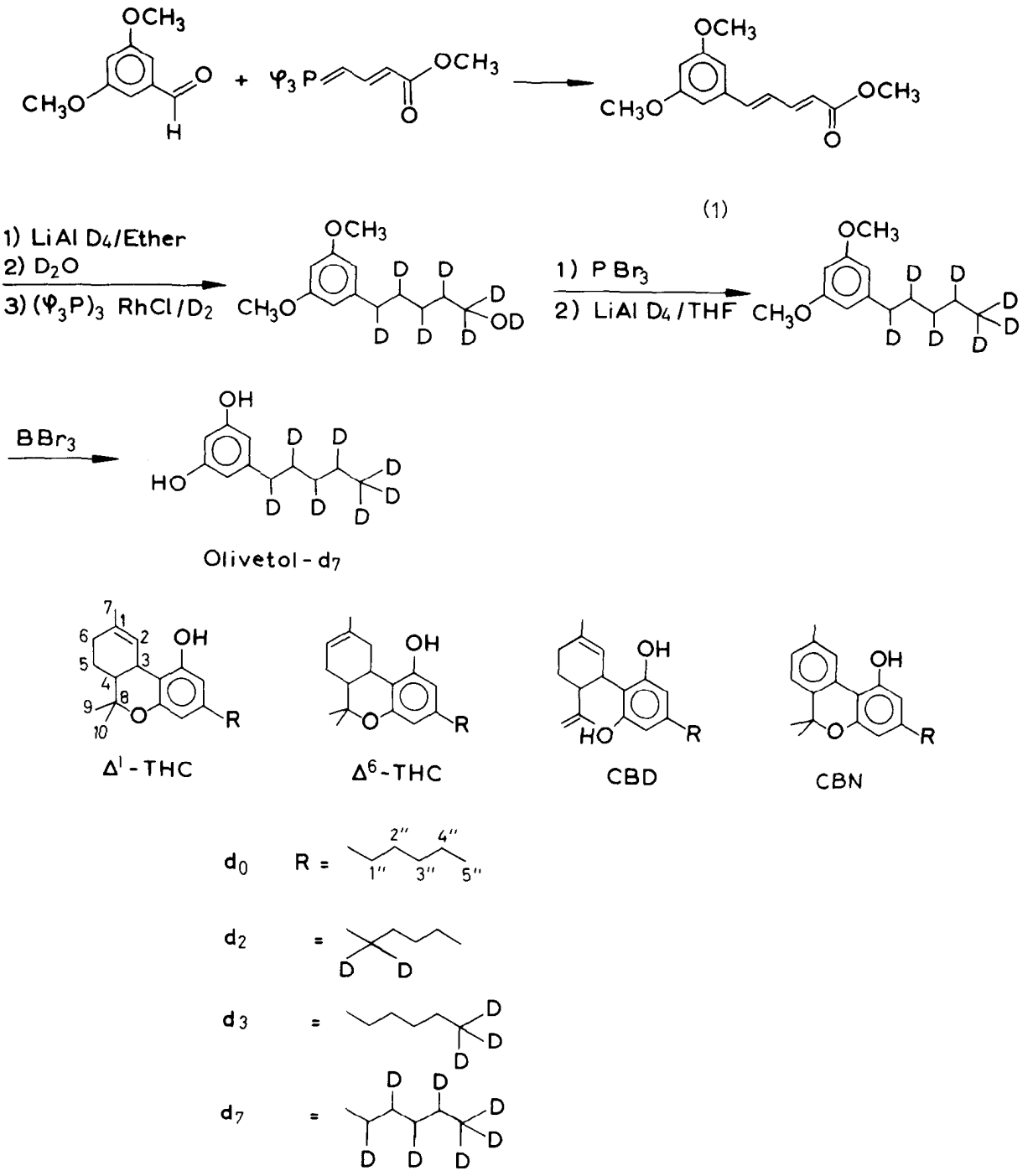

Fig. 1. Scheme for *synthesis* of *olivetol-d_7. Formulas of cannahinoids and deuterium containing internal standards.*

Olivetol-d_3 was prepared as described by Pitt *et al.* (1975). Compound 1 (see olivetol-d_7) was hydrogenated in the presence of Pd and the product reduced with $LiAlD_4$. Subsequent reactions with PBr_3, $LiAlD_4$ and BBr_3 giving olivetol-d_3 were as described for olivetol-d_7.

Δ^1-THC-d_3 and CBN-d_3 were synthesized as described for the d_7-analogues. Since larger amounts were prepared Δ^1-THC-d_3 was separated from Δ^6-isomer on a column of Florisil using 2% ether in light petroleum as solvent. Δ^1-THC-d_3 was 95% pure according to GLC and the deuterium content (m/e *314-317:* d_0 2%, d_1 6%, d_2 12%, d_3 *100%.*

CBN-d_3 eluted with 2-10% ether in light petroleum from the Florisil column was 95% pure according to GLC with a deucerium content (m/e 310-313): d_0 2%, d_1 5%, d_2 15%, d_3 100%.

Olivetol-d_2. The methyl ester of 3,5-dimethoxybenzoic acid was reduced with $LiAlD_4$ in tetrahydrofuran and the resulting alcohol was reacted with PBr_3. (See olivetol-d_7). The bromide crystallized from ether-light petroleum was mixed with Cu(I)I in ether and butyl lithium was added drop-wise to the mixture (Petrzilka *et al.,* 1969). The obtained 0,0-dimethylolivetol-1',1'-d_2 was distilled at 110° C, 0.15 mm Hg and showed the following deuterium content (m/e 180-182): d_0 0.3%, d_1 4%, d_2 100%. After demethylation with hydriodic acid (cf. Agurell *et al., 1973)* olivetol-d_2 was purified on a silica column with methanol-chloroform of increasing polarity as eluent (from 3% methanol).

CBD-d_2, Δ^1-THC-d_2 CBN-d_2. CBD-d_2 was synthesized as described for the d_7-analogue and crystallized from hexane. The CBD-d_2 was 97% pure by GLC and showed a deuterium content (m/e 314-316): d_0 8%, d_1 12%, d_2 100%.

Δ^1-THC-d_2 was prepared as Δ^1-THC-d_3 and more than 95% pure by GLC: Deuterium content (m/e 314-316): d_o 10%, d_1 20%, d_2 100%. CBN-d_2 was prepared from Δ^6-THC-d_2 by dehydrogenation with sulphur at 260°C (Petrzilka *et al.,* 1969). The deuterium content (m/e 310-312): d_0 12%, d_1 46%, d_2 *100%.*

Δ^6-THC-d_4. The synthesis of this compound (98.5% pure) was described previously (Agurell *et al.,* 1974); deuterium content (m/e 314, 318): d_0 1.5%, d_4 100%.

Non-labelled cannabinoids. (Δ^1-THC, Δ^6-THC, CBN, CBD) were synthesized according to standard procedures and carefully purified and dried before use. Stock solutions were maintained in ethanol (1-5 mg/ml, 4° C, stored in the dark).

GLC was carried out on a Varian 2100 FID gas chromatograph using 2 mm (i.d.) x 180 cm glass columns with 2% SE-30 ultraphase on Gas Chrom Q (100-120 mesh) at 250°C.

Analysis of THC in blood plasma

The following procedure was used for Δ^6-THC (Agurell *et al.,* 1974). Analogous procedures utilizing the proper deuterated internal standard and mass numbers are applicable for Δ^1-THC, CBN, and CBD. The procedure for Δ^1-THC has been published (Agurell *et al.,* 1973).

Blood samples. Blood samples (10 ml) were collected as desired in heparinized tubes after smoking of the Δ^6-THC sample.

Plasma is obtained by centrifugation and stored in silanized glass tubes at -20°C until analysed.

Extraction. To a 5.0 ml plasma sample is added 100 ng (THC levels above 5 ng/ml) or 20 ng (below 5 ng/ml) of deuterated internal standard (Δ^6-THC-d_4) dissolved in 50 µl ethanol. The plasma sample is extracted three times with an equal volume of light petroleum containing 1.5% isopentanol in a glass stoppered centrifuge tube. After centrifugation the light petroleum is drawn off and the combined organic extracts evaporated under nitrogen at 50°C almost to dryness. This extract is quantitatively transferred to the Sephadex LH-20 column using three 0.2 ml portions of the elution solvent.

Liquid chromatographic purification
Mantled silanized glass columns (1 x 40 cm, void vol. ca. 15 ml) operated at 12°C (const. temp. water cooling system) containing Sephadex LH-20 and eluted with light petroleum-chloroform-ethanol (10:10:1) were used for purification of plasma extracts. Each column was provided with a 200 ml solvent reservoir and after each purification the column was washed with 40 ml solvent, If not in use, the columns were washed twice weekly with fresh solvent to maintain constant elution volumes.

The elution volume for Δ^6-THC was determined by calibration with ng amounts of Δ^6-THC-^{3}H (Fig. 2). The calibration can also be carried out by GLC analysis of

Figure 2

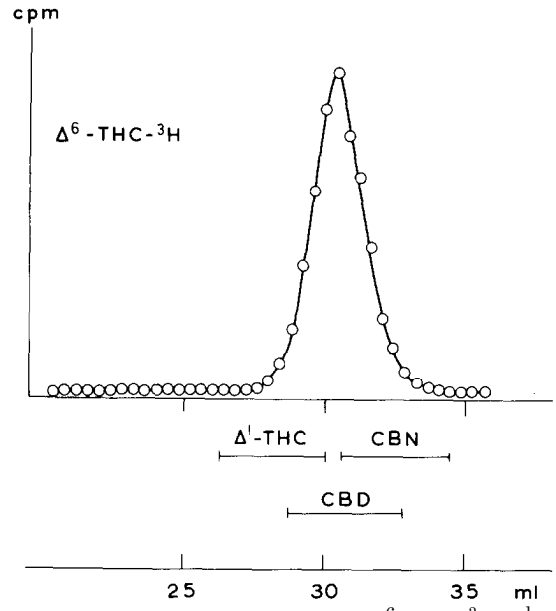

Fig. 2. Elution pattern for Δ^6-THC-^3H, Δ^1-THC, CBD and CBN from a Sephadex: LH-20 column.

the concentrated fractions after application of 10 µg quantities to the column. The column was run at 0.2 ml/min and the pertinent 7-8 ml fraction was collected and evaporated under nitrogen. The residue was dissolved in ethanol and transferred to a 50 µl conic vial (Reactivial, Pierce) dried, and finally dissolved in 10-15 µl of ethanol and stored at 4°C in the dark until analysis. This solution was subjected to mass fragmentography.

Mass fragmentography was carried out using an LKB 9000 GC-MS instrument. The column was a 1.4 m x 2 mm i.d. silanized glass column containing 3% OV-17 on Gas Chrom CLP 100/120 mesh. Temperatures were in the column 180-210°, flash heater *250°,* and source 290°. Helium was carrier gas (25 ml/min) and typical retention times are: CBD 3.4 min, Δ^6-THC 4.0 min, Δ^1-THC 4.5 min, CBN 6.1 min. For mass fragmentography a multiple ion detector was added (Elkin *et al.,* 1973) and used

at 50 eV. For Δ^6-THC, the mass spectrometer was set to continously record the intensities of m/e 314 (molecular ion of non-labelled Δ^6-THC) and m/e 318 (internal standard, Δ^6-THC-d_4) as well as m/e 299, 303 as shown in Fig. 3.

Figure 3

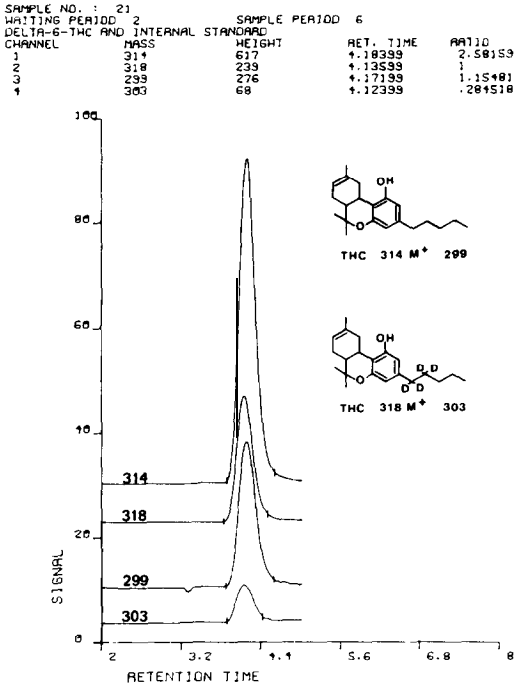

Fig.3. Mass fragmentation of Δ^6-THC-d_4(Δ^8-THC-d_4) as internal standard (m/e 318, 303) and Δ^6-THC (m/e 314, 299) from purified plasma extract.

Standard curves were prepared by adding known amounts of Δ^6-THC (0.5-100 ng/ml) to blank plasma samples and carrying out the described procedure (Fig. 4). The correctness of the standard curves were checked during the day.

Figure 4

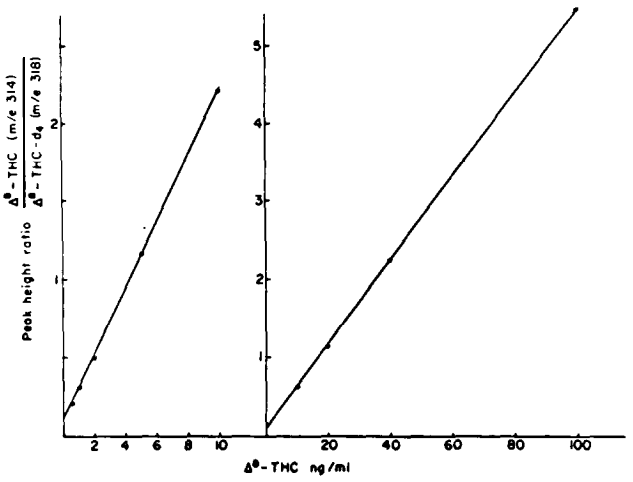

Fig. 4. Standard curves for Δ^6-THC (Δ^8-THC) in plasma. 0-1- ng/ml (left), 10-100 ng/ml (right).

RESULTS AND DISCUSSION

Principles

The described method for the determination of Δ^6-THC *(cf.* Agurell *et al.,* 1974) in blood is basically identical to the one published for Δ^1-THC (Agurell *et al.,* 1973). The method is based upon the addition of the proper deuterated internal standard (Δ^6-THC-d_4) due to the blood plasma sample. After extraction with light petroleum, the Δ^6-THC containing fraction is purified by liquid chromatography on a Sephadex LH-20 column. The pertinent fraction containing Δ^6-THC (absorbed by smoking a spiked Cannabis sample) and Δ^6-THC-d_4 (added as internal standard) is collected. The relative amounts of the two compounds are determined by mass fragmentography monitoring the molecular peaks (m/e 314 and 318, respectively; Fig. 3.).

As subsequently discussed, this method can also be used for the analysis of other cannabinoids, such as THC:s of different molecular weight, CBD, and CBN. However, at present less information is available with regards to these latter compounds.

There are generally only ng-amounts of cannabinoids present in blood plasma and although the deuterated internal standards serve as carriers, the method requires scrupulously clean, silanized glass ware and redistilled solvents.

Reference cannabinoids

In all methods pure cannabinoids are necessary for identification and calibration purposes. Since cannabinoids are often rather unstable and, with few exceptions, not crystalline - to obtain and maintain the purity of cannabinoids is not an entirely easy task. However, purification on pre-washed TLC plates as described previously (Agurell *et al.,* 1974) followed by careful drying and storage in ethanol solution at 4° in the dark provides cannabinoid standard solutions of satisfactory stability.

The syntheses of the deuherium labelled compounds were accomplished by condensation of suitable deuterated olivetols and (+)-*trans-p*-mentha-2,8-dien-1-ol according to standard methods (Mechoulam et al., 1976) - Fig. 1.

Δ^6-THC-d_4. The synthesis of this internal standard was described previously (Agurell *et al.,* 1974). The label is located in the 1"- and 2"-positions of the pentyl side chain (Fig. 3).

Other deuterated cannabinoids. A synthesis of Δ^1-THC-d_4 was reported earlier (Agurell *et al.,* 1973). In the present paper the syntheses of olivetol-d_7, olivetol-d_3, and olivetol-d_2 - mainly based upon the procedures of Pitt *et al.* (1975) - are outlined. From these intermediates, correspondingly labelled Δ^1-THC, CBD, and CBN can be prepared as exemplified in Fig. 1.

The sensitivity in the final mass fragmentographic assay may be limited by the amount of non-labelled (d_0) compound present in the deuterated internal standard. This interferes with the non-labelled cannabinoid present in the plasma. Thus, we have tried to minimize this interference by preventing exchange reactions, increasing the number of hydrogens substituted with deuterium, and by limiting the amount of internal standard in samples containing low amounts of cannabinoids (Fig. 5). Thus, the present limit of sensitivity (*ca.* 0.3 ng THC/ml plasma) is partly due to the amount of THC-d_0 in the internal standard and not due to chromatographic or mass spectrometric problems *per se*. As expected, d_7-containing Δ^1-THC, CBD, and CBN showed, together with Δ^1-THC-d_3, the least contamination with d_0-analogues (2%). This is in the same range as found for Δ^6-THC-d_4 (1.5%; Agurell *et al.,* 1974).

The mass spectra of Δ^1-THC-d_7 and non-labelled Δ^1-THC are shown in Fig. 5 together with Δ^1-THC-d_3.

Figure 5

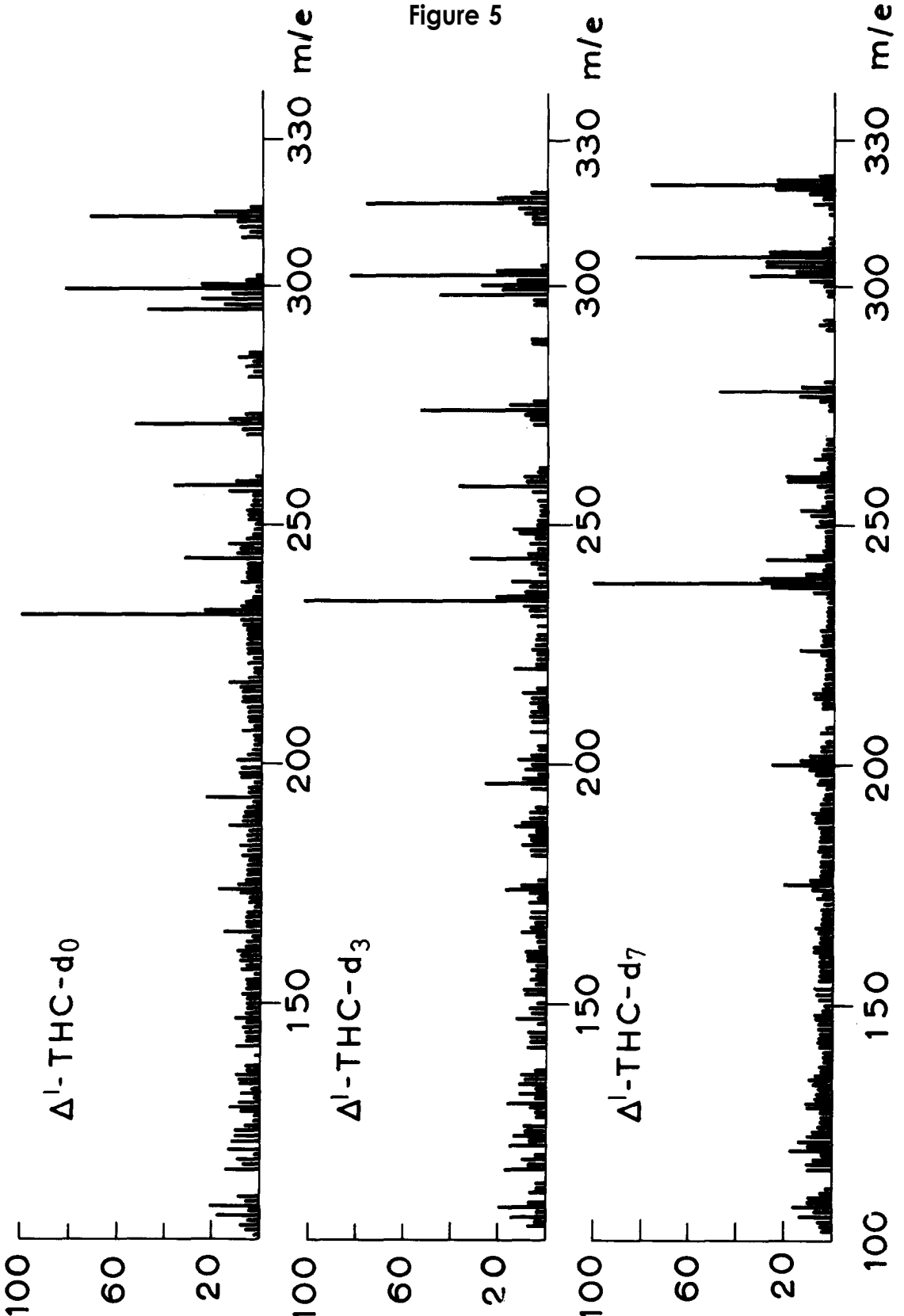

Fig. 5. Mass spectra of Δ^1*-THC,* Δ^1*-THC-*d_3*, and* Δ^1*THC-*d_7 .

Extraction and purification

Δ^6-THC and its Δ^1-THC isomer appear to be stable in human plasma for months if stored at -20°C i silanized glass tubes.

The extraction and purification procedures for Δ^1-THC, CBD, or CBN are analogous.

The extraction procedure for Δ^6-THC, as revealed by experiments with Δ^6-THC-^{3}H, is quite efficient and the recovery after both extraction and liquid chromatography is usually over 80%. Also. early studies on Δ^1-THC showed recoveries of 70 ± 6% (s.d.) after the column purification (Agurell *et al.,* 1973).

The Sephadex LH-20 separation is essential in removing interfering lipids and metabolites before mass fragmentography. The elution volume for *e.g.* Δ^6-THC is stable for months if the column is operated at low const. temp. (12°C) and, when not in use, washed with fresh solvent regularly. Our present set-up contains five columns which can be handled by a technician (10 samples per day). However, such systems can be automated (Sippell *et al.,* 1975). Over 90% of the Δ^6-THC peak is eluted in a 5 ml volume (Fig. 2) but a 7-8 ml fraction is collected. As a precaution *ca.* 3-4 ml on each side of the peak is collected and stored.

We have investigated the elution patterns of different cannabinoids and their metabolites on the Sephadex LH-20 column (Fonseka *et al.,* 1976). This is shown in Fig. 6.

Figure 6

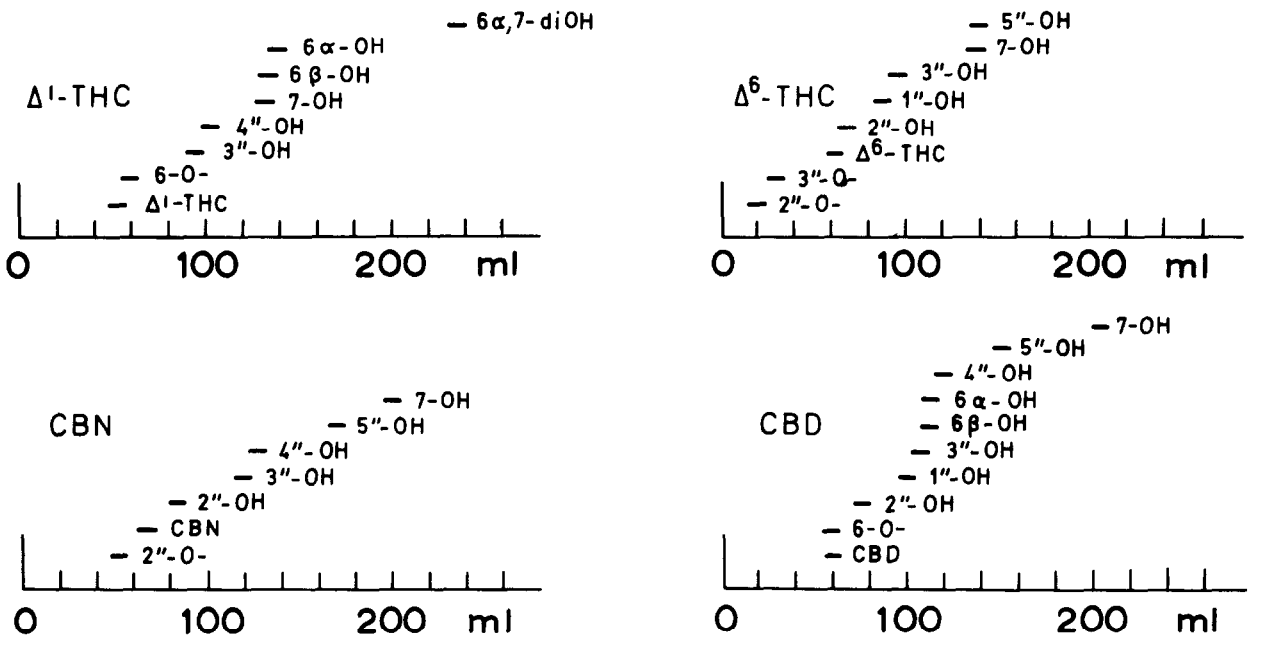

Fig. 6. Elution patterns of Δ^1-THC, Δ^6-THC, CBN, and CBD and their mono-oxygenated derivatives on Sephadex LH-20. Abbreviations as exemplified by the Δ^1-series 6-0 = 6-oxo-Δ^1-THC, 3"-OH= 3"-hydroxy-Δ^1-THC, 6α, 7-dihydroxy-Δ^1-THC, etc.

The complexity of cannabinoid metabolism in man and animal is indeed great and the requirements for specificity when assaying a certain cannabinoid are consequently considerable. To give an impression of the complexity, we have in a review (Agurell *et al.,* 1976 b) listed 35 metabolites of Δ^1-THC, Δ^6-THC, CBN, and CBD, which have been isolated in our and other laboratories and are substituted in the pentyl side chain. To make a complete list an equal number of metabolites oxygenated in the terpene nucleus *(cf.* Mechoulam *et al.,* 1976) should be added.

Mass fragmentography

The sensitivity achieved in the quantification of Δ^6-THC and other cannabinoids is partly due to the liquid chromatography clean-up of blood plasma extract. Mainly, however, the sensitivity and specificity is dependent upon the mass fragmentographic analysis.

We have used the deuterium labelled analogue Δ^6-THC-d_4 for the analysis of Δ^6-THC in blood plasma from four male, casual Cannabis smokers who had smoked 8 mg of Δ^6-THC. Such a mass fragmentogram is shown in Fig. 3.

Two standard curves were prepared (0-10 and 10-100 ng/ml) by adding known amounts of Δ^6-THC to blank plasma samples and carrying out the described procedure. Peak heights of Δ^6-THC-d_4 (m/e 318) were plotted against known amounts of Δ^6-THC (Fig. 4). Since the same ratios were obtained simply by mixing known amounts of Δ^6-THC and Δ^6-THC-d_4, usually such standard curves were used. The correctness of the standard curves should be checked occasionally during the day of analysis.

There are advantages as well as disadvantages in the use of deuterium labelled analogues as internal standards. They are carriers for the minute amounts of cannabinoids present in biological samples and, being almost identical to the analyzed compound they can be added to the original plasma sample and will then compensate for variations in extraction and purification recoveries. A disadvantage is that deuterium labelled standards have to be synthesized. Hopefully these might be provided by NIDA.

The present method for Δ^6-THC can be used down to 0.3 ng/ml. As pointed out (see discussion on "Reference cannabinoids") the sensitivity is partly limited by the small amount of Δ^6-THC-d_0 present in the deuterated internal standard. Thus, we have tried to eliminate d_o-contamination in the internal standards for Δ^6-THC, Δ^1-THC, CBD, and CBN - with best results in the d_7-*analogues.*

The d_3-compounds can also be used as standards but were mainly synthesized to study single dose pharmacokinetics of Δ^1-THC, CBD, and CBN in heavy hashish users. These users presumably have a high "steady-state" blood level of cannabinoids and their metabolites. By the use of a d_3-labelled single dose of *e.g.* Δ^1-THC, the kinetics of a particular Δ^1-THC dose can be followed in spite of high background levels of non-labelled Δ^1-THC (Fig. 7a). The d_7-labelled Δ^1-THC is then used as internal standard (Fig. 7b).

Δ^1-THC and CBD can both be determined in the same mass fragmentogram using the same channels - m/e 314 and *e.g.* 321 for internal standard - whereas CBN is simultaneously assayed using m/e 310 and 313 (Fig. 7c).

