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Quantitative Determination of Δ^9-Tetrahydrocannabinol in Cadaver Blood

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A long-term program involving the quantitative identification of cannabinoids in biological fluids made it clear that there was a significant need for the development of an accurate and reliable assay procedure for the determination of Δ^9-tetrahydrocannabinol (Δ^9-THC) in blood samples obtained from autopsy. It is frequently of considerable concern to know whether a subject was under the influence of marijuana at the time of death, and since Δ^9-THC is the major psychoactive agent present in marijuana, a definitive identification of Δ^9-THC in blood from an autopsy sample presents direct and unquestionable evidence of the subject's use of this drug.

The principal methods by which Δ^9-THC can be identified in biological fluids are gas-liquid chromatography (GLC), thin-layer chromatography (TLC), immunological methods, high-pressure liquid chromatography (HPLC), and gas chromatography-mass spectrometry (GC/MS).

The GLC methods were among the earliest techniques used for the identification of Δ^9-THC [*1,2*]. Some of these methods are extremely sensitive, particularly when electron capture detectors are employed [*3-5*]. The use of this detector is somewhat less direct because it normally requires the preparation of a derivative of Δ^9-THC, which adds an additional step to the procedure. Further improvements in GLC methods involve a dual-column system with combined flame ionization and electron capture detection [*6*]. Regardless of the sensitivity achievable, GLC alone lacks the capability of providing the requisite selectivity for the qualitative identification of Δ^9-THC since, in the extract of a biological fluid, there is considerable risk that some component may be present which is not Δ^9-THC but which possesses a GLC retention time similar to that of Δ^9-THC. In such a case, Δ^9-THC could be confused with such components and a misidentification would occur.

The TLC methods usually involve the identification of the Δ^9-THC by radioactivity measurements on a TLC plate [*7-9*]. This procedure is therefore only suitable under laboratory conditions where ^{14}C- or tritium-labeled Δ^9-THC can be administered with the drug, and even then there are potential health hazards associated with this technique.

Immunological procedures for determination of Δ^9-THC can be grouped into two types: radioimmunoassay (RIA) and homogeneous enzyme immunoassay (the EMIT® method). Both of these methods show promise as potentially sensitive and relatively inexpensive ways to identify Δ^9-THC: RIA techniques have been developed for various cannabinoids [*10-13*] both in urine and in plasma, and the EMIT technique, which involves the competitive inhibition of various enzymes such as lysozyme or malate dehydrogenase with

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specific anti-hapten antibodies in homogenous phase, has also been adapted to Δ^9-THC [*14,15*].

The problem with these procedures from the forensic science standpoint is that the reagents and the substrates are derived from biological sources and are frequently subject to variation from batch to batch and to degradation with time. Forensic science samples are often contaminated with unexpected or abnormal metabolic products resulting from traumatic injury to the subject. These could possibly give rise to erroneous results. In addition, the imunological procedures invariably involve antibodies that show cross-reaction with a number of related cannabinoid antigens, and the possibility for a misidentification is substantial.

The HPLC procedure provides a very sophisticated and powerful method for separating Δ^9-THC in biological fluids [*16*]. Like GLC, the method must be coupled with mass spectrometry to provide the necessary confidence to the forensic science investigator of the correctness of the Δ^9-THC identification [*17*]. This procedure undoubtedly could be developed for use with autopsy blood samples; however, the method described here makes use of simpler chromatographic techniques and requires only low resolution mass spectrometry, which is far more widely available in forensic science laboratories.

For a variety of reasons, therefore, all of these methods have some limitation in their application to the analysis of Δ^9-THC for legal applications.

The GC/MS method depends on the coincidence of the specific molecular ions of the substance analyzed and one of its labeled isotopes with the precisely defined retention volume of that component, a combination which yields the ultimate reliability of identification, accuracy, and precision for Δ^9-THC analysis [*18-20*]. For application to Δ^9-THC analysis of cadaver blood samples, an adaptation of a method originally developed in this laboratory has been made to accommodate the specific biological medium. This communication describes this new procedure in detail.

Materials

The Δ^9-THC was prepared synthetically by the Arthur D. Little Co., Cambridge, Mass., under contract to the National Institute on Drug Abuse (NIDA). Quantitative GLC analysis showed it to be 98% pure. Deuterated Δ^9-THC was prepared at the Research Triangle Institute under contract HSM-42-71-108 to NIDA. The deuterated Δ^9-THC was 97% pure by GLC analysis and showed a composition of 96% d_3, 2.6% d_2, 1.4% d_1, and $<0.5\%$ d_0 by low resolution MS analysis with an MS-902 mass spectrometer.

