Picogram Analysis of Tetrahydrocannabinol and Application to Biological Fluids

Keyphrases ☐ Tetrahydrocannabinol in biological fluids—GLC-electron-capture analysis, picogram quantities ☐ GLC–electron-capture detection—analysis, tetrahydrocannabinol in biological fluids

Sir:

A major goal in marijuana research is to quantify those compounds and metabolites in biological fluids responsible for psychoactive effects (1). Clinical investigations of Δ^9-tetrahydrocannabinol (2, 3), a major active component of marijuana, and 11-hydroxy-Δ^9-tetrahydrocannabinol (4), an active metabolite, have relied on analysis by radioactive labeling. However, the study of distribution, metabolism, and excretion of the drug and its metabolites under chronic or "street" conditions demands nonradioactive analytical procedures suitable for analysis in biological fluids of the picogram quantities of Δ^9-tetrahydrocannabinol typically found under clinical conditions (2, 3).

Detection of larger amounts of tetrahydrocannabinol is possible using several procedures (5) including GLC with flame-ionization detection, but the sensitivity for the underivatized tetrahydrocannabinols does not exceed 0.8 ng. (Fig. 1). An antibody detection method was recently developed (6), and submicrogram amounts of Δ^9-tetrahydrocannabinol and metabolites may be quantified by mass fragmentography (7) or fluorometry (8).