Figure 7a

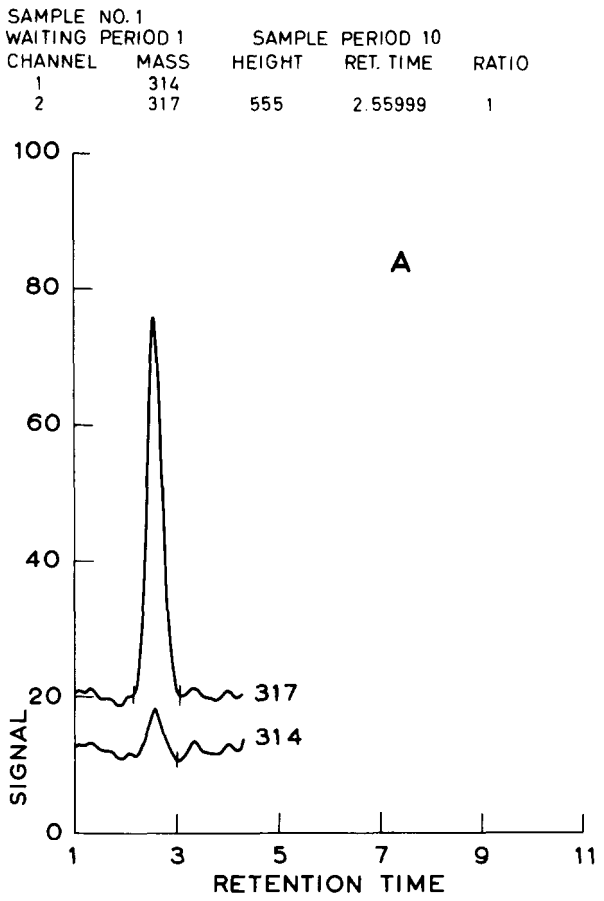

Fig. 7 a. Mass fragmentation of purified plasma extract containing Δ^1-THC (0.9 ng/ml plasma, m/e 314) and Δ^1-THC-d_3 as internal standard (m/e 317). Column temp. 210°C.

Figure 7b

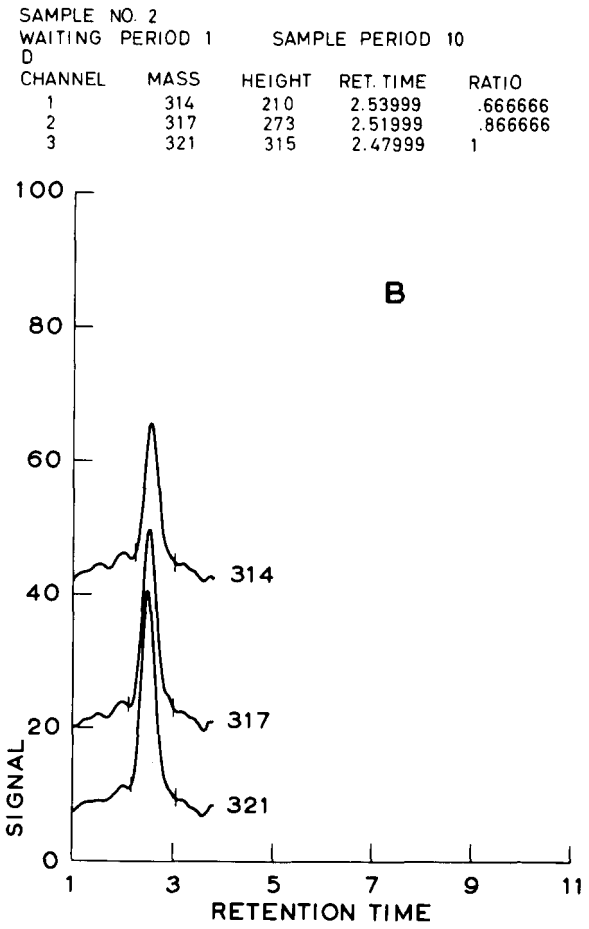

Fig. 7 b. Mass fragmentogram of Δ^1-THC, Δ^1-THC-d_3, and Δ^1-THC-d_7 (ca. 5 ng/ml plasma of each). Column temp. 210°C.

So far we have not developed assays for any of the possibly important Δ^1-THC metabolites, *e.g.* 7-hydroxy-Δ^1-THC. However, it is likely that if quantitative metabolic Studies in man warrant, this metabolite could also be quantitated after elution and derivatization using mass fragmentography.

It is also possible that cannabinoids monohydroxylated in the side chain can be assayed as their trimethylsilyl ethers using specific fragments (Binder *et al.,* 1974). Side chain hydroxylation seems to be a general metabolic route and of importance in at least some species (Agurell *et al.,* 1976 a, Harvey & Paton, 1976). Hydroxylation in the 3"- and 4"-positions are favored - reactions which also seem to confer high psychotomimetic activity in the rhesus monkey (Agurell *et al.,* 1976 a).

Figure 7c

SAMPLE NO. 24
WAITING PERIOD 1 SAMPLE PERIOD 10

CHANNEL	MASS	HEIGTH	RET. TIME	RATIO
1	314	453	4.06000	.628294
2	310	186	5.03999	.257975
3	313	135	4.02000	.187239
4	316	249	2.93999	.345353
5	317	721	4	1

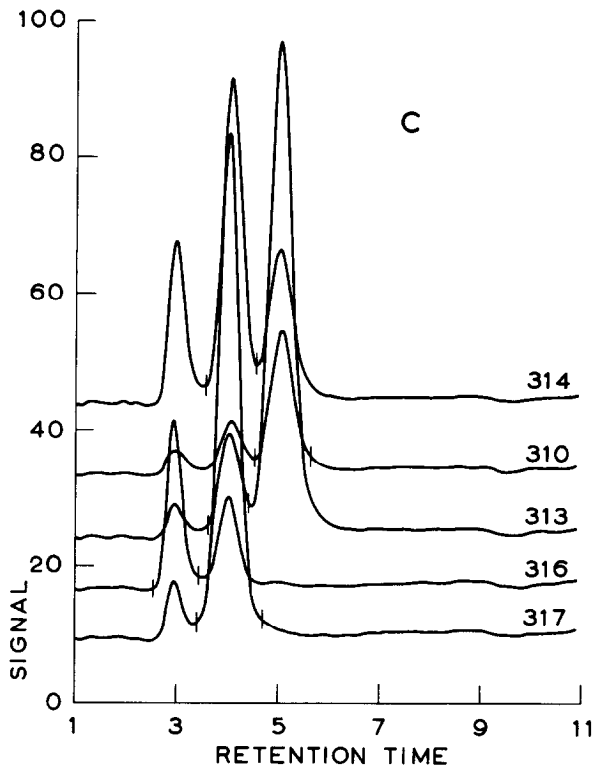

Fig. 7 c. Mass fragmentograms of CBD (m/e 314) *and internal standard CBD-d_3 (m/e 316, retention time 3.0); Δ^1-THC (m/e 314) and internal standard Δ^1-THC-d_3* (m/e 317, re*tention time* 4.0); *and CBN (m/e 310) and internal standard CBN-d_3 (m/e 313, retention time 5.0). Column temp. 220° C.*

Plasma levels

The plasma levels of Δ^6-THC in man after smoking 8 mg Δ^6-THC - of which about half is absorbed in the lungs - are shown in Fig. 8. Immediately after smoking high values (>100 ng/ml) of Δ^6-THC are recorded but drop rapidly to 10-20 ng/ml at 0.5 hour and are about 1 ng/ml at 4 hours. The present sensitivity of 0.3 ng/ml, thus allows THC levels to be followed for 12-24 hours. This is more than enough to study any correlations of Δ^6-THC levels with physiological and psychological effects (Agurell *et al.,* 1974) but not enough to establish a true elimination phase. Lemberger and co-workers (1971) have estimated elimination phase half-lifes in man of 1-2 days.

Garrett and Hunt (1976 b) have shown that the terminal half-life of Δ^1-THC in the dog is reached only slowly. They also found that the return of Δ^1-THC from the tissues is the rate determining step of the drug elimination process after the initial distribution and metabolism.

Similar plasma levels of Δ^1-THC as for Δ^6-THC (Fig.8) have earlier been found in man by us (Agurell *et al.,* 1973) and Rosenfeld *et al.* (1974) using mass fragmentography, and by e.g. Galanter *et al.* (1972) using Δ^1-THC-^{14}C. Wall and co-workers (1974) have published plasma levels of both Δ^1-THC, CBD, and CBN and certain metabolites as well as subjective psychological effects after i.v. administration of the labelled drug.

Hence, the sensitivity requirements for the determination of Δ^1-THC are indicated in Fig. 8. Plasma levels will obviously be modified by the amount of THC absorbed and by the rate of absorption but if levels are to be measured later than 4 hours after administration a sensitivity of 1 ng/ml is required. Such a sensitivity with sufficient specificity is perhaps limited to the mass fragmentographic tech-

Figure 8

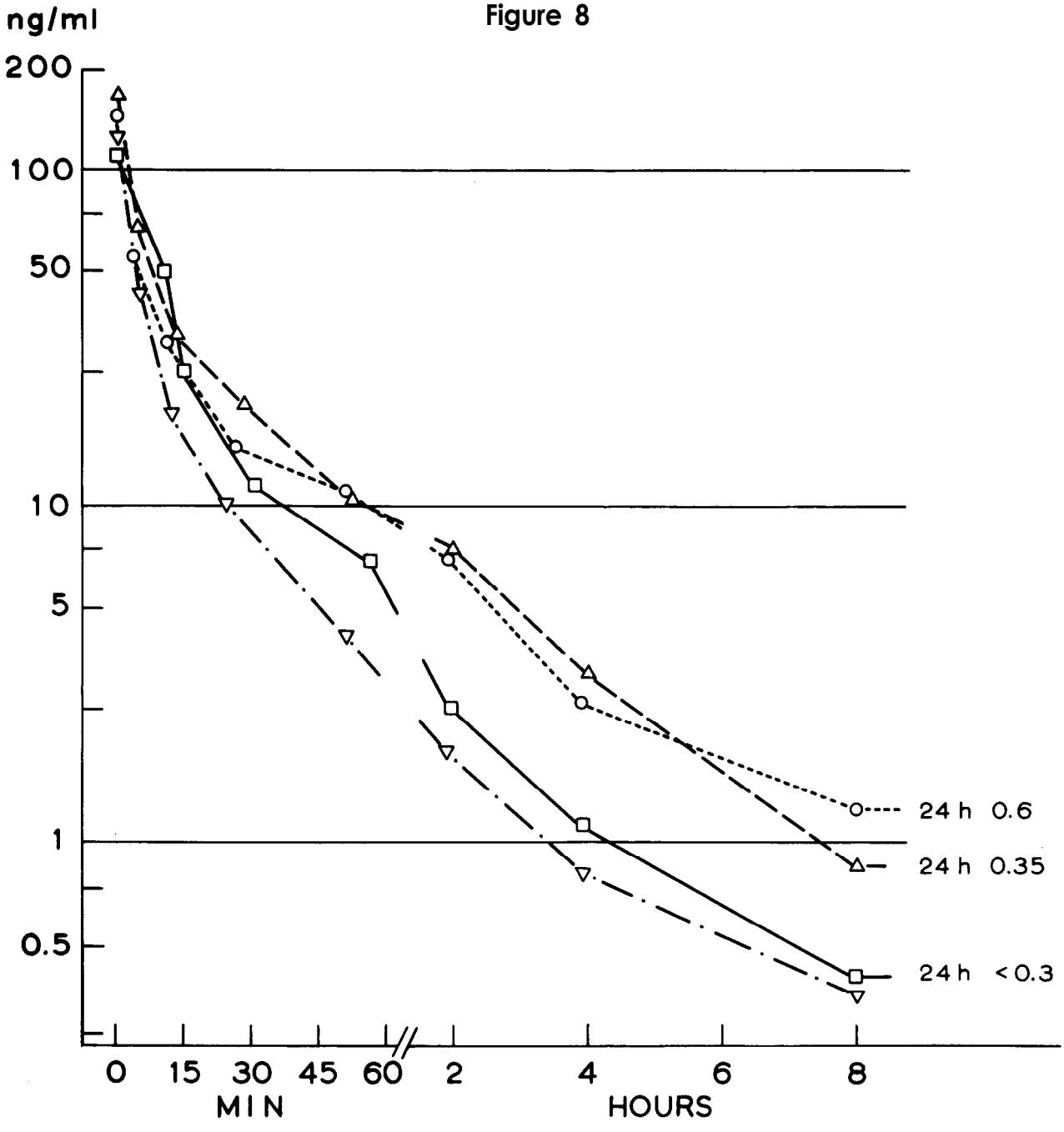

Fig. 8. Plasma levels of Δ^{6}*-THC after smoking* 8.3 *mg* Δ^{6}*-THC.*

niques. We have so far encountered little interference in the mass fragmentographic determination of Δ^{1}-THC provided redistilled solvents, particularly ethanol, and all silanized glass ware are used.

Radioimmuno assay procedures, where also certain metabolites cross-react, would clearly be applicable to the qualitative identification of Cannabis users. However, the potential cross-reactivity to *e.g.*

steroids and other drugs also has to be ascertained.

The capacity of the mass fragmentographic technique is more limited than the radio-immune assay procedure, the main time requiring step being the liquid chromatography purification. With the non-automated system now in use, a technician can process about ten plasma samples per day. This might be improved by automation, by high pressure liquid chromatography or by using the double extraction technique of Rosenfeld *et al.* (1974).

ACKNOWLEDGMENTS

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A METHOD FOR THE IDENTIFICATION OF ACID METABOLITES OF TETRAHYDROCANNABINOL (THC) BY MASS FRAGMENTOGRAPHY

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INTRODUCTION

Plans to identify Cannabis users by the detection of Δ^1-THC in urine had to be abandoned after the reports by Lemberger *et al.* (1970, 1971). Their experiments with humans given ^{14}C-Δ^1-THC i.v. and later work by Wall *et al.* (1974) showed that the drug is almost completely metabolized and the metabolites excreted mainly via faeces. Most of the urinary radioactivity derived from acidic metabolites. The contents of unchanged Δ^1-THC were less than 0.02% of the administered dose. Only small amounts of 7-hydroxy-Δ^1-THC, the major metabolite of Δ^1-THC *in vitro,* were indicated in the urine by TLC (Lemberger *et al.,* 1970). In contrast, the faecal contents of 7-hydroxy-Δ^1-THC are high, about 20% (Lemberger *et al.,* 1971; Wall *et al.,* 1974). The low amounts of Δ^1-THC and also 7-hydroxy-Δ^1-THC excreted in the urine seem to be a result of further metabolism yielding more polar compounds with the 7-methyl group being further oxidized to a carboxyl group *via* the aldehyde (Ben-Zvi & Burstein, 1974) with or without introduction of additional hydroxyl group(s). The Δ^1-THC-7-oic acid has been identified as a major metabolite in urine, faeces, and plasma from humans given Δ^1-THC or 7-hydroxy-Δ^1-THC i.v. (Wall *et al.,* 1974).

A method for detection of Δ^1-THC or 7-hydroxy-Δ^1-THC in urine or in plasma samples taken more than 12 h after the inhalation of a normal occasional dose of Δ^1-THC would have to be extremely sensitive. The plasma level of unchanged drug is then less than 0.3 ng/ml, which is the limit of our method for detection of THC. 7-Hydroxy-Δ^1-THC, 6β-hydroxy-Δ^{11}-THC, 6α-hydroxy-Δ^1-THC and 6,7-dihydroxy-Δ^{11}-THC are all minor metabolites in plasma with concentrations much lower than that of the parent compound at all times, according to Wall *et al.* (1974). On the other hand, the amount of Δ^1-THC-7-oic acid in plasma is almost equal to that of Δ^{11}-THC within 50 min and then remains slightly higher for more than 24 h.

The possible occurrence of other major acid metabolites must not be overlooked, as only Δ^1-THC-7-oic acid is identified in man so far. The major ones isolated from rabbit urine - 1"- and 2"-hydroxy-Δ^1-THC-7-oic acid (Burstein et *al.,* 1972) and 4",5"-bisnor-Δ^1-THC-7,3"-dioic acid Nordqvist *et al.,* 1974) could also be expected to be formed in man by further hydroxylation and oxidation of 7-hydroxy-Δ^1-THC. The unidentified part of metabolites in human plasma, urine, and faeces classified as more polar acids by Wall *et al.* (1974) are probably similar to the hydroxylated THC-oic acids identified by Harvey and Paton (1976) in mice.

Our proposed method for the identification of Cannabis intoxication is based upon the finding that Δ^1-THC-7-oic acid is a major metabolite. For forensic purposes, when the identification but not accurate quantitation is required, it should be a practical method, if gas chromatography - mass spectrometry (GC-MS) equipment is available. It is related to our method for detection of THC in blood plasma (Agurell *et al.,* 1973) and can as described here be used for qualitative identification and semiquantitative assay of a major acidic metabolite in urine and plasma from Cannabis users. This method is probably with slight modifications applicable also to related acid metabolites.

METHODS

Analysis of THC-7-oic acid in blood plasma

The procedure used was a modification of that described for Δ^1-THC (Agurell *et al.,* 1973). All glass ware was carefully washed and silanized before use. The solvents were of analytical grade and those constituting the eluent mixture were distilled twice.

Extraction. 50 ng Δ^6-THC-7-oic acid (Mechoulam *et al.,* 1973) dissolved in 25 µl ethanol was pipetted into a glass stoppered centrifuge tube containing 2.5 ml human plasma. It was equilibrated and 2.5 ml 1 M citrate-HCl buffer pH 4.1 was added 5 min before extraction with 10 ml diethyl ether. After centrifugation for 10 min at 4000 rpm the ether layer was drawn off, transferred into a conic tube and evaporated to dryness under a stream of nitrogen at 40°.

Methyl ester formation. The residue was dissolved in 100 µl methanol, an etheral solution of diazomethane was added in excess and the solution allowed to react for 10 min before evaporation of reagent and solvent under nitrogen.

C!olumn chromatography. The resulting residue was transferred to a jacketed Sephadex LH-20 column (1 x 55 cm, V_0 = 17 ml) using three consecutive 200 µl portions of the elution solvent light petroleum-chloroform-ethanol (10:10:1). At the elution rate 0.2 ml/min and the temperature 12° the methyl ester of Δ^6-THC-7-oic acid was eluted between 37 and 47 ml. This fraction was collected in a 10 ml conic tube. The solvent was evaporated under nitrogen, the residue dissolved in ethanol and transferred to a' 1.0 ml conic vial, where it was stored at 4' in the dark until analysis.

Silylation. On the day of analysis the methylated extract was dried under nitrogen, dissolved in 25 µl dry acetonitrile, mixed with 10 µl silylating agent [N,0-bis-(trimethylsilyl)acetamide] and kept at 50-60° for 10 min. It was then dried again under nitrogen and redissolved in 25 µl acetonitrile.

Mass fragmentography. The silyl ether of Δ^6-THC-7-oic acid methyl ester was subjected to mass fragmentography (3% SE-30 Gas Chrom Q. 100/120 mesh, 220°). The mass spectrometer (LKB Model 9000) was adjusted to record the intensity of m/e 430 (M^+), 415, and 374 at 70 eV on three different channels. For further details, see previous paper in this volume (Fig. 1).

Analysis of THC-7-oic acid in urine

The procedure described above for analysis of human plasma can also be applied to urine. In a preliminary study we equilibrated 100 ng ^{3}H-Δ^6-THC-7-oic acid for 20 min with 5.0 ml human urine and added 2.5 ml 1 M citrate-HCl buffer to maintain pH 4.1 during the extraction with equal volume of diethyl ether (7.5 ml x 1).

Figure 1

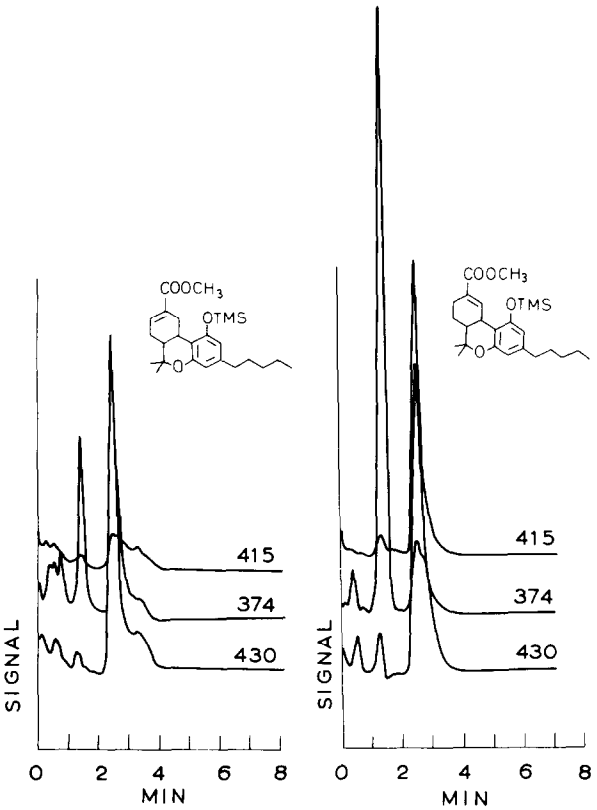

Fig. 1. Mass fragmentograms of the silyl ethers of the isomeric THC-7-oic acid methyl esters from purified plasma extracts.

RESULTS AND DISCUSSION

The partition of ^{3}H-Δ^6-THC-7-oic acid (prepared by acid catalyzed exchange) between buffer solutions of different pH and an organic phase (diethyl ether, light petroleum with 1.5% isopentanol, and toluene, respectively) was studied to determine the combination of pH and extraction solvent giving the best recovery. Diethyl ether provedtobe the most efficient solvent (log D = 2.2, pH 4.1). The recovery from

plasma of added ^{3}H-Δ^{6}-THC-7-oic acid (in the range of 50-200 ng) was over 85% after extraction with a double volume of ether. The recovery from urine was over 90% using equal volumes. The major in vivo metabolite Δ^{1}-THC-7-oic acid is expected to behave almost identically.

The diazomethane was effective in esterifying the Δ^{6}-THC-7-oic acid but the corresponding acid metabolite of Δ^{1}-THC was more difficult to esterify. However, the proceduredescribed using a 10 min incubation with excess of reagent was applicable to both acids. The elution volume on Sephadex LH-20 of the methyl ester of Δ^{6}-THC-7-oic acid was determined by calibration with ng amounts of tritiated compound. From earlier experiments Δ^{1}-THC is known to have an elution volume slightly higher than that of Δ^{1}-THC. In our study the delayed elution of the ester of Δ^{1}-THC-7-oic acid, in comparison to its Δ^{6}-isomer, had to be compensated for by a wide fraction of collection. Total recovery of ^{3}H-Δ^{6}-THC-7-oic acid in plasma after extraction, methylation, and column purification was around 70%. The silylated methyl ester of Δ^{6}-THC-7-oic acid is not fully separable from the Δ^{1}-isomer on the conventional GLC columns tested (SE-30, SE-52, OV-17, JXR, XE-60). When not silylated, the methyl esters were resolved but that of the Δ^{1}-isomer partly decomposed on the column. Thus, the silylated methyl ester of Δ^{6}-THC-7-oic acid was used as an external standard for the Δ^{1}-isomer since both compounds have very similar retention times but different intensities in the mass fragmentograms (Fig. 2, 3). The Δ^{6}-isomer can also serve as a reference for semiquantitative estima-

Figure 2

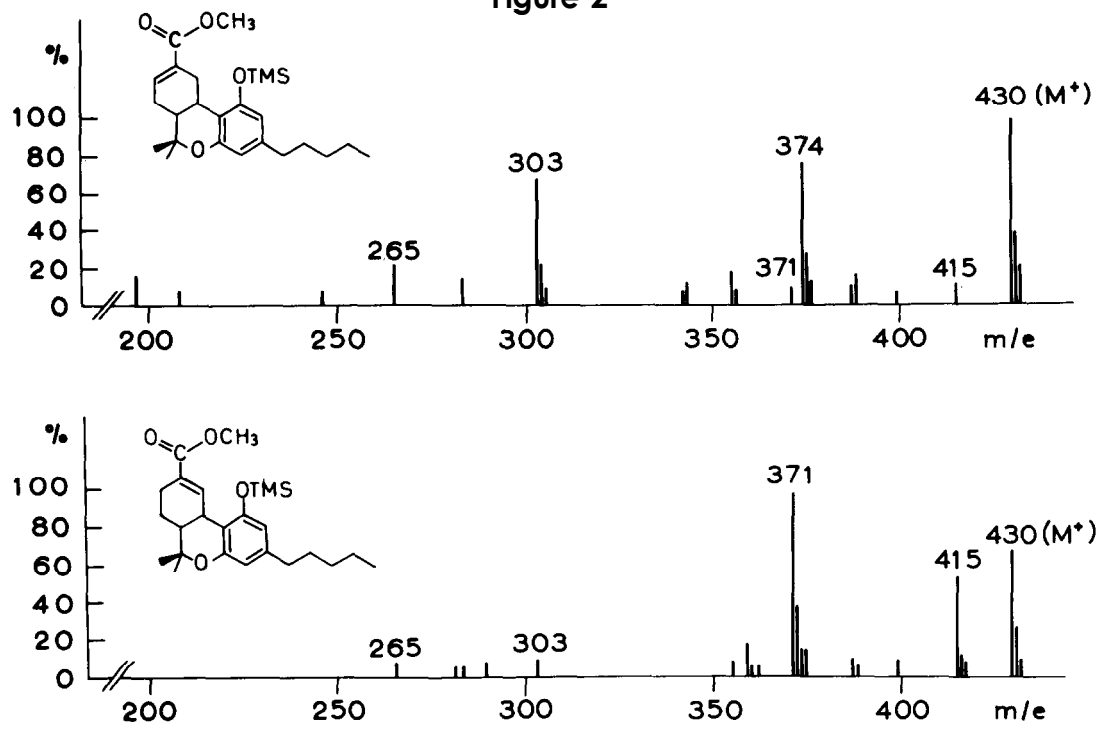

Fig. 2. Mass spectra of the silyl ethers of Δ^{6}- (upper panel) and Δ^{1}-THC-7-oic acid methyl ester (lower panel).

Figure 3a

SAMPLE NO. 1
WAITING PERIOD 1 SAMPLE PERIOD 10
DELTA-1-SYRA

CHANNEL	MASS	HEIGHT	RET. TIME	RATIO
1	415	570	4.18000	5.22935
2	374	109	4.18000	1
3	430	564	4.18000	5.17431

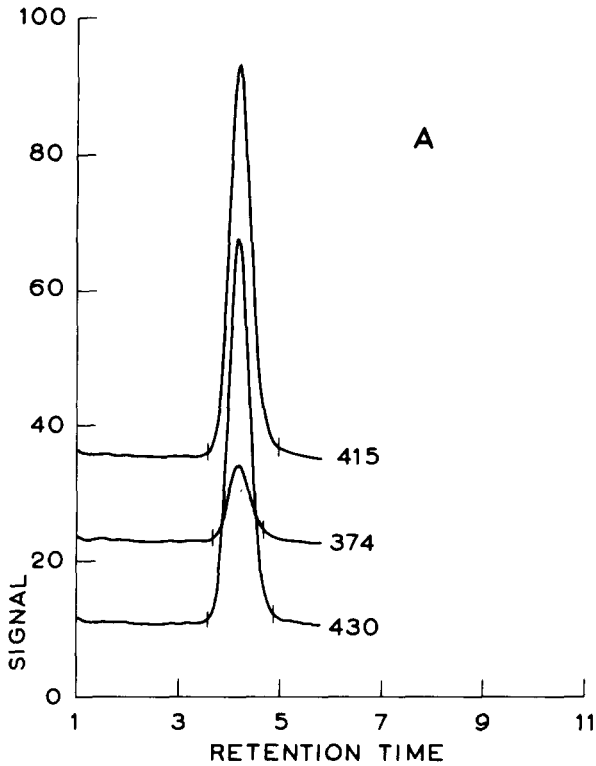

Fig. 3. a. The silyl ether of Δ^1*-THC-7-oic acid methyl ester (m/e 430, 374, 415).*

Figure 3b

SAMPLE NO. 2
WAITING PERIOD 1 SAMPLE PERIOD 10
DELTA-6-SYRA

CHANNEL	MASS	HEIGHT	RET. TIME	RATIO
1	415	75	4	9.84251E-2
2	374	762	4.02000	1
3	430	690	4.02000	.905511

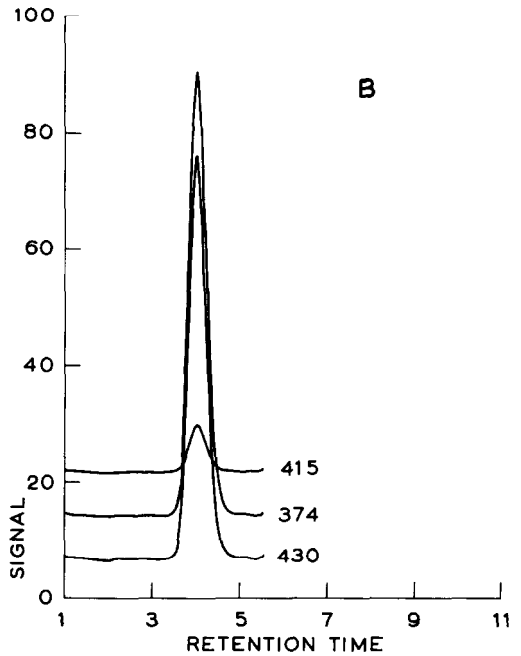

Fig. 3. b. The silyl ether of Δ^6*-THC-7-oic acid methyl* ester.

tions of Δ^1-THC-7-oic acid in plasma or urine. The possible concomitant occurrence of Δ^6-THC-7-oic acid in plasma or urine samples from smokers should be of little significance since Δ^{t6}-THC at most occurs in 2% of the amount of Δ^1-THC in Cannabis (Ohlsson *et al.,* 1977). There is only a limited need for an accurate quantitation method for Δ^1-THC-7-oic acid since pharmacological test with Rhesus monkeys (Edery et al., 1971) have shown that the isomeric Δ^6-THC-7-oic acid is inactive in doses up to 10 mg/kg i.v. If necessary, the deuterium labelled Δ^1-THC-7-oic acid can be synthesized as an internal standard according to methods described by Pitt and Wall (1974) and Pitt *et al.,* (1975).

