Determination of Δ^9-Tetrahydrocannabinol in Blood by Electron Capture Gas Chromatography

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Electron capture gas chromatographic determination of Δ^9-tetrahydrocannabinol (Δ^9-THC) in blood serum is described. The compound is detected as the heptafluorobutyrate on a dual column–dual oven gas chromatograph utilizing a capillary column as the final resolving component. The limit of detection is below 100 pg per ml with excellent reproducibility using hexahydrocannabinol as an internal standard. Blood serum concentrations in experimental animals injected with 0.1 mg/kg Δ^9-THC were determined with levels below 1 ng/ml four hours after administration.

An accurate method for the determination in blood of Δ^9-tetrahydrocannabinol (Δ^9-THC), the major active component of marihuana (*1*), is important in studies of the physiological and behavioral effects of this drug. Because of the extremely low concentration of Δ^9-THC circulating in the blood following administration of a pharmacological dose, investigators have relied upon the sensitivity of radioactive tracer techniques to estimate relative blood levels (*2–4*). The obvious hazards of employing radioactively labeled compounds in human subjects, particularly in studies involving chronic administration, have prompted searches for alternative analytical methods of sufficient sensitivity to permit quantitation in the nanogram per milliliter range or lower. Detection of cannabinoids in biological samples by mass fragmentography has been reported (*5, 6*) and immunological assay has been suggested as a possible method of determination (*7*), but these approaches, although promising, have yielded little in quantitative data at present.

Electron capture gas chromatography is well known for the extreme sensitivity of detection which can be obtained with compounds having a high affinity for thermal electrons (*8*). Although Δ^9-THC does not in itself possess an electrophoric structure (*9*), it is capable of forming a variety of electron capturing derivatives through reaction at the phenolic hydroxyl group. Enhanced sensitivity through derivatization does not necessarily ensure successful analysis, particularly when the compound in question is present at the parts-per-billion level or lower in blood. Compounds having hydroxyl or amine groups which can react with derivatizing reagents are ubiquitous in samples of biological origin and, for this reason, selectivity rather than sensitivity is often the limiting factor in achieving an analytical method of practical significance.

Gas chromatography is inherently a selective analytical technique within the limits imposed by the efficiency and resolution of the particular instrumental system. In applications involving electron capture detection of derivatized compounds obtained from biological materials, the resolution of most packed chromatographic columns is insufficient for isolation of the compound of interest without rigorous cleanup procedures prior to the gas chromatographic analysis. Efforts to obtain a relatively pure isolate by solvent partitioning, thin-layer or column chromatography, etc. frequently lead to low recoveries when the compound is present originally in submicrogram amounts.

In order to improve resolution while retaining sensitivity to the point that Δ^9-THC could be determined accurately at the low concentrations anticipated, a gas chromatographic system was designed utilizing a packed precolumn for initial cleanup followed by 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 could be introduced to the capillary with minimum loss of efficiency. Similar systems have been described (*10, 11*) as a means of introducing relatively large sample volumes into capillary column instruments. The principal advantage of the chromatograph employed in the present study is in the magnification of a specific portion of a conventional chromatogram with respect to both resolution and sensitivity.

An additional feature of this instrument design is the capability for temperature programming, a technique seldom employed in electron capture systems because of excessive base-line drift due to the sensitivity of the detector to column bleed. In the dual column–dual oven system described here, the pre-column can be temperature programmed while the capillary column is maintained at a fixed temperature. This, in effect, permits rapid and efficient separations on the pre-column without disturbing the base-line response of the electron capture detector.

A number of electron capturing derivatives of Δ^9-THC were compared on the basis of sensitivity, retention, ease of formation, etc. (Table I). The heptafluorobutyrate was selected for use with blood serum extracts, and concentrations as low as 0.1 ng per ml of serum were determined accurately and reproducibly using the dual column instrument.

