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# MARIJUANA METABOLITES IN URINE OF MAN

## X. IDENTIFICATION OF MARIJUANA USE BY DETECTION OF $\Delta^9$-TETRAHYDROCANNABINOL-11-OIC ACID USING THIN-LAYER CHROMATOGRAPHY

SAUL L. KANTER*, LEO E. HOLLISTER and MICHAEL MUSUMECI

*Veterans Administration Medical Center, Palo Alto, CA 94304 (U.S.A.)*

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## SUMMARY

Marijuana use can be determined by detecting $\Delta^9$-tetrahydrocannabinol-11-oic acid (THC-11-oic acid) in urine. For this, we describe a procedure for its chemical detection by using sequential thin-layer chromatography on a single plate for rapid isolation and identification. A volume of urine containing 50 mg of creatinine is concentrated by evaporation to 10 ml, the concentrate is enzymically hydrolyzed for 30 minutes and extracted with ether, and the extract is purified by treatment with $NaHCO_3$, then chromatographed in an alkaline and an acidic solvent sequence. The plate is sprayed with Fast Blue Salt B, and THC-11-oic is identified by its characteristic mobility and its characteristic colour reaction. The sensitivity is 0.5 $\mu$g. THC-11-oic acid has been detected in urines collected after the smoking of one standard cigarette containing 16–18 mg of $\Delta^9$-tetrahydrocannabinol and in 34 of the first 100 tests of spontaneously collected urines of patients in a hospital drug-abuse treatment program. Multiple samples are easily carried through this extraction procedure. Evaporative concentration takes about 20 min per sample, and the analysis of eight concentrated samples takes about 5.5 h.

## INTRODUCTION

Recent use of marijuana can be determined by testing for $\Delta^9$-tetrahydrocannabinol (THC), but only by using the extreme sensitivity of radioimmunoassay (RIA), mass spectrometry or far-ultra-violet spectrophotometry coupled with high-performance liquid chromatography (HPLC)[1,2,3]. In this report, we show that marijuana use can be determined by testing for $\Delta^9$-tetrahydrocannabinol-11-oic acid (THC-11-oic acid), the major urinary metabolite of THC in man, using equipment normally found in the clinical laboratory. Although the presence of THC-11-oic acid in urine cannot establish the time of ingestion of marijuana, as it is excreted for periods varying from hours to several days, depending on the amount and the route of ingestion of the drug[1,4], its detection is of value as a definite indication that

marijuana has been used. Thus, THC-11-oic acid in urine can support other evidence of intoxication.

Previously published methods for the determination of THC-11-oic acid are primarily research-oriented. To ensure specificity, some workers use multi-step extraction procedures to separate the THC-11-oic acid from other metabolites of THC, others use mass fragmentography preceded by derivatization with silylating agents, still others employ HPLC followed by RIA and some use RIA alone[1,4-7]. Of these methods, only RIA and the enzyme-multiplied immunoassay technique (EMIT), each of which measures THC-11-oic acid and other THC metabolites, are suitable for clinical work[8].

The RIA method requires a liquid scintillation counter and readily available antisera. Conditions to set up and perform the EMIT procedure are far less restrictive, but the initial outlay for the equipment may still be prohibitive. The procedure we present has none of these disadvantages. The most extraordinary laboratory equipment required is a vacuum rotary evaporator. Thus, it is the most easily set up of any currently available means for determination of cannabinoid use.