There are no studies on the urinary excretion rates of Δ^1-THC-7-oic acid in humans after Cannabis smoking. However, the studies on Δ^1-THC metabolism in humans by Wall *et al.* (1974) suggest that identification of this acid may be a practical method to identify Cannabis users. The sensitivity of the method has not been thoroughly tested yet but is at least in the range of a few ng/ml. Further studies are in progress.

ACKNOWLEDGMENTS

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QUANTITATION OF CANNABINOIDS IN BIOLOGICAL SPECIMENS USING PROBABILITY BASED MATCHING GC/MS

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INTRODUCTION

The major effort in our laboratory in the development of THC analytical methodology has been on evaluating the efficacy of extraction procedures, on the TLC fractionation of individual *in vivo* metabolites, and on the development of an automated GC/MS quantitative procedure. For the first two aspects, we have relied heavily upon ^{14}C-studies. In California it is almost impossible to administer ^{14}C-labeled drugs to humans for research purposes, so our model animals were usually the higher primates, Rhesus and baboon (PPA). We found that the excretion rates of total radioactivity in both urine and feces for Rhesus monkey and for man (Lemberger *et al,* 1971) were very similar (Figures 1 and 2), so most of our early development efforts employed Rhesus specimens.

EXPERIMENTAL

Baboon feces

Extractions: Fecal specimens, although of no direct practical importance, were useful for orientation and for methods develop-

Figure 1

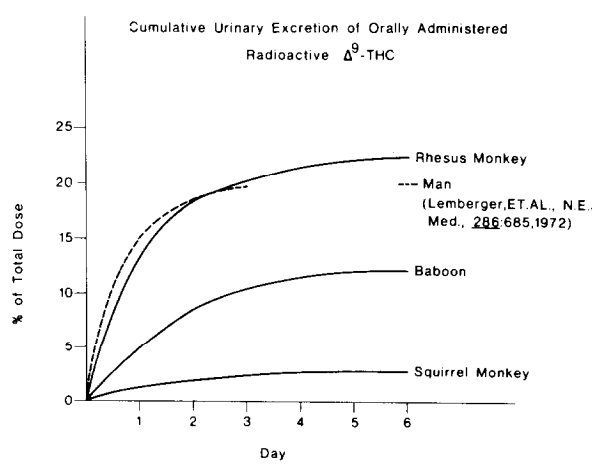

Figure 1. Comparative urinary excretion of Δ^9-THC and its metabolites in man and three primates.

Figure 2

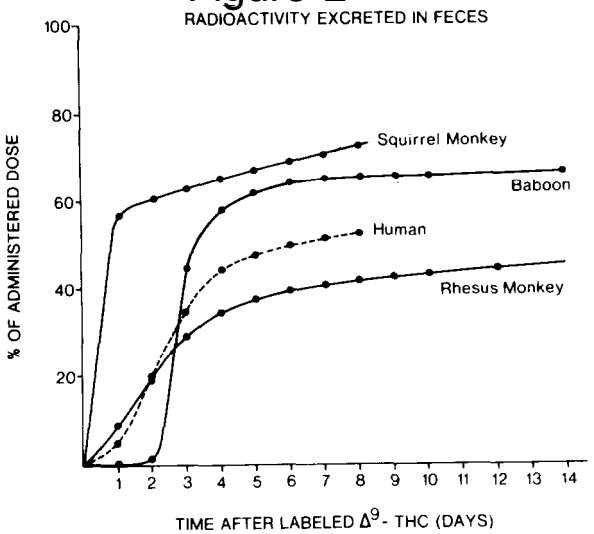

Figure 2. Comparative cumulative fecal excretion of parent drug and metabolites after oral administration of Δ^{9}-THC in man and three primates. (Human data from Lemberger et al, 1971)

Table 1

Continuous Liquid/Liquid Extractions of Soxhlet-Extracted Residues

Petroleum Ether (neutral pII)	80.2%
Diethyl Ether (pII 10)	3.2
Diethyl Ether (pII 2.5)	6.4
Total Recovery	89.8%

Figure 3

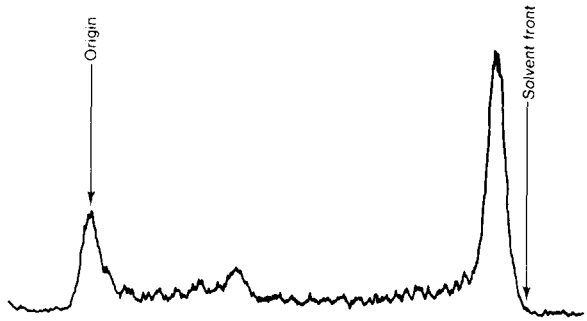

Figure 3. Thin layer radiochromatogram of petroleum ether extract of baboon feces. (Silica gel, petroleum ether/diethyl ether 1:1, 3 passes.)

ment because they contained copious quantities of the parent drug and its less extensively metabolized forms. (In this latter sense, they can serve as a general model for THC in blood.) In order to minimize the un pleasantness of working with feces, a general technique, based upon continuous extractions, was developed. In typical experiments in which ^{14}C-Δ^{9}-THC was administered to chronically THC-dosed baboons, 24-hour fecal collections were homogenized in methanol using a Waring Blender and then transferred into a soxhlet thimble where overnight extraction with methanol removed more than 99% of the total counts.

Methanolic extracts from individual specimens were evaporated to dryness and the residues were suspended in distilled water and extracted for 48 hours with petroleum ether in a liquid/liquid extractor (Fraction I). The aqueous phases were adjusted to pH 10 and extracted with diethyl ether for 18 hours (Fraction II). After adjusting the aqueous phases to pH 2.5, final ether extractions were continued for another 18 hours (Fraction III). These three extractions removed nearly 90% of the original counts as shown in Table 1 (Green *et al,* 1975).

Fraction I, which contained 4/5 of the total radioactivity, was separated by TLC into three main groups of compounds: A non-polar band (42%) near the Rf of Δ^{9}-THC; a moderately polar diffuse region (9%) in the approximate position of mono-hydroxy-Δ^{9}-THC's; and some highly polar compounds near the origin (16%) (Figure 3). A corresponding non-radioactive petroleum ether extract - obtained from feces collected on the day prior to radio-label dosing - was separated by preparative TLC (on silica gel with 3 passes in chloroform) into 5 Fast Blue B-reacting bands: Band I-A, Rf 0.88; Band I-B, Rf 0.68; Band I-C, Rf 0.14; Band I-D, Rf 0.12; and Band I-E, Rf 0.08.

High pressure liquid chromatography: An aliquot of Band I-A was dansylated and a small portion was analyzed using a gradient, high pressure liquid chromatograph (Varian Aero-Graph Model 8500) equipped with a 25 cm NH_2-bonded 10 μ silica column and an experimental filter fluorometer detector (Figure 4)

Figure 4

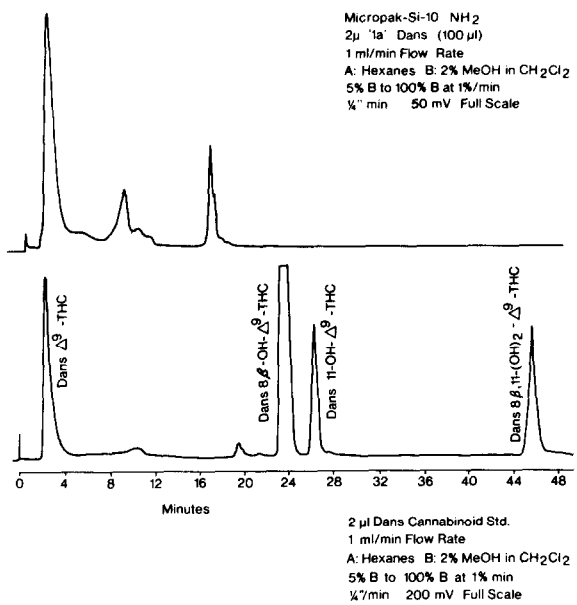

Figure 4. High pressure liquid chromatography of Dansylated Fraction I-A from petroleum ether extract of baboon feces (top) and mixture of Dansylated standards (bottom).

Figure 5

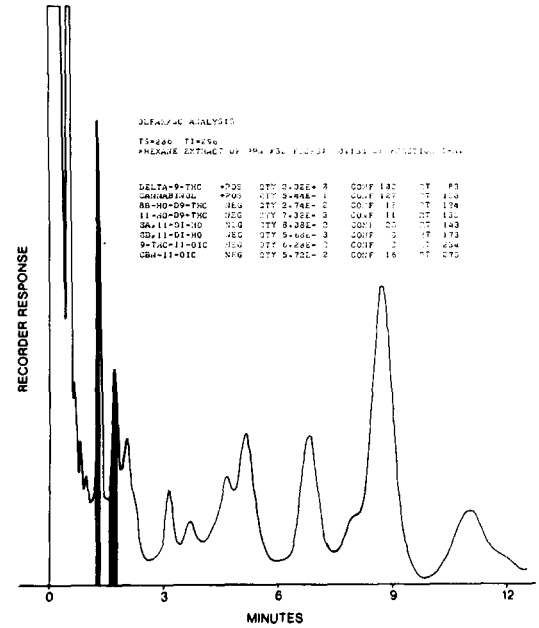

Figure 5. FID Chromatogram of silylated Fraction I-A of petroleum ether extract of baboon feces. (Column: 6' X 2 mm ID pyrex with 1.5% OV-17 on 100/120 mesh HP Chromasorb G; 30 ml nitrogen/min.; 275°C.) Inset: Automated GC/MS analytical data. Shaded areas indicate retention time intervals during which positively identified components were scanned.

(Loeffler *et al,* 1975). From this chromatogram, it can be seen that I-A had a major component with the same retention time as Δ^9-THC. Minor components at the approximate retention times of CBN and CBD were also present.

Gas chromatography/mass spectrometry: A similar aliquot of Band I-A was silylated with BSTFA and analyzed by GC/MS (Figure 5). The automated GC/MS report (the inset in Figure 5) shows that Δ^9-THC and CBN were abundantly present (2.02 μg and 544 ng, respectively), but that little, if any, of the common mono- and di-hydroxylated or carboxy metabolites were present in this TLC band.

On our instrument, the automated GC/MS analyses can also be generated and displayed in a more complete and accurate mode, if desired. The actual mass fragmentographic data from which the analytical results are derived can be printed for any single individual compound at that point on the GC peak at which the best mass spectral match between the experimental sample and the library spectrum occurred. This type of data for the two compounds which were judged to be present in Band I-A are shown in Figures 6 and 7 which show that eight characteristic ions from the spectrum of $^9\Delta$THC were present in the proper intensity ratios (and at the correct retention time) and that nine such ions were recorded for CBN. As an illustration, the five highest mass ions from the Δ^9-THC contracted spectrum were monitored as a function of time (Figure 8) and it can be clearly seen that the GC peak is entirely homogeneous with respect to Δ^9-THC. The ion currents reveal that the typical pattern was already recognizable at about 10 ng.

The quantities of Δ^9-THC and CBN in these individual compound assays represent a total daily output of 1.48 mg and 390 μg, respectively. The first of these figures indicates that about 8.5% of the total Δ^9-THC which was administered was excreted as unmetabolized drug at "steady state."

The value obtained from CBN helps to settle the question as to whether CBN, whose presence has been reported in plasma (Wilman *et al,* 1975; McCallum *et al,* 1975), is truly a meta-

Figure 6

```
OLFAX/GC ANALYSIS

TS-207  TI-295
>HEXANE EXTRACT OF PPA #30 FECES: 0.23% OF FRACTION I-A.


DELTA-9-THC    +POS    QTY 3.08E+ 3     CONF 102     RT    81

MASS   INTENSITY

 67 C   2.22E- 7
 93 C   6.16E- 8
181     5.68E- 9
196     3.52E- 9
219     8.38E- 9
231     6.32E- 9
246     1.20E- 8
330     1.49E- 8
343     2.78E- 8
371     8.03E- 8

PLOT Y OR N?Y
# OF DECADES=1

FS=2.22E- 7


      0    1    2    3    4    5    6    1    8    9    10
      +----+----+----+----+----+----+----+----+----+----+
      +
      +
  67C+>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>
  93C+>>>>>>>>>>>>>>
181  +>
196  +>>
219  +>>
231  +>
246  +>>>
331  +>>>
343  +>>>>>>
371  +>>>>>>>>>>>>>>>>>>
      +
      +
      +
```

Figure 6. Single compound GC/MS analysis of Fraction I-A of petroleum ether extract of baboon feces indicating 3.08 ng of Δ9-THC with a Confidence Index of 102 at a retention time of 81 secs. Note that only 2 ions *(m/e* 67 and 93) out of 10 are contaminated.

Figure 7

```
OLFAX/GC ANALYSIS

TS=209  TI=298
>HEXANE EXTRACT OF PPA #30 FECES: 0.23% OF FRACTION I-A.


CANNABINOL    +POS    QTY 8.08E- 1     CONF 136     RT   109

MASS   INTENSITY

165 C   7.68E- 9
178     6.84E- 9
238     9.36E- 9
295     1.34E- 3
310     2.05E- 3
323     7.68E- 9
367     2.27E- 7
368     8.20E- 8
369     2.24E: 3
382     1.04E- 6

PLOT Y OR N?Y
# OF DECADES=1

FS=2.27E- 7


      0    1    2    3    4    5    6    7    8    9    10
      +----+----+----+----+----+----+----+----+----+----+
      +
      +
165C+>>
178  +>>
233  +>>
295  +>>>
313  +>>>>>
333  +>>
367  +>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>
368  +>>>>>>>>>>>>>>>>>>
369  +>>>>>
382  +>>
      +
      +
      +
```

Figure 7. Single compound GC/MS analysis (Confirmation mode) of Fraction I-A of petroleum ether extract of baboon feces indicating 0.808 μg of CBN with a Confidence Index of 136 at a retention time of 109 secs. (only m/e 165 is not present in the required relative abundance for pure CBN.)

bolic product or arises as the excretion of a CBN impurity in the administered THC. Our work with standards indicates that there is usually a maximum of about 3% CBN in the Δ^9-THC obtained from NIDA. If this were also true of the lot which was used to dose this baboon, a maximum quantity of about 0.5 mg could have been administered. The finding of nearly 0.4 mg in this specimen indicates a recovery of nearly 80% of the maximum amount of impurity possibly present. Leo Hollister's group, with which we have collaborated closely for several years in their intensive studies of the urinary excretion of various metabolites of Δ^9-THC, CBN, CBD, and 11-HO-Δ^9-THC, has shown that orally administered CBN is very extensively metabolized (Kanter, 1975). Thus it seems unlikely that more than half of the "administered" CBN should be recoverable from the feces in a single fraction especially after ail the precessing this sample received - and therefore it would appear that CBN is a genuine metabolite of Δ^9-THC. The remaining four bands derived from the petroleum ether extraction contained none of the eight compounds in our test panel.

The continuous ether extraction at pH 10 (Fraction II) contained 54 μg 11-HO-Δ^9-THC (total daily output) and 210 μg cannabinol-11-oic acid (providing further evidence for the metabolic origin of CBN), but the presence of Δ^9-THC-11-oic acid could not be unequivocally demonstrated. The ether extract obtained at

Figure 8

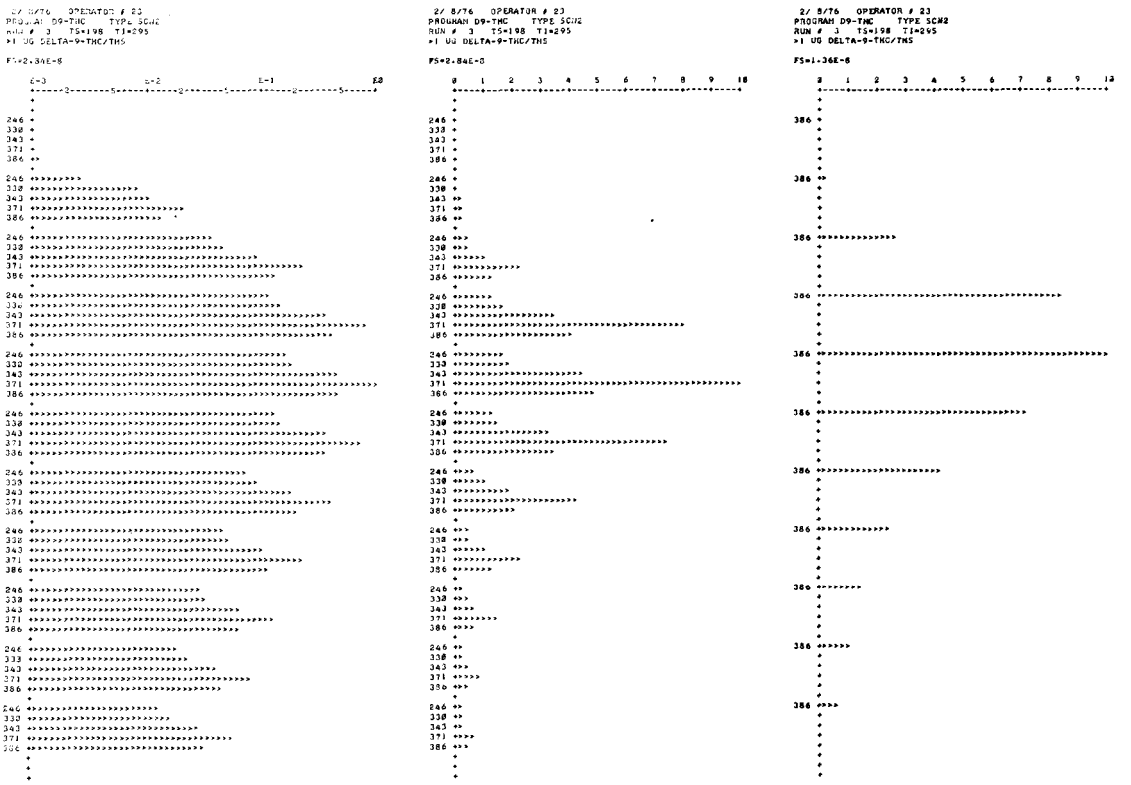

Figure 8. Top: Single ion mass chromatogram *(m/e* 386) for 1 μg Δ⁹-THC. Center: 5-Mass selected ion record for 1 μg Δ⁹-THC (5 high. est masses used in the automatic assay program). Bottom: Same as center, but with 3-decade logarithmic presentation.

pH 2.5 (Fraction III) contained 93 μg of 8β,11-dihydroxy-Δ⁹-THC, but no other metabolite in our panel could be conclusively identified.

The foregoing GC/MS results were obtained using a computer-managed Olfax II spectrometer system (Universal Monitor Corp., Pasadena, CA) which utilizes Processor McLafferty's "Probability Based Matching" (PBM) algorithm (McLafferty *et al,* 1974). In the PBM procedure, reverse sear& logic (Abramson, 1975) is used to compare a many-line mass spectral pattern derived from an experimental sample with a corresponding spectrum stored in a limited library. In the present study, the library was specially tailored to the Problem of differentiating between various cannabinoids and their metabolites.

Probability based matching

McLafferty's PBM technique was specifically designed to monitor individual compounds in mixtures by "resolving" their merged mass spectra and therefrom to automatically generate two kinds of information: 1) quantitation and 2) an estimate of the degree of specificity of the determination. Both of these analytical values are derived from an ability to automatically ascertain whether or not any particular mass spectral line (fragment ionj is contaminated by the simultaneous presence of an impurity. Such judgments are possible because of one particular characteristic physical property of mass spectra; when two or more different compounds contribute an identical molecular weight fragment ion to a merged spectrum. the

combined ion currents are always additive. This undirectional variation can be used by the computer to detect an artificially enhanced abundance of any individual ion in the mass spectral pattern of a pure substance which arises as result of a contribution from a second substance.

Since Probability Based Matching concerns the manner of specific ion recording and the processing of these data, a brief description of the PBM algorithm may help in understanding the type of results which it produces. In the reverse search process, it is not necessary to scan an entire mass spectrum, but only to measure a set of ions which are particularly characteristic of the target compound. A principal advantage of this concept is that a complete search for a limited, or "contracted," spectrum can be accomplished in less than a second and therefore the contracted spectra of several different compounds can be accurately recorded repetitively during the elution of even very sharp GC peaks.

In the Olfax II GC/MS system a microprocessor directs the spectrometer to record the ion currents for each of the pre-selected ions in the contracted spectrum and subtracts the instrument background from these values (Hertel *et al,* 1975). It then automatically computes the relative intensities and compares the resulting ratios with the stored library spectrum in order to detect any contaminated ions; it simultaneously compares the absolute intensities of each ion with the intensities of the corresponding lines in the background and disregards any ions which are so weak that they are approaching background levels. All contaminated lines and those which are near background intensity are ignored in subsequent calculations.

Confidence Indices: From the remaining uncontaminated lines, a statistical probability is automatically computed which reflects the quality of match between the experimental spectrum and the reference spectrum of the pure compound. McLafferty calls this probability a "Confidence Index" (K-score). Its magnitude depends mainly upon the absolute number of uncontaminated lines, the relative importance of each specific uncontaminated line, and the "dilution" of the sample.

Of these parameters, the concept of relative importance of a particular ion - "line value" - is unique to the PBM algorithm. The line value (or V-score) is a combination of the uniqueness of that particular mass number (frequency of occurrence in McLafferty's library of 18,800+ compounds) and the relative abundance of that particular ion in the entire mass spectrum of that specific compound (Pesyna *et al,* 1975). Unequivocal identification of a spectrum is obviously easier when using unusual mass peaks than when one is forced to rely on common peaks and when using strong ions rather than weak ones. Actually, it is often better to have a weak unique ion than a strong common ion. V-scores provide a convenient way to automatically make such judgments without being experienced in the interpretation of mass spectra.

Quantitation: All of these criteria (and more, which time does not permit mentioning) are incorporated into the K-score which is calculated each time the contracted spectrum is scanned (i.e., several times a second). Simultaneously, the absolute intensity of each uncontaminated ion is compared with the absolute intensity of each corresponding ion - resulting from a given quantity of the pure material and stored as a part of the computer library - to produce an independent mass fragmentographic quantitation for each uncontaminated ion. All of these scaled ion currents are then averaged to provide an estimate of the quantity, Q, of substance present at that point in time at which the contracted spectrum was scanned. A significant aspect of PBM quantitation is that the computer automatically selects the uncontaminated ion which represents the lowest quantity of the target compound (based upon the required relative abundances) and therefore is a self-adaptive system which reports the maximum quantity of that compound which could possibly have been present in the sample. Since the K-score reflects the mass spectral specificity of the uncontaminated ions upon which quantitation is based, its magnitude is helpful in establishing the confidence one can place in the quantitative results.

A highly unusual feature of the Olfax system is that it is entirely digital; the only truly analog output it provides is a conventional FID record from the GC. The manner in which data are recorded is shown in Figure 9. Each repetitive set of 5 ions constitutes a single cycle (i.e., one scan of the contracted spectrum) which is compared against the library values and for each cycle a new K and Q are calculated. The computer reports only the very best fit observed during any series of scans (highest K-score) and the maximum ion currents generated (highest Q), e.g., cycle 5 in this illustration.

To show how these digital data relate to conventional specific ion recording, a set of data like those of Figure 9 have been plotted in Figure 10. (In normal operation, at least three to five cycles are scanned between each

Figure 9

```
 2/ 8/76 OPERATOR # 23
PROGRAM D9-THC TYPE SCN2
RUN    #    2    TS=197    TI=298
>0.75 UG DELTA-9-THC/TMS

MASS INTENSITY

246   3.76E-12
330   8.28E-12
343   2.26E-12
371   1.11E-11
386   1.81E-11

246   3.66E-11
333   3.52E-11
343   1.94E-10
371   3.84E-10
386   3.04E-10

246   5.44E-10
330   5.52E-10
343   1.58E- 9
371   3.84E- 9
386   2.40E- 9

246   2.43E- 9
330   3.40E- 9
343   7.00E- 9
371   1.65E- 8
386   7.88E- 9

246   3.28E- 9
330   4.26E- 9
343   9.92E- 9
371   2.34E- 3
386   1.09E- 8

246   2.72E- 9
330   3.48E- 9
343   6.24E- 9
371   1.78E- 8
```

Maximum Cycle

Figure 9. Representative digital data recorded during elution of 0.75 μg Δ^{9}-THC GC peak. (Intensities are in amperes, e.g. *m/e* 371 in maximum cycle is 2.34 X 10^{-8}A.)

Figure 10

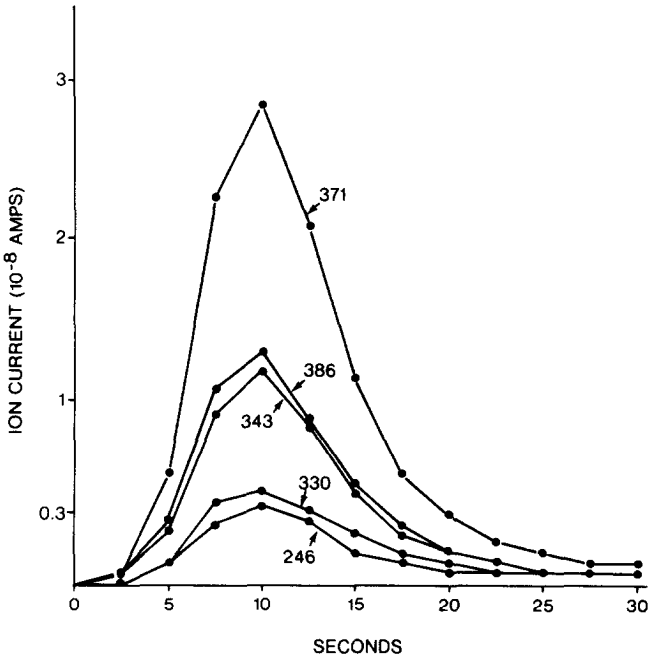

Figure 10. Plot of typical digital data (like that of Fig. 9) for 0.9 μg Δ^{9}-THC.

of the points depicted in Figure 10 - and a typical cycle contains 10 to 15 ions, rather than 5 as shown here.) Figure 11 shows three modes in which an Olfax contracted spectrum may be displayed. At the top is a single ion mass chromatogram; in the middle is a fairly conventional specific ion record (note the recurring "pattern" of relative ion currents); and at the bottom is a logarithmic presentation of the same data as in the middle (note how much more easily the relative abundances can be compared from cycle to cycle and also that the correct pattern can be readily discerned at a very low level of signal - i.e., at less than 10 ng).

Selection of specific fragment ions

The PEN reverse search technique is such a powerful pattern matching procedure that almost any set of ions selected from the spectrum of a compound will give acceptable qualitative and quantitative results. However, for sets of compounds having the similarity of the cannabinoids, especially where high sensitivity is desired, selection of the optimum mass lists requires careful consideration of many factors.

The main types of differential criteria which can be incorporated into a PBM assay program are: 1) positive attributes of the target compound (namely, high V-scores); 2) negative attributes of challenges (compounds which are likely to be encountered at similar retention times); and 3) absolute intensities of the selected ions relative to their background intensities (which affect the minimum detectable quantity at a given mass number). Thus one must attempt to maximize the K-score for the target compound while simultaneously minimizing the K-score for challenges (by deliberately selecting lines which result in contaminated or weak ions for the challenges) and all the while keep in mind the background ion currents for each mass being considered.