EXPERIMENTAL

Apparatus. A diagram of the dual column gas chromatograph is shown in Figure 1. The instrument was constructed with two separately controlled ovens (Antek Instruments, Houston, Texas), the oven housing the packed column having linear temperature programming capability, and the capillary oven being operated

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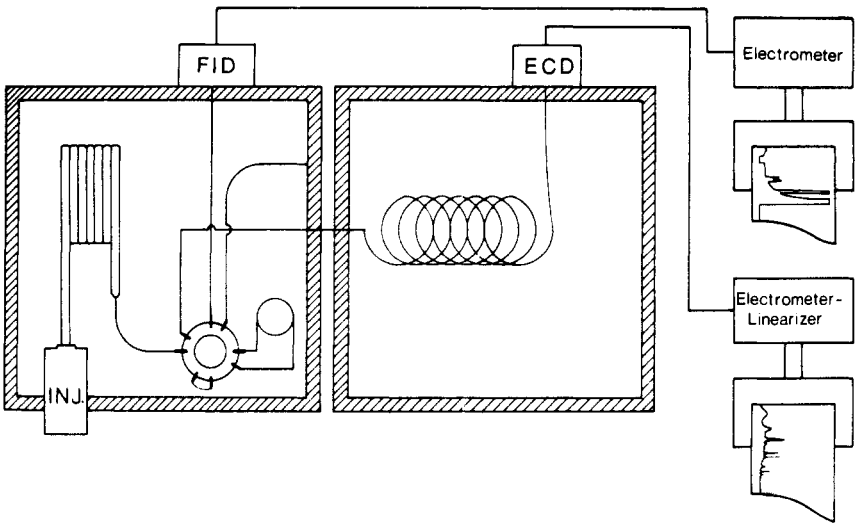
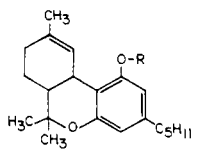


Figure 1. Dual oven–dual column gas chromatograph

Table I. Comparison of Electron Capturing Derivatives of Δ^9-THC



R	REAGENT	SENSITIVITY	RELATIVE RETENTION TIME
$-COCF_2Cl$	CHLORODIFLUOROACETIC ANHYDRIDE	1 pg	2.66
$-COCF_3$	TRIFLUOROACETIC ANHYDRIDE	5 pg	1.04
	N-TRIFLUOROACETYL-IMIDAZOLE		
$-COC_3F_7$	HEPTAFLUOROBUTYRIC ANHYDRIDE	2 pg	1.00
	N-HEPTAFLUOROBUTYRYL-IMIDAZOLE		
$-COC_6F_5$	PENTAFLUOROBENZOYL CHLORIDE	1 pg	11.91
	PENTAFLUOROBENZOYL IMIDAZOLE		
$-COC_2F_5$	PENTAFLUOROPROPIONIC ANHYDRIDE	2 pg	1.00
$-COC_6H_4CF_3$	M-TRIFLUOROMETHYLBENZOYL CHLORIDE	10 pg	25.52
$-Si(CH_3)_2(CH_2Cl)$	CHLOROMETHYLDIMETHYL-CHLOROSILANE	100 pg	8.11
$-CH_2C_6F_5$	α-BROMOPENTAFLUORO-TOLUENE	2 pg	11.22

isothermally. The packed column is coiled 6-ft × 2-mm i.d. glass arranged for on-column injection and containing 5% SE-30 on 100/120 mesh Gas Chrom Q. The effluent from this column leads to an eight-port, high temperature, low dead volume switching valve (CV-8-HTa, Valco Instruments, Houston, Texas). As shown in Figure 2, the gas flow from the first column leads to a flame ionization detector (Varian Aerograph, Walnut Creek, Calif.) when in the normal operating position. In this position, carrier gas to the capillary column flows through the trap. The trap consists of 1 meter of ⅛-in. stainless steel tubing to which is brazed longitudinally a 2-meter length of 0.32-in. o.d. × 0.020 i.d. nickel-200 capillary tubing. This assembly is coiled in approximately 4-in. diameter loops and suspended in the first oven by bulkhead fittings which lead to a water supply and waste. When the valve is in the trapping position, the effluent from the packed column flows through the nickel capillary and then to the flame ionization detector while the capillary column gas supply flows directly to that column. Cooling water flows through the ⅛-in. tubing during this interval. The trapped material is swept into the capillary by returning the valve to the original position and shutting off the water supply. Oven heat is sufficient to reheat the trap rapidly to the packed column oven temperature. A heated (*ca.* 240 °C) nickel transfer line leads to the capillary column which consists of 280-ft × 0.02-in. i.d. nickel-200 needle stock (Handy and Harmon, Norristown, Pa.). This column is coated with 10% OV-17 solution in chloroform after lightly etching the interior of the nickel tubing with 20% nitric acid solution and thoroughly washing with water, acetone, and chloroform.