The marijuana cigarettes were prepared by reconstituting ethanol-extracted plant material with pure Δ^9-THC. Each cigarette was then hand-rolled with 1 g of plant material that contained 17 mg of Δ^9-THC.

Absolute ethanol for stock solutions was U. S. Pharmacopeia reagent quality (U. S. Industrial Chemical Co., New York). Petroleum ether (30 to 60°C) was obtained from Fisher Scientific Co., Pittsburgh, Pa. The hexane was ultraviolet-grade glass-distilled from Burdick and Jackson, Muskegon, Mich. Human plasma was obtained from paid volunteers and was stored in glass containers.

All glassware used was first treated with 5% dimethylchlorosilane in toluene for 15 min, then rinsed three times with reagent-grade toluene, three times with methanol, and finally with glass-distilled hexane.

Experimental Procedure

Sample Procurement

The sampling protocol was designed to compare a new scheme devised to simulate the autopsy situation with the methods previously developed in our laboratory [*21*].

Two experienced, adult, male marijuana users were the subjects in this study. Each subject was given one marijuana cigarette to smoke. Absorption of the Δ^9-THC was about 40%, resulting in a total dose of about 6.8 mg. From previous work it was known that blood samples withdrawn 10 min and 1 h after smoking would contain high and low levels of Δ^9-THC, respectively [22,23]. Therefore each subject had 10 ml of blood withdrawn at 10 min and at 1 h after smoking. Each sample was immediately divided into two 5-ml portions. One portion was treated in a manner chosen to simulate a postmortem situation: the blood was immediately drawn into an evacuated nonheparinized container in such a way as to totally exclude air from the system. The sample was stored at room temperature for 3.5 h and then refrigerated at about 5°C for an additional 16.5 h. At this point the sample was opened to air and 100 mg of solid sodium fluoride was immediately added. The vial was then stirred in a vortex generator. The sample was transferred to a screw-cap vial and stored at room temperature without special precautions to exclude air. After four days the sample was transferred to a refrigerator until the time of analysis, ten days after the experiment began.

The other 5-ml portion of each sample was used as a control to compare with the simulated autopsy sample. The control sample was collected in a heparinized container and centrifuged immediately at approximately 4000 rpm for 15 min. The plasma was stored in a freezer for 18 days.

Sample Extraction

The simulated autopsy sample was extracted on the tenth day after collection, by which time the blood was entirely hemolyzed. To 3 ml of the sample was added 250 ng of 11-d_3-Δ^9-THC from a stock solution made up at 5 ng/μl in absolute ethanol. The mixture was extracted successively with two 25-ml portions of acetone. After each addition of acetone, the sample was centrifuged until a clear supernatant resulted. The supernatant was decanted after each extraction and the two acetone extracts were combined. The acetone was then evaporated in vacuo, and the aqueous residue was extracted with three successive 6-ml portions of petroleum ether. The petroleum ether extracts were combined, evaporated in vacuo, and chromatographed on a 1- by 40-cm column of Sephadex LH-20 packed in petroleum ether/chloroform/ethanol (10:10:1) at 26°C. The freeze-dried extract was dissolved in a minimum quantity of this solvent and the chromatography was carried out without varying the solvent concentration (isocratically). The first 25 ml collected was discarded. The next 10 ml was collected and contained the entire Δ^9-THC fraction. The column was then regenerated by the addition of at least 50 ml more of the same solvent. The fraction containing the Δ^9-THC and the 11-d_3-Δ^9-THC was evaporated in vacuo and made up to 30 μl with hexane for GC/MS analysis.

Extraction of Control Sample

The fraction that had been centrifuged immediately and frozen was put aside as a control sample to compare with the simulated autopsy sample. This fraction was worked up in a manner similar to that previously described [*19*]. The extraction procedure involved the addition of 50 ng/ml of d_3-Δ^9-THC to the plasma as an internal standard, followed by extraction with petroleum ether and chromatography on Sephadex LH-20 just as in the simulated autopsy sample.

All samples were stored in closed containers over anhydrous calcium sulfate until the GC/MS analysis was performed. In this form the samples were stable for at least one week.

