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Note

Detection and quantitation of tetrahydrocannabinol in serum using thin-layer chromatography and fluorometry

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Much work is being performed in the cannabis field due to the popularity of marijuana among the young and because marijuana is being used in medical research in the areas of cancer and glaucoma treatment. The concentration of Δ^9-tetrahydrocannabinol (THC), the active principle in marijuana, in blood is believed to correlate with some psychological and physiological responses [1, 2] and thus, an analysis of THC in blood or serum is important for pharmacological studies. Recent evidence [3] indicates that of approximately 1800 blood specimens from motorists stopped by the California Highway Patrol for erratic driving, analysis revealed that 14.8% were positive for THC. Analysis of THC would be useful for forensic purposes since its concentration in plasma is related to impaired motor function [4].

THC occurs in plasma in very low concentrations (ng/ml) with the peak level of about 50 ng/ml about 10 min after smoking [5]. The physiological effects persist for several hours after smoking when the plasma levels are only about 2 ng/ml. Thus, a method is needed which is capable of detecting and quantitating THC down to 1 ng/ml. There are several analytical methods available for analysis of THC in serum. These have been recently reviewed [6] and include gas chromatography, radioimmunoassay, mass spectroscopy, and high-performance liquid chromatography. Most recently, an immunoassay technique has come into widespread use for urine analysis but lacks the sensitivity and specificity to be used for blood or serum [7].

Thin-layer chromatography (TLC) has been shown by us to be a sensitive and selective qualitative method for detecting THC in serum [8]. We wish to report that a modification of this TLC procedure followed by solution fluorometry is suitable for quantitative analysis of THC in serum.

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EXPERIMENTAL

Reagents and chemicals

Tetrahydrocannabinol (THC) was obtained as a solution in ethanol from the National Institute of Drug Abuse and was 99+% pure as analyzed by gas chromatography. The derivatizing reagent, 2-*p*-chlorosulfophenyl-3-phenylindone (DIS·Cl) was available from Polysciences (Warrington, PA, U.S.A.). Acetone and benzene were distilled-in-glass solvents from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). Dimethylformamide, methanol, hexane, isoamyl alcohol and acetonitrile were Fisher reagent grade (Pittsburgh, PA, U.S.A.). Potassium methoxide was also obtained from Fisher. Dibenzo-18-crown-6 was available from Aldrich (Milwaukee, WI, U.S.A.).

Supplies and instrumentation

Membrane filters were Alpha Metricell-450 (25 mm) from Gelman (Ann Arbor, MI, U.S.A.). Bakerflex IB2 silica gel sheets, 5 × 20 cm or 20 × 20 cm (J.T. Baker, Phillipsburg, NJ, U.S.A.) were used for the chromatography. All glassware was siliconized with Dri-Film SC-87 from Pierce (Rockford, IL, U.S.A.). Reacti-Vials were also obtained from Pierce. The fluorescence was measured with an uncorrected SPF-125 fluorometer (American Instruments, Silver Springs, MD, U.S.A.).

Extraction from serum

Methanol (4 ml) was added to 2.0 ml of sample serum and vortexed for 1 min in a screw-capped siliconized test tube. The tube was then centrifuged for 5 min with a tabletop centrifuge. The clear solution was decanted into another tube. The remaining precipitate was washed with 4 ml of methanol and the centrifugation and decantation repeated. The pooled extracts were evaporated to a volume of 3 ml in an 80°C water bath with a slow stream of air. The THC was then extracted with 8 ml of hexane—isoamyl alcohol (97:3) by vortexing followed by centrifugation. The hexane layer was transferred to another tube and evaporated to dryness. A 2-ml volume of a modified Claisen alkali [8] (37 g of potassium hydroxide in 20 ml of water followed by addition of 100 ml of methanol) was added to dissolve the dried residue. The solution was washed with 1 ml of hexane which was discarded. The Claisen alkali was evaporated to 0.5 ml and 2 ml of water were added. Then 8 ml of hexane were added and the mixture vortexed. The hexane layer was quantitatively removed and evaporated to dryness. The residue was transferred to a 0.3-ml Reacti-Vial with hexane which was then evaporated. The extraction was repeated on a pooled serum blank which was known to be free of THC.

