Separation and Analysis of Δ^9-Tetrahydrocannabinol in Biological Fluids by High-Pressure Liquid Chromatography and GLC

EDWARD R. GARRETT [x] and C. ANTHONY HUNT *

Abstract □ High-pressure liquid chromatographic (HPLC) systems were developed to separate quantitatively Δ^9-tetrahydrocannabinol from heptane-extractable lipoidal and other endogenous substances in biological fluids. These substances interfered with the quantitation by flame-ionization GLC of the unmodified compound and by electron-capture GLC of the pentafluorobenzoyl derivative. Reversed-phase HPLC elution, with 47% acetonitrile in water, and normal-phase HPLC with 25% chloroform in heptane separated Δ^9-tetrahydrocannabinol from 11-hydroxy-Δ^9-tetrahydrocannabinol and other monohydroxylated tetrahydrocannabinols. These systems also purified stock solutions of Δ^9-tetrahydrocannabinol from accompanying contaminants. The various monohydroxylated tetrahydrocannabinols were resolved from each other in the normal phase, 80% chloroform in heptane. The Δ^8- and Δ^9-tetrahydrocannabinols were separable in the normal phase with 5% tetrahydrofuran in hexane. The GLC analysis of pentafluorobenzoylated Δ^9-tetrahydrocannabinol had a sensitivity of 1 ng/ml of plasma, with an estimated 5% standard error with the developed extraction and GLC procedures. Radiochemical analysis of the HPLC-separated fraction had a sensitivity of 0.2 ng/ml of plasma, with an estimated 2% standard error. 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. Radiolabeled compounds can be added to plasma samples as internal standards to determine the recovery efficiencies of the several procedures in the analysis of unlabeled tetrahydrocannabinol.

Keyphrases □ Δ^9-Tetrahydrocannabinol—GLC and high-pressure liquid chromatographic analyses, biological fluids □ GLC—analysis, Δ^9-tetrahydrocannabinol in biological fluids □ High-pressure liquid chromatography—analysis, Δ^9-tetrahydrocannabinol in biological fluids

A GLC method for tetrahydrocannabinol in biological fluids was developed previously (1) 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 (1, 2) and rubber stoppers (2) 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[1]. In the case of organic solutions, the tetrahydrocannabinol could be reincorporated from the glass into solution by 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 hr after the administration of 0.1 mg of pure tetrahydrocannabinol/kg (1). However, when the same method was applied to nonfasting animals, a significant increase in the GLC background from interfering plasma constituents was observed, particularly within 4 hr 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 pharmacokinetic studies were contemplated in both dogs and humans over longer periods 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 cleanup 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 interfering endogenous materials of biological fluids with subsequent analysis by various appropriate methods.

EXPERIMENTAL

Materials—Δ^9-Tetrahydrocannabinol[2], cannabinol[3], cannabidiol[4], 11-hydroxy-Δ^9-tetrahydrocannabinol[5], 8α-hydroxy-Δ^9-tetrahydrocannabinol[6], 8β-hydroxy-Δ^9-tetrahydrocannabinol[7], ^{3}H-11-hydroxy-Δ^9-tetrahydrocannabinol[8], and ^{14}C-Δ^9-tetrahydrocannabinol[9] were used.

Liquid Scintillation—Two different instruments were used. In one system[10], two of three channels were set specifically to read tritium and carbon-14. In the other system[11], all three channels were used. All samples were counted for 10 min with a blank inserted at the start of every 50 samples.

The degree of quench of each sample was estimated from the channel ratios of the sample and from known standards. When quench was indicated, it was calculated after addition of a ^{14}C-toluene or tritiated water standard. All samples were allowed to adapt to the dark, and this adaptation was checked by recounting selected samples.

[1] Rinsing glass for 30 min with a 1% (v/v) solution of Sil-Prep (Applied Sciences Laboratories, State College, PA 16801) in dichloromethane reduced adsorption to less than 5%. This rinsing was followed by a solvent rinse of dichloromethane and drying.

[2] Unless specified differently, obtained from the Department of Health, Education, and Welfare, Public Health Service, National Institute of Mental Health; Lot SSC-61591, 1 g/ml of ethanol.

[3] Lot NMH-IV-65c.

[4] Lot NMH-IV-65c.

[5] Lot DD-I-45A.

[6] Lot DD-I-61A.

[7] Lot RLH-III-39b.

[8] Lot SS-II-110A, 169.5 μCi/5 mg.

[9] Sample 3168-145-27, 3817 μCi/ml, R. T.I., Research Triangle Park, N.C.

[10] Model 3003, Packard Instruments, Downers Grove, Ill.

[11] Liquid scintillation system, Beckman Instruments, Fullerton, Calif.

GLC—A gas chromatograph[12], fitted with both ^{63}Ni- and flame-ionization detectors, was used. Detectors were maintained between 270 and 285°. The ^{63}Ni-detector had a separate nitrogen gas flow of 35 ml/min, and the glass columns were 30 cm or 1.8 m (6 ft) long (2.0 mm i.d.). The conditions were: column, 210–230°; injection port, 245°; 3% OV-225 on 100–120-mesh Gas Chrom Q or 3% OV-17 on 100–120-mesh Gas Chrom Q; nitrogen gas flow, 35–45 ml/min; standing current (^{63}Ni), 60–90% at 2×10^{-9}; and background noise, 2–3% at 1×10^{-10}.

HPLC—A chromatograph[13], equipped with a 6000-psi constant-flow pump, a 254-nm UV detector, and a differential refractometer detector, was used. The columns were either a low polarity 0.61-m (2-ft) × 0.3-cm (0.125-in.) Bondapak-C_{18} Corasil [with a $(CH_2)_{17}CH_3$ surface functionality], designed for reversed-phase chromatography, or a high polarity 30-cm μ-Porasil (with a SiOSiOH surface functionality), designed for normal-phase chromatography. All on-column injections were made under constant-flow conditions. Special high quality solvents[14] were used.