Calibration Runs

Prior to the analysis of unknown samples, standard calibration mixtures were prepared by using various concentrations of d_0-Δ^9-THC along with a constant amount of d_3-Δ^9-THC

in plasma to determine a standard calibration curve. To determine the full range of Δ^9-THC levels, calibration standards were prepared by using 200, 100, 50, 20, 10, 5, 2, 1, and 0 ng/ml of d_0-Δ^9-THC along with the fixed amount of 50 ng/ml of d_3-Δ^9-THC. Sometimes it was possible to obtain satisfactory calibration curves with a smaller number of standard points, particularly if duplicate analyses were carried out. In every case the ratio of ion intensities (*m/e* 314 to *m/e* 317) was determined from peak area or peak heights of single ion plots. After the standard ratios were obtained, the d_0 concentration in ng/ml was plotted against the *m/e* 314/317 ratio on a log/log plot. For highest accuracy, the calibration runs were carried out in triplicate. For a proper calibration run, the data thus plotted were linear. A typical calibration curve is shown in Fig. 1.

Gas Chromatography/Mass Spectrometry

The GC/MS analysis was carried out with either a magnetic sector or a quadrupole mass spectrometer. The instruments were an LKB-9000 fitted with a peak-matching attachment and a Finnigan 3300 instrument controlled by a DEC PDP-12 computer. The resolutions of the instruments were set up in the range of 300 to 400 ($M/\Delta M$, 10% valley definition).

Gas chromatography was carried out by using 1.8-m by 4-mm inside diameter Pyrex® columns that were acid-cleaned, washed, and treated with 1% hexamethyldisilazane in toluene for 5 min followed by a methanol wash and oven drying prior to packing. The column packing was 2% OV-17 on 100-120 mesh acid-washed dimethyl dichlorosilane-treated high-performance Chromosorb W or equivalent (Supelco, Inc., Bellefonte, Pa.). The column was packed with a 10-mm plug of silane-treated glass wool at each end. Prior to use, columns were conditioned for at least 24 h at 250°C with a slow stream of helium.

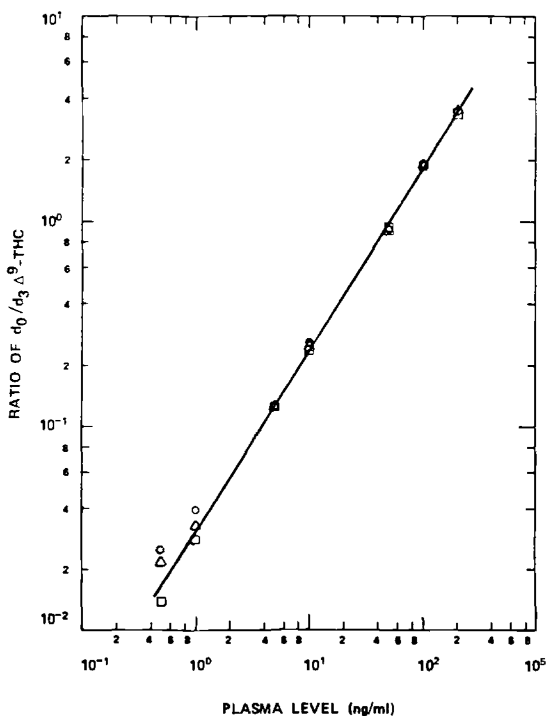

FIG. 1—*Calibration curve for Δ^9-THC (triplicate analyses).*

The column operating conditions that gave good transfer rates for Δ^9-THC were as follows: injector port, 270°C; column, 250°C; transfer line and separator, 260°C; and ion source, 270°C. Gas chromatography was carried out isothermally.

To carry out an analysis, the helium flow rate was set to 30 to 40 ml/min and the mass spectrometer was set up for selected ion monitoring of the exact masses 314.22 and 317.24, corresponding to the molecular ions of d_0-Δ^9-THC and d_3-Δ^9-THC, respectively. At these masses, there were no observable interferences from other plasma constituents or from metabolites of Δ^9-THC. It was important that focusing stability be maintained to within 0.1 AMU during the course of the entire series of experiments. Under these conditions, the desired components eluted from the column in 2 to 5 min. A typical computer printout for the intensity of the two selected ions versus time is shown in Fig. 2. It should be noted that under the described conditions the elution pattern showed no perceptible tailing. It also may be noted that the d_3 derivative elutes just slightly before the d_0.

Further experimental details of the GC-MS conditions have been reported previously [*21*].