The procedures described herein permit the quantitative analysis of the total Δ^9- and Δ^8-tetrahydrocannabinols in picogram amounts by preparing the pentafluorobenzoate ester for analysis by GLC with electron-capture detection. A gas chromatograph[1], fitted with both ^{63}Ni and flame detectors, was used. Detectors were maintained at 285°. The ^{63}Ni detector had a separate nitrogen gas flow of 35 ml./min., and the glass columns were 30 cm. long (2.0 mm. i.d.). The conditions were: column 210–230°; injection port, 245°; 3% OV-225 on Gas Chrom Q, 100–200 mesh; nitrogen gas flow, 35 ml./min.; standing current (^{63}Ni), 60–90% at 2 × 10^{-9}; and background noise, 2–3% at 1 × 10^{-10}. The minimum detectable quantity of tetrahydrocannabinol was 1.65 × 10^{-14} mole. The minimum detectable quantity is defined as that amount giving a signal three times the background noise at maximum sensitivity (1 × 10^{-10}). When tetrahydrocannabinol was extracted from various solutions prior to derivatization, the minimum detectable quantity was a function of the efficiency of the extraction processes and the nature and magnitude of the nonionizable contaminants present and ranged between 40 and 125 pg./ml. of plasma for a 5-ml. blood sample.
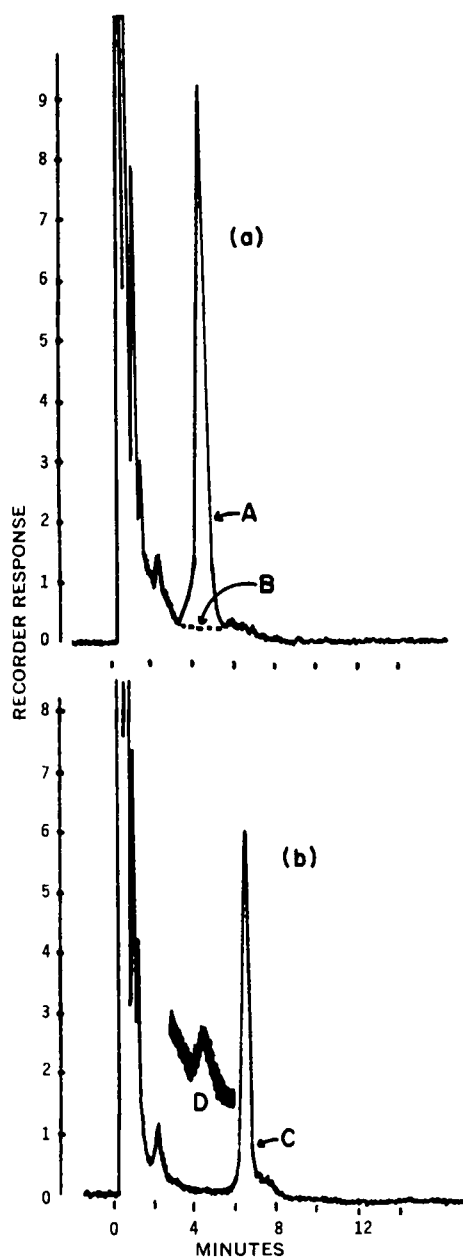
To extract tetrahydrocannabinol from blood, a 5-ml. blood sample was centrifuged and a 2.5-ml. aliquot of
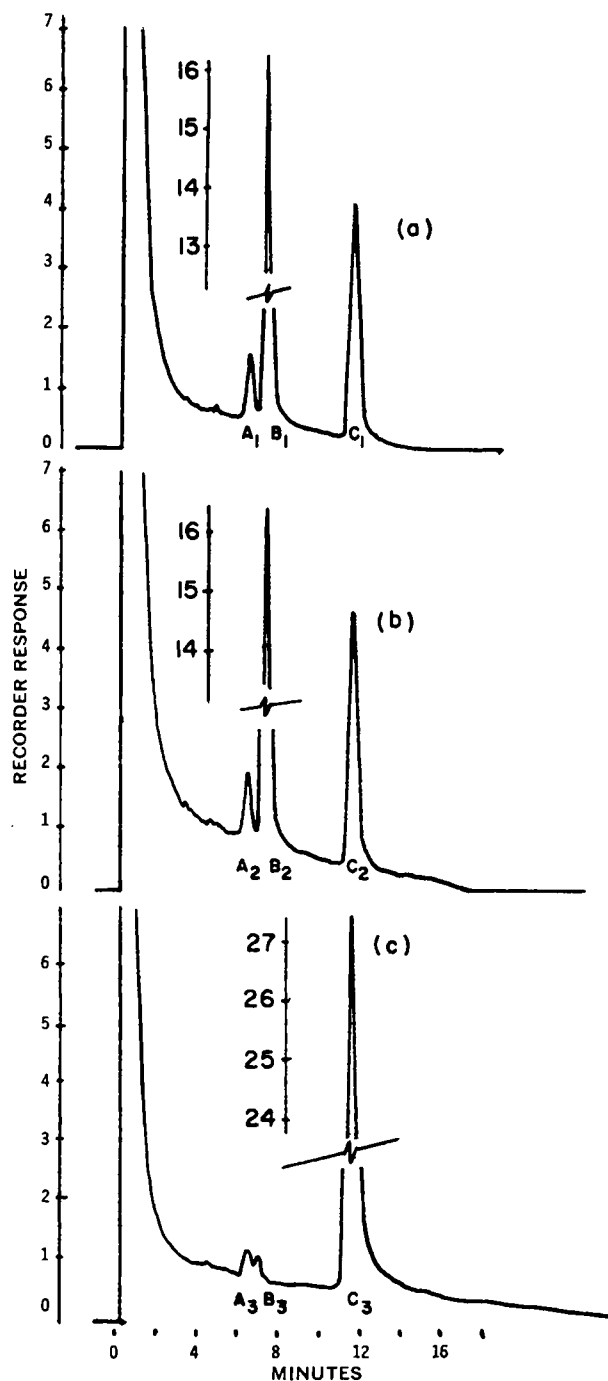
Figure 1—(a) *GLC analysis by flame-ionization detection of 1.3 µl. of a toluene solution containing 5 mg./ml. tetrahydrocannabinol and 1 mg./ml. cannabinol. Key: A_1, Δ^8-tetrahydrocannabinol (0.4 mcg.); B_1, Δ^9-tetrahydrocannabinol (6.5 mcg.); and C_1, cannabinol (1.3 mcg.). The tetrahydrocannabinols were from the same stock solution (see text), and A_1 accounted for 6.2% of the total tetrahydrocannabinols. (b) Tetrahydrocannabinol, 50 mcg., from the stock solution was added to 50 ml. water and treated as described in the text for extraction of tetrahydrocannabinol from blood. Cannabinol was added after the last step (prior to derivatization). After these various treatments, A_2 accounted for 7.0% of the total tetrahydrocannabinols. (c) GLC analysis (using flame-ionization detection) of the cannabinol used as internal standard. The amount of Δ^8- and Δ^9-tetrahydrocannabinols (A_3 and B_3, respectively) present as contaminants was approximately 1%. C_3 represents 5.2 mcg. cannabinol.*