Extraction of ^{14}C-Δ^9-Tetrahydrocannabinol from Plasma—A 2-ml plasma sample containing the ^{14}C-Δ^9-tetrahydrocannabinol was transferred to a silylated 50-ml glass centrifuge tube. The pH was adjusted between 9.5 and 11.0 by addition of 0.1 *N* Na_2CO_3 (0.2–0.5 ml) prepared from water purified by HPLC using a clean Bondapak-C_{18} Corasil column. The final volume was adjusted to 2.5 ml by addition of water, and 15 ml of heptane[14] containing 1.5% isoamyl alcohol[14] was added. The tube was stoppered[15], shaken for 15 min, and centrifuged. It was not necessary to denature the plasma protein as described previously (1).

An aliquot (5–14 ml) of the organic layer to be used for HPLC was transferred to a glass centrifuge tube, and a second aliquot (0.5–1.0 ml) was transferred to a 20-ml glass liquid scintillation vial. Both vial and tube contents were dried under a stream of nitrogen in a water bath (50°).

Six milliliters of scintillation solution[16] was added to the vial contents, and the sample was analyzed by liquid scintillation spectroscopy. Samples with low counts (less than 400 cpm) were counted twice and averaged.

HPLC Separation of Δ^9-Tetrahydrocannabinol—The sides of the 5-ml tube were rinsed with 100 μl of ethanol, and the contents were dried again. The residue was dissolved in 15–21 μl of ethanol, and an aliquot (10–14 μl) was used for separation of Δ^9-tetrahydrocannabinol by the normal-phase HPLC system, which used a 20–25% chloroform in heptane solvent system at a constant flow rate (either 1.0 or 1.5 ml/min). The tetrahydrocannabinol fraction was collected over the predetermined collection range in a 5-ml tube and dried. The residue was dissolved in 0.5 ml of ethanol and stored in a freezer until time for derivatization and analysis by GLC with electron-capture detection.

After one to three injections of plasma extracts, it was necessary to remove from the column those components of the extract that had high affinities with the packing material. When normal-phase HPLC was used, the solvent system was changed to heptane, 4 ml of 10% heptane in tetrahydrofuran was injected, the flow rate was adjusted to 2 ml/min, and the heptane was replaced after 3 min by the original solvent system. No further injections were made until the system operated for 10 min at the original specifications.

The HPLC system was checked for reproducibility in collecting fractions of administered tetrahydrocannabinol prior to a series of injections on a given day. Known amounts of HPLC-purified ^{14}C-Δ^9-tetrahydrocannabinol were dissolved in 21 μl of ethanol, and 14 μl was injected in the chromatograph. The remainder of each sample was counted for total carbon-14. At the beginning of each series of HPLC collections, two reference standards of approximately 5 and 500 ng, each originally dissolved in 21 μl and of equal activity, were injected and collected as stated to assure that the collection range used was proper.

The collected fractions were counted for total carbon-14, and the collection efficiency was considered as the fraction injected recovered. It was shown previously (1) that Δ^9-tetrahydrocannabinol did not degrade significantly under the mild conditions of these analytical procedures (1–3). All glassware was silylated as described (1). It is possible to analyze eight samples/day, including the determination of the reproducibility of the HPLC system.

Derivatization of Samples for Sensitive Electron-Capture Detection (1)—Prior to GLC analysis, each stored sample was dried under a stream of nitrogen in a water bath (50°). One hour was a satisfactory drying time. The residue was dissolved in 0.1 ml of dry benzene. A 100 *M* excess of pyridine in benzene was added to the benzene solution of Δ^9-tetrahydrocannabinol. A 100 *M* excess of pentafluorobenzoyl chloride[17] in 5–20 μl of benzene was also added. The amounts were estimated from the known amount of radiolabeled tetrahydrocannabinol in the heptane extract. The tube was stoppered and allowed to stand for 15 min, and 100 μl of 0.1 *M* Na_2CO_3 was added. The mixture was shaken for 1 min on a vortex shaker and centrifuged.

The aqueous layer was removed, 200 μl of benzene was added, and the tube was centrifuged again. An aliquot, approximately 250 μl, of the benzene layer was transferred to a 5-ml centrifuge tube and dried under nitrogen in a water bath. The residue was dissolved in an appropriate volume, 0.2–2.0 ml, of toluene, usually containing 50 pg of cannabidiol pentafluorobenzoate (the internal standard)/ml. The optimum amount of internal standard to give an area ratio with tetrahydrocannabinol pentafluorobenzoate of about unity was predicted from the radiolabeled tetrahydrocannabinol concentration in the heptane extract of plasma. Aliquots of 0.5–1.5 μl of this final solution were analyzed by GLC.

Preparation of Internal Standard—Cannabidiol for use as the GLC internal standard was purified by injecting 1 mg into the normal-phase HPLC system with a 20% chloroform in heptane eluant. The central third of the peak was collected, dried, and dissolved in 10 ml of dry benzene. The concentration of the solution was determined by flame-ionization GLC (2, 3). Cannabidiol pentafluorobenzoate was prepared by adding 2 μl each of pyridine and pentafluorobenzoyl chloride to 1 ml of this solution. After 5 min, 1 ml of 0.1 *M* Na_2CO_3 was added, the solution was shaken for 1 min, and the organic layer was transferred to a 5-ml tube and dried.

The stable residue was dissolved in dry toluene to yield 50–500 pg/ml when needed for use as an internal standard. A given volume of this solution was analyzed by electron-capture GLC, and the area under the peak was determined. This approach provided a measure of the volume of internal standard solution necessary to add to the tetrahydrocannabinol sample to be analyzed to obtain the proper area ratio even without an exact knowledge of the internal standard concentration.