Discussion

There has been a long-standing need for an accurate procedure for the determination of Δ^9-THC in autopsy samples. The major problem in the development of an accurate method is that active physiological levels of this drug are usually quite low, rarely exceeding 100 ng/ml of whole blood [*22,23*]. Autopsy samples are most likely to be very much lower than this. A useful assay should be sensitive to a level below any significant physiological activity, in this case to the 0.5 ng/ml level. In addition, the assay should be specific and characteristic for only Δ^9-THC to be valid in forensic science applications. The method previously developed in our laboratory fulfilled all of these requirements except that it had been worked out for plasma analysis and not for whole, postmortem blood. Since it was difficult for logistical reasons to obtain postmortem samples containing known levels of Δ^9-THC, it was decided to simulate postmortem conditions on a sample obtained from a living subject. This approach had the additional advantage of allowing

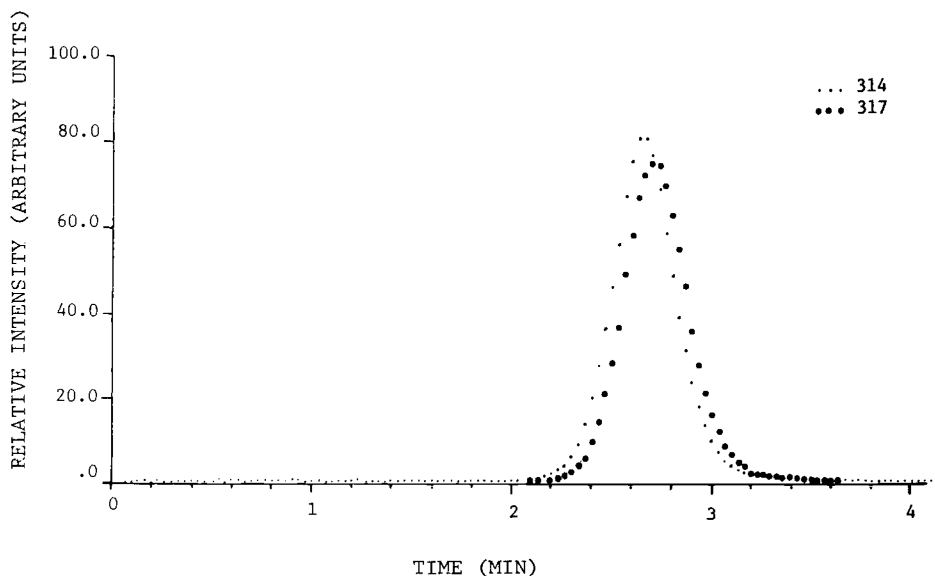

FIG. 2—*Typical single ion plots for d_0-Δ^9-THC and d_3-Δ^9-THC.*

the sample to be divided into an unknown and a control portion, and the latter could be analyzed by a well-known, previously developed method.

The procedure for treatment of the simulated sample was developed after consultation with personnel from the North Carolina Medical Examiner's Office, who gave us a typical set of conditions under which their postmortem samples are normally handled. We therefore chose a sampling protocol that most closely simulated these conditions and used these samples to obtain the test results described.

Autopsy specimens of blood are normally obtained from cadavers between 2 and 24 h after death. Blood samples are usually obtained by the pathologist by heart puncture with a hypodermic syringe. The blood is by this time totally hemolyzed. However, since it has normally not been in contact with air, clotting does not occur. Since circulation ceases at death, the metabolism of Δ^9-THC, which occurs in the liver [*24,25*], would be expected to be halted, and the Δ^9-THC levels determined by analysis should reflect those present at death. This assumption has been confirmed; the levels of Δ^9-THC observed in a simulated autopsy specimen could be reproducibly analyzed and were comparable to those obtained from a preparation analyzed from a fresh plasma specimen.

After the autopsy blood specimen has been taken, the usual practice is to transfer the blood directly into a nonheparinized vial containing enough sodium fluoride to bring its final concentration in the blood to 1 or 2%. After the addition of sodium fluoride, the blood sample is normally sent to the medical examiner's office without special refrigeration, and analyses are performed up to two weeks after the time of death.