plasma was transferred into a 50-ml. glass centrifuge tube. One milliliter of 5% trichloroacetic acid was added dropwise while shaking to denature proteins. Ten
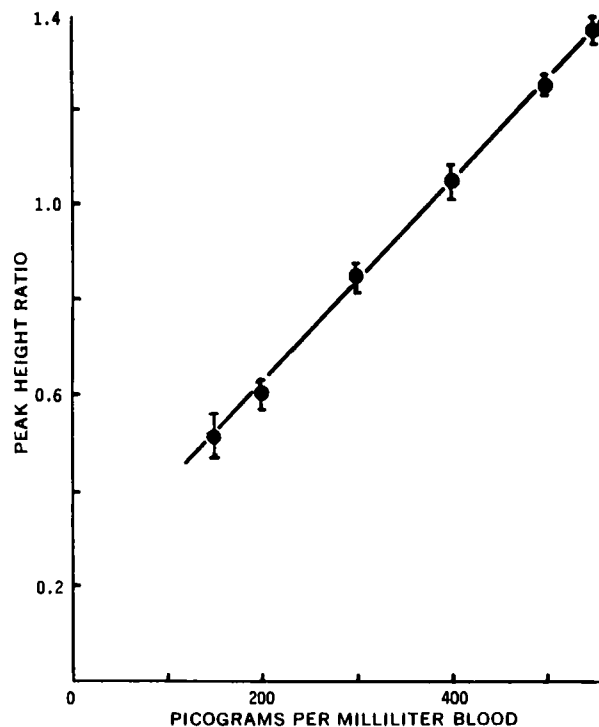
Figure 3—*Calibration curve obtained for* Δ^9-*tetrahydrocannabinol added to fresh heparinized dog blood. Five-milliliter blood samples were spiked with tetrahydrocannabinol (15 mcg./ml. in ethanol) and allowed to stand 10 min. prior to extraction and derivatization. The peak height ratios are plotted as the mean of three replicate extractions, and the vertical lines represent the standard deviations.*

Figure 2—*(a) GLC analysis by electron-capture detection of tetrahydrocannabinol pentafluorobenzoate (A). The chromatogram is for 200 pg. and was prepared from a 5-ml. plasma sample containing 1000 pg./ml. Sensitivity* $= 8 \times 10^{-10}$ *(B is for a plasma blank). (b) Chromatogram of the extracted and derivatized plasma blank to which derivatized cannabinol (C) was added. The insert (D) shows the chromatographic response for the minimum detectable quantity (1.65 × 10^{-14} mole) of tetrahydrocannabinol pentafluorobenzoate at a sensitivity of 1 × 10^{-10}.*

milliliters of a 1% isoamyl alcohol solution in dichloromethane[2] was added. The mixture was shaken for 15 min. and centrifuged. The organic layer was transferred into another 50-ml. test tube and the extraction process was repeated. The combined organic layers were evaporated to approximately 5 ml., and 25 ml. pH 8.5 buffer (50 ml. 0.1 *M* tromethamine plus 14.7 ml. 0.1 *N* HCl) was added. The mixture was shaken for 5 min. and then centrifuged. The dichloromethane layer was transferred

quantitatively to a third 50-ml. test tube containing 25 ml. of 0.1 *N* HCl. This mixture was shaken for 5 min. and centrifuged. The organic layer was transferred quantitatively to another 50-ml. test tube. The tetrahydrocannabinol solution was then ready to be derivatized.

These procedures neither isomerized Δ^9-tetrahydrocannabinol to Δ^8-tetrahydrocannabinol nor the converse. This was shown by adding 50 mcg. of Δ^9-tetrahydrocannabinol [from a 5-ml. sample[3] containing 1.0 g. of (−)-Δ^9-*trans*-tetrahydrocannabinol in 99% ethanol, assayed as 93%] to 50 ml. of water and treating in the exact manner already described. This included the addition of trichloroacetic acid and the various treatments with acid and buffer. The relative amounts of Δ^9- and Δ^8-tetrahydrocannabinols were compared for the material before and after these treatments.