Preparation of Calibration Curves—Calibration curves of peak area ratios of drug to internal standard *versus* plasma concentration for monitoring plasma levels of tetrahydrocannabinol in pharmacokinetic studies were usually prepared from duplicate 2-ml plasma samples containing 1, 5, 10, 50, and 100 ng of ^{14}C-labeled or unlabeled Δ^9-tetrahydrocannabinol. After heptane extraction of the samples, normal-phase HPLC and electron-capture GLC assay of the derivatized material was effected.

The amount in the plasma sample could also be calculated from a calibration curve of the ratios of the peak area of the tetrahydrocannabinol pentafluorobenzoate prepared from pure tetrahydrocannabinol in toluene to that of the cannabidiol pentafluorobenzoate when the dilution, reconstitution, and separate efficiencies of the extraction and HPLC elution procedures were taken into account. The extraction efficiency was established for each batch of plasma by adding known amounts of ^{14}C-Δ^9-tetrahydrocannabinol to 2 ml of plasma and determining the fraction of the label recovered after extraction in 15 ml of heptane.

The HPLC eluant of the labeled Δ^9-tetrahydrocannabinol fraction was also collected in vials (20 ml), dried, and analyzed for total carbon-14, using liquid scintillation spectroscopy. The amount of tetrahydrocannabinol present was calculated from the known dilutions, reconstitutions, and evaluated efficiencies of the extraction and HPLC elution procedures.

Purification of Δ^9-Tetrahydrocannabinol by HPLC—Aliquots of the stock ^{14}C-Δ^9-tetrahydrocannabinol solutions were analyzed for radiolabel purity by HPLC with a reversed-phase column, using a 45 or 51% acetonitrile–water solvent system at a constant pressure below 3000 psi at a flow rate of 1.5 ml/min, or with the normal-phase column, using a 20 or 25% chloroform–heptane solvent system at a flow rate of 2.5 ml/min. The eluate was monitored for UV absorbance at 254 nm and collected in volumetric tubes at 0.5- or 1-ml intervals.

Each fraction was analyzed by GLC and by liquid scintillation counting for total carbon-14; its activity, corrected for background, was plotted against the cumulative volume for that fraction, defined as the volume eluted at a time after injection minus the "dead volume" of the system. The pooled volumes containing the separated ^{14}C-Δ^9-tetrahydro-

[12] Models 2100 and 20, Varian Aerograph, Walnut Creek, Calif.
[13] Model ALC 202, Waters Associates, Milford, Mass.
[14] Burdick and Jackson Laboratories, Muskegon, Mich.
[15] Kimble, polyethylene, Sargent-Welch, Birmingham, Ala.
[16] Handifluor, Mallinckrodt Chemical Works, St. Louis, Mo.
[17] PCR, Inc., Gainesville, Fla.

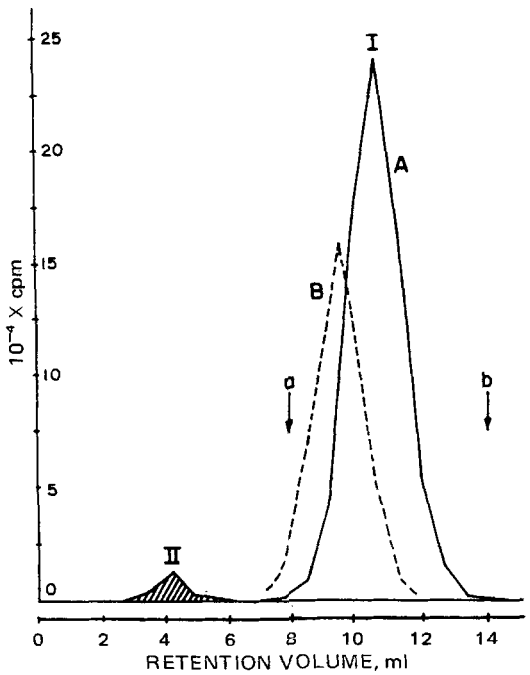

Figure 1—*Reversed-phase HPLC of stock* ^{14}C-Δ^9-*tetrahydrocannabinol. The total radioactivity per eluate fraction was plotted* versus *retention volume (curve A). Peak I is* ^{14}C-Δ^9-*tetrahydrocannabinol, and peak II is an unknown radioactive contaminant. Curve B is a plot of the mean UV detector response (arbitrary units)* versus *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. The column was Bondapack* C_{18}*; the eluant was 45% acetonitrile in water at 1.5 ml/min. Each fraction was corrected for background counts per minute.*

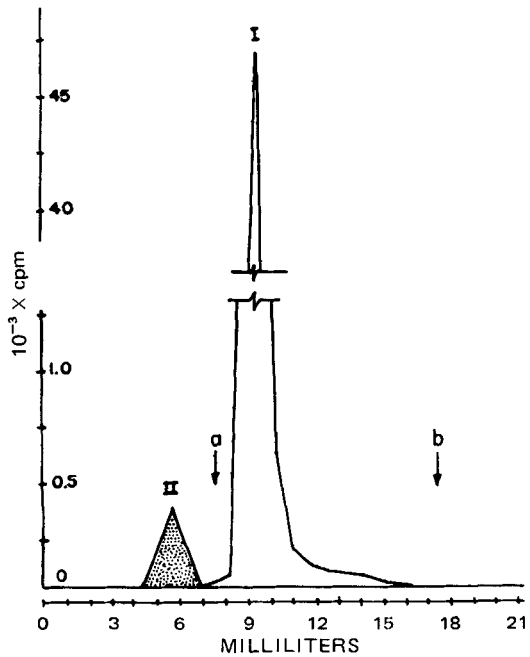

Figure 2—*Normal-phase HPLC of stock* ^{14}C-Δ^9-*tetrahydrocannabinol. The total activity per eluate fraction was plotted* versus *milliliters of eluate. Peak I is* ^{14}C-Δ^9-*tetrahydrocannabinol, and peak II is an unknown radioactive contaminant. Collection between volumes* a *and* b *recovered 97.5%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

cannabinol (peak I, Figs. 1 and 2), as evidenced by the observed single peak, contained the material used in the quantification of the efficiencies of the various separation and analytical methods.