A low volume coaxial electron capture detector containing a 300-mCi scandium tritide source, described previously (*12*), is mounted externally to the capillary oven. The detector is operated in the pulse collection mode, usually at a pulse interval of 500 μsec or more if the cleanliness of the chromatographic system so warrants. Output from the EC detector is linearized before recording (*13*).

Various electron capturing derivatives of Δ^9-THC were studied using a Victoreen Model 400 gas chromatograph equipped with a coaxial nickel-63 electron capture detector, and response again was linearized prior to recording.

The various Δ^9-THC derivatives were verified using a DuPont Model 491 mass spectrometer interfaced with a Varian Aerograph Model 1200 gas chromatograph.

Reagents. All solvents were either of chromatographic quality or redistilled in glass. Derivatizing reagents were used without further purification and included the following: chlorodifluoroacetic anhydride (Pfalz and Bauer); trifluoroacetic anhydride, pentafluoropropionic anhydride, heptafluorobutyryl imidazole, pentafluorobenzoyl chloride, pentafluorobenzoyl imidazole, *m*-trifluoromethylbenzoyl chloride, (Pierce Chemical); α-bromopentafluorotoluene (Aldrich). Reagents and solvents used in blood serum extraction and derivatization were 1.0*M*, pH 6.0 phosphate buffer, 0.1*N* HCl, and 0.1*N* sodium hydroxide. A solution of trimethylamine was prepared by allowing a stream of the trimethylamine gas from a lecture bottle to bubble slowly into 50 ml of benzene with constant mixing for approximately 1 minute. This reagent was prepared for each group of serum extracts. Synthetic (−) - Δ^9-tetrahydrocannabinol (Δ^9-THC), Δ^8-THC were furnished by the National Institute of Mental Health, Rockville, Md. Tritium-labeled Δ^8-THC was obtained from New England Nuclear, Boston, Mass.

Hexahydrocannabinol was prepared from Δ^8-THC by hydrogenation over platinum-charcoal catalyst and purified by column chromatography on Florisil.

Blood Serum Extraction and Derivatization. All glassware used in the extraction and derivatization of Δ^9-THC was acid washed and silanized using Dri-Film SC-87 siliconizing fluid

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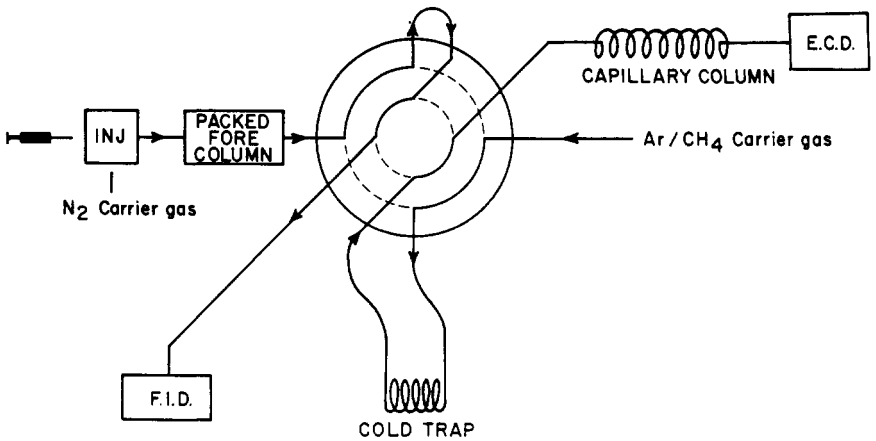

Figure 2. Flow diagram of eight-port valve. Normal operating flow shown by solid lines; flow during trapping shown by broken lines