Standard

The pure DIS·THC derivative was prepared as follows. A 14-μl volume of 1.0 *M* sodium hydroxide was added to 4 mg of THC. To this mixture was added 0.6 ml of an acetone solution of 4.9 mg of DIS·Cl. After 5 min, the product was precipitated with water, filtered and taken up in 1 ml of methanol. The solution was spotted as a band on a 20 × 20 cm silica gel IB2 plate which was then developed for 10 cm in methanol—water (95:5). The yellow band at

4.8 cm was removed and eluted with acetone. The gummy residue after evaporation was recrystallized twice from methanol, dissolved in benzene, and stored at −15°C until use.

Derivatization and chromatography

Of a solution of 1 mg/ml of DIS·Cl in acetonitrile 10 μl were added to the sample extract and blank extract. The vials were vortexed to insure proper mixing. After adding 5 μl of a 0.2 *M* aqueous sodium carbonate solution, the vials were sealed. The mixture was allowed to react for 30 min at 40°C. Inorganic solids were precipitated by the addition of 25 μl of acetone to the reaction mixture. After centrifugation, the salt-free solution was spotted with a microsyringe on a 5 × 20 cm or 20 × 20 TLC plate about 1 cm from the bottom of the plate. A 20-ng amount of pure DIS·THC (10 μl of a 2 μg/ml solution) was also spotted on the plate as a standard.

After drying of the spots, the TLC plate was developed for 3 cm in an unsaturated chamber with dimethylformamide—water (80:20). The plate was removed, dried with a hair dryer, and then redeveloped for 6 cm. The drying was repeated and the plate redeveloped for 9 cm. The plate was dried and then sprayed lightly with a fresh solution of 4 g of clean sodium dissolved in 100 ml of methanol. The plate was immediately observed under long-wave ultraviolet light. The blank contained two spots, a reagent spot near the solvent front and slightly below an unknown endogenous material. The DIS·THC had an R_F of 0.68 and fluoresced yellow—green. If the sample was positive, then the DIS·THC standard, and the DIS·THC spot were lightly circled with a lead pencil.

Elution and fluorometry

A 1-cm^2 section of the silica gel plate was carefully scraped into a siliconized centrifuge tube. Sections were taken for DIS·THC standard, samples positive for THC and blank serum. For the latter, the silica gel was scraped at the same R_F as the DIS·THC standard. Each silica gel section was vortexed for 1 min with 1 ml of acetone. After centrifugation, the supernatant was decanted onto a membrane filter and the vacuum applied. The filter was previously washed with 5—10 ml portions of acetone. The filtrate was collected directly in a fluorometry tube. The residual powder was washed twice with 1 ml of acetone. The combined filtrates were evaporated with a slow stream of air in an 80°C water bath.

The fluorometry reagent was prepared by dissolving 0.2 g of dibenzo-18-crown-6 in 100 ml of benzene. To a 10-ml portion of this solution was added 0.1 g of potassium methoxide. The solution was allowed to stand for 1 h, centrifuged and transferred to a screw-capped tube. This final solution was prepared daily.

To the fluorometry cells was added 1 ml of fluorometry reagent by means of a pipette. The fluorescence of samples and standard were read immediately at an excitation of 430 nm and an emission of 500 nm using a mercury lamp. The blank serum plate eluate was used to zero the fluorometer.

RESULTS AND DISCUSSION

The necessity of siliconizing glassware when analyzing for trace quantities of THC was shown by Garrett and Hunt [9] who found that 20—40% of THC in aqueous solution bound to glass. Plastics and rubber were found to be even more effective than glass. Thus, all glassware was siliconized in this work and contact with plastics was minimized.

The extraction procedure was a modification of that published by Rosenfeld et al. [10]. The addition of methanol to serum effectively extracts the THC while precipitating the proteins which bind the THC. The Claisen alkali [8] selectively extracts phenols such as THC while leaving the lipids in the hexane. The absolute extraction efficiency was found by spiking duplicate serums and comparing the fluorescence with THC standards which were also derivatized, chromatographed and eluted. The results are shown in Table I. The extraction efficiency was found to be 96.4 ± 6.5%.