Fugure 11

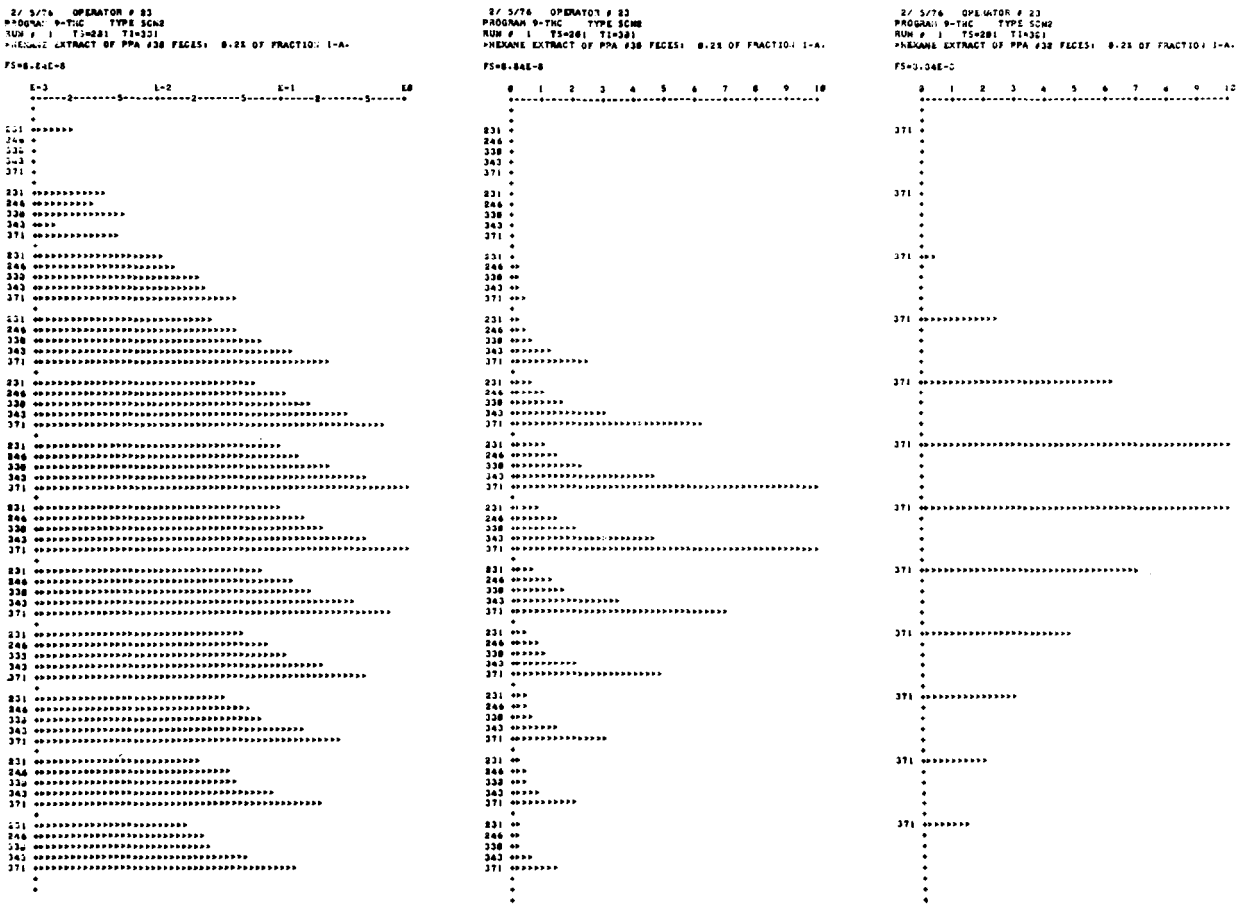

Figure 11. Top: Mass chromatogram for Δ^9-THC in Fraction I-A of petroleum ether extract of baboon feces. Center: 5-Ion selected ion record for the same sample (linear presentation). Bottom: Same as center, but with 3-decade logarithmic presentation. (5-Highest masses used in automatic assay program.)

The closer the challenge comes to the retention time of the target, the more critical it is to select contaminated or weak ions. Thus for 11-hydroxy- and 8α,, 11-dihydroxy-Δ^9-THC/TMS, the retention times differed by less than 1% and it became more important to screen against the challenging compound than to screen for the target. Because their spectra are quite different this could be accomplished, but only by sacrificing some sensitivity. The pair 8α,, 11-dihydroxy- and 8β,, 11-dihydroxy-Δ^9-THC/TMS had similar spectra but differed markedly in retention time so that they could be easily differentiated by GC alone. Unfortunately, a different situation was encountered in the pair 8α-hydroxy- and 8β-hydroxy-Δ^9-THC/TMS which have nearly identical spectra and nearly identical retention times; therefore even PBM was unable to produce unique assays for the individual compounds. Accordingly, a single program was produced which was labeled "8β-hydroxy-Δ^9-THC," but which was in reality an 8β- and/or 8α-hydroxy-Δ^9-THC assay.

Responses of the PBM algorithm

The performance of the PBM algorithm can best be understood by looking at the manner in which it responds to specific single compounds. If the Confidence Index is monitored as a function of sample size, a decreasing value is observed with decreasing quantity of sample (Figure 12). This is due primarily to two effects. At the upper end of the plot, it decreases due to a "dilution" correction be-

Figure 12

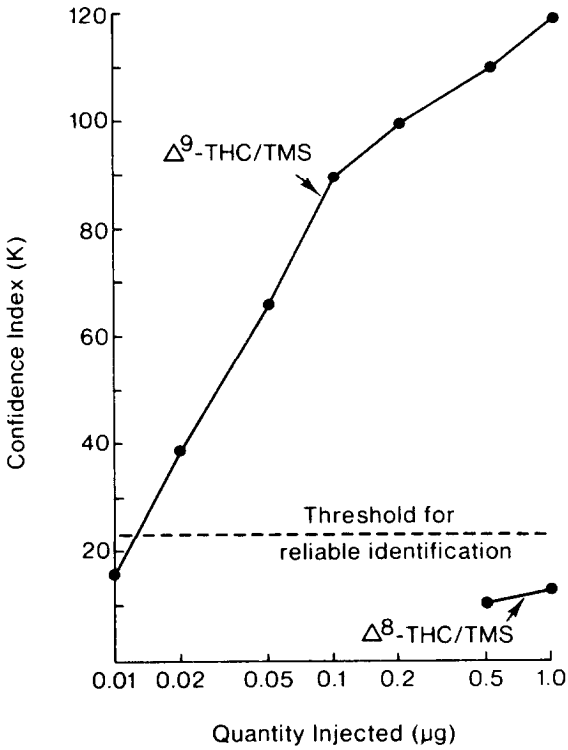

Figure 12. Effect produced on PBM Confidence Index by varying the quantity of pure Δ^9-THC injected.

cause it is assumed that low ion currents are frequently the result of dilution with impurities and, if this is truly the case, such readings are statistically less valid. At the bottom end of the curve, in addition to the dilution correction, the weaker lines approach background levels, and are dropped from consideration because they are too weak to measure reliably.

Numerically, K is a binary logarithmic representation of the probability of an accidental (random) match of the observed quality between the unknown spectrum and the library spectrum. A rough approximation of the significance of the K-scores is that 2^{Kth} spectra, chosen at random, would have to be compared with the library spectrum before one encountered a spectrum which would match as well as does the experimental spectrum. Thus a K of 10 means roughly that 2^{10} (approximately 1,000) spectra would have to be examined in order to find as good a fit.

Obviously, the numbers grow very rapidly - K=20 is approximately one million - so that only a qualitative meaning can be given to very large K-scores. As the result of two years of experience with the method, we can say that a K of 30 to 40 is somewhat equivocal, but a Confidence Index of 60 to 70 is virtual certainty of proper identification, within the limits of low resolution mass spectrometry (even without retention time considerations). Differential K-scores are of great significance as is easily seen in Figure 12 where Δ^8-THC, when used to challenge the pattern for the Δ^9-THC program, gives a K-score of 11, more than 100 points lower than does Δ^9-THC (the target compound). Differentials in K-score are likewise used to establish the "thresholds for reliable identification" for each program. Somewhat arbitrarily, this threshold is set at 10 units greater than the maximum K-score generated by any known challenging compound or control specimen (i.e. a "safety" factor of 1,000). In the case of Δ^9-THC shown in Figure 12, the Confidence Index threshold for reliable identification was set at 21, indicating that one can be reasonably certain that it is Δ^9-THC which is actually being monitored in any analysis which results in a K of 22 or greater.

The ability of these PBM assay programs (all of which contained ten fragment ions) to distinguish between closely related compounds can be seen in the matrix shown in Figure 13. The Confidence Index (the upper number in each box) for each target compound is compared with that of the THC metabolites eluting immediately before it and after it. In most cases, the difference (in K-scores) between the correct compound and its challenge is greater than 100. Besides this Confidence Index screening, many adjacent pairs can be differentiated by their GC retention times (the lower number in each box).

As one gains experience with these Confidence Indices, the analytical significance of this statistical parameter becomes "abundantly clear". Thus, for each and every quantitation, the likelihood that the data are valid is immediately apparent by inspection.

Along with the computation of the Confidence Index, the PBM algorithm provides a quantitative response which is linear for well over two decades (as may be seen in Figure 14). Olfax II quantitation usually has less than a 10% error and its precision has typically a 2-5% coefficient of variation.

For the test panel used in the present study, the 10 best lines which were found for each of the compounds, along with some of the other essential parameters for the Olfax algorithm

Figure 13

COMPOUND MONITORED / COMPOUND INTRODUCED	DELTA-9-THC	CANNABINOL	8B-HO-D9-THC	11-HO-D9-THC	8A,11-DI-HO	8B,11-DI-HO	9-THC-11-OIC	CBN-11-OIC
DELTA-8-THC	14/81							
DELTA -9-THC	116/85	8/109						
CANNABINOL	10/85	144/111	7/121					
8B-HO-D9-THC		12/119	127/126	10/147				
11-HO-D9-THC			0/131	122/149	9/155			
8A,11-DI-HO				13/154	101/155	9/197		
8B,11-11-DI-HO					2/163	119/197	11/254	
9-THC-11-OIC						8/198	112/253	9/306
CBN-11-OIC							11/261	100/308

Figure 13. Confidence Indices and retention times for nine cannabinoids. Reported as $\frac{K}{RT(sec)}$ for 1 μg samples. Column temperature 275°C.; nitrogen carrier flow rate 30 ml/min.)

Figure 14

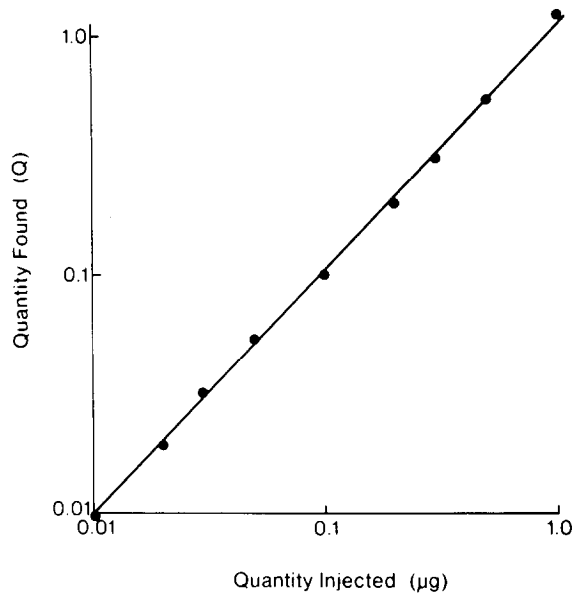

Figure 14. Quantitation reported by the mass spectrometer (PBM algorithm) as a function of the quantity of Δ^9-THC actually injected into the GC.

Table 2

OLFAX II ASSAY PROGRAMS

COMPOUND	TITLE	K_T	T_S	LINE NO.	1	2	3	4	5	6	7	8	9	10
Δ^9-THC/TMS	DELTA-9-THC	21	210	m/e	371	343	330	246	231	219	196	181	93	67
				V	14	11	11	8	8	8	6	7	3	3
Cannabinol/TMS	CANNABINOL	21	210	m/e	367	368	369	382	323	310	295	238	178	165
				V	15	14	13	13	11	12	9	9	7	4
8β-HO-Δ^9-THC/TMS	8B-HO-D9-THC	20	210	m/e	343	371	386	369	341	330	328	286	196	107
				V	13	12	11	11	10	9	10	7	5	2
11-HO-Δ^9-THC/TMS	11-HO-D9-THC	23	210	m/e	371	384	341	329	303	289	265	75	73	45
				V	15	12	11	10	8	8	8	5	6	2
8α,11-$(HO)_2$-Δ^9-THC/TMS	8A,11-DI-HO	31	210	m/e	339	371	341	330	317	303	265	145	129	93
				V	9	11	9	7	9	6	6	2	3	2
8β,11-$(HO)_2$-Δ^9-THC/TMS	8B,11-DI-HO	23	210	m/e	369	383	371	343	339	303	293	223	131	129
				V	12	12	12	10	10	7	7	6	4	3
Δ^9-THC-11-oic Acid/TMS	9-THC-11-OIC	23	225	m/e	371	355	305	303	299	297	289	265	246	75
				V	14	11	7	7	6	7	6	7	6	4
Cannabinol-11-oic Acid/TMS	CBN-11-OIC	27	225	m/e	353	350	321	309	296	249	235	220	219	
				V	11	10	10	7	6	6	6	5	6	

Table 2. Confidence Index "thresholds for reliable identification" (K_T), molecular separator temperatures (T_S), and line-values (V) for the ions (m/e) used in the 8 cannabinoid assay programs.

Figure 15

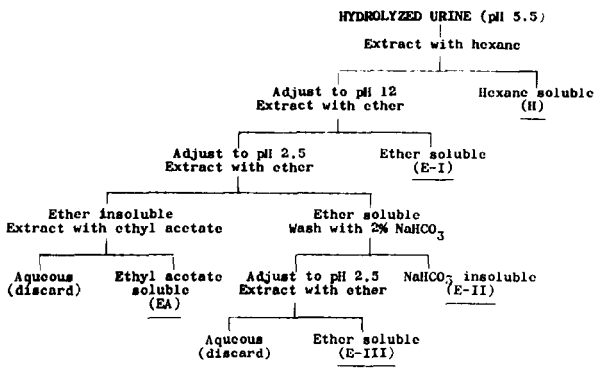

Figure 15. Extraction scheme used for β-glucuronidase / aryl sulfatase hydrolyzed Δ^9-THC urines indicating derivation of the H, E-I and E-II Fractions.

(V-scores for the individual lines, Confidence Index "thresholds", and optimum temperatures for the molecular separator), are listed in Table 2.

Human urines

Although working with feces is, relatively speaking, easier than with most other biological specimens, no great practical value can be derived from their analysis. Therefore, we have concentrated our efforts upon investigating urine, which would appear to be the most practical biological specimen, provided that some metabolite could be identified and monitored which follows the time course of physical impairment due to THC. (We have some preliminary evidence that suggests that we have seen such a metabolite.)

Extractions: Four years ago, we developed a rather elaborate procedure for fractionating THC metabolites according to their polarities and acidities using simple changes of solvents and/or pH (Figure 15) (Forrest *et al,* 1972; Melikian *et al,* 1973). This scheme separated the total radioactivity in enzyme-hydrolyzed Rhesus urine into six approximately equal fractions: Hexane extractable, H (non-polar neutrals); ether (pH 12) extractable, E-I (weakly polar neutrals); ether (pH 2.5) extractable, E-II and E-III (weakly polar acids); ethyl acetate (pH 2.5) extractable, EA (moderately polar acids); and tetrahydrofuran (pH 2.5) extractable, THF (highly polar acids). Figure 16 shows the relationships between the sizes of these fractions in hydrolyzed, as well as unhydrolyzed, urines. The actual values are given in Table 3. Subsequently, this procedure

Figure 16

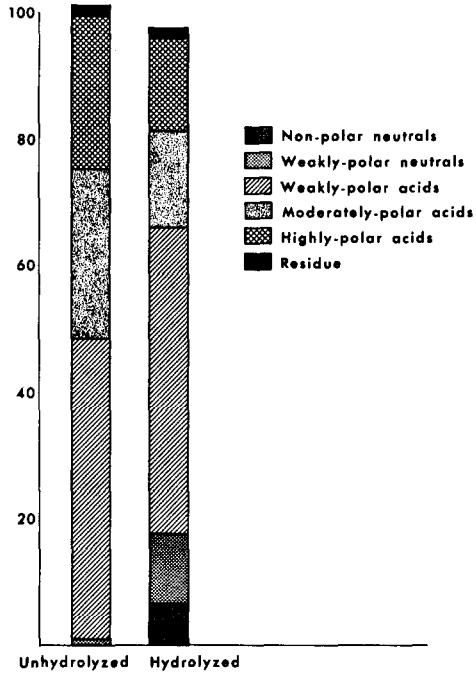

Figure 16. Distribution according to polarity (extractability) of Δ^9-THC metabolites in vivo (hydrolyzed and unhydrolyzed Rhesus urine).

Non-polar neutrals = extractable with hexane at neutral pH
Weakly polar neutrals = extractable with ether at pH 12
Weakly polar acids = extractable with ether at pH 2
Moderately polar acids = extractable with ethyl acetate at pH 2
Highly polar acids = extractable with tetrahydrofuran at pH 2.

Table 3

Extraction Efficiencies of Various Solvents (Percent of total counts extractable from Rhesus monkey urine)

Solvent and pII	Unhydrolyzed Urine	Hydrolyzed Urine
Hexane (natural pII)	0.1%	6.7%
Ether (pII 12)	0.9%	11.0%
Ether (pII 2)	47.8%	48.3%
Ethyl acetate (pII 2)	26.8%	15.2%
Tetrahydrofuran (pII 2)	23.9%	14.6%
Residue	1.4%	1.3%
Total	100.9%	37.1%

was refined by resolving the weakly polar acids (the largest single fraction) into strong and weak acids according to their solubility in $NaHCO_3$ (E-III and E-II, respectively)(Kanter *et al,* 1974).

Most of our efforts have been concentrated on the three least polar fractions, H, E-I, and E-II, which contain the parent cannafioids and the mono- and di-hydroxys, as well as the "11-oic" acids. Standards do not distribute cleanly between these categories; Δ^9-THC and 11-hydroxy-Δ^9-THC, for instance, distribute in the ratio of about 4:1 between the hexane fraction and the ether at pH 12 fraction (E-I). 8,11-Dihydroxy-Δ^9-THC, on the other hand, is found predominantly in the E-I fraction (80%) with only about 5% each in fractions H, E-II and E-III.

Hollister's group has applied this fractionation scheme very extensively to urines obtained from human volunteers after oral administration of pure standards of Δ^9-THC, CBN, CBD, 11-HO-Δ^9-THC, singly and in combinations (Hollister, 1973; Kanter, 1975). They have observed many interesting phenomena which can best be summarized briefly as: 1) the existence of a multitude of drug-related metabolites; 2) very few matches with available standards; and 3) the extreme persistance (10-15 days) of highly polar metabolites after a single administration of drug.

High dose specimens: As part of our own work using these same procedures, our group was very-fortunate to be able to obtain urine specimens from subjects used in some unusual experiments last spring at the University of California Langley Porter Clinic in San Francisco (Jones *et al,* 1975). These subjects received the extremely high oral doses of 210 milligrams of Δ^9-THC per day.

The urine was hydrolyzed with β-glucuronidase/aryl sulfatase and fractionated according to the foregoing extraction scheme. Aliquots of the dried extracts were derivatized with BSTFA and screened by GC/MS. All the "positives" which were detected, and any determinations which showed high K-scores (although "negative"), were re-run in the more comprehensive single compound mode.

The hexane fraction showed only CBN on initial screening (Figure 17), but the confirmation mode re-run showed a marginal positive for Δ^9-THC at 10 ng/ml (Figure 18). Since this positive identification was achieved with only two ions. a more conventional mass chromatogram was run (Figure 19). This showed clearly that there indeed was a GC component with the highly unique *m/e* 371 which eluted at the retention time of Δ^9-THC.

In addition to the Δ^9-THC, a confirmation mode re-run showed a fairly clear-cut presence of CBN (K=28) at a level of 12 ng/ml (Figure 20).

The weakly polar neutral fraction (E-I) showed a very marginal positive for CBN at 0.8 ng/ml, which probably was due to a slight carry-over from the hexane fraction, and a fairly strong positive for 8α,11-dihydroxy-Δ^9-THC at 24 ng/ml in the screening run (Figure 21). When fraction E-I was re-run in the single compound mode, it no longer showed a definite positive although the 8α,11-dihydroxy-Δ^9-THC was a near-miss with three weak ions in the correct ratios and corresponding to a maximum concentration of 22 ng/ml. The screening run for the weakly polar, weak acid fraction (E-II) indicated positives for Δ^9-THC (K=24), Δ^9-THC-11-oic acid (K=94) and CBN-11-oic acid (K=37) (Figure 22)) but only the THC acid could be unequivocally confirmed (Figure 23). The Δ^9-THC-11-oic acid concentration corresponded to 206 ng/ml in a highly specific determination. That these intensity ratios were in fact derived from a single substance which eluted at the proper retention time can be clearly seen in Figure 24 (top), as can the fact that the observed intensity ratios do, in fact, correspond to those of authentic Δ^9-THC-11-oic acid (bottom).

One aspect of PBM quantitation should be stressed in connection with the concentration of Δ^9-THC-11-oic acid which was measured. The PBM algorithm provides an upper limit to the possible quantity present. From this we must conclude that a maximum quantity of about 0.4 mg of this acid could have been excreted in the 24 hours during which the intake was 210 mg of Δ^9-THC. Since the urinary portion of the total excretion is about 20% (Lemberger *et al,* 1971), there would probably have been about 40 mg of metabolites in all forms. Therefore the Δ^9-THC-11-oic acid represents something of the order of 0.5 to 1% of the total metabolite pool. Allowing for dose-dependent variations of up to 10-fold, this particular acid could amount to no more than 10%, at best, of the total drug excreted in urine. This finding is entirely consistent with the TLC data which have been obtained in our laboratory and in Hollister's laboratory (Kanter, 1975), as well as in Jones' laboratory (Ellman, 1975). There are copious quantities of many acidic metabolites, but the Δ^9-THC-11-oic acid per se is definitely a minor component.

Moderate dose specimen: An examination of a slightly more typical urine specimen was carried out on some extracts provided to us by Saul Kanter of Hollister's group. These fractions, H, E-I, and E-II, were extracted from the urines of an individual before and after he received a single oral dose of 30 mg of Δ^9-THC; specimens were collected 12 hours prior to drug intake and during the interval of 12 to 24 hours post-drug.

Figure 17

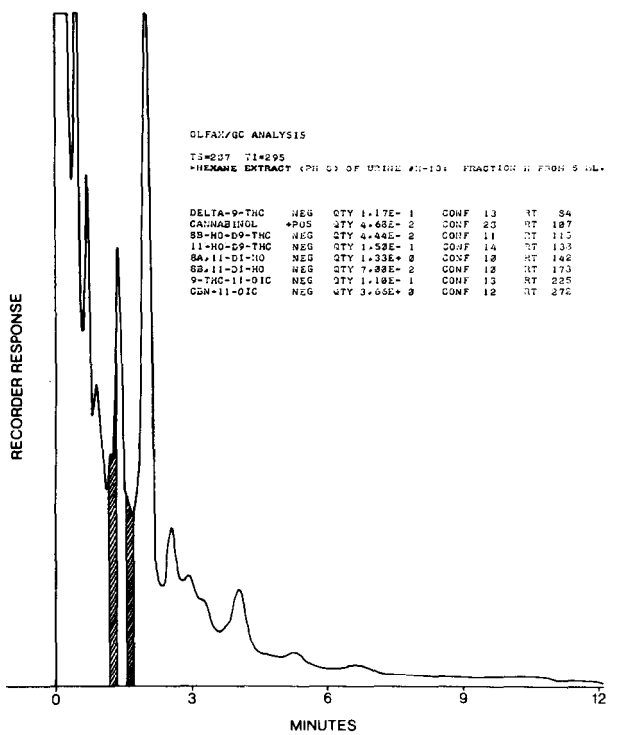

Figure 17. FID Chromatogram of silylated non-polar neutrals (H) fraction of human urine (enzyme hydrolyzed).- GC conditions same as Fig. 5. Inset: Automated GC/MS analytical data (Autoscan mode).

Figure 18

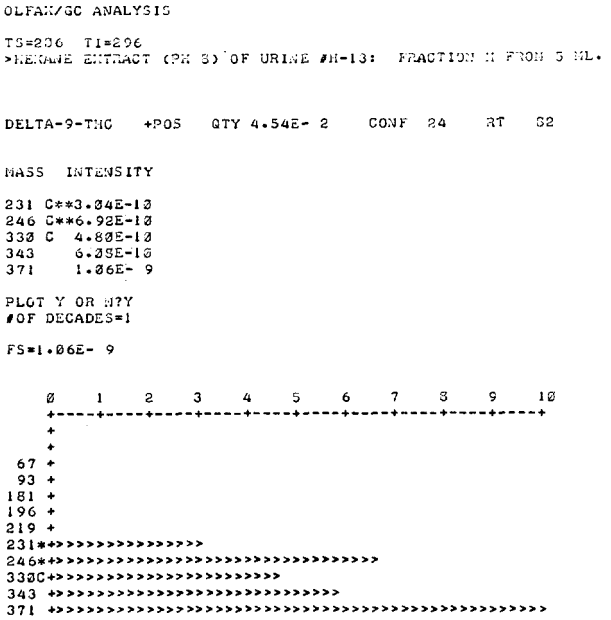

Figure 18. Single compound GC/MS analysis (Confirmation mode) of Fraction H from hydrolyzed human urine (same as Fig. 17) indicating 45.4 ng of Δ^9-THC in 5 ml urine with K=24 at a retention time of 82 secs. Note that the computer elected not to scan the lowest 5 masses because it knew that they would be too close to background levels and that the computer judged 3 of the remaining ions to be contaminated, i.e. their relative abundances were too high (c. f. pattern in Figs. 8, 9 and 11).

Figure 19

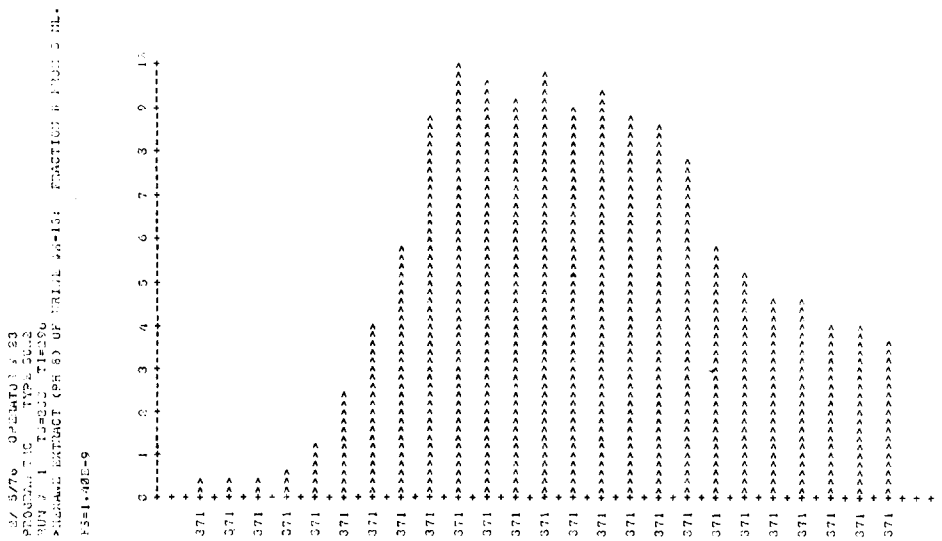

Figure 19. Single ion (m/e 371) mass chromatogram for same sample as Figs. 17 and 18. Note that this ion becomes cleanly measurable at about 5 ng.

Figure 20

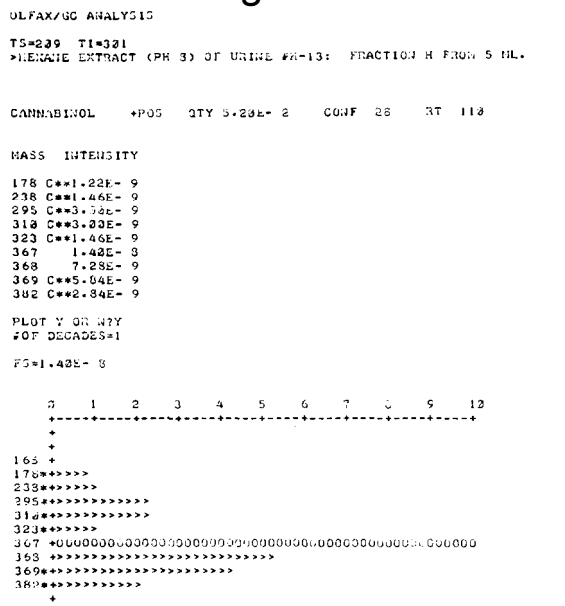

Figure 20. Confirmation mode GC/MS analysis of Fraction H from hydrolyzed human urine (same as Fig. 17) indicating 52.0 ng of CBN in 5 ml urine with K=28 at a retention time of 110 secs. Nine out of the 10 masses were scanned, but 7 were contaminated.