(Pierce Chemical Co.). Pipet a 1.0-ml portion of the serum into a 50-ml conical, glass stoppered centrifuge tube, add 10 ng of hexahydrocannabinol as an internal standard and 10 ml of heptane/1.5% isoamyl alcohol. Mix thoroughly on a vortex mixer for approximately 30 seconds, and centrifuge for 5 minutes. Transfer the heptane layer together with most of the emulsified interface to another 50-ml centrifuge tube and reduce the volume under a stream of dry nitrogen by heating at 60 °C, taking care to prevent the solution from going to dryness. Re-extract the plasma with an additional 10-ml portion of heptane/1.5% isoamyl alcohol as above and add the heptane layer to the original extract and reduce the volume to approximately 4 ml. Add 2 ml of 0.1*N* sodium hydroxide, vortex, and centrifuge. With a disposable Pasteur pipet, remove most of the aqueous layer and discard. Add 2 ml of 0.1*N* hydrochloric acid, vortex, centrifuge, and transfer the heptane layer to a 5-ml Reacti-vial (Pierce Chemical Co.). Evaporate to dryness at 60 °C under a stream of dry nitrogen. Dissolve the residue in 0.5 ml of benzene containing trimethyl amine, add 0.2 ml of heptafluorobutyric anhydride, cap tightly, and allow to react 10 minutes at room temperature. Blow to dryness, add 1 ml of pH 6.0 phosphate buffer and 5 ml of heptane; mix by shaking, centrifuge, and transfer the heptane layer to a 5-ml Reacti-vial. Blow to dryness at 60 °C, cool, and dissolve the residue in 50 μl of isooctane for injection into the gas chromatograph.

Gas Chromatography. In order to determine the exact time interval at which trapping of the effluent of the packed column is to occur, inject a sample of Δ^9-THC-heptafluorobutyrate (Δ^9-THC-HFB) with the column temperature at 180 °C. Program to 260 °C at 2 °C/minute and monitor the chromatogram using the flame ionization detector. Under the conditions outlined under the section on instrumentation, the retention time of the HFB-THC is approximately 13 minutes. Before beginning a high sensitivity chromatogram, inject 5 μl of pure solvent onto the packed column at 180 °C and program to 260 °C to remove any traces of the THC-HFB standard.

Inject 5 μl of the derivatized serum extract solution, and temperature-program as above. Approximately 30 seconds before trapping the effluent, cool the trap by turning on the water supply. Switch the gas flow with the eight-port valve for the interval required to trap the Δ^9-THC-HFB, return the valve to normal operating position, and shut off the cooling water supply to the trap allowing the trap to return to oven temperature and the trapped material to be transferred to the capillary columns.

RESULTS AND DISCUSSION

Most derivatizing reagents recommended for forming electron capturing products from phenolic compounds yielded derivatives with Δ^9-THC to which the electron capture detector responded with adequate sensitivity as shown in the table. The chloromethyldimethylsilyl ether, however, was eliminated from further study on the basis of low sensitivity. Although the perfluorinated aromatic derivatives gave excellent compounds on the basis of sensitivity, the relative retention times were substantially higher than those of the aliphatic derivatives and would necessitate capillary column temperatures which could produce excessive bleed for use with the sensitive detector employed in this study. Consequently, the heptafluorobutyrate was selected as the appropriate derivative for this particular determination.

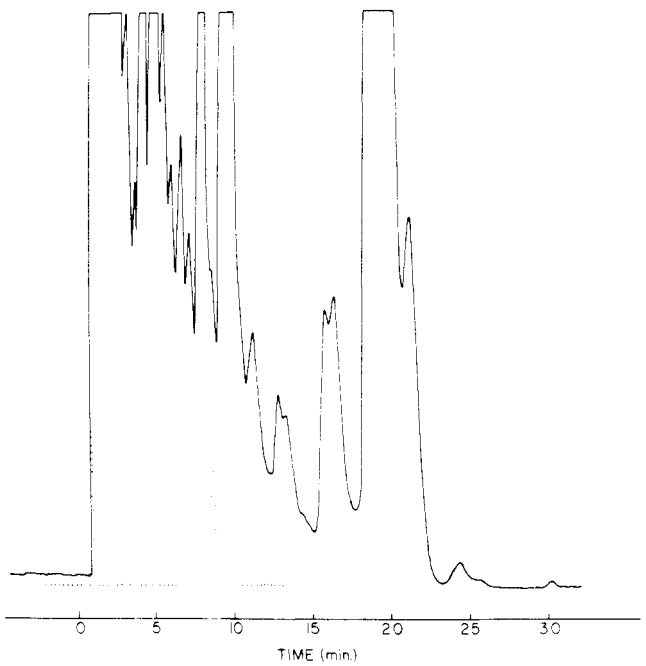

Figure 3. Electron capture gas chromatogram of human blood serum extract after derivatization with heptafluorobutyric anhydride