Quantitation of Efficiency of HPLC Separation of Δ^9-Tetrahydrocannabinol—The described procedure was repeated on this purified material after the collected eluates containing the Δ^9-tetrahydrocannabinol fraction were dried under nitrogen at 50° and reconstituted in absolute ethanol to yield 2×10^4 cpm/μl. The fractions collected were analyzed for total carbon-14. Thus, a collection range for tetrahydrocannabinol was established that would contain at least 98% of the recoverable label. The eluates within this established collection range were analyzed for total carbon-14 when various aliquots of the purified ^{14}C-tetrahydrocannabinol in ethanol were injected on the two HPLC systems (Tables I and II). The effect of varying percentages of acetonitrile in the eluting solvent for the reversed phase was also studied (Fig. 3 and Table I). Similarly, an authentic sample of ^{3}H-11-hydroxy-Δ^9-tetrahydrocannabinol was purified, and its collection range for 98% recovery was determined for reversed-phase HPLC (Fig. 3).

The HPLC-purified ^{14}C-Δ^9-tetrahydrocannabinol, in known various amounts, was added to aliquots of fresh dog plasma to determine the efficiency of HPLC drug recovery from plasma. The tetrahydrocannabinol was extracted, the pertinent fraction of drug was collected on the HPLC system used, and the total carbon-14 activity of each collection was determined. A 45% acetonitrile–water solvent system was used in the reversed phase; a 20% chloroform–heptane solvent system was used in the normal phase.

As stated previously, it was necessary to remove components of the plasma extract that had high affinities with the packing material of the column after one to three injections of plasma. The cleanup procedure for normal-phase HPLC was given previously. When reversed-phase HPLC was used, 4 ml of 10% methanol in acetonitrile was injected or the eluant was changed to 100% acetonitrile, the flow rate was adjusted to 1 ml/min, and the flow rate was increased after 5 min to 3 ml/min for 3 min.

A 2-ml plasma sample was spiked with 400 ng of unlabeled Δ^9-tetrahydrocannabinol and 400 ng of 11-hydroxy-Δ^9-tetrahydrocannabinol, extracted, and prepared for HPLC. The sample was injected on

Table I—Recovery of Radioactivity of ^{14}C-Δ^9-Tetrahydrocannabinol[a] from Reversed-Phase HPLC

Eluting Solvent, % Acetonitrile in Water	n[b]	Average 10^{-4} cpm Injected[c]	Average 10^{-4} cpm Collected[d]	Average Recovery, %
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 average	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: 4×10^3 cpm/μg and 2.003×10^4 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 II—Recovery of Radioactivity of ^{14}C-Δ^9-Tetrahydrocannabinol[a] from Normal-Phase HPLC[b]

n[c]	Average 10^{-3} cpm Injected[c]	Average 10^{-3} cpm Collected[d]	Recovery[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 was 25% chloroform in heptane. [c] Fifteen microliters of n replicates of each solution was 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 the mean. [f] Standard error of the mean where 4.23 is the standard deviation.

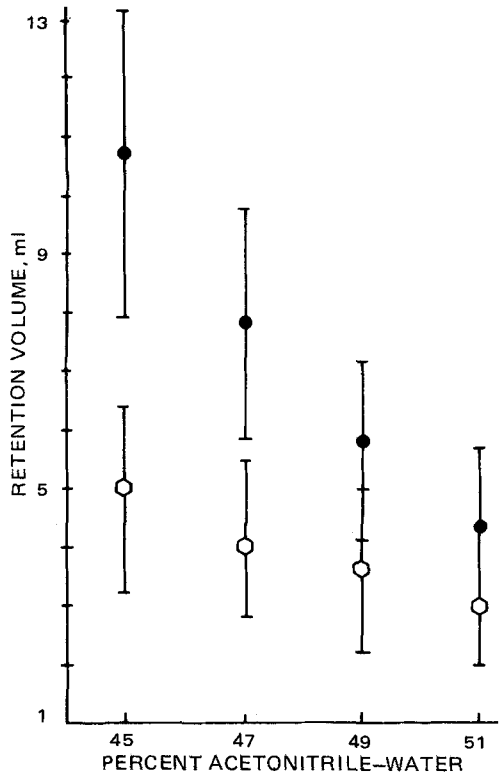

Figure 3—*Retention volumes for the peak amounts of Δ^9-tetrahydrocannabinol (●) and 11-hydroxy-Δ^9-tetrahydrocannabinol (○) for reversed-phase HPLC plotted* versus *solvent composition. Each point represents the mean peak retention volume for two determinations. The vertical bars represent the ranges of retention volumes that contained approximately 98% of the area under the plot of recovered radioactivity* versus *retention volume.*

the reversed-phase HPLC system, and the eluate was collected over the range for a 51% acetonitrile–water solvent system that would collect both compounds. The collected fraction was dried under nitrogen in a water bath (50°), reconstituted in 50 μl of chloroform, and analyzed by GLC, using flame ionization as previously described (2, 3).