The assay was performed by GLC analysis with flame-ionization detection in a system capable of quantifying both the Δ^8 and Δ^9 species. The GLC system and conditions were the same as given for the electron-capture detection, except that a 1.8-m. (6-ft.) column was used and the nonderivatized material was chromatographed and detected by flame ionization. Cannabinol (from a 1-ml. sample[4] containing 10 mg. of cannabinol in 99% ethanol, assayed as 99%) was used as the internal

[2] Nanograde, Mallinckrodt Chemical Works, St. Louis, MO 63160

[3] S.S.C. Lot 61591, furnished by the Department of Health, Education, and Welfare, Public Health Service, National Institute of Mental Health, Bethesda, MD 20014
[4] S.S.C. Lot 61656, furnished by the Department of Health, Education, and Welfare, Public Health Service, National Institute of Mental Health, Bethesda, MD 20014
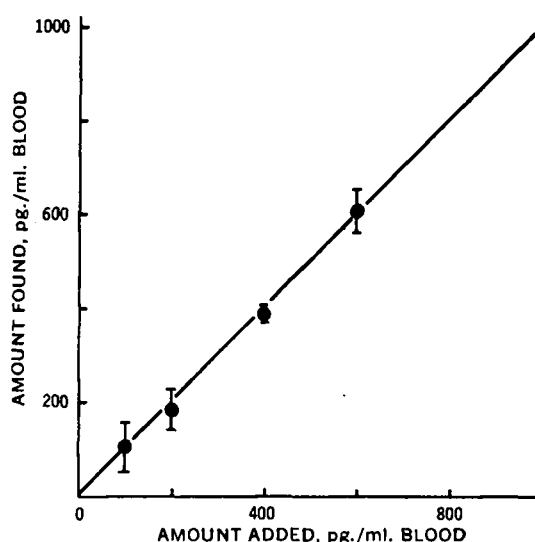
Figure 4—*Demonstration of the validity of the calibration curve and dilution technique. Sixty milliliters of fresh heparinized dog blood (same blood as used in Fig. 1) was divided into five 12-ml. samples, and one of each set was spiked with 100, 200, 400, 600, and 1000 pg./ml. with tetrahydrocannabinol (30 mcg./ml. in ethanol). The points represent the mean of three replicate extractions from 4-ml. aliquots. The vertical lines represent the standard deviations for the values shown, and the standard deviations ranged from 14.0 to 51.3 pg./ml. The slope and intercept of this plot were not statistically different from the expected values of 1.0 and zero, respectively.*

standard. The chromatograms of the Δ^9-tetrahydrocannabinol are given in Figs. 1a and 1b before and after the extraction processes, respectively. The original sample showed 6.2% Δ^8 (Fig. 1a); after the various treatments, there was 7.0% Δ^8, which demonstrated negligible conversion of Δ^9 to Δ^8 in the procedures. Although the cannabinol had about 1% of Δ^8- and of Δ^9-tetrahydrocannabinol impurities (Fig. 1c), this contribution to the peaks in Figs. 1a and 1b was below the sensitivity for these compounds at the level and conditions used with cannabinol as an internal standard.

The extractions of the tetrahydrocannabinols were made from dog blood, and the calibration curves obtained for known amounts of tetrahydrocannabinol in the blood from different dogs were significantly different. Significant differences among individuals should be assumed until shown otherwise. If electron-capture detector analysis of a derivatized blank plasma sample shows significant amounts of nonionizable compounds (steroids, etc.), a TLC or liquid chromatography cleanup . step may be required. This was not necessary with the dog plasma used.

The solution of tetrahydrocannabinol in dichloromethane in the 50-ml. glass centrifuge tube was reduced to dryness under nitrogen and reconstituted in 0.2 ml. of dry dichloromethane containing 50 μl. of triethylamine. Ten micrograms (at least a 10-meq. excess) of pentafluorobenzoyl chloride[5] in 0.2 ml. of dry dichloromethane was added dropwise over 1 min. The solution was constantly stirred and allowed to react for 5 min. After the reaction was complete, 10 ml. of 0.1 N NaOH was added and the mixture was shaken for 5 min. The

solution was centrifuged and the dichloromethane layer was transferred quantitatively to another 50-ml. test tube containing 10 ml. of 0.1 N HCl.