Conditions: 6-ft × 2-mm i.d., glass column packed with 3% OV-17 on Gas Chrom Q. Oven temperature: 180 °C. Flow: 20 ml/min. Chromatogram of 2 ng THC-HFB standard, dotted line, equivalent to amount contained in extract

A variety of extraction solvents were investigated to determine greatest recovery of THC from blood serum by using tritium-labeled Δ^8-THC. These solvents included petroleum ether, hexane, heptane, diethyl ether, ethyl acetate, benzene, and heptane containing various amounts of isopropanol, *n*-butanol, *n*-amyl alcohol. The heptane/1.5% isoamyl alcohol recommended by Lemberger *et al.* (2) gave the best recoveries, often in excess of 90%.

Early in this study attempts were made to achieve resolution of Δ^9-THC-HFB at low concentrations in blood serum extracts on packed chromatographic columns. The excessive interference of other derivatized materials is illustrated in Figure 3. The usual alternative in dealing with interference of this magnitude is to subject the serum extract to thin-layer or column chromatographic cleanup. We found that the introduction of other interfer-

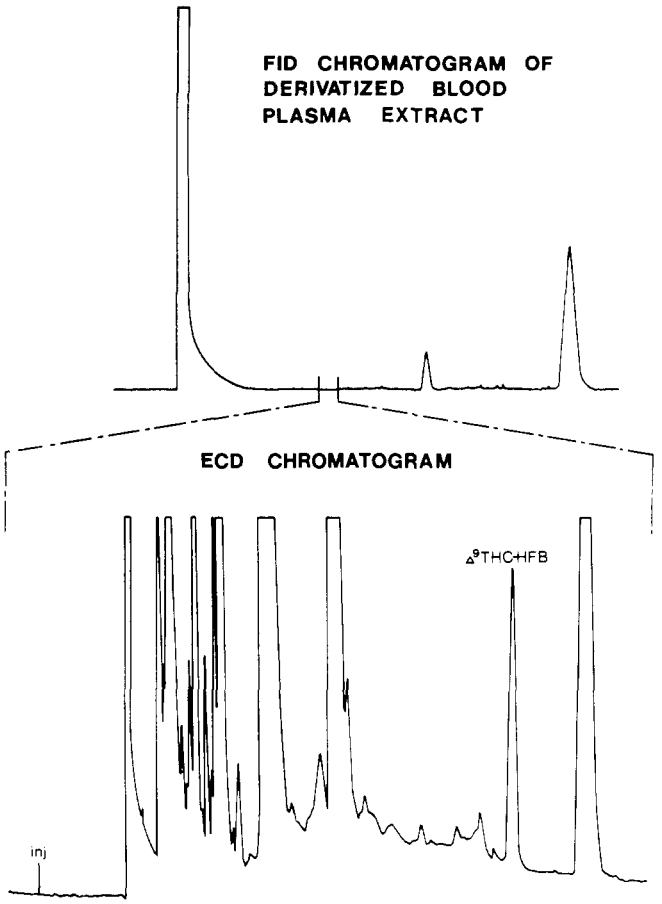

Figure 4. Gas chromatograms of blood serum extract containing 0.5 ng Δ^9-THC

Upper chromatographic trace obtained by flame ionization detection of packed column effluent. Lower trace obtained by electron capture detection of trapped portion of packed column effluent resolved by capillary column. Packed column programmed from 180 to 260 °C at 2 °C/min with 20 ml/min nitrogen flow. Capillary column operated at 215 °C with 3.5 ml/min argon/methane flow. Total flow through EC detector with purge gas: 12 ml/min

ing substances and/or low recovery of Δ^9-THC resulting from these procedures militated against extensive liquid chromatographic manipulation of the sample.