Variability in Extraction, HPLC, and Electron-Capture GLC Analytical Procedures for Δ^9-Tetrahydrocannabinol in Plasma—The total error and the sources of error for such analyses were evaluated

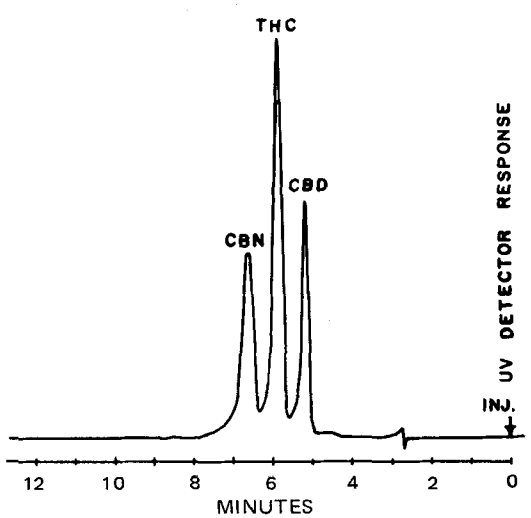

Figure 4—*Normal-phase HPLC separation of cannabinoids. The UV detector response (arbitrary units) was plotted* versus *retention time after injection (inj.) of a mixture of cannabidiol (CBD), Δ^9-tetrahydrocannabinol (THC), and cannabinol (CBN). The amounts of Δ^9-tetrahydrocannabinol and cannabidiol injected were approximately twice the amount of the cannabinol. The column was μ-Porasil; the eluant was 20% chloroform in heptane at 1.5 ml/min.*

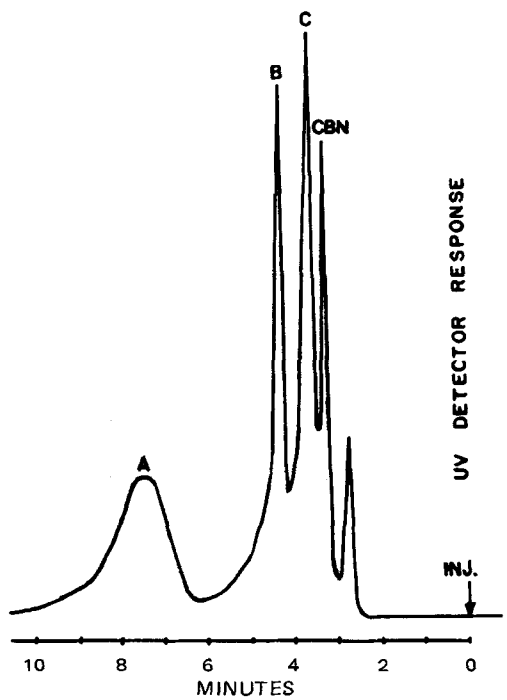

Figure 5—*Normal-phase HPLC separation of Δ^9-tetrahydrocannabinol metabolites. The UV detector response (arbitrary units) was plotted* versus *retention time after injection (inj.) of a mixture of cannabinol (CBN), 11-hydroxy-Δ^9-tetrahydrocannabinol (C), 8α-hydroxy-Δ^9-tetrahydrocannabinol (B), and 8β-hydroxy-Δ^9-tetrahydrocannabinol (A). The column was μ-Porasil; the eluant was 80% chloroform in heptane at 1.5 ml/min.*

for the overall analytical scheme in three separate experiments.

1. Each of three 9-ml plasma samples was spiked with 2.25, 22.5, or 225 ng of ^{14}C-Δ^9-tetrahydrocannabinol. Four 2-ml aliquots were removed from each sample, the tetrahydrocannabinol was extracted from each, and 14 of the 15 ml of the heptane extract was dried and analyzed for total carbon-14.

2. Three 9-ml plasma samples were prepared and extracted as already described. The tetrahydrocannabinol in the dried heptane extract was separated by normal-phase HPLC, and the tetrahydrocannabinol fraction was collected directly in counting vials and analyzed for total carbon-14.

3. Three 9-ml plasma samples were prepared as described, extracted, and separated by HPLC (normal phase). The proper eluant fraction was analyzed by GLC.

These three studies were carried out on different days and used fresh plasma from different dogs.

HPLC Separation of Δ^9-Tetrahydrocannabinol from Selected Cannabinoids and Metabolites—Normal-phase HPLC (20% chloroform in heptane) was tested for the degree of separation obtainable between cannabinol, cannabidiol, and tetrahydrocannabinol by injection of an aliquot of a mixture of the three cannabinoids at a flow rate of 1.5 ml/min. The compounds were monitored by UV detection. When an aliquot of a mixture of cannabinol and three monohydroxylated metabolites of tetrahydrocannabinol, 8α-hydroxy-Δ^9-tetrahydrocannabinol, 8β-hydroxy-Δ^9-tetrahydrocannabinol, and 11-hydroxy-Δ^9-tetrahydrocannabinol, was injected into the same HPLC system, no peaks for the three monohydroxy compounds were observed prior to 15 min. Thus, this system widely separated Δ^9-tetrahydrocannabinol, with its retention volume of 9 ml (Fig. 2), from these compounds. HPLC was obtained for the three metabolites in an 80% chloroform–heptane solvent system at a 1.5-ml/min flow rate. Δ^9-Tetrahydrocannabinol had a retention value equivalent to cannabinol in this system.

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 was reportedly[18] free of radiolabeled

[18] National Institutes of Health, personal communication in reference to Contract HSM-42-71-108.

