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Note

Semi-quantitative thin-layer mass-screening detection of 11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid in human urine

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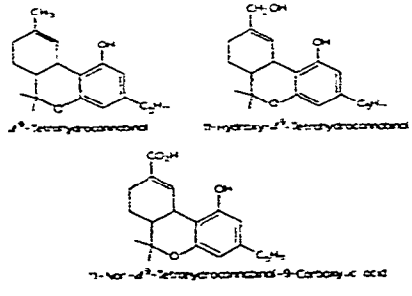
Marijuana is the term used to present the various preparations derived from the plant *Cannabis sativa* (family Cannabinaceae). Marijuana is not a simple drug; it is a complex mixture of over 400 individual chemicals[1]. Marijuana contains four constituents of similar structure, *i.e.*, Δ^9-tetrahydrocannabinol (Δ^9-THC); cannabidiol (CBD); cannabinol (CBN); and cannabichromene. The widespread abuse of marijuana throughout the world is primarily via smoking. Δ^9-THC is the most active of the principal constituents of marijuana and is contained in various parts of the plants in amounts varying from only trace to as high as 4% (w/w)[2]. Initial metabolism of cannabinoids in marijuana smoke takes place in the lungs, whereas initial cannabinoid metabolism of orally ingested marijuana takes place in liver. Major lung metabolites are usually side-chain hydroxylated metabolites, whereas major liver metabolites are usually hydroxylated derivatives of the cyclohexene ring system. There are over 35 metabolites of Δ^9-THC, 22 metabolites of CBD, and 22 metabolites of CBN known. Metabolism of Δ^9-THC in humans has been reported by several groups, with findings that 11-hydroxy-Δ^9-tetrahydrocannabinol and 11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid are the principal metabolites. The identification of either of the above metabolites in body fluids will be the evidence that Δ^9-THC had been present. Because of the very small amounts of THC and 11-hydroxy-THC which appear in human plasma and urine, the major urinary metabolite, THC-9-carboxylic acid (THC-9-acid), can be used as an indicator of recent cannabis use. The concentration of this metabolite in plasma exceeds that of THC within 50 min



after smoking and then remains higher for more than 24 h. This acid seems to be the result of further metabolism of the 11-methyl group, which is oxidized to a carboxyl group via the aldehyde[3,4]. Wall *et al.*[5] suggested that identification of this acid may be a practical method to identify cannabis users. Green *et al.*[6] also pointed out that nearly half of the total THC-9-acid present in urine can be extracted without hydrolyzing the specimen. In fact they postulated that unconjugated free THC-9-acid may be a practical indicator of the degree of physical impairment caused by the smoking of marijuana. Qualitative and/or quantitative procedures for the determination of carboxy-THC in body fluids have been reported using gas chromatography–mass spectrometry by Foltz *et al.*[7], Green *et al.*[6], Green[8], and Nordquist *et al.*[4]; high-performance liquid chromatography (HPLC) alone[9,10] or combined with radioimmunoassay (RIA)[11], RIA alone[12,13], and homogeneous enzyme immunoassay[14] techniques, have also been reported.

The purpose of this communication is to report a very sensitive thin-layer chromatographic TLC procedure which can be used as an indicator of a recent marijuana use in the urine of marijuana smokers. The procedure can detect the presence of marijuana in the urine of smokers who either smoke a few puffs through a tobacco pipe by burning small amounts of street marijuana (*ca.* 200 mg) or smoke *ca.* 1/4th of a marijuana cigarette for recreational purposes. The procedure is not only simple but also very specific and reproducible. It permits mass testing at very low cost since it neither needs sophisticated and costly equipment nor imposes special requirements such as (i) prior silylation of glassware, (ii) evaporation of extraction solvent under vacuum or at low temperatures under stream of nitrogen, or use of expensive reagents within a stipulated period of time. The total laboratory cost per specimen is less than US $ 2.00 including the labor of one skilled technician who performs 40 complete tests per day.

The test involves total extraction of free and conjugated 11-nor-tetrahydrocannabinolic acid (THCA). The urine specimen is subjected to a very mild alkaline hydrolysis and total acid is extracted at a pH of *ca.* 4.0 (3.0–4.0) with cyclohexane–ethyl acetate. The evaporation of the extraction solvent is accomplished either by employing a boiling water bath or in the air-circulated oven maintained at 80–85°C. A sample of standard 11-nor-Δ^8-THC-9-carboxylic acid in control urine is carried concomitantly through the assay procedure to be used as a standard for TLC along with unknown specimens. TLC separation is achieved on a 20 × 20 cm Gelman precoated silica gel glass microfiber sheet. The sensitivity of this proposed procedure is 50–100 ng/ml of urine and the volume of urine needed is 20 ml. An immunochemical test called the enzyme multiplied immunoassay technique (EMIT) is already on the market for the measurement of cannabinoid metabolites in human urine[14,15]. It is the most sensitive to 11-nor-Δ^9-THC-carboxylic and 11-hydroxy-Δ^9-THC, the predominant metabolites of Δ^9-THC. The detection limit of the assay is 50 ng/ml of urine[16]. The procedure is rapid and semi-quantitative, but any positive should be confirmed by an alternate non-immunological technique of comparable sensitivity. This laboratory analyzed more than 100 urine specimens using the EMIT system and all the positives obtained by the EMIT system were analyzed using the proposed TLC procedure. The results were 100% in agreement for the positives shown by the EMIT system. The authors are of the opinion that the proposed TLC procedure is suitable for mass testing, and only the positive urines need to be confirmed by the EMIT System in situations where a punitive action on a urine specimen is contemplated.