These considerations led to the design of the dual oven chromatographic system described above. Figure 4 shows the results achieved with this design when applied to an extract of blood serum with 10 ng of Δ^9-THC added to the blood sample. The upper chromatographic trace is the flame ionization detector record of the packed column effluent. Switching and trapping of a small portion of the effluent with subsequent release to the capillary column yielded the lower chromatogram as detected by the low volume electron capture detector. The peak due to the presence of Δ^9-THC-HFB actually represents 1 ng of Δ^9-THC, because a one-tenth aliquot of the sample was injected into the gas chromatograph.

Uncertainties in recovery of extracted drug from the serum and extent of derivatization, and errors in sample introduction into the chromatograph necessitated the inclusion of an internal standard to the serum sample for accurate quantitation of Δ^9-THC. A compound was thus required which was chemically and physically similar to Δ^9-THC and would have approximately the same retention time as Δ^9-THC on the packed column. Further, this standard could not be a compound which might be a metabolic product of Δ^9-THC or other naturally occurring

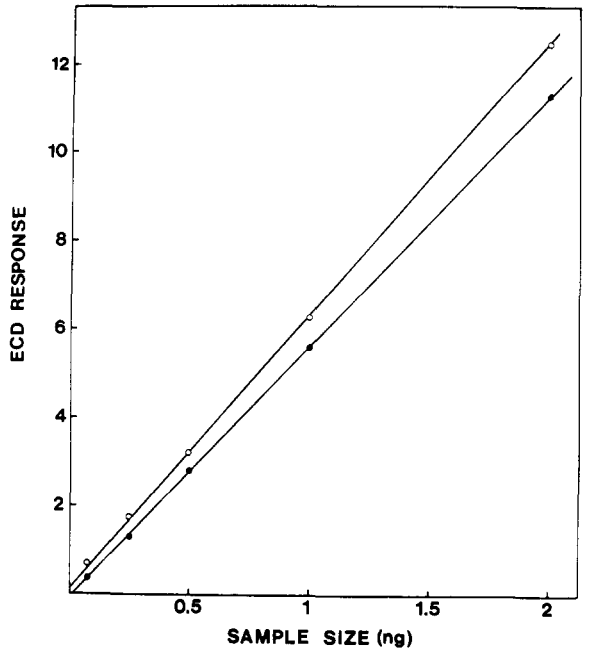

Figure 5. Electron capture calibration curves of Δ^9-THC and HHC heptafluorobutyrates on dual oven system

● Δ^9-THC-HFB; O HHC-HFB

cannabinoids for obvious reasons. Hexahydrocannabinol formed by the catalytic hydrogenation of either Δ^9- or Δ^8-THC fulfilled these requirements. The hydrogenation of the substituted cyclohexane ring of THC yields two geometrical isomers, but these are readily separated by the chromatographic system and offer no problem in the analysis.

The derivatization of blood serum extracts was first conducted by reacting heptafluorobutyric anhydride directly with the extraction residue. This method eventually proved unsatisfactory because of occasional partial isomerization of Δ^9-THC to Δ^8-THC, presumably caused by small amounts of free heptafluorobutyric acid in the reagent. By employing the method of Ehrsson *et al.* (*15*) where trimethylamine is added to the reaction mixture, this problem was avoided.

Standard preparations of both Δ^9-THC-HFB and hexahydrocannabinol heptafluorobutyrate (HHC-HFB) were chromatographed on the dual system and the calibration curves are shown in Figure 5. The essentially linear response over the range of 100 pg to 2 ng with a correlation coefficient of linear regression of better than 0.999 in each case gave assurance of quantitative recovery of the samples through the entire chromatographic system. The response values on the ordinate of this graph refer to arbitrary units of peak height.

Studies of a series of human serum samples, to which had been added known amounts of Δ^9-THC in the range anticipated to be present after minimal dosage, showed the recoveries relative to the internal standard to be 91.4% with a standard deviation of 0.05. The absolute recovery of Δ^9-THC through the entire procedure was 70%.