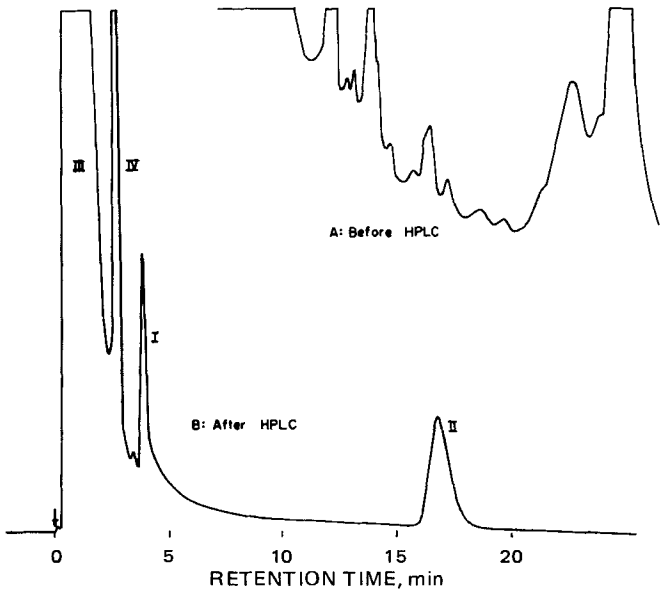

Figure 6—*Gas–liquid chromatograms (flame-ionization detection) of an extract of 2 ml of plasma containing Δ^9-tetrahydrocannabinol, I (200 ng/ml), and 11-hydroxy-Δ^9-tetrahydrocannabinol, II (200 ng/ml), before (A) and after (B) reversed-phase HPLC separation of both cannabinoids over a range of predetermined collection volumes (Fig. 1). One microliter of 18 μl of extract was injected into the gas chromatograph prior to HPLC (A). Ten microliters of 18 μl of the extract was injected into the liquid chromatograph, the collected fraction was reconstituted in 10 μl of chloroform, and 1 μl was injected into the gas chromatograph (B). Peak III is the solvent; peak IV is an unknown from plasma. For HPLC, the column was Corasil C_{18}; the eluant was 51% acetonitrile in water at 1.5 ml/min. For GLC, the column was 1.5 m (5 ft) × 2 mm, 1.9% OV-225 at 245°, with a nitrogen flow of 24 ml/min. An attenuation of 8×10^{-12} was used for both chromatograms. The initial baselines and injection times for both chromatograms are superimposed for comparison.*

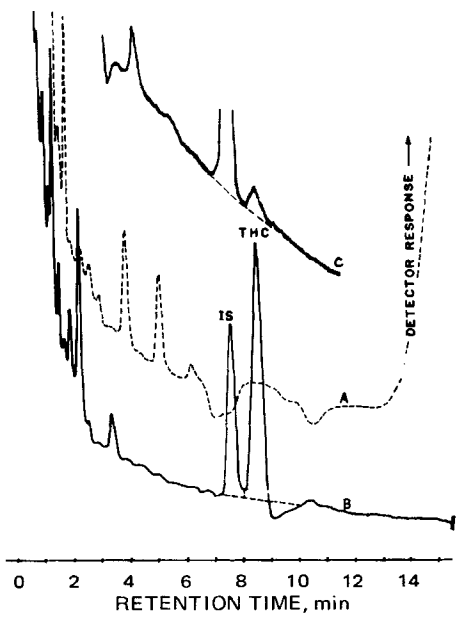

Figure 7—*GLC (electron-capture) analysis of derivatized samples. The chromatograms represent: (A) the injection of 1 of 300 μl from a derivatized extract of 2 ml of blank plasma without HPLC purification; (B) the injection of 1 of 200 μl of the final solution of derivatized compound, which was 70% of the total amount extracted from 2 ml of dog plasma containing 200 ng of Δ^9-tetrahydrocannabinol/ml, with extraction and normal-phase HPLC collection efficiencies of 91 and 95%, respectively; and (C) the injection of 1 of 200 μl from a derivatized extract of 2 ml of plasma containing 1.3 ng of Δ^9-tetrahydrocannabinol/ml. All other factors were the same as for chromatogram B. The peaks for the internal standard, cannabidiol pentafluorobenzoate (IS), and Δ^9-tetrahydrocannabinol pentafluorobenzoate (THC) are appropriately labeled. Typical estimated baselines are shown. The GLC conditions were: 1.8-m (6-ft) OV-17 column, 225°; detector, 280°; injector, 255°; nitrogen flow, 45 ml/min; and attenuations, 4×10^{-9} (A and B) and 8×10^{-10} (C).*

contaminants by TLC. However, reversed-phase HPLC (Fig. 1) revealed the presence of a labeled contaminant. The major peak, I, contained Δ^9-tetrahydrocannabinol (>96%) and Δ^8-tetrahydrocannabinol (≃3%), quantified by flame-ionization GLC (2, 3). A labeled contaminant was also found (Fig. 2) with a normal-phase column. The contaminant eluted prior to Δ^9-tetrahydrocannabinol in both cases. Since the more polar compounds elute first on reversed-phase HPLC whereas the least polar compounds elute first on normal-phase HPLC, the contaminants observed in the two systems probably were not the same. The contaminants were not analyzed further.

When the tetrahydrocannabinol under peak I (Figs. 1 and 2) was collected, dried, reconstituted in ethanol, and reanalyzed on the same HPLC system, only the single peak I was observed. All of the stock Δ^9-tetrahydrocannabinol used underwent this purification procedure. The percent of the total injected radioactivity under peak I recovered was 97.6 ± 0.7% (*SEM*) (Table I) in the reversed-phase system for the ranges of volumes collected (Figs. 1 and 3) and 96.3 ± 1.0% (Table II) for 1–1000 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 labeled 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-tetrahydrocannabinol. The retention volume and the appropriate collection range increased with decreasing solvent polarity (less percent acetonitrile) due to peak spreading (Fig. 3).

The quality of the water used in the eluting solvent, acetonitrile–water, in reversed-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 eluant. Additional evidence of these contaminants was demonstrated when the GLC background varied significantly when distilled water from various sources was collected after reversed-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—Δ^9-Tetrahydrocannabinol, cannabinol, and cannabidiol were resolved by the normal-phase HPLC system (Fig. 4). If 98% of the Δ^9-tetrahydrocannabinol were to be collected after normal-phase HPLC in this system, the chosen range (Fig. 2) would also collect cannabinol and cannabidiol. The retention volumes of cannabinol and cannabidiol relative to Δ^9-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 reversed-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.

Both 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% tetrahydrofuran 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.

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 reversed-phase HPLC. The reduction in potential contaminants from plasma observable on GLC was demonstrated by flame-ionization GLC analysis (2, 3) both before and after the HPLC treatment (Fig. 6).