EXPERIMENTAL

Materials

11-nor-Δ^8-tetrahydrocannabinol-9-carboxylic acid and 11-nor-Δ^9-THC-9-carboxylic acid, Δ^9-THC, CBN, and 11-hydroxy-Δ^9-THC were obtained from the National Institute of Drug Abuse (Rockville, MD, U.S.A.).

Solvents

All solvents used for the extraction and TLC separation were of reagent grade as used in a standard analytical laboratory. The extraction solvent was cylohexane–ethyl acetate (96:4), and the TLC development solvent was chloroform–methanol–concentrated ammonium hydroxide (28–30%) (85:15:2, v/v/v).

Reagents

Potassium hydroxide (10%) solution in methanol, and glacial acetic acid. The detection reagent was Fast Blue RR, 0.5% (w/v) solution in an equal volume of methanol and water (Fast Blue RR must be highly pure, the one specially prepared for histochemical phosphatase determination by Calbiochem-Behring Corporation, Cat. No. 34134, mol.wt. 272, should be used. The reagent of lower purity could give false positives.)

Thin-layer plates

Gelman precoated silica gel glass microfiber sheets (ITLC) with a layer thickness of 250 μm were used. The use of these plates results in high sensitivity since a colored spot could form either on one side or both sides of the plate, and even a spot of a very minute intensity can be seen visually.

Extraction procedure

A 20-ml aliquot of urine (10–25 ml depending on the volume of urine available) is transferred to a 4-oz wide-mouth glass jar, and 2 ml of a methanolic solution of potassium hydroxide are added (10%, w/v). The contents are swirled, the mouth of the jar is covered with tinfoil, and the jar is placed in the air-circulating oven previously maintained at 100°C. After 12 min heating (10–15 min range), the rack containing jar (S) is removed and the contents are allowed to cool. The pH is then adjusted to a range of 3–4 by the addition of 3 ml of glacial acetic acid and 15 ml of cyclohexane–ethyl acetate (96:4) solution are added. The rack containing jar (S) is placed on a reciprocating shaker (Eberbach table model shaker) and after shaking for 10 min at low speed, the upper layer is pipetted out into a 40–50 ml plain conical glass centrifuge tube in a steel rack. The extraction is repeated twice more (total 3 × 15 ml extractions) and all the extracts are combined into the same test-tube. If the results are needed on the same day, the steel rack is placed in the boiling water bath and then evaporation of first extract is continued until the second extract is ready to be pipetted out. It is recommended that the second extract is pipetted out into the same tube when the solvent of the first extract has evaporated to *ca.* 5 ml and the third extract is pipetted out similarly. The steel rack containing the tube (S) is taken out when the solvent has just evaporated to dryness. Alternatively, all three extracts are combined and the rack containing tube (S) is placed in the air-circulating oven maintained at

80–85°C (the rack is placed a little farther from the air-circulating fan). The evaporation is carried out just to dryness, and the sides of the test-tube are washed with 0.5 ml of methanol, vortexed, and rinsed again with a few drops of methanol. The methanol is removed as above.

It is imperative that the standard of 11-nor-Δ^8-THC-9-carboxylic acid (THCA) must be carried through the assay procedure concomitantly with the unknown specimens(s). This standard should be used for the TLC identification of unknown specimens. A 20-ml aliquot of controlled urine is spiked with 2–4 μg of the tetrahydrocannabinolic acid* (100 ng/ml of urine), 3 ml of methanolic potassium hydroxide are added and the assay is carried out as above. Another 20-ml aliquot of the same controlled urine without spiking with the acid is also recommended to be carried through the assay procedure (blank controlled urine).

Thin-layer chromatography

One specimen of standard THCA (THCA which has been carried through the assay procedure in controlled urine) and 8–10 unknown specimens are spotted on a 20 × 20 cm ITLC Gelman precoated silica gel glass microfiber sheet. The residue obtained from the extraction procedure is spotted dexterously as follows. Methanol (10 μl) is added into conical tip of the tube, the tube is vortexed with swirling for 20–30 sec, and methanol is spotted on the plate using a 5-μl capillary tube. The process is repeated twice more. The sides of the tube are then washed with 0.5 ml of methanol, vortexed, sides rinsed with a few drops of methanol, the methanol is evaporated to dryness on a water bath or in the oven as described under *Extraction procedure.* Methanol (10 μl) is added and transferred to the plate on to the same spot as above. The process is repeated again with 10 μl of methanol (in all five 10-μl spottings are done). The spotting may be done under nitrogen or without nitrogen. The plate is dried in the oven at 90°C for 5 min after the standard and unknown specimens are all spotted. It is then placed in the freshly prepared development solvent (chloroform–methanol–concentrated ammonium hydroxide (85:15:2, v/v/v)**, shaken well until it forms a clear solution) and taken out after the solvent has travelled a distance of 15 cm or more (time taken to travel 15 cm is *ca.* 55–60 min).