In order to verify the applicability of this analysis to experimental subjects, Δ^9-THC was injected into the ear vein of New Zealand white male rabbits and blood was collected at timed intervals by heart puncture. The THC was injected as a suspension in PVP/saline solution (*16*) at a dose level of 0.1 mg/kg which would be considered a

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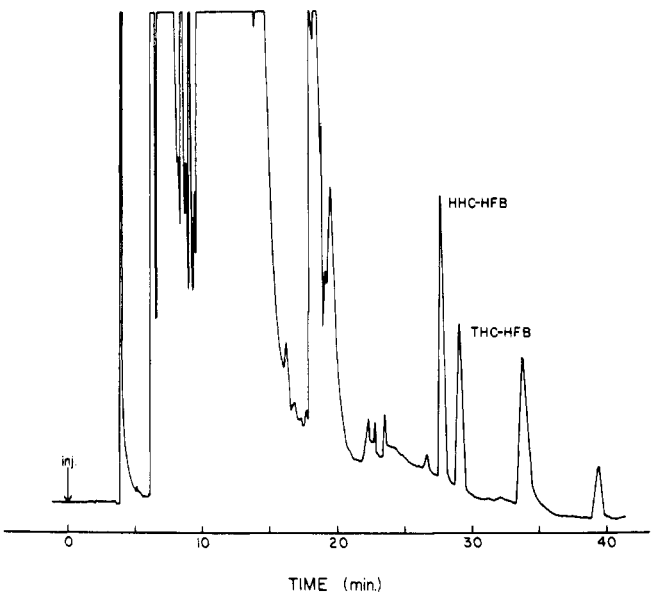

Figure 6. Rabbit blood serum extract 1 hour after injection of 0.1 mg/kg Δ^9-THC

Attenuation: 128. HHC internal standard: 10 ng. Calculated serum level of Δ^9-THC: 6.5 ng/ml

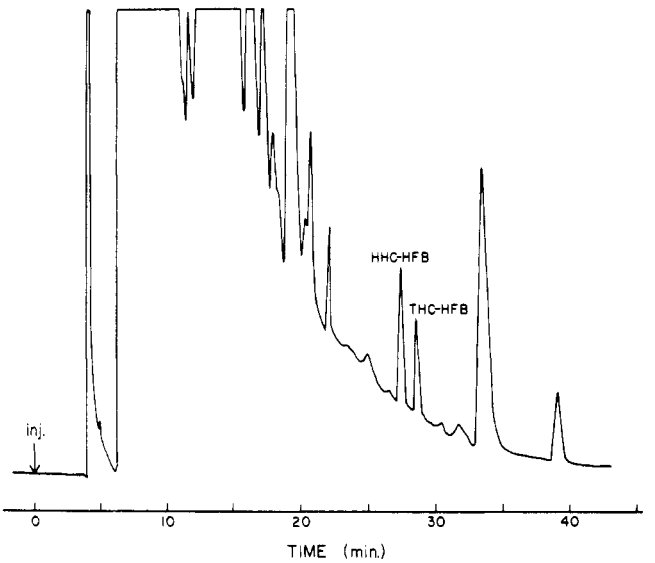

Figure 7. Rabbit blood serum extract 4 hours after injection of 0.1 mg/kg Δ^9-THC

Attenuation: 32. HHC internal standard: 1 ng. Calculated serum level of Δ^9-THC: 0.77 ng/ml

moderately low dose by most previous studies (*17, 18*). Representative chromatograms are shown in Figures 6 and 7 where samples were prepared from blood drawn 1 hour and 4 hours after administration. The Δ^9-THC level falls rapidly during the first 30 minutes and then decreases much more slowly. This observation agrees with previous studies where radioactively labeled Δ^9-THC was employed. Samples were collected as long as 7 hours after drug administration and Δ^9-THC could still be detected. With proper choice of columns and conditions, background interference did not present a problem at concentrations of Δ^9-THC as low as 100 pg per ml of blood.

Although some metabolites of Δ^9-THC may serve as a more reliable correlate to the physiological and behavioral effect of cannabis preparations, this method demonstrates the applicability of electron capture gas chromatography to analysis of cannabinoids in biological materials. One metabolite which has been identified as being physiologically active is 11-hydroxy-Δ^9-tetrahydrocannabinol (*19*). Preliminary studies of this compound indicate that accurate analysis is feasible using the method described for Δ^9-THC as should be the case for any of the cannabinoids capable of derivatization with electrophoric reagents. In addition, use of dual column electron capture gas chromatography could prove highly advantageous in the ultramicroanalysis of numerous compounds of biological interest such as steroids, prostaglandins, catecholamines, etc.

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