Figure 21

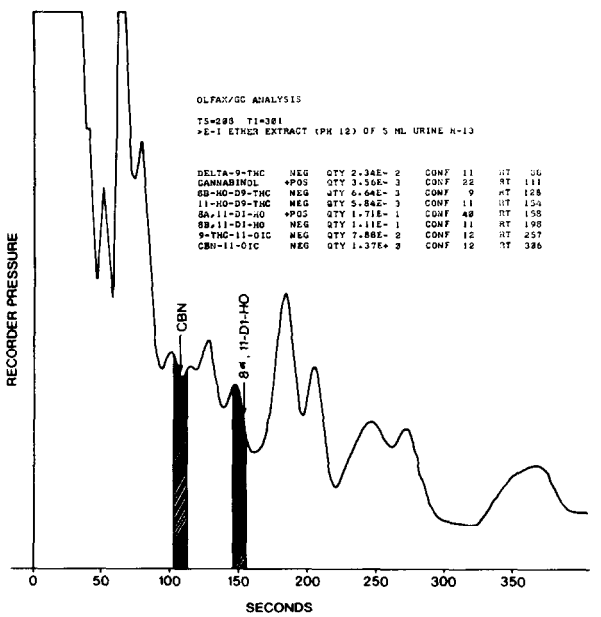

Figure 21. FID Chromatogram of silylated weakly polar neutrals (E-I) fraction of human urine (enzyme hydrolyzed). GC conditions same as Fig. 5. Inset: Automated GC/MS analytical data (Autoscan mode).

Figure 22

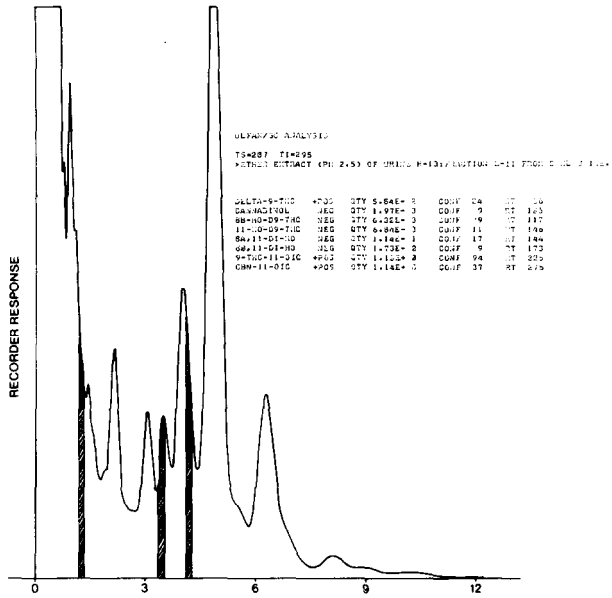

Figure 22. Chromatogram of silylated weakly polar, weak acids (E-II) fraction of human urine (enzyme hydrolyzed). GC conditions same as Fig. 5. Inset: Autoscan mode GC/MS data.

Figure 23

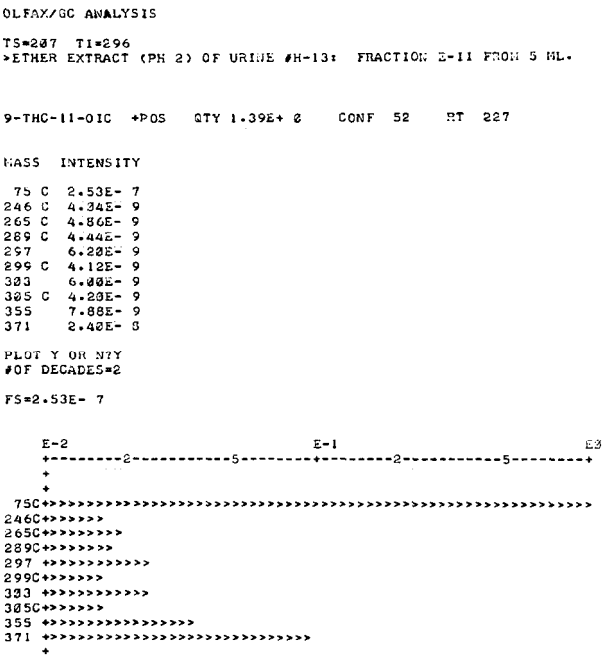

Figure 23. Δ^9-THC-11-oic acid/TMS analysis of Fraction E-II (same as Fig. 22) showing 1.39 µg/5 ml hydrolyzed urine with K=52 at a retention time of 227 secs. All 10 lines were scanned, but 6 were contaminated.

Figure 24

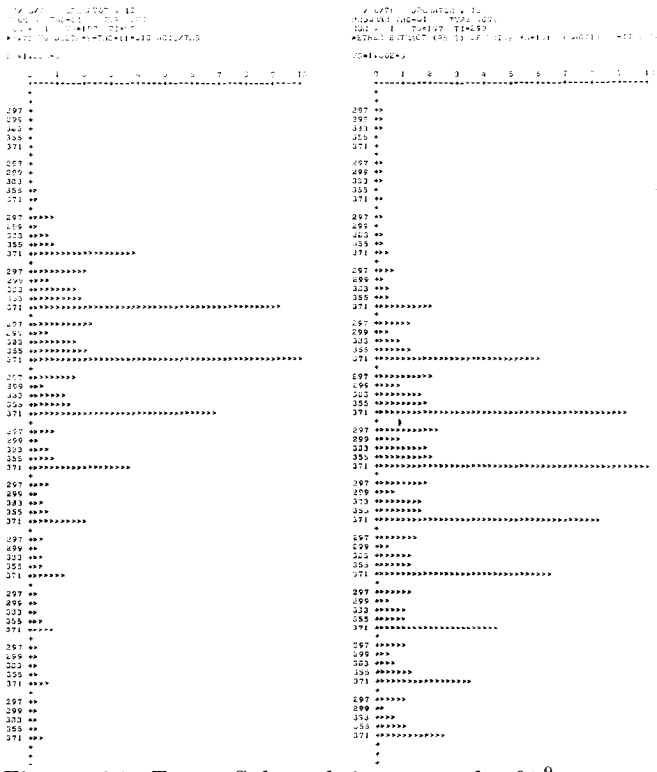

Figure 24. Top: Selected ion record of Δ^9-THC-11-oic acid GC peak from Fraction E-II (same sample and quantity as Figs. 22 and 23). Bottom: Same kind of selected ion record of 0.75 µg of authentic Δ^9-THC-11-oic acid.

Figure 25

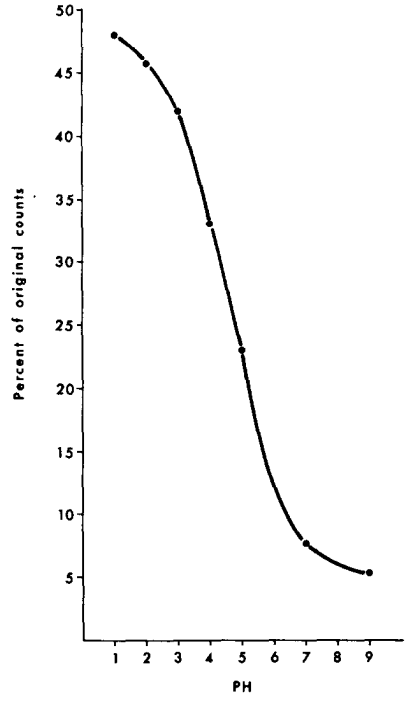

Figure 25. Effect of pH on extractability of Δ^9-THC *in vivo* metabolites from unhydrolyzed Rhesus urine.

We found a maximum quantity of about 50 ng of Δ^9-THC per 10 mg creatinine in the hexane fraction, but the Confidence Index was too low to identify the compound with certainy. The CBN, however, was clearly evident (K=59) at a level of about 100 ng/10 mg creatinine;

The E-I fraction (weakly polar neutrals) gave a very marginal confirmation for 8β-HO-Δ^9-THC at a level of about 60 ng/10 mg creatinine and a suspiciously high reading of nearly 0.6 µg of 8α, 11-dihydroxy-Δ^9-THC per 10 mg creatinine with a K-score of 35. The E-II fraction (weakly polar, weak acids) from this specimen, surprisingly, failed to give a positive for Δ^9-THC-11-oic acid.

Unhydrolyzed urines: Four years ago, we reported that an appreciable proportion of the total counts in Rhesus urine could be extracted with ether, even without prior hydrolysis (Forrest *et al,* 1972; Melikian *et al,* 1973). This extraction was highly pH-dependent with the curve's inflection point being near pH 4 (Figure 25) indicating that it was probably carboxylic acids which were being removed by the ether.

As one part of an experiment on urines obtained from the Langley Porter Clinic Study, we extracted and another specimen with ether before and after treatment with enzyme at pH 5.5. Unfortunately this pH was far from the optimum pH for removal by ether, but the results are interesting, nevertheless. A highly significant Confidence Index (K=68) for Δ^9-THC-11-oic acid was obtained along with a quantitation of 36 ng/ml (Figure 26). Had the extraction been made at pH 2.5, a quantity of about 90 ng/ml would probably have been extracted, if the compound follows a normal ionization curve for carboxylic acids. (This level suggests that about half of the Δ^9-THC-11-oic acid which is present in urine can be extracted directly, without hydrolyzing the specimen.)

Single specific compound analysis confirmed the screening results as did a selected ion record, as shown at the bottom of Figure 27.

Figure 26

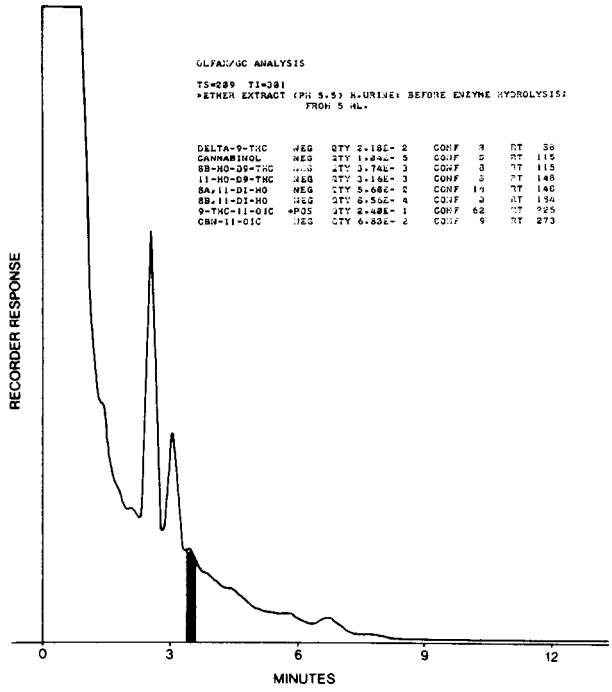

Figure 26. Silylated ether extract (pH 5.5) of human urine prior to enzyme hydrolysis. Inset: Autoscan analysis.

Figure 28

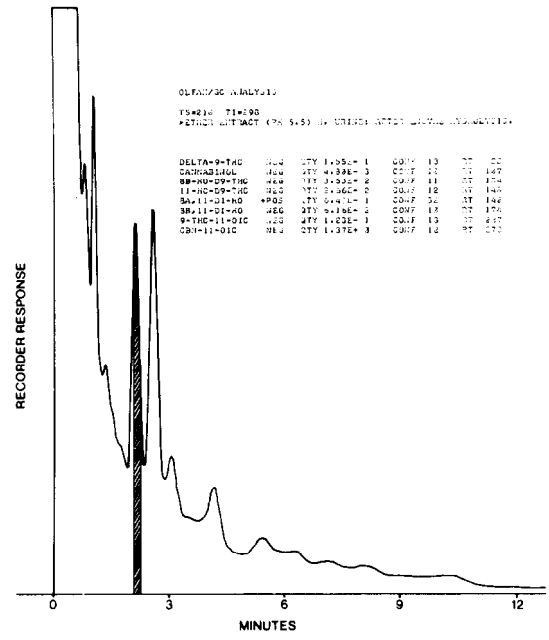

Figure 28. Silylated ether extract (pH 5.5) of human urine after enzyme hydrolysis. Inset: Autoscan analysis.

Figure 27

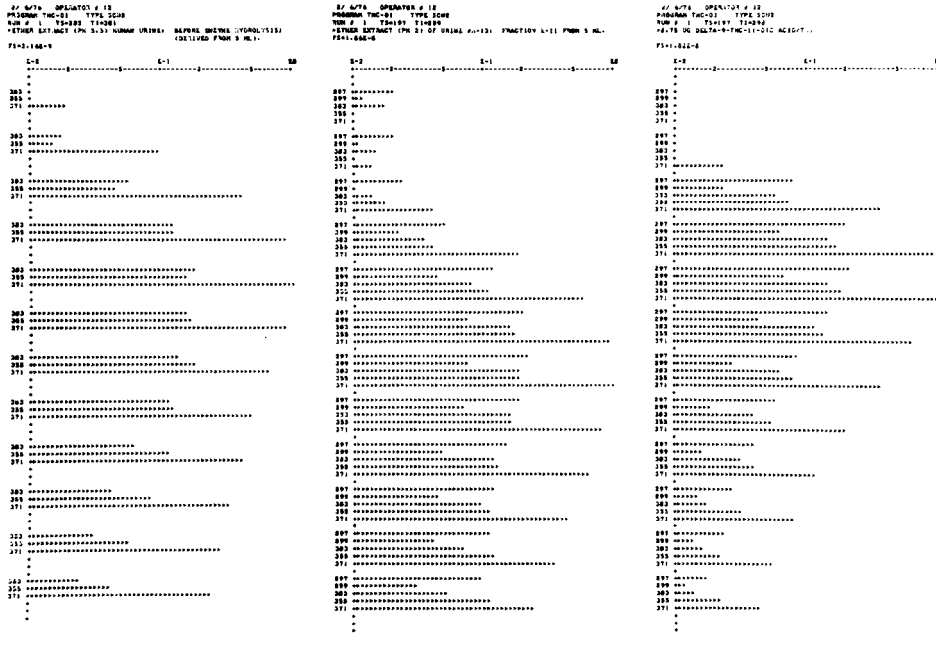

Figure 27. Top: Selected ion record of 0.75 µg of authentic Δ^9-THC-11-oic acid. Center: E-II Fraction of hydrolyzed human urine at RT of Δ^9-THC-11-oic acid. Bottom: Ether extract of unhydrolyzed human urine at RT of $\Delta^{(9)}$-THC-11-oic acid.

Figure 29

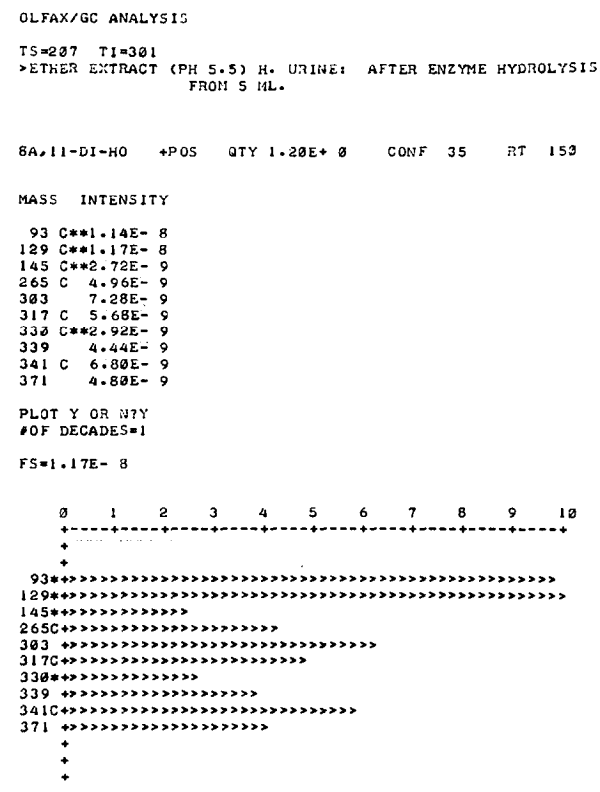

Figure 29. 8α,11-Dihydroxy-Δ^9-THC/TMS analysis of ether extract of human urine after enzyme hydrolysis showing 1.20 µg/5 ml with K=35 at a retention time of 150 secs. Three ions are uncontaminated.

This selected ion record also shows that one ion *(m/e* 355) becomes contaminated shortly after the peak has eluted and is the type of situation which would have been rejected by the computer if it had occurred a few seconds earlier. The center record was obtained from the E-II (weak acids) fraction of the previously discussed enzyme hydrolyzed urine, and the top record is authentic Δ^9-THC-11-oic acid.

Figure 28 shows the results from the post-hydrolysis ether extraction. Only 8α,11-dihydroxy-Δ^9-THC appeared to be present, but its identification was very marginal (K=32) although it was slightly better in the confirmation mode (K=35) (Figure 29).

SUMMARY

The results from these urine studies and a few other isolated extracts we have run may be generalized as follows: The hexane fractions showed low levels of Δ^9-THC (ca. 10 ng/ml) with marginal Confidence Indices (25-30), but both the quantities and the K-scores were well above the values seen in extracts of control urines; the hexane fractions showed similar levels of CBN (ca. 12-25 ng/ml) with significant K-scores (60-80); the ether-extractable neutral fraction contained 8α,11-dihydroxy-Δ^9-THC (25-150 ng/ml) with low confidence and sometimes a suggestion of 8β-hydroxy-Δ^9-THC (15 ng/ml) with a marginal identification; and the E-II fraction (ether-extractable, weak acids usually contained a strong indication (CONF 60-95) of Δ^9-THC-11-oic acid (100-200 ng/ml).

CONCLUSIONS

Obviously, the limited scope of these data which I have presented cannot be considered to be much more than a feasibility study, but the results are quite promising. Three metabolites of Δ^9-THC are consistently observed and Δ^9-THC *per se* is seen and quartitated with a degree of certainty which requires only slight improvement. Since the Δ^9-THC determinations were not entirely satisfactory, suggestions for future efforts aimed at improving this particular assay might include partially shifting the burden of identification from the mass spectrometer to the GC by improving the GC separation using such means as operating at a lower column temperature or increasing the length of the column. The effectiveness of the PBM assay program might be improved by processing a larger sample to decrease the effect of weak ions in the algorithm or by improving the sensitivity of the mass spectrometer itself. Also, since 11-hydroxy-Δ^9-THC was not encountered in any urine samples, the 8α,11-dihydroxy-Δ^9-THC program should be modified so as to make it better able to withstand general challenges even though in the process it becomes less specific with respect to 11-hydroxy-Δ^9-THC.

In all of the work described here, a standard test panel of TMS derivatives of Δ^9-THC and seven of its metabolites was employed. Assay programs for additional metabolites, such as the side-chain hydroxylated compounds described by Professor Agurell, could easily be added to the panel if appropriate reference samples were made available to us.

The Olfax method, in which GC retention time screening is combined with the PBM mass spectrum matching technique, appears to be a very

promising procedure for the highly specific quantitative analysis of cannabinoids because it automates and makes practical the best features of mass fragmentography. It cannot do anything which would not be possible using conventional GC/MS systems, but its degree of automation provides an advantage in speed and convenience while retaining the inherent specificity of mass fragmentography. The extractions employ solvents and apparatus which are available in any laboratory, the labor and analysis time have been minimized, and the final assay uses a commercially available, completely automated instrument which any skilled technician can operate and which does not require prior experience with mass spectrometers or an ability to interpret mass spectra.

ACKNOWLEDGMENTS

Parts of this work were supported by Contract HSM-42-71-100 and by USPHS Grants DA 00424-01 and DA 00748-02.

The baboon specimens were very kindly supplied by Eva E. K. Killam of the University of California (Davis) whose cooperation and interest is greatly appreciated. Special thanks are expressed to George L. Ellman of the University of California (San Francisco) for providing the unique urine specimens and also to Saul L. Kanter of this Hospital for providing special urine extracts. I wish to thank Richard Schneider of Syva Corporation for the sample of CBN-11-oic acid and Robert H. Hertel of Universal Monitor Corporation for valuable discussions during the course of this work. The very able technical assistance of Fu-Chuan Chao and Kay O. Loeffler is gratefully acknowledged.

Finally, most special thanks are due to Duane P. Littlejohn and the Universal Monitor Corporation for the loan of a prototype Olfax II GC/MS instrument system, without which this work would not have been possible.

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QUANTITATION OF Δ^9-TETRAHYDROCANNABINOL IN BODY FLUIDS BY GAS CHROMATOGRAPHY/ CHEMICAL IONIZATION -MASS SPECTROMETRY

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As part of a program for the development of methodology for quantitation of cannabinoids in body fluids, we have validated an extraction and GC/CI-MS analysis for the determination of Δ^9-THC in plasma. The procedure has proven suitable for the analysis of relatively large numbers of samples, yet has sufficient sensitivity and selectivity for analysis of Δ^9-THC concentrations as low as 0.5 ng/ml in plasma. Using this procedure, we are currently analyzing a variety of body fluid samples from different sources submitted by outside researchers.

Previous work at Battelle and other laboratories indicated that substantial clean-up of the body fluid extract was necessary prior to GC/MS analysis in order to achieve adequate sensitivity. Although several clean-up procedures have been used successfully, many of these are cumbersome and not well suited for analysis of large numbers of samples. The procedure we are currently using is a modification of the RTI solvent extraction procedure (Rosenthal, 1975). The analysis scheme is essentially an organic solvent extraction and clean-up, concentration and derivatization of the Δ^9-THC, followed by quantitation by GC/CI-MS analysis.

Specifically, the plasma sample is transferred with a calibrated pipet to a culture tube. To this is added pH 7.0 buffer solution and a known amount of Δ^9-THC-d_3 internal standard. The mixtures is vortexed to assure complete mixing, then extracted twice with hexane. The combined hexane extract is successively washed with 0.1 N NaOH and 0.1 N HCl. The hexane extract is transferred to an evaporation tube and evaporated to dryness under a stream of nitrogen using a tube heater maintained at below 50 c. The tube is rinsed with

pentane and the pentane solution transferred quantitatively to a Reacti-Vial(R). After removal of the pentane, BSTFA [N,O-bis-(trimethylsilyl)-trifluoroacetamide] with 1% TMCS is added, and the vial is heated for a minimum of 30 minutes at 75 C.

The concentration of Δ^9-THC is determined using the technique of selected ion monitoring (SIM) with a GC-MS. A 6 ft x 2-mm glass column packed with 3% OV-17 on 100/120 mesh Gas Chrom Q is temperature programmed from 180 to 280 C at 10°/minute. Methane is used as the carrier gas while a small amount of ammonia is bled into the ion scource through a make-up gas inlet. The masses monitored are m/e 387, the protonated molecule ion of the trimethylsilyl ether (Δ^9-THC-TMS) and m/e 390, the protonated molecule ion of the TMS ether of the trideuterated internal standard (Δ^9-THC-d_3-TMS).

Previously we used methane as the reagent gas for chemical ionization of the GC effluent. We have now found that addition of ammonia to the ion source improved the signal strength for a specific quantity of Δ^9-THC-TMS by a factor of about 3. Furthermore, ammonia being a very mild reagent gas is more selective than methane so that there tends to be less interference from other components of body fluid extracts. Also, the relative intensity of the M-2 peak is significantly lower when ammonia is used rather than methane. Therefore, the Δ^9-THC-d_3-TMS will make less of a contribution to the MH^+ ion current of Δ^9-THC-d_0-TMS. Figure 1 shows the methane and methane-ammonia CI mass spectra of Δ^9-THC-TMS, while

Figure 1

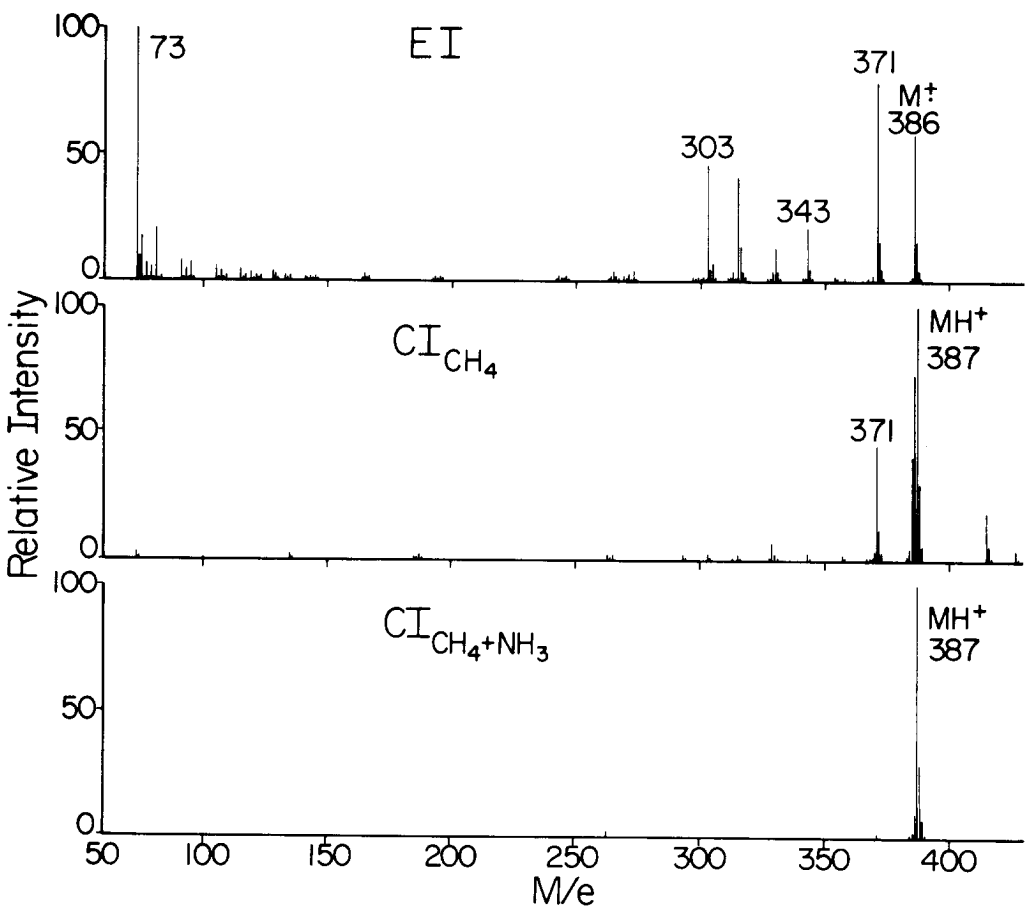
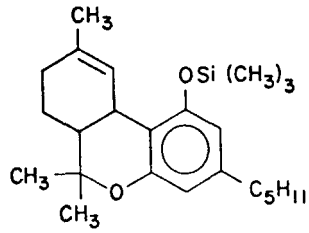


Figure 1. Comparison of mass spectra of Δ^9-THC-TMS

Table 1



Mol. Wt. = 386

Ionization Method	m/e Monitored	% of Sample Ion Current	Relative Response Per Unit Weight of Δ^9-THC
EI	386 (M^+)	7	50
CI (CH_4)	387 (MH^+)	24	20
CI (CH_4+NH_3)	387 (MH^+)	67	100
CI (N_2+NH_3)	387 (MH^+)	67	80

TABLE 1. RELATIVE RESPONSES FOR PROMINENT IONS IN THE EI AND CI MASS SPECTRA OF Δ^9-THC-TMS

Table 1 compares the response per unit weight of Δ^9-THC for different modes of ionization.

A disadvantage of using ammonia as the reagent gas is the lack of a second prominent ion which can be monitored as a check on possible contributions to the MH^+ ion currents by other components of the body fluid extracts. Chemical ionization with methane gives several prominent fragment ions in addition to the MH^+. However, methane is a less selective reagent gas than ammonia, and in our experience intefering peaks are more of a problem with methane than with ammonia. Of course, it is also important to carefully select the GC column and operating conditions to minimize interference from endogenous components of the body fluids. We have found it necessary to use temperature programming of the GC column.