The extent of THC derivatization with DIS·Cl was found to be extremely dependent on the solvent and the base used to ionize the THC. With sodium hydroxide, the derivative forms rapidly and degrades rapidly as evidenced by extra spots on the TLC plate. Sodium bicarbonate gave a slower reaction than sodium carbonate and thus the carbonate was chosen as the base. As a solvent, acetonitrile gave a reaction rate more than twice as fast as acetone which is the commonly used solvent in the analogous dansyl chloride derivatizations. In acetonitrile, using sodium carbonate as the base, the reaction is complete after 30 min at 40°C. Reaction times of up to 12 h have shown no degradation of the DIS·THC derivative. The extent of derivatization of amounts of THC varying from 4—50 ng is shown in Table II. The results were calculated by comparing the amount of fluorescence produced by THC which was derivatized with DIS·Cl, chromatographed and eluted to that of DIS·THC which was also chromatographed and eluted. The average derivatization was 87.3 ± 4.3%.

TABLE I

DETERMINATION OF THC EXTRACTION EFFICIENCY OF THC FROM SERUM

Spiked THC concentration (ng/ml)	Found THC concentration (ng/ml, ± S.D.)
2.0	1.9 ± 0.1
10.0	9.5 ± 0.6
50.0	49.0 ± 6.2

TABLE II

EXTENT OF DERIVATIZATION OF THC WITH DIS·Cl USING SODIUM CARBONATE IN ACETONITRILE FOR 30 min AT 40°C

Amount of THC (ng)	Percent derivatized (± S.D.)
4.0	87.0 ± 1.6
20.0	90.7 ± 1.9
50.0	84.3 ± 4.8

Recovery of DIS·THC from the plate was very critical in order to obtain the maximum sensitivity of the method. It was found that acetone, among the many solvents tried, provided the maximum recovery with a minimum of contamination from the silica gel. More polar solvents gave slightly greater recoveries but left behind a white residue on evaporation which was probably the silica gel binder. This residue caused a large amount of blank fluorescence. Distilled-in-glass acetone was used to minimize fluorescence due to solvent impurities. The average recovery of 10 ng of THC as DIS·THC which was spotted four times, chromatographed and eluted was 93.0 ± 3.9%.

The search for a suitable, strongly basic reagent to render the DIS·THC fluorescent in solution proved to be a difficult task. The reagent used to visualize the DIS·THC on the plate, sodium dissolved in methanol, was found to give too great a blank fluorescence. Potassium methoxide in methanol, a reagent used to render the DIS derivative of vitamin B_6 fluorescent in solution [11], also gave too high a blank fluorescence. Sodium methoxide in dimethyl sulfoxide, an extremely basic medium, produced reasonable fluorescence of DIS·THC but the solution was quite viscous and was difficult to pipet and a search for other solvents was begun. It is known that fluorescent intensity is favored by a solvent of low viscosity. Hydrocarbons are of extremely low viscosity but it is impossible to dissolve strong base in them. However, Pedersen [12] has found that potassium hydroxide could be solubilized in toluene using compounds known as crown ethers. These macrocyclic polyether compounds complex with the cation leaving a "naked" anion dissolved in the hydrocarbon. Dibenzo-18-crown-6 is a specific crown ether for potassium ion. This crown