The protocol chosen was designed to simulate these conditions, and a control sample was processed in an entirely parallel manner. The volunteer subjects for these experiments were experienced marijuana users to assure that the drug levels obtained were reproducibly in the physiological range. Each subject smoked one marijuana cigarette for this experiment. A 10-ml blood sample was taken 10 min after the onset of smoking to obtain a high level, and another sample was taken at 1 h after to obtain a sample containing a low Δ^9-THC level. Each sample was divided into two portions, the first used for the simulated autopsy run and the second as a control. The control sample was put into a heparinized container to protect against clotting, and the plasma, which contains most of the free Δ^9-THC,[2] was immediately extracted by centrifugation. The simulated autopsy sample was first allowed to stand at room temperature for several hours in the absence of air to simulate conditions within a cadaver. Then sodium fluoride was added to the sample at a level of 2% (w/v) after 19 h, as might occur during an autopsy. After this, the sample was allowed to stand in the presence of air at room temperature for four days. This corresponds to a typical time between taking an autopsy specimen in the field and sending it to the North Carolina Medical Examiner's Office for analysis. The sample was stored an additional ten days in the refrigerator, as might occur in the laboratory before the extraction and MS analysis.

Although it is recognized that, in general, the exact sampling protocol in the field will be different from that described above, it was thought that the procedure chosen was fairly typical, and if anything, was a more rigorous test of method than would be applied in actual practice.

The extraction procedure involves the use of acetone in the initial phase and petroleum ether subsequently. This procedure represents the major departure for the method developed for plasma analysis that is described in great detail elsewhere [*21*]. These changes were necessary because the medium, hemolyzed blood, is far more difficult to handle and to process than plasma, and it can give rise to emulsion problems, incomplete extraction, and other difficulties. The experiments were undertaken to develop a method, using hemolyzed blood, that would yield an extract of Δ^9-THC that could be analyzed to produce quantitative results comparable with those achieved by our previous work with plasma.

[2]This fact was determined independently in our laboratories.

In addition, due consideration was given to the problems of stability of the Δ^9-THC in various solvents [*26,27*].

Results

After the samples had been treated they were extracted and analyzed by GC/MS. The extraction procedure for the hemolyzed blood was modified to ensure that complete extraction took place. Since the extraction was carried on whole blood samples instead of plasma, they were of necessity somewhat more cumbersome than those carried out on the control samples. However, the procedure is entirely adequate for carrying out the final GC analysis. The GC/MS analysis was done on both analytical and control samples individually and the absolute level of Δ^9-THC present in each sample was determined from the calibration curve prepared in the normal manner. The results of the analyses are shown in Table 1.

The observed correlation between the Δ^9-THC analyses of the simulated postmortem sample and those of the control is excellent. In every case the amount of Δ^9-THC found in the hemolyzed blood samples was lower than that of the plasma extracted samples. This is entirely understandable, and indeed expected, when one recognizes that the bases for calculating the concentrations of the plasma samples and of the hemolyzed samples are different. For normal blood samples, the usual practice is to report Δ^9-THC levels in units of ng/ml of plasma. For the hemolyzed samples this was impossible because there is no practical way of separating the plasma from the cells at this stage. Accordingly, the concentrations were based on the volume of whole blood used in the analysis. It is possible, however, to correct the plasma levels of the standard to correspond to the autopsy whole blood samples if one knows the percentage of cells in a given whole blood volume.

Comparison of the values obtained in this study with the corrected plasma levels, assuming a normal hematocrit of 0.45 [*28*], are in extremely close agreement, thereby validating this method.

Since this work was originally performed our laboratories have had numerous occasions to carry out these determinations in actual autopsy cases. In most cases the observed levels were very low or entirely absent. In one case in which there was a high likelihood that the deceased had been a marijuana user, a finite but very low level (about 0.5 ng/ml of plasma) was detected and the test was reproducible. Since this level of Δ^9-THC is far below that which would be psychoactive, we feel that this procedure provides the sensitivity normally required in forensic science.

Summary

We have described a highly sensitive, accurate, and selective method for the determina-

TABLE 1—*Results of analyses for Δ^9-THC under simulated postmortem conditions.*

Subject	Sample Time After Onset of of Smoking	Δ^9-THC/ml of Plasma Extract, ng	Δ^9-THC/ml Blood (Found), ng	Δ^9-THC/ml Blood (Calculated),[a] ng
A	10 min	37	18.5	20.3
A	1.33 h	3.4	2.5	1.87
B	8 min	130	75	71.5
B	1.17 h	19	11	10.45

[a] The calculated value was based on the amount found by using the plasma extract technique corrected for an assumed hematocrit of 0.45.

tion of Δ^9-THC in postmortem blood. Although the method requires the use of fairly complex and sophisticated equipment, the procedure is straightforward and has been reproduced many times in our laboratories. It is thought that this procedure will serve as a useful adjunct to the forensic scientist in his armamentarium for determining physiological levels of important, abused drugs in postmortem analysis.

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