Quantitation of the Δ^9-THC is achieved by monitoring ion masses associated with the Δ^9-THC-TMS and the deuterated internal standard, and measuring the peak height ratios of the ion currents. The actual amount of Δ^9-THC is determined by comparison of the ratio to a standard curve established at the time of each set of analyses. The standard curve is prepared by simply mixing aliquots of standard solutions of Δ^9-THC and Δ^9-THC-d_3, removal of the solvent by evaporation, derivatization, and SIM analysis. Figure 2 shows a typical calibration curve prepared in the above manner. The slope is 1.03 and the zero intercept corresponds to a peak height ratio of 0.0074 or a Δ^9-THC concentration of 0.3 ng. The curve has a correlation coefficient of 1.000. In almost all cases the standard curve has been linear over the concentrations measured, and the

Figure 2

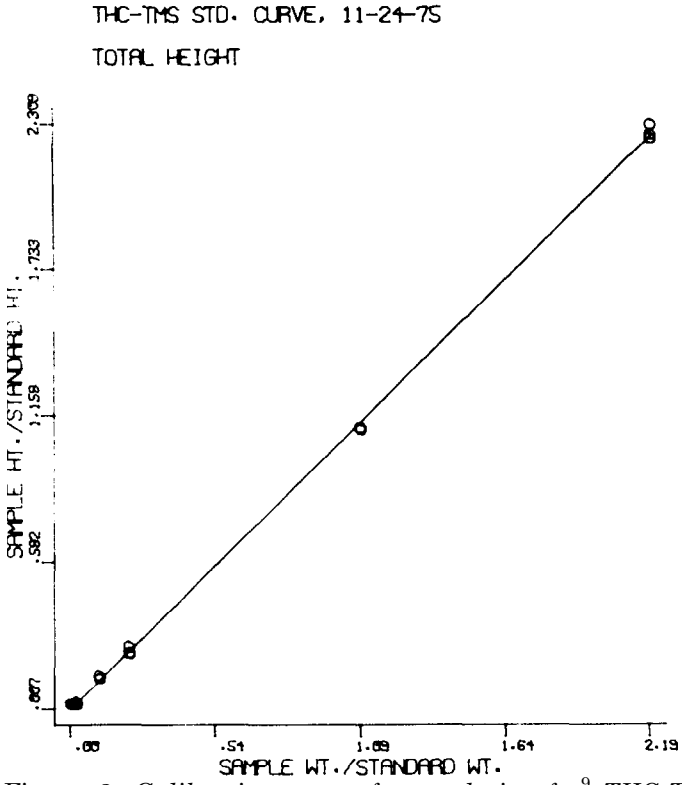


Figure 2. Calibration curve for analysis of Δ^9-THC-TMS

Table 2

Δ^9-THC Added	Sample Ht./Standard Ht. Theoretical	Sample Ht./Standard Ht. Experimental (Average)	Relative Standard Deviation	Number of Determinations
96.8 ng	2.20	2.28	1.23	3
48.4	1.10	1.11	0.32	3
9.7	0.22	0.23	6.08	3
4.8	0.11	0.13	6.65	3
1.0	0.02	0.03	14.00	2
0.5	0.01	0.02	1.80	2
0.0	0.00	0.02		1

TABLE 2. RELATIVE STANDARD DEVIATIONS FOR POINTS ON CALIBRATION CURVE (44.1 NG of Δ^9-THC-d_3 ADDED TO EACH)

relative standard deviation at any given peak height ratio has typically been less than 20, even for the lower concentrations. The relative standard deviations for the points on this particular calibration curve are listed in Table 2. Changes in the slope and intercept of the curve have been observed, but the greatest differences in the Δ^9-THC values assigned to a sample by using different calibration curves have been less than 10%. To allow for changes due to instrument settings or to a change in the concentration of the internal standard solution, we continue to establish a calibration curve for each set of samples.

Table 3 summarizes the precision and accuracy data for a series of analyses. Known quantities of Δ^{9}-THC and Δ^{9}-THC-d_3 were added to 1-ml portions of plasma. The final volume of each derivatized extract was about 25 µl. Injections into the GC were between 2 and 4 µl. The actual quantity of Δ^{9}-THC injected on column is therefore approximately one-tenth of the amount of Δ^{9}-THC added to each ml of plasma. Figure 3 is a typical computer plot of an extract of 1 ml of plasma containing 1 ng of Δ^{9}-THC. In this example the peak in the m/e 387 ion current curve corresponds to about 100 pg of Δ^{9}-THC-TMS injected on column. The corresponding peak in the m/e 390 ion current curve corresponds to a Δ^{9}-THC-d_3 concentration of 44.1 ng/ml. The m/e 390 ion current signal is attenuated by a factor of 10 relative to the m/e 387 ion current signal.

Figure 3

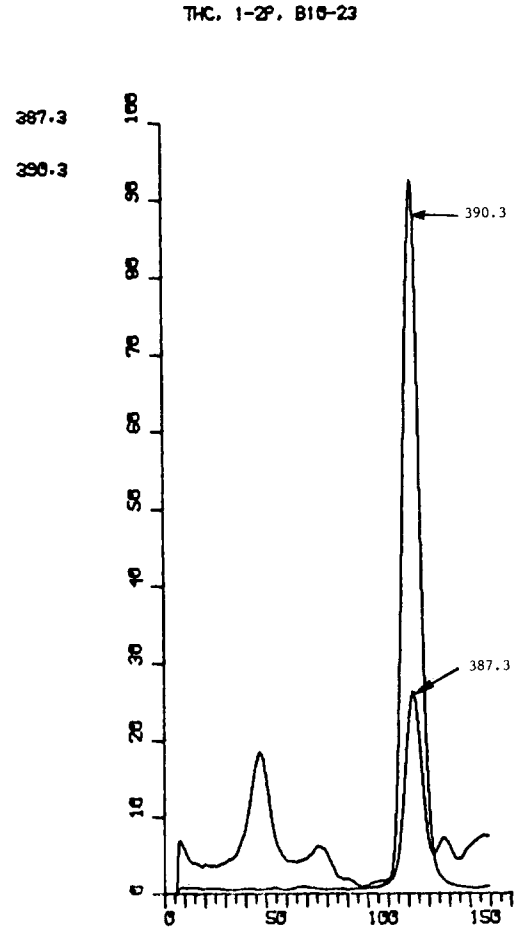


Figure 3. Computer plot of ion currents for m/e 387 and 390 corresponding to 1 NG/ML of Δ^{9}-THC and 44 NG/ML of Δ^{9}-THC-d_3. The m/e 390 ion current curve is attenuated by a factor of 10.

Table 3

Δ^{9}-THC Added (ng/ml)	Δ^{9}-THC Measured (Average)	Relative Standard Deviation	Number of Determinations	Percent Error
9.7	7.7	1.7%	10	21%
4.8	3.8	2.5%	10	21.%
1.0	0.65	6.4	9	35%
0.5	0.28	19.0%	10	44%
0	-0.13[(a)]	15.0%	8	-

(a) The average of the measured weights corresponded to a value below the zero intercept of the calibration curve.

TABLE 3. PRECISION AND ACCURACY DATA FOR MEASUREMENT OF Δ^{9}-THC ADDED TO PLASMA (44 NG OF Δ^{9}-THC-d_3 ADDED PER ML OF PLASMA AS INTERNAL STANDARD)

Figure 4

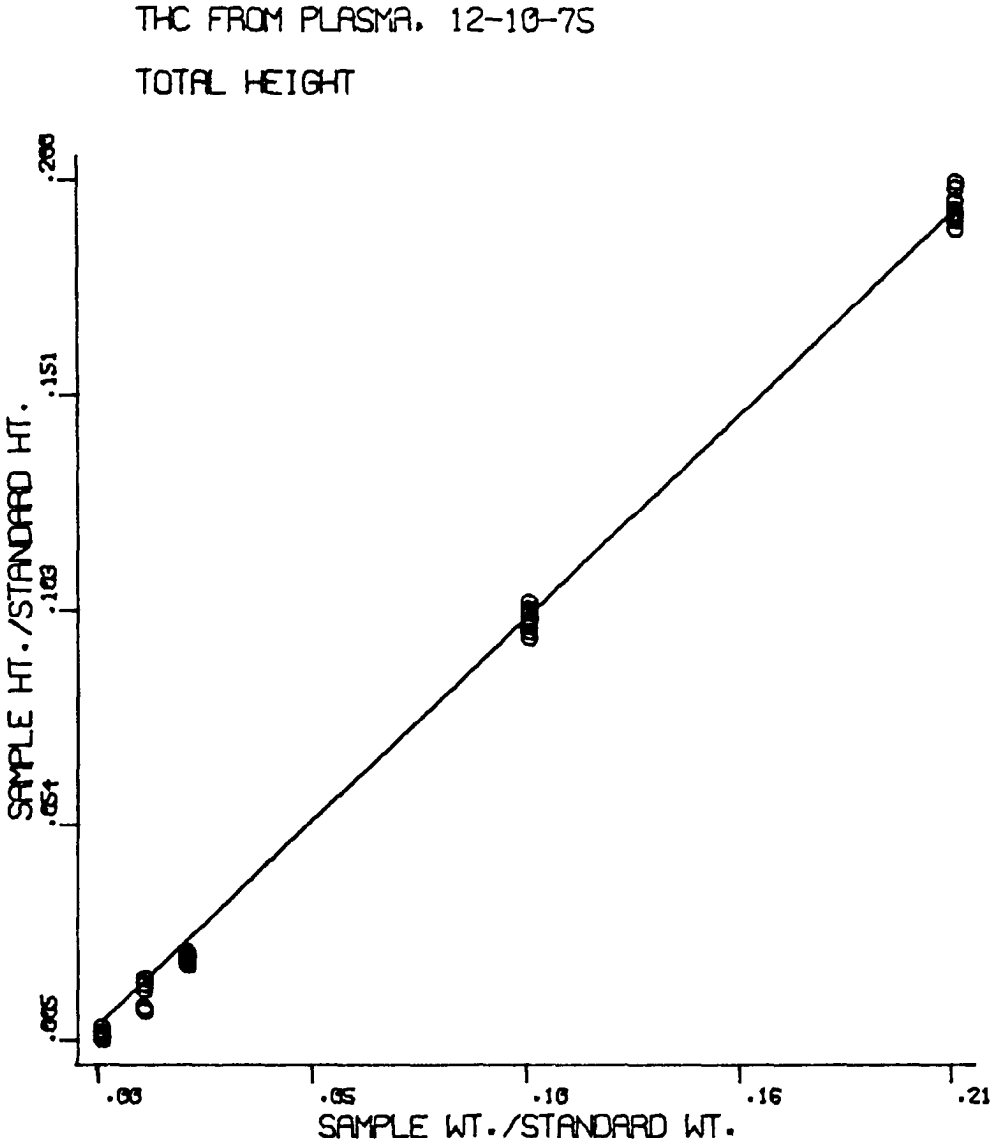


Figure 4. Plot of data points from SIM analysis of extracts of 1-ML plasma samples containing 0 to 9.7 NG of Δ^9-THC and 44.1 NG of Δ^9-THC-d_3.

Finally, Figure 4 plots data points corresponding to 50 separate SIM determinations on 2 sets of extracts from plasmas to which known amounts of Δ^9-THC and Δ^9-THC-d_3 were added. The Δ^9-THC concentrations ranged from 0 to 9.7 ng/ml of plasma.

Using this procedure, we are currently analyzing a variety of plasma samples submitted through NIDA by other researchers. These have included rat, monkey, and human plasma samples with volumes in the range of 0.25 to 2.0 ml. Levels of Δ^9-THC in the samples have been typically in the range of 5-50 ng/ml of plasma, although samples containing concentrations of Δ^9-THC as high as 400 ng/ml of plasma have been analyzed. The actual procedure for analyzing the unknowns is as previously described. To increase our confidence in the integrity of the results for any given set of samples, spiked plasma

samples containing a known amount of Δ^9-THC are analyzed at the same time. These have been useful in pointing out various problems with interferences, and help to establish some confidence limits for the measured values, particularly at the lower levels. In addition, a GC column resolution check and a sensitivity check of the GC-MS are made at the beginning of each day to pinpoint any instrumental problems.

Although further modifications in the analysis scheme are likely, the procedure presently being used has proven suitable for the analysis of Δ^9-THC in plasma to concentrations as low as 0.5 ng/ml and can be conveniently used for relatively large numbers of samples.

Experimental Section

The plasma sample (usually ∿1 ml) is transferred to a 16-ml culture tube using a calibrated pipet and the volume is recorded. To this is added 1.0 ml of pH 7.0 buffer solution and 100 µl of 441 ng/ml solution of Δ^9-THC-d_3 in ethanol. The mixture is vortexed for 30 seconds. The plasma is then extracted with 5 ml of Distilled-in-Glass(R) hexane for 30 minutes on a rotator. The mixture is centrifuged and the hexane extract transferred to a second tube. The hexane extraction is repeated and the extracts combined. The combined hexane extract is successively washed with 2.5 ml 0.1 N NaOH, and 2.5 ml 0.1 N HCl. The hexane extract is transferred to an evaporation tube and evaporated to dryness under a stream of nitrogen using a Kontes tube heater maintained at below 50 C. The tube is rinsed with 1 ml of Distilled-in-Glass(R) pentane and the pentane solution transferred quantitatively to a 1.0-ml Reacti-Vial(R). The pentane is removed under a stream of nitrogen at room temperature. The sides of the vial are washed down with 100 µl of pentane, and the pentane is again evaporated. To the vial is added 25 µl of BSTFA with 1% TMCS. The vial is capped using a Teflon liner and heated for 2 hours at 75 C.

The concentration of Δ^9-THC is determined using the technique of selected ion monitoring with a Finnigan 3200 GC-MS. A 6 ft x 2-mm glass column packed with 3% OV-17 on 100/120 mesh Gas Chrom Q is temperature programmed from 180 to 280 C at 10°/minute. Methane is used as the carrier gas while a small amount of ammonia is bled into the ion source through a make-up gas inlet. The partial pressure of ammonia in the ion source is 100 µ. The flow rate of methane is 20 ml/min giving a partial pressure in the ion source of about 700 µ. Other mass spectrometer parameters are: electron energy, 210 v; ion energy, ∿15 v; lens voltage, ≈30 v; electron multiplier voltage, 1.4 to 1.6 KV; and filament emission, 0.8 to 1.0 ma.

Cross-contamination from the syringe used for injecting the samples into the gas chromatography is avoided by rinsing 10-20 times with pentane between injections.

For *every* 5 unknowns, a spiked sample containing 9.7 mg Δ^9-THC/ml of plasma is prepared by adding 100 µl of a 97 ng/ml solution of Δ^9-THC in ethanol to 1 ml of human plasma. This is extracted in the same manner and at the same time as the unknowns.

A standard curve is established at the time of each set of analyses by adding 100 µl of the 441 ng/ml Δ^9-THC-d_3 solution in ethanol and 100 µl of one of various concentrations of Δ^9-THC in ethanol directly to a Reacti-Vial(R). A series of four such standards with d_0/d_3 ratios ranging from 0-1 for ∿50 ng of Δ^9-THC-d_3 are prepared. The ethanol is removed at below 40 C, and the THC derivatized in the same manner as the unknowns: 25 µl of BSTFA with 1% TMCS is added, and the vial is heated at 75 C for 2 hours. These standard samples are analyzed by monitoring the m/e 387 and 390 peaks of the mass spectrum. Since there should be no interferences in these standards, the gas chromatographic column is used isothermally at 205 C. The values for the ratio of d_0/d_3 are used to prepare a standard curve.

All reusable glassware used in the analysis is rinsed with methanol, cleaned with detergent and water, rinsed with water, soaked in hot sulfuric and nitric acid solution, rinsed successively with tap water, distilled water and methanol, and finally dried in an oven at >100 C.

Aliquots of standard Δ^9-THC and Δ^9-THC-d_3 solutions are measured out

using a 100-µl Eppendorf pipet with disposable tips. Syringes used for measuring out BSTFA or injecting samples into the GC-MS are cleaned in methanol. The hexane and pentane used are high-purity, Distilled-in-Glass(R) solvents.

The concentration of the Δ^9-THC-d_3 stock solution in ethanol was determined by comparison with a primary standard of Δ^9-THC in ethanol using SIM analysis.

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HPLC-MS DETERMINATION OF Δ^9-TETRAHYDROCANNABINOL IN HUMAN BODY SAMPLES

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INTRODUCTION

Social and chronic abuse of cannabis is believed to occur throughout the United States. However, most information on the societal use of marijuana, in particular, comes from question-response type surveys. Precise quantitative data obtainable via body specimen analysis has been unavailable due mainly to the lack of an accurate biological assay technology for the chemical constituents found in cannabis.

The marijuana commonly smoked in this country contains four principal constituents, viz., Δ^9-tetrahydrocannabinol (Δ^9-THC), cannabidiol (CBD), cannabinol (CBN) , and cannabichromene (CBC) (Doorenbos, et al., 1971). One of these constituents, Δ^9-THC is believed to be responsible for the psychomimetic properties of marijuana (Edery, et al., 1971). Likewise some of the physiological responses in man have been shown to change following smoking of cigarettes containing Δ^9-THC and these correlate with plasma levels of Δ^9-THC and its metabolites (Galanter, et al., 1972). However, such studies were accomplishable only by administering radiolabeled Δ^9-THC to the study subjects and following its deposition in accessible body fluids.

More recently there have been a number of methods proposed for determination of Δ^9-THC in blood plasma. A glc procedure which utilized an electron capture detector was detailed and reported to be capable of detectins 0.5 nq per 1 ml of blood plasma (Fenimore; et al., 1973). This method which required precolumn derivatization and a dual oven apparatus was not used to assay the blood plasma of actual cannabis smokers. Another reported method employed glc-mass fragmentography with d_2-Δ^9-THC as the internal standard (Agurell, et al., 1973). This later method was employed to analyze 5 ml samples of blood plasma from three volunteers each of whom smoked a cigarette containing 10 mg of Δ^9-THC. A chromatographic elution of the blood plasma

on a Sephadex column was required prior to glc-ms analysis. Blood samples were taken from the volunteers at 0, 0.2, 0.5, 1 and 2 hours following smoking. Peak levels of Δ^9-THC were found to be 19-26 ng/ml at 10 minutes following smoking. The levels declined to 5 ng/ml or less at 2 hours. Another method which has been reported to be capable of detecting Δ^9-THC is based upon radioimmunoassay procedures (Teal, et al., 1974 and Gross, et al., 1974).

The method reported herein makes use of a high pressure liquid chromatography-mass spectrometry (hplc-ms) technique for determining ng/ml quantities of Δ^9-THC in human body specimens. Problems associated with direct coupling of the hplc to the ms have been circumvented by collecting fractions of the mobile phase eminating from the hplc and subsequently analyzing the fraction via the direct insertion probe of the ms. Inherent in this method is the use of d_3-Δ^9-THC for controlling extraction efficiency, as a marker for collection of the hplc effluent and as a convenient internal standard for ms quantification. Thus by this method the body specimen has enough d_3-Δ^9-THC added to allow its detection by a u.v. spectrophotometer connected to the output of the hplc. Once the d_3-Δ^9-THC is detected, fractions of the mobile phase eluant are collected and introduced to the ms via the direct insertion probe for quantification. This method was then validated in various human body samples over the concentration range of 1-100 ng/ml or ng/g depending upon the specimen of interest.

The new assay technology was then used to determine the blood plasma levels of Δ^9-THC in eleven male volunteers during a 24 hour period following smoking of a marijuana cigarette. Also the new procedure has been used to determine the presence of Δ^9-THC in exhaled breath and saliva of volunteers following marijuana smoking. Similarly, a correlation has been made, using the method, between the blood plasma, bile and brain levels of Δ^9-THC in human post-mortem specimens. The method has also been used to analyze human blood samples from a Δ^9-THC aerosol inhalation study on a blind basis.

EXPERIMENTAL

High pressure liquid chromatography

All hplc analyses were conducted on a Varian 8520 gradient elution liquid chromatograph utilizing a Varian 635M spectrophotometer set at 273.7 mμ as the detector. The column was a 10 μ silica gel (Varian Si-10) , 25 cm X 2 mm (i.d.). A gradient elution program was developed using heptane and methylene chloride. For a satisfactory separation of the cannabinoids as well as to assure accurate ms quantitation, it was necessary to routinely record the u.v. spectrum of each lot of heptane and methylene chloride prior to its use in the gradient program. Only those lots of heptane and methylene chloride which gave minimum absorbance in the region of 260-280 mμ were used.

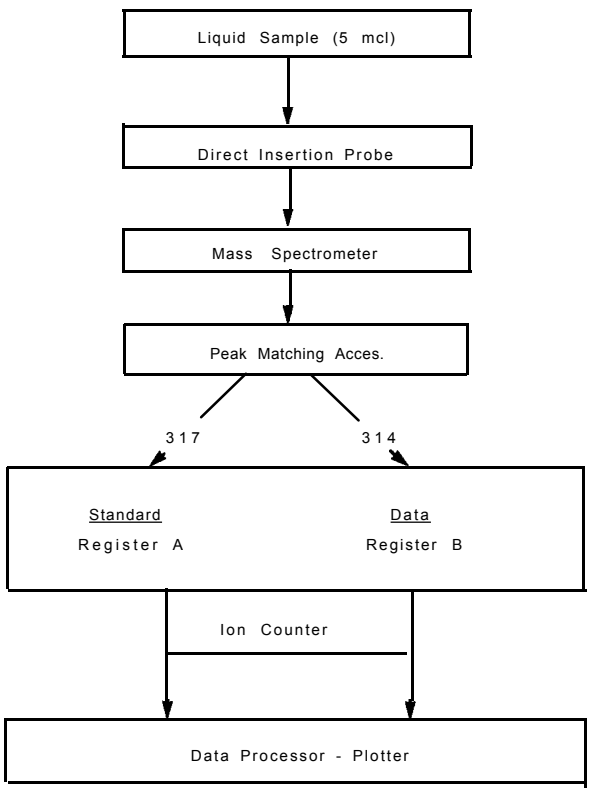
The gradient elution program, developed for this application, began at 95:5%, heptane:methylene chloride and proceeded to 95:5%, methylene chloride:heptane over 9 minutes. The program was reversed, i.e., from 95:5% methylene chloride:heptane to the initial 95:5% heptane:methylene chloride mixture, thereby regenerating the column. A solvent flow rate of 120 ml/hr was used for all determinations. By employing these conditions Δ^9-THC as well as d_3-Δ^9-THC were found to have a retention time of 4.7 minutes, i.e., they appear at a gradient elution mixture of 52:48 methylene chloride:heptane. The other major constituents of marijuana, CBD, CBN and CBC were found to have retention times of 4.4, 4.6 and 5.6 minutes, respectively. The amount of d_3-Δ^9-THC added to the body sample was sufficient to allow u.v. detection of Δ^9-THC (labeled plus unlabeled) as it eminated from the column. 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 passing through the flow cell.

Mass spectrometer quantification

All ms analyses were accomplished using a Varian MAT SMI-B high resolution, double-focus mass spectrometer. A new ion-counting technique

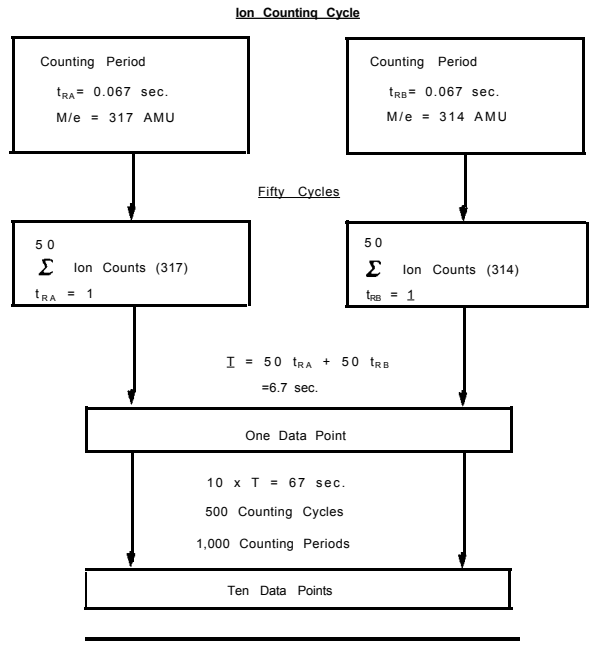
was developed in conjunction with the peak matching accessory which provided for a rapid comparison between data from the internal standard (d_3-Δ^9-THC, mass 317) and the assayed compound (Δ^9-THC, mass 314). Each sample from the hplc was introduced into the ms via the direct insertion probe. The instrument was 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 1.

Figure 1



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 Princeton Applied Research Model SSR1110 dedicated computer. 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

Figure 2



stored separately by the counter as shown schematically in Figure 2. 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 combined with a probe temperature of 65° gave optimum results. Thus in 67 seconds the alternating cycle is repeated 500 times yielding 1000 bits of data for comparison and quantification. With time the sample is depleted but the ratio based on the internal standard remains linear and provides for dependable quantification as shown in Figure 3. The curve shown in this figure was obtained from a Hewlett-Packard 9820 computer-plotter by using the data from register B for the ordinate and register A for the abscissa and determining the least square best straight line. The slope of the data line gives the 314/317 ratio. The amount of Δ^9-THC (mass 314) is determined by multiplying this ratio times the known amount of internal standard added. In actual practice the d_3-Δ^9-THC employed contained a small amount of undeutrated Δ^9-THC (mass 314). A ms determination of this amount of mass 314 was made by analyzing 10 samples of 1.6 µg d_3-Δ^9-THC which had been added to 1 ml of blood plasma by the

Figure 3

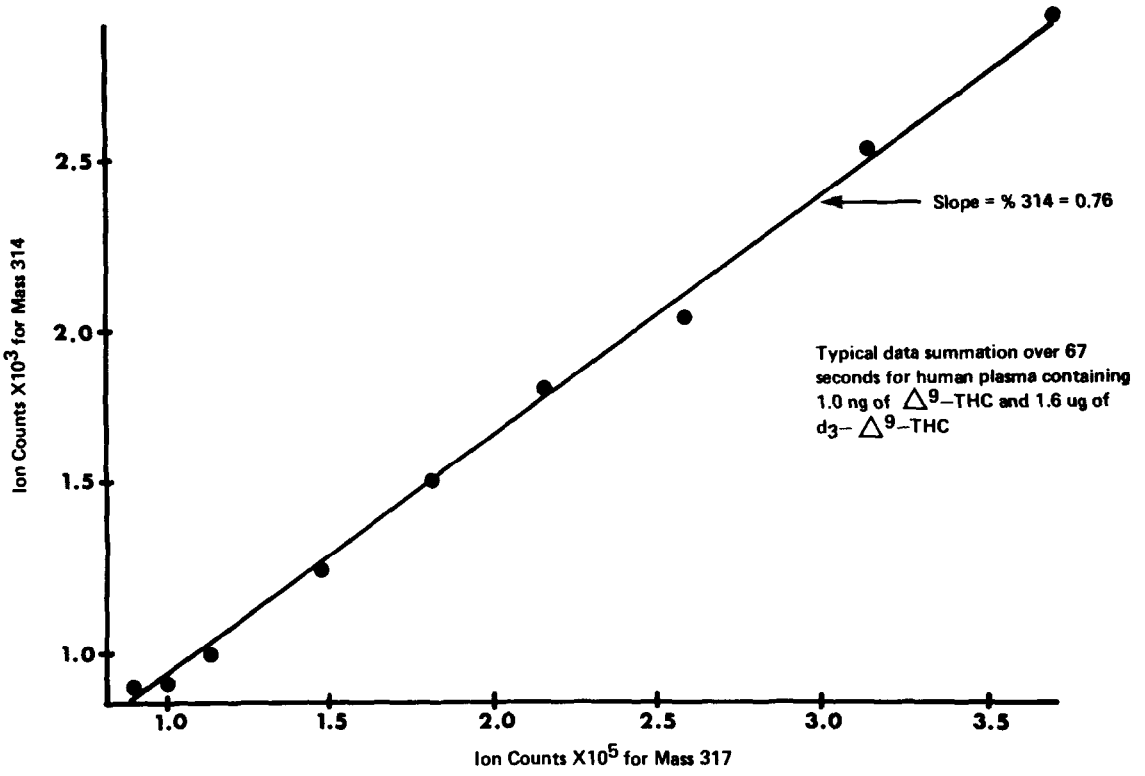


Table 1

Table 1. Precision and Accuracy in Recovery of Δ^9-THC Added to Human Plasma.