This mixture was shaken for 5 min. and centrifuged, and the dichloromethane layer was transferred quantitatively to a 3-ml. glass-stoppered test tube and stored overnight at 0° until time for GLC analysis.

The sodium hydroxide and hydrochloric acid washings were designed to hydrolyze and remove unreacted acid chloride and triethylamine catalyst. These removals were imperative for successful quantitative analysis.

At the time of GLC analysis, the final dichloromethane solution was dried under a stream of nitrogen and redissolved in at least 25 μl. of toluene containing the derivatized internal standard, cannabinol pentafluorobenzoate.

Although the cannabinol used had about 1% of tetrahydrocannabinol as an impurity (Fig. 1c), at the levels used for the addition of the derivatized cannabinol as cannabinol pentafluorobenzoate internal standard, no peaks assignable to these impurities were observable on electron-capture detection (Fig. 2b). Figure 2b shows the chromatogram for the internal standard obtained from 200 pg. of cannabinol. The chromatogram of derivatized Δ^9- and Δ^8-tetrahydrocannabinols appeared as one peak under our conditions (Fig. 2a) and was well separated from the peak assigned to the internal standard, cannabinol pentafluorobenzoate. This chromatogram was for the derivatized tetrahydrocannabinol obtained from 200 pg. of the original material.

Calibration curves over small concentration ranges were preferred since the ^{63}Ni detector has a small linear detectable range. Whole blood samples with known amounts of tetrahydrocannabinol added (e.g., 150–550 pg./ml. plasma) were extracted and derivatized as already described. At the same time, 1 mcg. (or more) of cannabinol (internal standard) was derivatized and reconstituted in clean, dry toluene such that 1 μl. of the cannabinol pentafluorobenzoate solution contained about 10 times the minimum detectable amount, 1.65 X 10^{-14} mole. A volume of this internal standard solution, 25–500 μl., was added to each of the dried tetrahydrocannabinol pentafluorobenzoate samples such that the midpoint concentration of the tetrahydrocannabinol samples gave a peak height ratio of about one when 1–3 μl. of the final solution of derivatized tetrahydrocannabinol was injected. The calibration curve in Fig. 3 was obtained in this manner. A zero intercept for the linear range of the calibration curve was not necessarily expected or needed.

Once the calibration curve was obtained, a known volume of the internal standard solution (starting with 25 μl.) was added to each dried, unknown tetrahydrocannabinol pentafluorobenzoate sample prepared as described. If, on injection of 1–3 μl. of this solution the peak height ratio did not fall within the calibration curve, an additional volume of the internal standard solution was added until the peak height ratio did. The actual blood level concentration was obtained by correcting the value obtained from the calibration curve for the excess internal standard used. If a peak height ratio of 1.0 corresponded to 375 pg./ml. (X_1) on the calibration curve when 75 μl. (Y_1) of internal standard solution

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[5] PCR, Inc., Gainesville, FL 32601
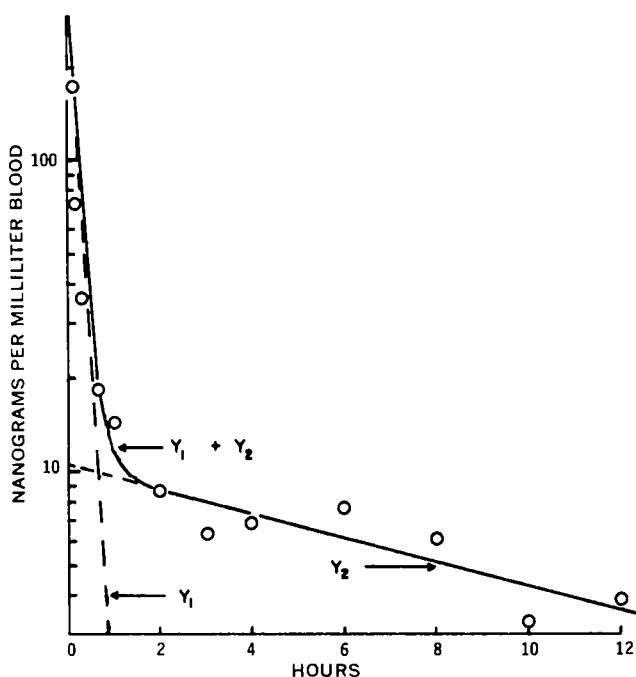
Figure 5—*Pharmacokinetic profile of tetrahydrocannabinol (0.1 mg./kg.) in blood from an 18.0-kg. dog. The calibration curve used is shown in Fig. 2. Five-milliliter blood samples were taken at 5, 10, 20, 40, and 60 min. and 2, 4, 6, 8, 10, and 12 hr. Urine was collected for 28 hr. but only a trace amount of tetrahydrocannabinol was found. The time course of this curve is fitted with the sum of two first-order exponential components, Y_1 and Y_2, where $Y_1 = 300\ (ng./ml.)\ e^{-0.17t}$ and $Y_2 = 10.05\ (ng./ml.)\ e^{-1.06t}$. The slower component, Y_2, has a $t_{1/2}$ of 8.0 hr. and the faster component, Y_1, has a $t_{1/2}$ of 7.5 min. The volume of distribution associated with Y_2 (equilibrated tissues) referenced to total drug in blood is 171 l. and the volume of distribution associated with Y_1 (central compartment) is 6.0 l.*