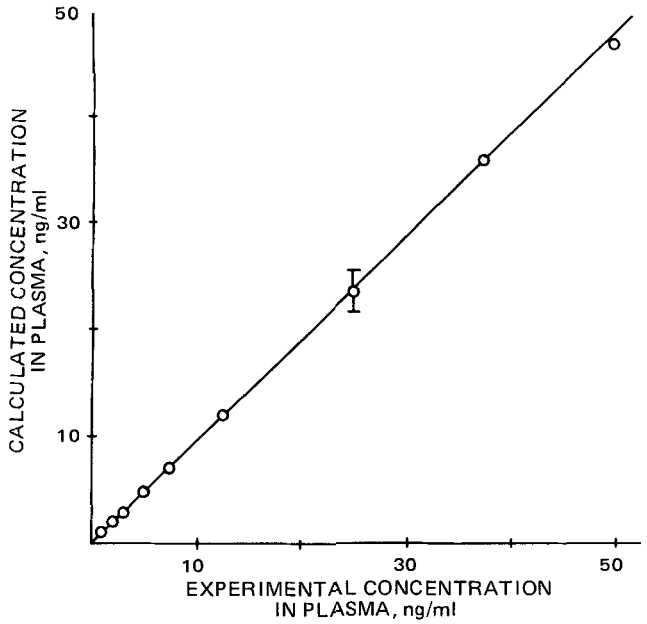

Figure 8—*Recovery of ^{14}C-Δ^9-tetrahydrocannabinol calculated as concentration in plasma corrected for an extraction efficiency of 91% against the experimentally prepared concentrations. The drug was extracted from plasma and collected over the proper volume range (Table I and Fig. 1) after reversed-phase HPLC with a 45% acetonitrile–water eluant. 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 ±1 SD (n = 4).*

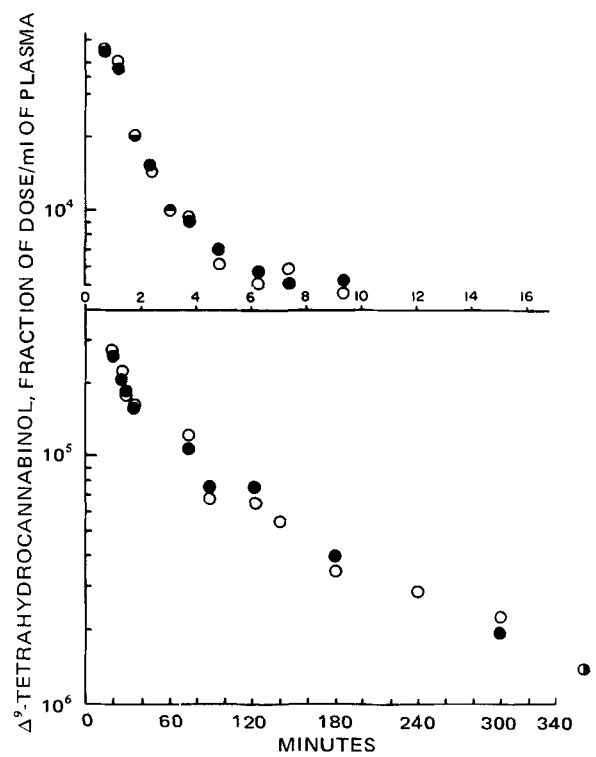

Figure 9—*Semilogarithmic plots of fraction of the Δ^9-tetrahydrocannabinol, 0.1-mg/kg dose/ml of plasma, against time for Dog A plotted from the liquid scintillation analysis of the total carbon-14 collected as Δ^9-tetrahydrocannabinol on normal-phase HPLC (○) and from the electron-capture GLC of the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

Normal-phase HPLC with 20% chloroform in heptane could separate Δ^9-tetrahydrocannabinol from monohydroxylated metabolites and 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 separated these nonpolar 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 Δ^9-tetrahydrocannabinol analyzed with electron-capture detection, as shown by a comparison of curves A and B or A and C in Fig. 7.

Plasma samples obtained from dogs administered Δ^9-tetrahydrocannabinol solutions intravenously were analyzed by electron-capture GLC in accordance with the modified procedures described here, including extraction, normal-phase HPLC separation, and derivatization, but no internal standard was added. These procedures would have included any cannabinol or cannabidiol in the HPLC collection volume range (Figs. 2 and 4) used. However, no peaks were seen at the retention times of cannabinol or cannabidiol pentafluorobenzoate, 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 (4–6), cannabidiol pentafluorobenzoate was chosen as the internal standard. Cannabinol is known to be a contaminant of degraded Δ^9-tetrahydrocannabinol (3).

The presented GLC methodology differed from the prior studies (1) 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 resolution of the cannabinols (7–9).

Efficiency, Reproducibility, and Sensitivity of 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% (*SEM*).

The recovery of Δ^9-tetrahydrocannabinol from the heptane extract of dog plasma by 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% *SE*) and electron-capture GLC analysis (84.0 ± 4.9% *SE*) of the derivatized tetrahydrocannabinol (Fig. 7). Both methods permitted estimation of

Table III—Percent Recoveries[a] of ^{14}C-Δ^9-Tetrahydrocannabinol in the Heptane Extract of Plasma and 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-Δ^9-Tetrahydrocannabinol[b], ng/2 ml of Plasma	Extracted by Heptane, %, Scintillation	Extract HPLC Collected[c], %		Overall Recovery[d], %	
		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	88 ± 4	81 ± 7
Overall average ± *SE*	90.6 ± 0.7	92	93	84 ± 2	84 ± 5

[a] Given as the mean from four separate plasma samples ± *SD*. 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 milliliters of plasma was extracted with 15 ml of heptane, and 14 ml was analyzed for total carbon-14. [c] Quotient divided by the extraction efficiency (0.91). Additional studies were conducted for 26 ng of ^{14}C-Δ^9-tetrahydrocannabinol/2 ml of plasma on 2 other days for four samples each, and the percents recovered on HPLC from the extract were 94 ± 4 and 92 ± 3% (*SE*), 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.