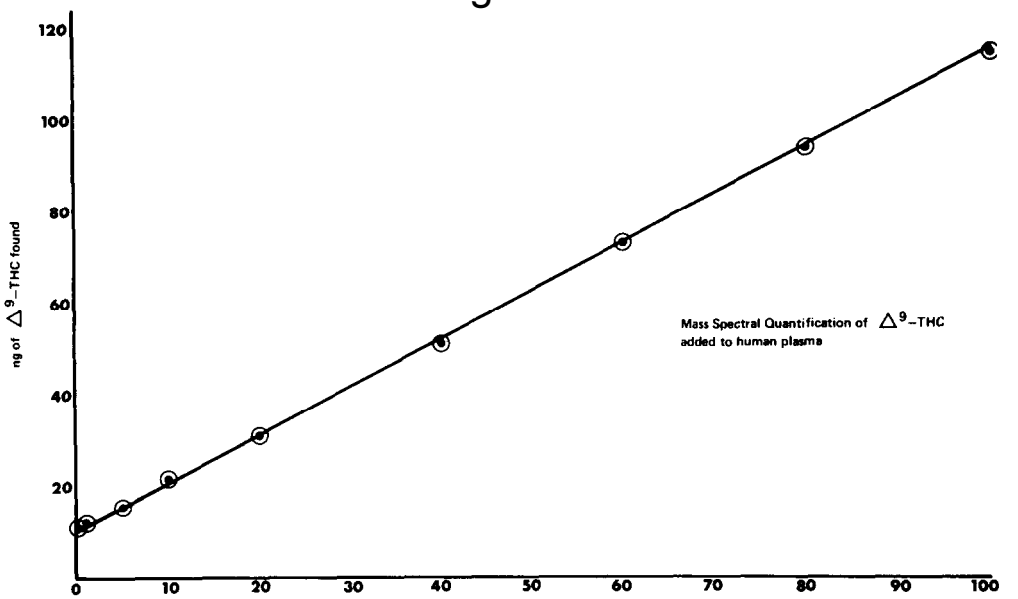
Added ng/ml	Found,[a] ng/ml		n	±RSD	RE
0	11.3	(9.35-13.6)[b]	10	11.15	--
1	1.2	(0.6-1.5)	3	43.3	20.0
5	4.5	(4.0-4.7)	3	9.0	-11.12
10	10.5	(9.5-12.4)	10	10.0	5.0
20	20.0	(19.1-21.1)	3	5.14	0.0
40	41.2	(39.6-42.5)	3	3.58	3.0
60	62.1	(61.3-62.8)	3	1.26	3.5
80	82.8	(74.9-90.7)	3	9.55	3.5
100	103.7	(99.3-106.0)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

[a] Average (range) of n determinations.

[b] This value represents the amount of unlabeled Δ^9-THC in 1.6 µg of d_3-Δ^9-THC. Other found values have been corrected for this amount.

method described above, cf. Table I. This background value for Δ^9-THC (mass 314), inherently present in the internal standard, is subtracted from the total amount of Δ^9-THC recorded to give the actual found value.

Figure 4



Methods of blood plasma analysis

All glassware used was silinized using a reported method (Garrett and Hunt, 1974). Whole blood was centrifuged at 2600 rpm for 20 minutes to obtain blood plasma. To 1 ml of blood plasma was added 1.6 µg of d_3-Δ^9-THC followed by three repetitive extractions with 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 injected into the hplc. A 100 µl wash of heptane was used on the vessel which contained the extracts and it was also injected into the hplc and the gradient elution program begun as described above. When the peak for Δ^9-THC was noted on the recorder, the eluant was collected in a silinized screw cap vial. Collection of the eluant was synchronized with the recorder tracing such that equal amounts were obtained from either side of the symmetrical peak. In general, approximately 1 ml of eluant was collected for each Δ^9-THC peak. Samples thus collected from the hplc were stored at -5° until ms analysis. Prior to ms analysis each sample was evaporated under nitrogen at room temperature to approximately a 10 µl volume. A microsyringe was used to transfer this solution in two portions to a 5 µl gold cup. The solution was allowed to air evaporate and the gold cup was introduced into the ms by attaching it to the direct insertion probe. Quantification was accomplished as described above.

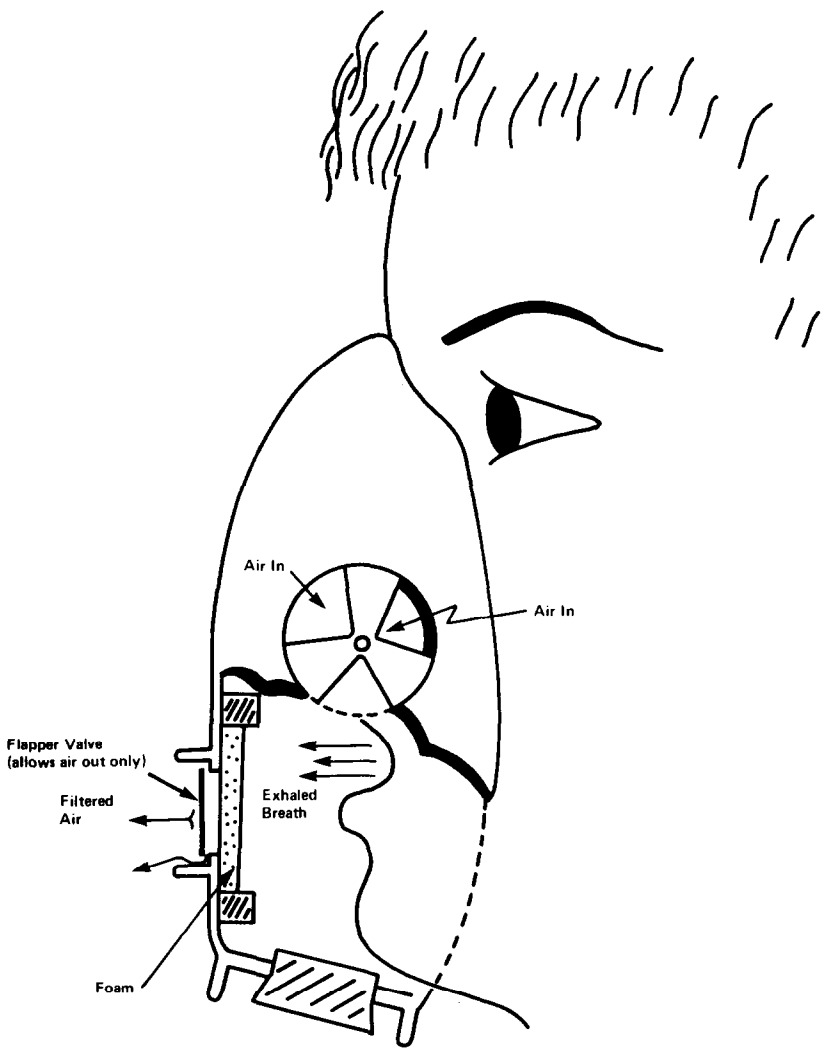
Validation of the assay method in blood plasma

Blood from 10 laboratory workers known to be non-users of marijuana was drawn and analyzed as detailed above. These blood plasmas constituted the control samples. Each sample contained some background amount of Δ^9-THC (mass 314) since the 1.6 µg of d_3-Δ^9-THC, added to each plasma sample, contained an average of 11.3 ng of Δ^9-THC (mass 314), cf. Table I. 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 I is a summary of this study. Figure 4 is a plot of the data given in Table I. As outlined above, the intercept in Figure 4 does not pass through the origin or zero value since some Δ^9-THC (mass 314) is present in the d_3-Δ^9-THC used as the internal standard.

Methods of breath analysis

For human breath collection a standard face mask (Welch model 7500-30G) was modified by placing a 2.5 cm diameter port directly in front of the mouth. On the interior of the mask was placed a 4.5 cm (o.d.) by 2.0 cm thick rubber ring of sufficent flexibility to firmly hold a specially molded polyethylene filter. The polyethylene was formed into a small wafer size, i.e., a disc 3.0 cm in diameter and 0.25 cm thick. This polyethylene wafer was thus held directly in front of the subject's mouth and approximately 1.5 cm away from the lips. Figure 5 gives a cross-sectional view of this modified mask. For actual breath collections each subject was asked to breathe for one minute with the mask positioned over the nose and mouth using deep inhalations and exhalations through the mouth. The polyethylene wafers were placed in and taken from the mask using disposable examination gloves. After breath collection, each sample was placed in 10 ml of methanol in a silinized beaker and then ultrasonicated for 30 minutes. To each extract was added 1.6 µg d_3-Δ^9-THC and the solution was evaporated to dryness at room temperature, under nitrogen. The residue was reconstituted in 300 µl of heptane and analyzed via hplc-ms as described above.

Figure 5



Method of saliva analysis

Saliva was collected from each volunteer in silinized collection tubes and immediately frozen until analysis. To 100 µl of saliva was added 1.6 µg of d_3-Δ^9-THC followed by extraction 3 times with 2 ml of petroleum ether for each extraction. The extracts were combined and evaporated to dryness under nitrogen at room temperature. The residue was reconstituted in 300 µl of heptane and analyzed by hplc-ms as discussed above.

Method of bile analysis

Bile taken at post-mortem examination was frozen until analysis. To 100 µl of the bile was added 1.6 µg of d_3-Δ^9-THC and 1 ml of pH 7.0 buffer. The mixture was extracted 3 times with 2 ml of petroleum ether for each extraction and the extracts combined and evaporated to dryness under nitrogen at room temperature. The residue was reconstituted in 300 µl of heptane and analyzed by hplc-ms as discussed above.

Method of brain analysis

Brain samples from the cerebrum taken at post-mortem examination were frozen until analysis. To 5 g of brain sample was added 20 ml of pH 7.0 buffer and 1.6 µg of d_3-Δ^9-THC. The entire mixture was homogenized then extracted 3 times with 10 ml of petroleum ether each time and the extracts combined and evaporated to dryness under nitrogen at room temperature.

Marijuana smoking studies

Eleven healthy male volunteers between the ages of 21 and 26 were used in the marijuana smoking studies. Each subject was within 10% of ideal body weight and received both medical and psychological exams prior to admission. Values for the following tests were determined prior to the study and each subject was required to be within the normal range: electrocardiogram, chest x-ray, creatinine, BUN, LDH, SGOT, 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 hrs. prior to smoking and they were not allowed any 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 silinized heparin vacutainer 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 removed and placed in another silinized tube and frozen for later analysis. This initial 5 ml of blood drawn prior to smoking constituted the 0 hr sample. Each subject was then allowed to smoke one marijuana ci arette which contained 10.8 mq 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 (5 ml) were withdrawn at 0.25, 0.5, 1, 2, 3, 4, 12 and 24 hours. Each blood sample was handled as described above. At the same time intervals saliva and breath samples were taken from each subject.

Δ^9-THC aerosol and oral administration studies

Two normal male volunteers, who met the same physical criteria listed for the marijuana smoking volunteers, were used. Each subject was administered an aerosol spray which contained 10 mg of Δ^9-THC. Blood samples were taken from each subject at 0, 0.25, 0.5, 1, 1.5, 2.0, 3.0, 4.0, 5.0 and 6.0 hours. Samples were supplied to our laboratory with a code number. In addition to these samples, some additional blood plasma samples from subjects in an oral cannabanoid study were also coded and submitted for analysis. All samples were analyzed as discussed above.

RESULTS AND DISCUSSION

Analysis of trace substances in a biological system is often limited by the sensitivity of an analytical method. This was precisely the case with the analysis of Δ^9-THC since prior to the present work there were

not many analytical methods which offered the selectivity and sensitivity needed. The newly developed method reported herein was shown to be reproducible, accurate and linear in the range of 1-100 ng/ml of human blood plasma. Use of hplc rior to ms quantification permits Δ^9-THC to be selectively separated from the other cannabinoids present in marijuana preventing an erroneous analysis with the other mass 314 cannabinoids, viz., CBD and CBC. Also when the ms analysis is performed the high resolution capability of the ms prevents any possible confusion of compounds, i.e., only Δ^9-THC will have the correct mass number.

Use of the stable isotopic' form of Δ^9-THC in the developed method allows for a control of extraction efficiency as well as a marker for peak collection and quantification. Thus the d_3-Δ^9-THC was added to the plasma prior to extraction and gave a control for any losses which might occur during extraction or chromatographic procedures. In the described method the plasma was extracted three times. Ordinarily three extractions would be redundant when an internal standard has been added. However, the main reason for using the multiple extractions is to introduce to the ms a sufficient amount of the stable isotope for accurate quantification while not using excessive amounts of the isotope for the internal standard. The other important use of the d_3-Δ^9-THC was to permit the total amount of Δ^9-THC (both labled and unlabled) to be detectable by the u.v. spectrophotometer attached to the output of the hplc since Δ^9-THC has a low extinction coefficient.

A human marijuana smoking study was conducted and blood samples taken at appropriate time intervals for 24 hours. Each sample was analyzed by hplc-ms and the results are given in Table II. As observed from the mean values in this table, the peak blood plasma concentration of Δ^9-THC occurs in all individuals, except L.G., at 0.25 hours. Also worthy of note in Table II is the fact that most subjects showed a level of Δ^9-THC in their control samples. This is readily explainable since all subjects were prior users of marijuana and most likely had smoked sometime before reporting to the study.

The breath samples from each subject in the controlled smoking study were also analyzed by the new method. Since the volume of exhaled air was not measured, no meaningful quantification data could be obtained. That is, the subjects were asked to breathe through the breath apparatus for a period of one minute and in this time the rate of exhalation in

Table 2

Table 2. Amount of Δ^9-THC (ng/ml) found in human blood plasma following marijuana smoking.

	TIME (hrs.)								
SUBJECTS	0	. 2 5	.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

Table 3

Table 3.
Blood Plasma Levels of Δ^9-THC

	Code No.[a]	Time (min) Following Δ^9-THC	ng of Δ^9-THC
SUBJECT 1	11A	0	7.4
	10A	5	30.3
	7A	15	9.6
	16A	30	4.3
	22A	60	14.6
	12A	90	15.2
	13A	120	14.9
	5A	180	lost[b]
	15A	240	24.5
	4A	300	20.3
	2A	360	4.9
SUBJECT 2	18A	0	2.2
	19A	5	10.4
	14A	15	13.3
	20A	30	11.8
	9A	60	no sample[c]
	3A	90	13.6
	17A	120	no sample[c]
	8A	180	7.1
	21A	240	7.6
	1A	300	1.4
	6A	360	lost[b]
ORAL CANNABANOID SUBJECTS[d]	3	0[e]	0.1
	4	300[f]	0.0
	5	300[g]	lost[b]
	6	300[e]	0.0
	7	300[e]	0.0
	8	0[f]	0.4
	9	0[g]	0.0
	10	0[f]	4.0

[a] Code number of sample as supplied by Dr. Tashkin.
[b] Laboratory accident resulting in sample loss.
[c] Sample broken in transit.
[d] All subjects were confined in the hospital ward for 4 days prior to receiving the oral formulation.
[e] Receiving oral CBN.
[f] Receiving oral CBD.
[g] Receiving oral Δ^9-THC.

each subject may have varied. More important, however, was the fact that a positive and statistically meaningful level of Δ^9-THC could be determined in the breath of these known marijuana smokers during the initial 60 minutes following smoking.

Saliva samples from marijuana smokers have to be quantitatively treated very much like the breath samples. That is, in the present study no attempt was made to accurately control the amount of saliva exudated. Thus any attempt to quantitatively assign levels of Δ^9-THC per unit volume would be meaningless. However, it was observed by analyzing the saliva samples that a positive, statistically significant level of Δ^9-THC could be detected in the saliva of known marijuana smokers during the initial 60 minutes following smoking.

Analysis of the blood samples which came from a Δ^9-THC aerosol study provided an opportunity to analyze samples on a blind basis. The data obtained from this study is shown in Table III. Positive levels of Δ^9-THC for the control samples (0 hour) is readily explainable since each subject was a marijuana user and was not confined prior to the study. In contrast, however, the oral cannabanoid subjects were confined for 4 days prior to the collection of their control samples. Also as shown in Table III, other cannabanoids were being administered which could have conceivably interfered with the Δ^9-THC determination. However, the hplc program successfully separated the Δ^9-THC fraction as was verified by correlation following the blind sample assays. For both studies there was a good correlation between the physiological states recorded following administration and the Δ^9-THC levels measured later by means of the quantification technology reported in this paper (Tashkin, 1976).

CONCLUSIONS

A method was developed for quantitatively determining Δ^9-THC in human body specimens. The method was shown to be accurate and precise over a concentration range of 1-100 ng/ml in human blood plasma. This methodwas used to assay blood plasma, breath and saliva of human subjects following smoking of a marijuana cigarette. Results from these studies demonstrated that during the early time intervals following marijuana smoking the levels of Δ^9-THC measured are of Sufficient magnitude to be clearly discernable from the amount of Δ^9-THC (i.e., 11.3 ng) added in the internal standard.

The newly developed method has also been used to study the relationship between Δ^9-THC levels in blood plasma, bile and brain of post-mortem samples. Such data has revealed that Δ^9-THC levels are higher in brain and bile than in blood plasma.

Data was also accumulated on blood plasma samples which came from subjects receiving either an aerosol formulation of Δ^9-THC or oral formulations of CBD, CBN and Δ^9-THC. Analysis of these samples on a blind basis provided further validation of the new technology and gave results which were consistant with the observed physiological responses.

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ANALYTICAL METHODS FOR THE DETERMINATION OF CANNABINOIDS IN BIOLOGICAL MEDIA

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ABSTRACT

A pharmacokinetic tudy of the blood plasma levels in man of $\Delta^{1,9}$-tetrahydrocannabinol, 11-hydroxy-$\Delta^{1,9}$-tetrahydrocannabinol and cannabinol has been carried out by means of combined gas chromatographic-mass spectral analysis. In some cases comparison of the data was obtained on the same sample using thin layer chromatography of radiolabeled samples and electron capture gas-liquid chromatography. For the mass spectral studies appropriately deuterium labeled analogs of the previously named compounds were used both as internal standards and as a carrier for the relatively small amounts of non-labeled drug present in plasma. Blood samples were obtained at periodic intervals up to 24 hours from volunteers receiving 4-5 m g $\Delta^{1,9}$-THC intravenously. After extraction and "clean-up" by Sephadex chromatography, the extracts were concentrated and subjected to glc-ms in the electron impact (ei) mode or alternatively with a chemical ionization (ci) source, in which case preliminary chromatography could be omitted. In all cases calibration curves were obtained from replicate analyses of spiked plasma containing the internal standard and various quantities of the cannabinoid under analysis. A typical biphasic elimination of the drug was observed with rapid elimination of $\Delta^{1,9}$-THC from the blood over a period of 40 min followed by a much slower elimination up to 24 hours. The experimental data show that 11-hydroxy-$\Delta^{1,9}$-THC is found in the plasma in quantities only of about one-twentieth to one-twenty-fifth the values found for $\Delta^{1,9}$-THC. Cannabinol was not found in significant quantities. Good agreement was obtained between the mass spectral analyses and the thin layer chromatography or electron capture gas-liquid chromatographic procedures.

INTRODUCTION

In recent years there has been a great increase in interest in the pharmacology, metabolism and biodisposition of the cannabinoids [for recent reviews cf. Mechoulam, Paton and Crown, Wall (1975) and Wall, et al. (1975)]. Until recently quantitation of the various cannabinoids in blood, urine, feces and other biological tissues could be carried

out only by means of the use of appropriately radiolabeled analogs of the cannabinoids under study (Wall, et al. (1975) and Lemberger) . Because of the widespread and increasing opposition to the use of radiolabeled isotopes in studies involving man and because many of the studies currently being conducted with various cannabinoids involve the use of large scale experiments in which radiolabeled cannabinoids are not used, the need for the development of non-radiolabeled quantitative methodology for certain key cannabinoids has become increasingly apparent. In addition, the use of radiolabeled thin layer chromatography techniques, while useful in initial studies, lacks sufficient accuracy in the sense that when-biological extracts are studied, separation of Δ^{9}-THC from cannabinol and 11-hydroxy-Δ^{9}-THC from other monohydroxylated analogs is poor. If such interfering substances are present in considerable quantity, one would obtain erroneously high values, This will increasingly be the case when one is analyzing biological materials obtained from marihuana smokers which contain Δ^{9}-THC, cannabinol, cannabidiol, and 11-hydroxylated analogs of these compounds.

Quantitative gas liquid chromatography combined with mass spectrometry (glc-ms) has been used with excellent results for the quantitative analysis of drugs in biological materials, combining as it does the separative powers of glc and the inherent sensitivity of ms detection. Pioneer studies by Hammar, Holmstedt and Ryhage introduced the concept of mass fragmentography [now also called multiple ion detection (MID)] and greatly increased the sensitivity of ms methodology so that it could be applied to the nanogram and picogram levels. The concept has been applied to many drugs and recently by Agurell, Holmstedt and co-workers to the determination of Δ^{9}-THC in blood plasma. We wish to present in this paper methods for the quantitative determination by combined glc-ms of Δ^{9}-tetrahydrocannabinol (Δ^{9}-THC), 11-hydroxy-Δ^{9}-THC and cannabinol (CBN) in human plasma and results on the same samples obtained by the tlc-radiolabeled or electron capture glc procedures.

METHODS

Clinical protocol

Human, male volunteers who were experienced marihuana users were administered 4.0-5.0 mg of Δ^{9}-THC by the intravenous infusion method of Perez-Reyes et al. The volunteers were kept under medical supervision for 24 hours in the Clinical Research Unit of the University of North Carolina, School of Medicine. Blood samples (10 ml approximately) were collected at periodic intervals over a period of 24 hours. Plasma was obtained by centrifugation, immediately frozen and stored in frozen condition until analyzed.

Internal standards

A key feature of our quantitative procedures was the use of appropriate deuterated analogs of the cannabinoids under study as both carriers for the small quantity of cannabinoids expected to be present in many cases and as internal standards for quantitation by mass spectrometry. The structures of the cannabinoids and their deuterated analogs used in these studies are shown in Figure 1. All of

Figure 1

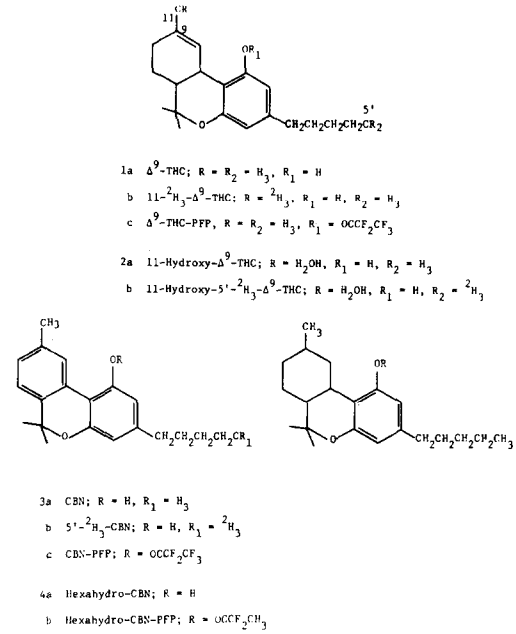

Figure 1.
Structure of Cannabinoids and Internal Standards

the compounds used were synthetic and were made available by the National Institute on Drug Abuse Synthesis Program.[1] Synthetic methods for the various deuterated cannabinoids utilized in these studies have been presented by Pitt, et al.

Analytical procedures prior to glc-ms

General Precautions.--Close attention must be paid to the precedural details presented below in order to obtain reproducible and quantitative data. In general in working with cannabinoids exposure of samples or extracts

to light or air should be minimized. All solvent evaporations should be conducted in vacuo or under nitrogen at low temperature. Cannabinoids in nanogram levels are subject to adsorption on the surface of glassware. In order to minimize this problem all glassware, including chromatography columns, were coated by silylation using 5% DMCS in toluene.

Extraction and Purification Prior to Analysis by GLC-MS in Ei Mode.--When the mass spectrometers were operated in this mode the molecular ions or charged fragments utilized for the quantitative analysis of underivatized cannabinoids were in a range of m/e of 320 or lower. Preliminary studies-with plasma extracts indicated that interference from endogenous plasma constituents would be encountered. This could be avoided by carrying out a preliminary cleanup by Sephadex LH-20 chromatography prior to the glc-ms step. The methods which are pretsented are for the combined determination of Δ^9-THC (1a), 11-hydroxy-Δ^{9}-THC (2a), and cannabinol (3a). The methods, of course, are equally utilizable for the determination of individual constituents. Deuterated internal standards (cf., Figure 1) were added to a sample of 3.0 ml of cold (not frozen) plasma as follows: 1b, 150.0 ng; 2b, 15.0 ng; and 3b, 15.0 ng. Each internal standard was added in 15-30 µl ethanol. Following addition of each internal standard the plasma sample was stirred for 3-5 seconds in a vortex agitator and then subjected to sonication (Cole-Parmer ultrasonic cleaner) for the same time. The plasma samples (contained in a screw capped centrifuge tube) were then extracted 4 times with 6.0 ml petroleum ether (bp 30-60°, Nanogram Grade or Burdick and Jackson) containing 1.5% isoamyl ether. The tubes were agitated 15 minutes each time in a vortex agitator and the layers separated by centrifugation after each extraction. The petroleum ether extracts were combined, evaporated in vacuo at room temperature and freeze dried overnight to remove water and isoamyl alcohol. The dried residue was dissolved in a minimal volume of petroleum ether/chloroform/ethanol (10:10:1) and chromatographed on 1 x 40 an water jacketed Sephadex LH-20 columns at 26°C in the same solvent mixture. Twenty-seven ml of column eluant were collected and discarded. Seven ml of eluant were then collected as the fraction containing Δ^{9}-THC. The next 8 ml of eluant were collected as the CBN-containing fraction. Thirty-eight ml of column effluent were then collected and discarded. Finally, 17 ml of eluant were collected as the fraction containing 11-hydroxy-Δ^{9}-THC. The Δ^{9}-THC and CBN fractions were evaporated to dryness and dissolved in 35 µl hexane. The 11-hydroxy-Δ^{9}-THC fraction was evaporated to dryness under vacuum and heated with 75 µl of Regisil (BSTFA + 1% TCMS) in a closed vial at 110° for 3 hours. The reagent was removed in vacuum and the residue dissolved in 20 µl hexane.

Extraction Prior to Analysis by GC-MS in CI Mode.--In this mode of operation Δ^{9}-THC and CBN were determined as their pentafluoropropionate ester derivatives, Fig. 1, 1c and 3c, respectively, using the PFP ester of hexahydrocannahinol, 4b as the internal standard for both analyses. It was found that endogenous plasma constituents did not interfere at the molecular ion positions utilized which were in the range of m/e 455-465. Hence preliminary purificatin of the extracts was not required. To a sample of 2.0-3.0 ml of plasma were added the internal standard hexahydrocannabinol 4b, 15 ng/ml plasma, under conditions described in the section above. The samples were extracted four times with hexane, 2.0 ml/ml plasma as described in the above section. The combined hexane extracts were washed sequentially with 2.0 ml 0.1 N sodium hydroxide, 2.0 ml 0.1 N hydrochloric acid, and 4.0 ml distilled water. The washed hexane extracts were evaporated and dried overnight in vacuo. To the dried extracts were added 0.2 ml hexane, 50 µl pyridine/benzene (5:95), and 50 µl pentafluoropropionic acid anhydride. After heating at 40°C for one hour, the reaction mixture was washed with 0.2 ml of saturated sodium bicarbonate solution, which was then backwashed with an additional 0.2 ml hexane. The hexane extracts were combined and evaporated under nitrogen. The derivatized extracts were dissolved in 20 µl hexane.

Gas chromatography conditions

LKB-GLC-MS.--With this instrument we utilized 2% OV-17 on Chromosorb W-HP, 80-100 mesh in 3' or 6' x 1/4" glass columns, temp. 220°C, the former being used for 11-hydroxy-Δ^{9}-THC-bis-TMS ether; the latter for both Δ^{9}-THC temp. 220°C; and CBN, temp. 240°C. Helium was used as the gas phase at a rate of 35 ml/min. Under the above stipulated conditions retention times of 4-6 minutes were observed for each compound.

Finnigan-GLC-MS.--Only Δ^{9}-THC and CBN were analyzed on this instrument in the ei mode. We used 6' glass columns containing 1% SE-30 on 100/120 mesh Chromatosorb W-HP, column temperatures 200-230°; He flow 30-35 ml/min. In the ci mode, which was used only with pentafluoropropionate derivatives, the same column was used at 180°.

Mass spectrometry

Only basic operating details are presented in regard to thems-computer systems described below. For a much more detailed report cf. Rosenthal et al.