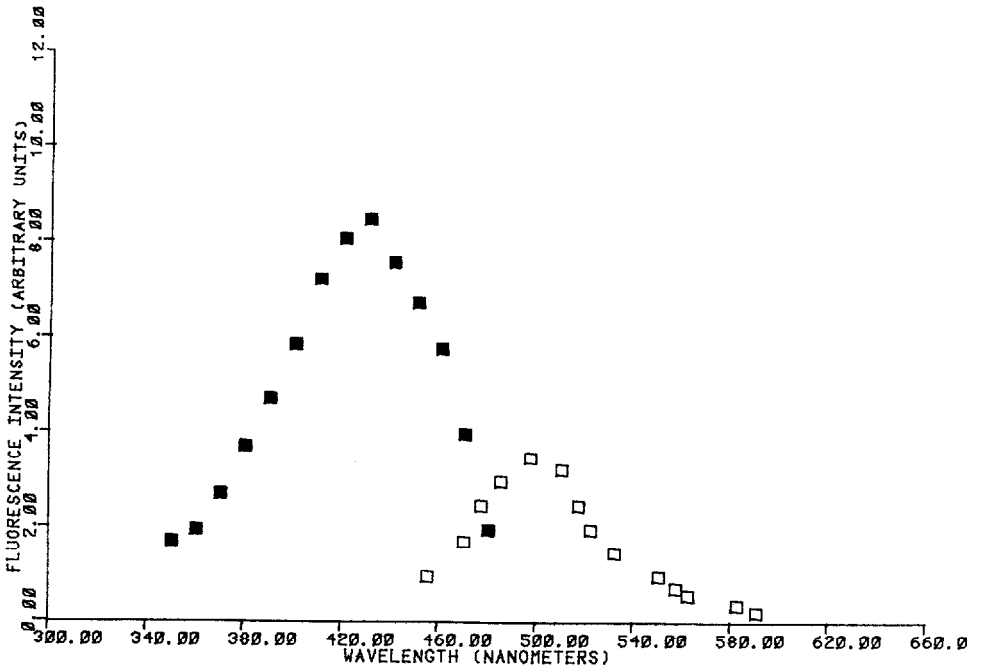

Fig. 1. Excitation (▪) and emission (▫) spectra of DIS·THC in a solution of sodium methoxide in benzene solubilized with dibenzo-18-crown-6.

ether is suitable for solubilizing potassium methoxide in benzene. With this system, the fluorescence of DIS·THC is about four times that in sodium methoxide in dimethyl sulfoxide. A 0.2% solution of the crown ether produces a maximal DIS·THC fluorescence with a minimum of blank fluorescence. The excitation and emission spectra of DIS·THC are shown in Fig. 1. A fluorescence calibration curve of 2—10 ng of THC as DIS·THC gives a straight line with a correlation coefficient of 0.9974 which passes through the origin.

Five "blind" serum samples from a human marijuana study were received from the National Institute of Drug Abuse. Each sample was analyzed by the reference gas chromatographic—mass spectrometric (GC—MS) method at the Battelle Institute and by the present THC procedure. The data are shown in Table III. A correlation plot gave a slope of 1.099 and a correlation coefficient of 0.981.

TABLE III

VALIDATION OF THE TLC METHOD FOR ANALYSIS OF THC IN SERUM AFTER SMOKING 10 mg OF THC

Time (h)	THC concentration GC—MS method (ng/ml)	THC concentration TLC method (ng/ml, ± S.D.)
Pre-smoking	0	0
0.25	22.0	25.5 ± 1.0
1	9.2	7.8 ± 0.2
2	2.1	5.5 ± 1.4
5.5	0	0

The TLC method has only minimal interferences. DIS·Cl has been shown to be specific for phenols [8]. It does not react under the experimental conditions to produce fluorescent derivatives with a variety of drugs which have amine functional groups such as amphetamines, tranquilizers, and psychoactives. Barbiturates and common analgesics such as salicylic acid, salicylamide and acetaminophen also do not react with DIS·Cl. Apomorphine, morphine, pentazocine, levorphenol and bufotenine, all of which have phenolic functional groups, give fluorescent derivatives with DIS·Cl but are either not extractable under the experimental conditions or give a different R_F value in the THC system and thus do not constitute an interference. No spots other than DIS·THC and the endogenous spot have been seen in serum samples analyzed to date. The metabolites of THC such as the hydroxy and carboxy compounds were not seen in any authentic sample positive for THC. They are probably too acidic to be extracted under the experimental conditions.

The detection limit for THC in serum by this method is approximately 1 ng/ml when 2 ml of serum are used. If no DIS·THC spot is seen after spraying, then the quantitation does not need to be performed — a considerable saving in time and labor. Other screening methods such as radioimmunoassay and GC require all samples to be put through the entire process in order to detect negatives.

In summary, the TLC procedure has been shown to be sensitive, selective and suitable for routine screening and quantitation.

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