Detection of tetrahydrocannabinolic acid

The plate is air-dried for 10 min and then dried at 100°C for 5 min and sprayed on both sides with a freshly prepared 0.5% Fast Blue RR solution (0.5%, w/v, in equal volumes of methanol and water). One pink spot of the acid compound is immediately seen at an R_F of *ca.* 0.25–0.38(3.8–5.8 cm). This spot becomes of greater intensity after the plate is allowed to stay in the air for 10–25 min. Respraying after 10–25 min also increases the intensity of the spot. In some specimens this pink spot is touching the greyish streak, but it is very distinct even when it is of the lowest intensity. A second pink spot simultaneously seen with the upper spot in some standards of tetrahydrocannabinolic acid carried through the assay procedure and in

* Prepare a solution of the standard 11-nor-Δ^8-THC-9-carboxylic acid by dissolving 10 mg of the acid in 10 ml of methanol (1 mg/ml) and use this solution for spiking. Store this in refrigerator.

** Since the quality of TLC plates differs from batch to batch, the volume of the solvent to be used for development must be weighed out in each batch, it varied from 45 to 100 ml.

unknown specimens, at the lower R_F value of *ca.* 0.093–0.23 (1.4–3.5 cm) should be ignored as it is sometimes seen in controlled urines as well. The positive unknown specimen can be semi-quantitated by scanning the thin-layer plate using Helna Quick Scan R and D TLC Densitometer. The areas under the peaks can be quantitated by attaching Quick Scanner out-put to the Hewlett-Packard Computer GC Terminal 5880A series.

RESULTS AND DISCUSSION

The proposed procedure is simple, specific, reproducible and cost effective. It is imperative that a controlled urine spiked with Δ^8-tetrahydrocannabinolic acid be carried through the assay procedure concomitantly with unknown urine specimen(s) since the Δ^8-tetrahydrocannabinolic acid not carried through the assay procedure gives a spot lower than the one carried through the assay procedure. The possibility that the acid might have decarboxylated to form tetrahydrocannabinol was eliminated since no tetrahydrocannabinol was seen on the thin layer plate. The tetrahydrocannabinolic acid (11-nor-Δ^8-THC-9-carboxylic acid) after it was carried through the assay procedure did not react with EMIT enzyme substrate, malate dehydrogenase, though it gave a positive reading prior to carrying through the assay procedure.

The reaction product needs rigorous purification since gas chromatography gave multiple peaks. The possibility of methyl ester formation* was also eliminated by forming the methyl ester and then performing gas chromatography on the ester, which gave a single peak as compared to the multiple peaks given by the reaction product. The acid product formed is highly specific and very sensitive to Fast Blue RR spray. No body metabolite or other known marijuana components gave this pink spot at the reported R_F value other than the THCA gone through the assay procedure. The efficacy and the sensitivity of the proposed TLC procedure were tested on the urine specimens which were collected in a controlled study at time intervals of 20, 24 and 35 h. In this study *ca.* 200 mg of street marijuana (equivalent to 1/4th a cigarette) was puffed four times using a tobacco pipe. Urine specimens collected at 20-, 24- and 35-h intervals gave strong positive test using the TLC procedure for the presence of 11-nor-Δ^9-THC-9-carboxylic acid; urine specimens collected at 4.0 and 10.0 h could not be tested owing to insufficient volumes. All of these specimens (including the 4.0-h specimen) gave positive readings on the EMIT system. The proficiency of the proposed TLC procedure was evaluated by applying this procedure to the urine specimens shipped by the Center for Disease Control** in its Fourth CDC Proficiency Testing Survey, 1980 and the first survey of 1981. Two of the ten specimens in each survey were spiked with 100 ng and 150 ng of 11-nor-Δ^8-THC-9-carboxylic acid per millilitre of urine, respectively. The spiked specimens in both

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* The gas chromatographic separation of the ester and of the reaction product was performed by Drs. Michael Schaffer and Reng-Lang Lin at the Cook County Medical Examiner's Laboratory, Chicago, IL, U.S.A.

** Center for Disease Control (CDC) Atlanta, GA, U.S.A., Department of Health and Human Service conducts a quarterly Proficiency Testing Program in Toxicology Drugs of Abuse. Each quarter, the center submits ten urine specimens each spiked with three or four commonly abused drugs.

surveys gave strongly positive tests for the acid on a 10-ml aliquot of each urine for the first survey and 14 ml of each urine for the second survey. A total of ten specimens, each spiked with a mixture of three or four commonly abused drugs, were submitted for each survey. This laboratory identified both of the spiked specimens in each survey with 100% accuracy.

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