LKB 9000 GLC-MS .--This instrument is of the magnetic sector type and was operated at ev in the ei mode. The instrument was fitted with a peak matching accessory, modified fran an instrument described by Klein, which permits detection of very law levels of compounds by the technique of acceleratig voltage alternation (AVA) (Hammar, 1968 and Agurell 1973). For Δ^9-THC the mass spectrum was set to focus alternately on the ions m/e 314 and 317 which correspond to the molecular ion of the compound and its trideuterio analog. For CBN the molecular ions were m/e 310 and 313. For analysis of 11-hydroxy-Δ^9-THC as the bis-tms-ether, the strong M-103 ion (Wall, et al., 1970, Wall, et al., 1976) at m/e 371 and 374 respectively was selected. The AVA accessory used with the mass spectrometer scans through each peak in turn; a hardware counter determines the area under the peaks each time they are scanned. The ratio of the selected response (either area or peak height) for the two ions in question is the value used to determine the quantity of unknown cannabinoid in the plasma. Appropriate calibration curves (Fig. 2, 3) are constructed by adding variable amounts of cannabinoid to a fixed quantity of corresponding trideuterio analog in human plasma. Extraction, preliminary purifcation, and glc are carried out as described above, Analysis by GLC-MS-EI.

Finnigan 3000-GLC-MS.--This instrument is a quadrupole mass spectrometer and was operated at 70 ev in the ei mode. Its scan is controlled by a dedicated PDP-12 camputer which also acquires data from the instrument. Although the computer operates on a different principle, the ratios of peak area or peak heights are determined as described above using the same molecular or fragment ions described in the previous section.

In the ci mode isobutane was used as the carrier gas at a pressure of 400 μ; Δ^9-THC and the internal standard HHC were determined as the corresponding PFP ester as the MH^+ ion at m/e 461 and 463, respectively. Similarly, for the case of CBN the MH^+ ions were m/e 457 and 463, respectively. Plasma calibration curves were obtained in the manner described previously.

Figure 2

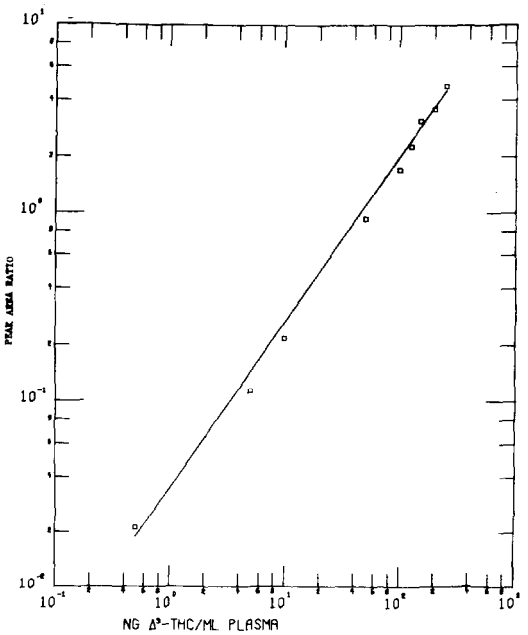

Figure 2.
LKB 9000-ei Plasma Calibration Curve for Δ^9-THC

Figure 3

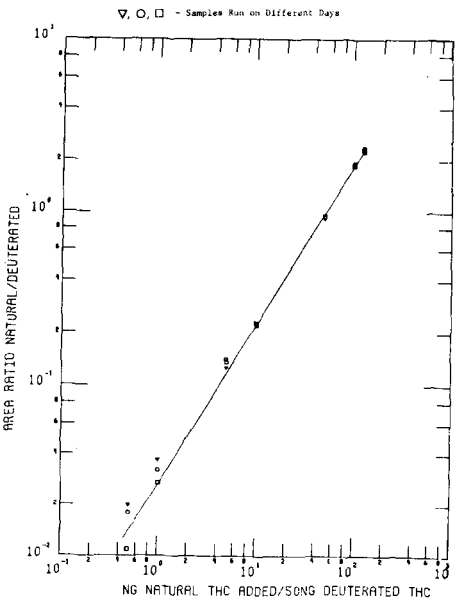

Figure 3.
Finnigan 3300-ei Plasma Calibration Curve for Δ^9-THC

TLC-radiolabel procedure

The volunteer subjects (described in Clinical protocol) all received 100 µCi of tritium labeled Δ^{19}-THC, along with the standard 4.0-5.0 mg IV dose. Two to three ml aliquots of plasma were analyzed by the procedure described by Wall, et al. (1975).

Electron capture-glc procedure

In this procedure Δ^{9}-THC was determined as the pentafluropropionate ester derivative using hexahydrocannabinol as internal standard. Extraction and derivatization were carried out as described under the procedure and extraction for gc-ms in ci mode. The instrumentation utilized was constructed at RTI by Dr. Edo Pellizzari as a modification of the design reported by Fenimore, et al.

A packed precolumn is utilized for initial cleanup of the sample followed by a packed column or a capillary column to furnish the requisite resolution. By interposing a valving system and cold trap between the two columns, a small portion of the effluent from the first column can be introduced to the second column with minimum loss of efficiency.

Furthermore, this instrumental design provides the capability for temperature programming which is seldom used in ec systems because of excessive base-line drift due to the detector's sensitivity to column bleed. In the reported dual column-dual oven system, the precolumn can be temperature programmed while the high efficiency column is maintained at isothermal temperature. Thus, in effect, rapid and efficient separations are achieved on the pre-column without disturbing the base-line response of the ec detector.

The instrument which we have assembled in our laboratory consists of two separately controlled ovens (Varian Model 1440, Walnut Creek, California). As shown in Figure 4 , one oven houses the packed columns and possesses the capability of linear temperature progamming; the second oven contains the capillary column or a second packed column and is operated isothermally. The effluent from the first packed column leads to an eight-port, high temperature, low dead volume switching valve (CF-8-HTa, Valco Instruments, Houston, Texas). The gas flow from the first column when in the normal operating position passes to the valve and through a capillary-loop trap and finally to a flame ionization detector. The trap consists of about 1 ft of 1/8 in stainless steel tubing to which is silver soldered a 2 meter length of 1/16 in O.D. x 0.20 i.d. Ni-200 capillary tubing. A water supply at 4°C is provided through the 1/2 in tubing for cooling purposes.

Figure 4

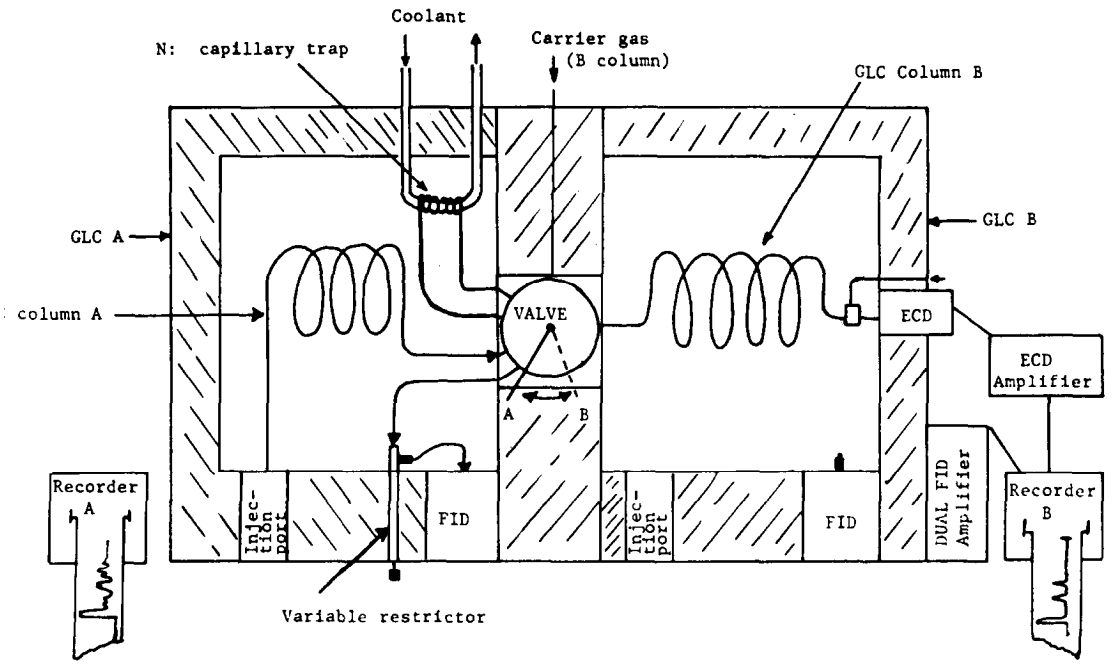

Figure 4. Dual Gas Chromatographic System

At the exact time interval (retention time) at which trapping of the constituents in the effluent from the packed column is to occur, the valve is rotated to the trapping position, and the coolant is passed through the 1/2 in tubing to cool the capillary loop. In this position, a second carrier gas flows to the high resolution capillary or second packed column; thus, the base-line is undisturbed. The trapped constituents are swept into the second oven and column by returning the valve to the original position and shutting off the ambient water supply. The heat in the first oven is sufficient to rapidly raise the temperature on the capillary trap for sample vaporation. A heated gold-plated Ni transfer line connects the packed column, valve and capillary or second column. The transfer line is also coated with the same stationary phase as in the final column.

Our system allows the utilization of flame ionization, alkali flame, or electron capture (ec) in the second oven for measuring drugs. The second oven will also accept conventional packed columns, metal (Ni) or glass capillaries. A linear ec amplifier (Hewlett Packard, Avondale, Pennsylvania) and a miniature ecd developed in our laboratories (Pellizzari, 1974) are employed in this dual gas chromatographed system.

Because of the low nanogram levels of cannabinoids found in human plasma, it was decided to use the electron capture detector to allow maximum sensitivity in the cannabinoid analysis. To make the analytical method more widely applicable by other researchers, it was decided to use packed columns in both ovens.

Glc conditions.--Plasma impurities were separated on a ten-foot column of 2% SE30 on Supelcoport (80/100 mesh) at 205°C with a carrier gas (N_2) flow rate of 15 ml/min. Column effluent was collected at the retention times of HHC and Δ^9-THC and transferred for quantitative analysis to a six-foot column of 2% OV225 on Supelcoport (100/120 mesh) at 178°C with a carrier gas (5% methane - 95% argon) flow rate of 9.5 ml/min.

RESULTS

Plasma calibration curves for Δ^9-THC obtained with the LKB and Finnigan mass spectrometers in the ei mode are shown in Figures 2 and 3; the calibration curve obtained with the Finnigan instrument in the ci mode is shown in Figure 5. In each case a linear calibration curve was obtained in range 1-100 nanograms Δ^9-THC/ml plasma. Detection of Δ^9-THC below the lower limit could be obtained (detection limits 0.1). 0.5 Ng/ml of plasma is regarded as the minimal concentration at

Figure 5

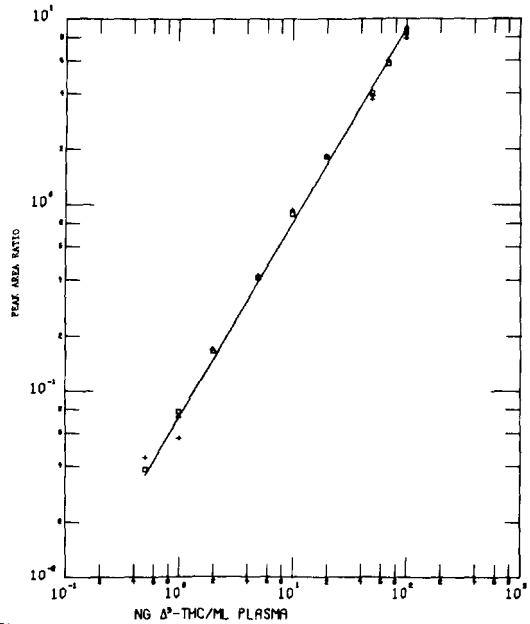

Figure 5.
Finnigan 3300-ci Plasma Calibration Curve for Δ^9-THC

which reliable data could be obtained. The standard error of estimation for LKB data as obtained by linear regression analysis was 0.114, and the correlation coefficient was 0.9970. For the Finnigan in ei mode, the comparable values were 0.034 and 0.9994, respectively. In the ci mode of the Finnigan mass spectrometer the corresponding values were 0.20 and 0.9977. Plasma calibration curves for CBN are shown in Figures 6 and 7, which respectively present the data obtained on the LKB-ei and Finnigan-ei. Linear curves on both instruments were obtained between 0.2-10.0 ng/ml plasma with detection limits about 0.1 ng/ml. The standard error of estimation for the LKB and Finnigan instruments was respectively 0.05 and 0.078; the correlation coefficients in each case were 0.998. Preliminary data for CBN determined in the ci mode as the PFP ester indicate that similar results are to be expected. The plasma calibration curve for 11-hydroxy-Δ^9-THC-bis-tms-ethrer obtained with the LKB-mass spectrometer is shown in Figure 8. The curve was linear in the range of 0.2-10.0 ng of 11-hydroxy-Δ^9-THC/ml plasma. As in previous cases the accuracy of the determination decreases below concentrations of 1.0 ng/ml. The standard error of the estimation was 0.027 and the correlation coefficient 0.9975.

Figure 6

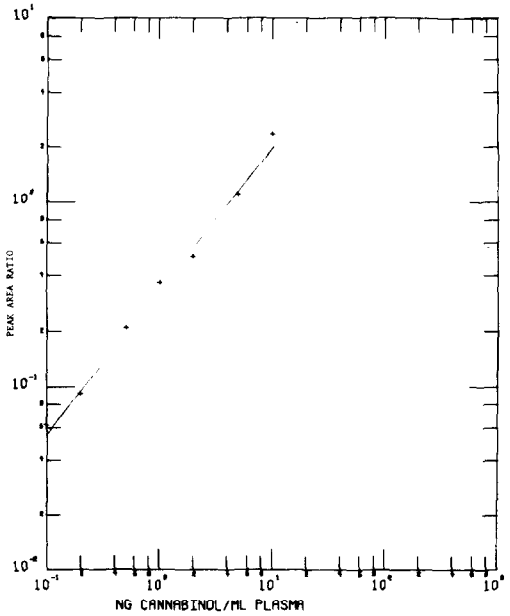

Figure 6.
LKB 9000-ei Plasma Calibration Curve for CBN

Figure 8

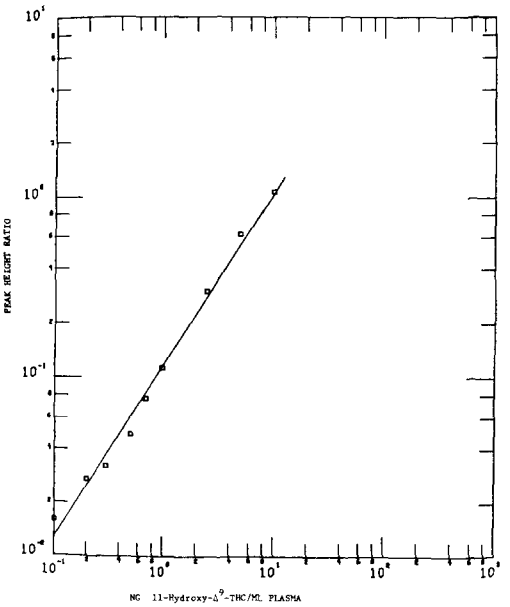

Figure 8.
LKB 9000-ei Plasma Calibration Curve for 11-Hydroxy-Δ^{9}-THC

Figure 7

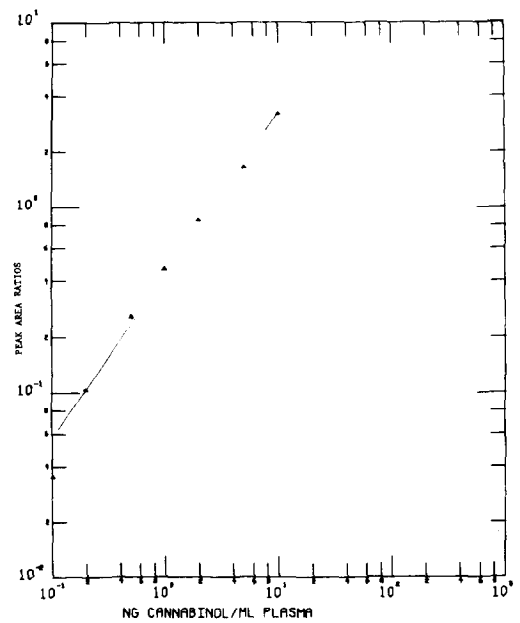

Figure 7.
Finnigan 3300-ei Plasma Calibration Curve for CBN

Figure 9

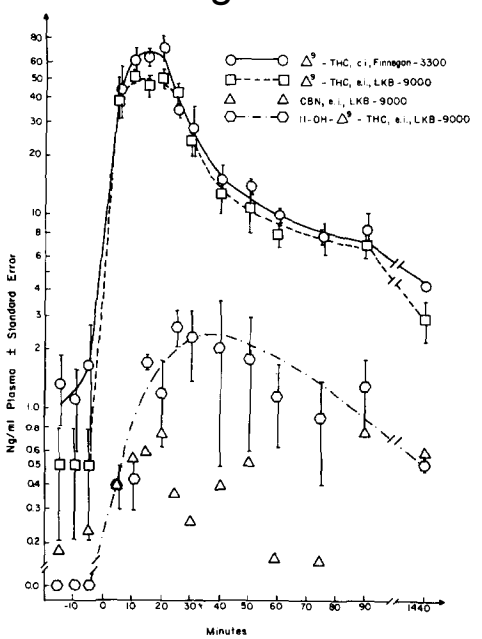

Figure 9.

Plasma Levels of Δ^{9}-THC, 11-Hydroxy-Δ^{9}-THC and CBN Found Over a 24-Hour Period in Human Plasma from Volunteers Receiving Δ^{9}-THC by IV Administration

Figure 9 presents the average values with standard error obtained for Δ^{9}-THC, 11-hydroxy-Δ^{9}-THC and cannabinol from plasma of male volunteers receiving Δ^{9}-THC by intravenous infusion. The measurements covered a 24 hour period. Δ^{9}-THC values obtained with the LKB-9000 ei source were in close agreement with the data obtained on the Finnigan 3300-ci source. Other data not presented here also show close agrerment between the LKB and Finnigan instruments when both were in the ei mode. Δ^{9}-THC values increased rapidly during the first 10-20 minutes, the peak values in the range of 50-60 ng/ml coinciding With the maximal psychomimetric activity. A typical biphasic elimination pattern was noted; the Δ^{9}-THC plasma levels decreased rapidly between 15-40 minutes and then fell at a much slower rate. With a particular group of volunteers (3 subjects) levels after 24 hours were between 3-5 ng/ml. Spot checks at lower levels utilizing the Finnigan MID program confirmed that the substance being evaluated was indeed Δ^{9}-THC and not instrument "noise".

Figure 1 compares the results obtained from the average of four subjects, analyzed by ms-ei tlc radiolabel and electron capture glc. Correlation coefficients are calculated in Figure 11. The results are in reasonable agreement, and in particular the glc-ms and electron capture glc procedures gave good agreement for most points over the whole curve. In the case of 11-hydroxy-Δ^{9}-THC much lower levels were found; peak values in the neighborhood of 2.0 ng/ml were noted between 30-40 minutes. The maximal values declined in a more gradual manner than was the case for Δ^{9}-THC, falling to a level of 1.0 ng/ml in 60-90 minutes and 0.5 ng/ml after 24 hours. The values for CBN are considered unreliable as obtained by the ei technique since for the most part the levels were well below 1.0 ng/ml and show no consistent pharmacokinetic pattern.

DISCUSSION

The basic objective of this investigation was to establish sensitive methodology, which would not depend on radiolabeling for the quantitative estimation of Δ^{9}-THC, its primary metabolite (Wall, 1970), 11-hydroxy-Δ^{9}-THC and cannabinol, which has been reported to be a metabolite of Δ^{9}-THC in the rat (McCallum, 1975). This objective has been realized, utilizing glc-ms with a variety of techniques and instruments. In addition, a specialized double glc procedure which utilizes one column for clean up and one for final estimation has been developed. Several aspects of our results merit detailed discussion.

Figure 10

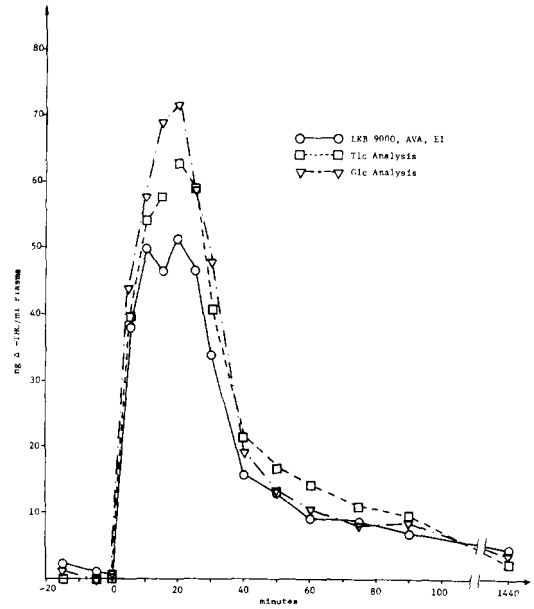

Figure 10.
Δ^{9}-THC Found in the Plasma Following Intravenous Administration of 5 mg Δ^{9}-THC, Average of 4 Subjects

Figure 11

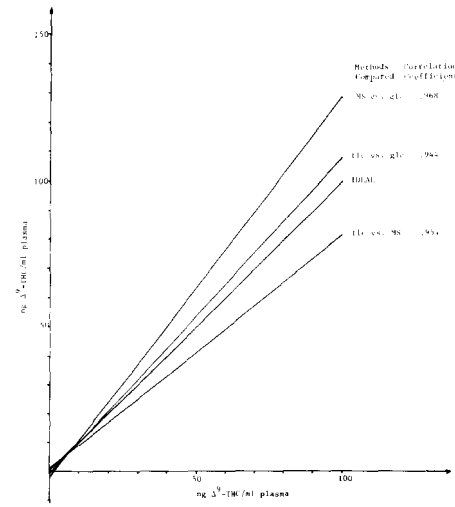

Figure 11.
Least-Squares Best Lines Comparing All Data Obtained by Each Two Methods of Analysis

Choice of instrument

Two completely different types of mass spectrometers coupled with different means for quantitation of data were utilized. One was a relatively old (1968) magnetic sector ms, the LKB-9000 which was coupled with an alternation voltage acceleration device (Rosenthal, in press: Klein, 1972) which permitted measurement of the ratio of the peak area of the unknown as compared with that of the internal standard. The other was a relatively new (1974) quadrupole ms, the Finnigan 3300 which was interfaced to a PDP-12 computer. The Finnigan ms has both ei and ci sources. As shown in Figures 2, 3, 6 and 7 and in the text of the Results section, both instruments in the ei mode gave virtually identical plasma calibration curves with identical linear range and quite similar stand error of estimation. Figure 9 gives pharmacokinetic data in man obtained on the LKB in the ei mode and the Finnigan in the ci mode. The results are quite similar. It is thus evident that a wide variety of mass spectrometers can be used with comparable results provided appropriate internal carriers and standards are added. Before concluding this discussion one word of caution should be given: the nature of the separators is most important; the LKB with the Ryhage separator and the Finnigan with a silylated glass jet separator gave appropriate sensitivity. On the other hand, another mass spectrometer which utilized a double glass coil separator showed poor sensitivity and could not be utilized for cannabinoid studies.

General analytical considerations

Internal standards.--As indicated previously, the final mass spectrometric measurements can be conducted with great accuracy. The key to success in the various analytical studies was the utilization of appropriate compounds which could be used as both carriers and internal standards. For this purpose deuterium labeled cannabinoids identical to the parent compound except for the label are ideal and were utilized for all of the ei studies. It is possible to use with equal success an internal carrier which is not isotopically labeled as long as its properties are very similar to that of the cannabinoid being studied but permit separation by glc. Hexahydrocannabinol was excellent fo this purpose and was used in ci studies of Δ^9-THC and cannabinol.

Ei vs ci source.--In recent years quantitative glc-ms using ci source has become more and more popular for estimation of drugs in biological materials. This is due to the fact that in many cases greater sensitivity can be obtained in the ci mode since there is little molecular fragmentation as compared to the situation when operating in the ei mode. In our studies we have found no particular advantage in terms of sensitivity as far as the two methods are concerned, the useable lower limits in both cases being about 1 ng/ml plasma and detection limits as low as 0.2 ng/ml. However there would seem to be a major advantage for utilizing the ci source if a choice is available. As noted earlier (cf., Methods section) a generalized background interference is found in human plasma in the region for determination of underivatized Δ^9-THC and cannabinol in the region of m/e 310-320. This endogenous interference cannot be removed by glc techniques alone but requires a preliminary "clean up", utilizing Sephadex LH-20 chromatography. This method which was described by Agurell et al. for $\Delta^{1,9}$-THC and extended by us to mixtures of $\Delta^{1,9}$-THC, 11-hydroxy-$\Delta^{1,9}$-THC and CBN, works well for the former two compounds but is time consuming. In addition, there is danger of some conversion of $\Delta^{1,9}$-THC to CBN because of the longer exposure period. All of these problems are avoided when the cannabinoids are converted to the corresponding pentafluoropropionate esters. The molecular ions are then in the region of m/e 455-465 and no interference was found after extraction of plasma with hexane, derivatization and glc-ms determination. The method at present has been applied to $\Delta^{1,9}$-THC and CBN which can be extracted with non-polar solvents. Whether more polar cannabinoids which require more polar solvents can be successfully analyzed without prior purification by the ci technique is an open question at this time.

Metabolic and pharmacokinetic data

The development of sensitive and accurate glc-ms methodology permitted a preliminary study in man utilizing these techniques for the precise determination of $\Delta^{1,9}$-THC, 11-hydroxy-$\Delta^{1,9}$-THC and CBN in plasma. Previously we have made an extensive stud (Wall, et al., 1975) of the metabolism of $\Delta^{1,9}$-THC in man using radiolabeled tracers and thin layer chromatography. The procedures utilized (in addition to the undesirability of a radiolabeled tracer in man) suffer from two potential sources of error. The method utilized would not permit separation of $\Delta^{1,9}$-THC from CBN, and in the case of 11-hydroxy-$\Delta^{1,9}$-THC, would not permit separation from other monohydroxy-metabolites which might be present (Wall, et al., 1973; Wall, et al. 1975). The data in Figure 9 for $\Delta^{1,9}$-THC are quite comparable to pharmacokinetic data obtained in earlier studies (Wall, et al., 1975). In both instances a biphasic elimination curve is noted with a sharp decline after the initial maximum level followed by a much more gradual decrease. Maximal values in the current studies were 50-60 ng/ml. After 24 hours, 3-5 ng/ml of $\Delta^{1,9}$-THC were still found in the plasma. Our

results for 11-hydroxy-Δ^9-THC are probably the most accurate data yet reported in man. The concentration of this active metabolite (cf. Figure 9) was only 2-3 ng/ml at peak levels declining at a slower rate than Δ^9-THC to 0.5 ng/ml after 24 hours. Although Δ^9-THC is readily converted to 11-hydroxy-Δ^9-THC in the liver (Wall, et al., 1975) only small quantities find their way into the blood.

Our interest in CBN was aroused by reports from McCallum (1975) and McCallum, et al. (1975) which indicate that CBN might be a transitory metabolite found at very early time periods after administration of Δ^9-THC.

As shown in Figure 9, the level of CBN was below our reliability limits in the ei mode. Other studies we have carried out by electron capture glc or glc-ms in the ci mode indicate the virtual absence of this substance at all time periods. Since we have found that CBN has the same general pharmacokinetic pattem as Δ^9-THC in man (Wall, 1975), we must conclude that CBN can be disregarded in terms of its importance as a metabolite in man.

Comparison of gc-ms with other procedures

As shown in Figures 10 and 11, the gc-ms procedures show reasonable agreement in the case of Δ^9-THC with data obtained by two independent procedures, involving respectively thin layer chromatography of radiolabeled cannabinoids, and a double glc-electron capture procedure. In preliminary studies on cannabinol levels of subjects who received Δ^9-THC, good agreement was found between the mass spectrometric glc-ci method and electron capture glc. In each case no cannabinol could be found. Studies on 11-hydroxy-Δ^9-THC levels by the glc-electron capture method are in progress. Initial attempts to use high pressure liquid chromatography as mother method indicate that the sensitivity by this procedure with current detectors is of the order 10 ng/rnl. Hence at present hplc techniques do not have requisite sensitivity.

ACKNOWLEDGMENTS

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FOOTNOTE

[1]Research Triangle Institute Contract HSM-42-71-95. Appropriately qualified investigators may obtain a variety of labeled and unlabeled cannabinoids by application to Dr. Robert Willette, Acting Chief, The Research Technology Branch, Division of Research, National Institute on Drug Abuse, Rockwall Building, 11400 Rockville Pike, Rockville, Maryland 20852.

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Quantitation of ^{9}*-Tetrahydrocannabinol* in *Body Fluids by Gas Chromatography/Chemical Ionization-Mass Spectrometry*

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HPLC-MS Determination of Δ^{9}*-Tetrahydrocannubinol in Human Body Samples*

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