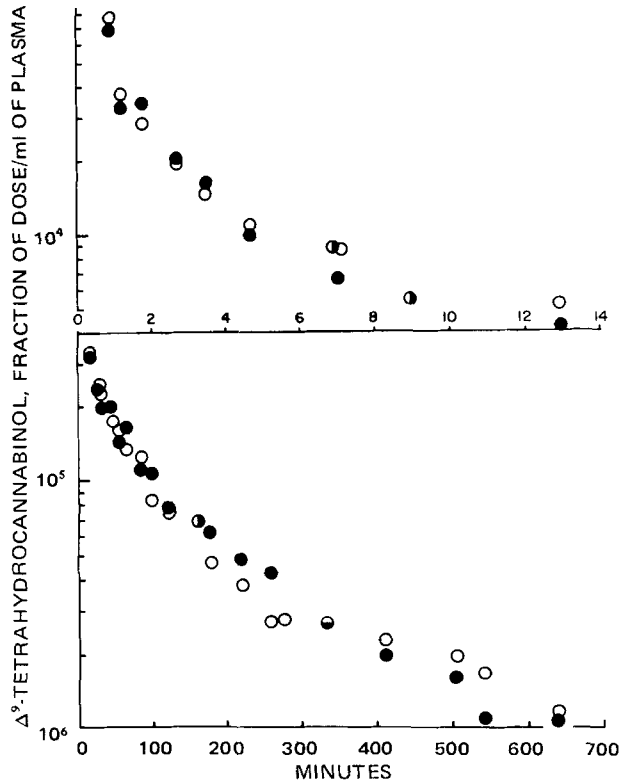

Figure 10—*Semilogarithmic plots of fraction of the Δ⁹-tetrahydrocannabinol, 2.0-mg/kg dose/ml of plasma, against time for Dog A plotted from the liquid scintillation analysis of the total carbon-14 collected as Δ⁹-tetrahydrocannabinol on normal-phase HPLC (○) and from the electron-capture GLC of the derivatized HPLC collected fraction (●). The values were corrected for the fractions of extracts and total collection range used.*

a 92.5% recovery of the amount in the heptane extract injected on 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 Δ⁹-tetrahydrocannabinol (Fig. 2 and Table II) since the procedure had to be modified to separate tetrahydrocannabinol from possible monohydroxylated or 11-hydroxy-Δ⁹-tetrahydrocannabinol metabolites in plasma. A slight overlap of their HPLC peak areas with Δ⁹-tetrahydrocannabinol would have occurred with the larger collection ranges.

A similar study of the reproducibility of collection of ^{14}C-Δ⁹-tetrahydrocannabinol in plasma assayed by liquid scintillation after extraction and reversed-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 was 95.7%.

Equivalency of Radiochemical Analyses of ^{14}C-Δ⁹-Tetrahydrocannabinol and GLC Electron-Capture Detection of Derivatized Material after Normal-Phase HPLC in Dog Plasma during Pharmacokinetic Studies—The plasma of a dog intravenously administered solutions of ^{14}C-Δ⁹-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.

The procedures for GLC analysis 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 of plasma (Table II). Similarly, the procedure for radiochemical analysis 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 finding demonstrated that all of the recovered radioactivity from the HPLC separation procedure could be assigned to the Δ⁹-tetrahydrocannabinol assayed specifically by electron-capture GLC. Thus, no significant amounts of radiolabeled metabolites were in the collected HPLC fractions.

It can be concluded that Δ⁹-tetrahydrocannabinol can be extracted from plasma and other biological fluids and 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 unlabeled Δ⁹-tetrahydrocannabinol in a solution of plasma were analyzed, the calculated recovery of known amounts of labeled Δ⁹-tetrahydrocannabinol added either to plasma prior to extraction or to the 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 ^{14}C-Δ⁹-tetrahydrocannabinol were used in pharmacokinetic studies, extracted, and separated liquid chromatographically, ^{3}H-tetrahydrocannabinol could be used as the appropriate internal standard to monitor the recovery efficiencies.

REFERENCES

(1) E. R. Garrett and C. A. Hunt, *J. Pharm. Sci.*, **62**, 1211 (1973).

(2) *Ibid.*, **63**, 1056 (1974).

(3) E. R. Garrett and J. Tsau, *J. Pharm. Sci.*, **63**, 1563 (1974).

(4) N. K. McCallum, *J. Chromatogr. Sci.*, **11**, 509 (1973).

(5) N. K. McCallum, B. Yagen, S. Levy, and R. Mechoulam, *Experientia*, **31**, 520 (1975).

(6) M. Widman, M. Nordquist, S. Agurell, J. E. Lindgren, and F. Sandberg, *Biochem. Pharmacol.*, **23**, 163 (1974).

(7) R. S. Fetterman and C. E. Turner, *J. Pharm. Sci.*, **61**, 1476 (1972).

(8) C. E. Turner and K. Hadley, *ibid.*, **62**, 251, 1083 (1973).

(9) C. E. Turner, K. W. Hadley, J. Henry, and M. L. Mole, *ibid.*, **63**, 1872 (1974).

ACKNOWLEDGMENTS AND ADDRESSES

Received September 19, 1975, from the *College of Pharmacy, University of Florida, Gainesville, FL 32610.*

Accepted for publication March 8, 1976.

Abstracted in part from a dissertation submitted by C. A. Hunt to the Graduate School, University of Florida, in partial fulfillment of the Doctor of Philosophy degree requirements.

Supported in part by Grant DA-0073 from the National Institute of Mental Health, Bethesda, MD 20014.

The technical assistance of Mrs. Kathleen Eberst and the contributions of Hendryk Roseboom are gratefully acknowledged.

*Present address: School of Pharmacy, University of California, San Francisco, CA 94143.

[x] To whom inquiries should be directed.