was used for all calibration curve values and a peak height ratio of 1.0 was obtained for an unknown amount of tetrahydrocannabinol (X_2) in blood when 25 μl. (Y_2) of internal standard was used, $X_2 = (X_1/Y_1)Y_2$. In this case, $X_2 = 125$ pg./ml. Figure 4 demonstrates this procedure and verifies its validity.

A number of critical variables affected the reliability and error of this procedure. Tetrahydrocannabinol bound extensively (15–40%) to glass from aqueous and organic solutions at low concentrations, and the degree of adsorption was time dependent. In the case of organic solutions, the tetrahydrocannabinol could be reincorporated from the glass into the solution on vigorous shaking. Thus, it was imperative to use vigorous shaking prior to any sampling. The degree of adsorption may be reduced if glassware is treated with an organic solution of a silyl reagent[6], followed by a solvent rinse of dichloromethane and drying. Storage times in aqueous or plasma solutions should be minimized and standardized since adsorption on storage in these media is unavoidable. Loss of tetrahydrocannabinol or a deriva-

tive from such solutions should not be assigned to instability until adsorption has been ruled out. Reagents of maximum purity must be used. Known amounts of tetrahydrocannabinol should be analyzed periodically to ensure reagent quality.

To demonstrate the applicability of this analytical technique, we determined the blood levels of tetrahydrocannabinol in a dog as a function of time. An 18.0-kg. dog was given a 1.8-mg. i.v. dose of tetrahydrocannabinol (0.1 mg./kg.) in 5 ml. of 50% aqueous ethanol. Blood samples were taken as a function of time over 24 hr. and urine was collected for 28 hr. The results are shown in Fig. 5 for tetrahydrocannabinol concentrations in blood as a function of time. Only a trace amount of unchanged tetrahydrocannabinol was found in the total urine collected when the urine was analyzed in the same manner as blood, and appropriate calibration curves were prepared. The calibration curves of Figs. 3 and 4 were prepared using blood from this dog and were used in this study. The apparent volumes of distribution of the central compartment referenced to the total concentration of the drug in blood was 6.0 l., whereas that of the rapidly equilibrated fluids was 171 l.

This pharmacokinetic profile shows an initial rapid distribution and metabolism phase (apparent half-life 7.5 min.) followed by a slow loss of tetrahydrocannabinol from blood (apparent half-life 8.0 hr.) and is consistent with the similar results reported for humans based on radiotracer studies (2).

Figure 5 demonstrates that this analytical procedure may be used to analyze blood levels of tetrahydrocannabinol. Although this procedure does not allow the separate quantitative analysis of Δ^8- and Δ^9-tetrahydrocannabinols at this sensitivity, preliminary results indicated that a similar procedure may be used to separate and quantify picogram amounts of other metabolites of tetrahydrocannabinol in blood and urine.

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[6] Rinsing glass for 30 min. with a 1% (v/v) solution of Sil-Prep (Applied Science Labs., State College, PA 16801) in dichloromethane reduces adsorption to less than 5%.