TEST PROCEDURE

A volume of urine containing 50 mg of creatinine is adjusted to pH 4.7 to 6.3 with 6 $N$ HCl and concentrated to approximately 5 ml in a Buchi rotary evaporator at 50 C. The concentrate is transferred to a 25 × 150 mm screw-cap culture tube (scct) and diluted to 10 ml with water, then the tube is stoppered with a PTFE-lined screw cap, and the contents are mixed and incubated with 0.1 ml of enzyme (Boehringer-Mannheim. $\beta$-glucuronidase–aryl sulfatase) at 55–60 C for 30 min. The hydrolysate is cooled to room temperature and, without further adjustment of pH, is extracted with anhydrous ethyl ether (2 × 15 ml). After each extraction, the tube is centrifuged at ca. 2000 RCF for 3 min to quickly separate the two phases and the ether extracts are transferred to another 25 × 150 mm scct. Between the extractions, the ether of the first extract is evaporated in a stream of nitrogen in a water bath at 50 C. After being washed with 5% NaHCO$_3$ solution and dried with anhydrous sodium sulfate, the ether is evaporated. The residue is streaked across 1.5 cm of a pre-coated silica gel G TLC plate, (250 $\mu$m, Analtech) with two 50-$\mu$l volumes of dichloromethane and chromatographed sequentially in two saturated TLC chambers, first with acetone–chloroform–triethylamine (80:20:1), then with petroleum ether–ether–glacial acetic acid (50:50:1.5). Between developments, the TLC plate is placed in a fume hood for 5 min. Approximately 5 min after the second development, the plate is sprayed with a freshly prepared cold 0.1% solution of Fast Blue Salt B in 2 $N$ NaOH. A positive response is indicated by a magenta colored zone of $R_F$ approximating 0.1 or corresponding to that of a reference standard.

EXPERIMENTAL

The experimental work was initially done with silylated glassware to ensure maximum recovery of THC-11-oic acid[6,9]. Untreated glassware was adopted for the test procedure when it was determined that it did not have a detectable effect on the recovery of THC-11-oic acid.

*Sample and sample size*

After removing an aliquot for determination of creatinine, the urine is stored either refrigerated, if the analysis will be done within 4 or 5 days, or frozen if analysis will be delayed longer.

Creatinine content rather than volume is used as the reference for amounts of sample, due to wide variations in concentration of urine. The use of creatinine content rather than volume as a reference for the sample vitiates the deliberate dilution of urine, which can lead to falsely negative tests.

A volume of urine containing 50 mg of creatinine approximates the maximum that can be used for analysis. Larger amounts frequently yield emulsions during extraction and "overloading" of the plate during TLC. Smaller amounts, such as 10 to 15 mg of creatinine, applied to the plate in a spot in order to obtain the same sensitivity as a 50-mg sample applied in a streak, gave poor results.

Concentration by evaporation rather than by column chromatography is used because our previous experience indicates that the solute content of the aqueous phase significantly affects the extraction of THC-11-oic acid by ether[4].

*Hydrolysis*

At pH 4.5–5, in which range $\beta$-glucuronidase of *Helix pomatia* has maximum activity, and with larger amounts of enzyme and incubation temperatures above $37.5^\circ C$[10–12], we found that conjugated THC-11-oic acid was completely hydrolyzed in 30 min at $55$–$60^\circ C$ using 0.1 ml of enzyme per 10 ml of aqueous phase. These conditions were used for the experimental work until recovery and sensitivity were evaluated. Then we determined that, at $55$–$60^\circ C$, hydrolysis was complete over the pH range 4.5 to 6.5. Thus, 4.7 to 6.3 is recommended in the test procedure and is used routinely.

*Ether extraction*

In an experiment similar to those previously reported[4], we showed that metabolic THC-11-oic acid, like THC-11-oic acid added to urine, was completely extracted into ether at pH values up to 10. Thus, the extraction could be done at the pH of hydrolysis.

After finding that successive prolonged extractions with ether[13] were not necessary, as almost all of the THC-11-oic was recovered in the first extraction, 10-ml aliquots of a hydrolyzed concentrated urine were extracted twice with 15-ml portions of ether by shaking on a reciprocal shaker with the long axis of the sect in the direction of shaking both vigorously (225 cycles per min) and gently (140 cycles per min) for various periods. All of the THC-11-oic acid was extracted by shaking vigorously for 1 min or gently for 5 min.

*Purification of ether extract*

Ether extracts of a hydrolyzed urine containing a moderate amount of metabolic THC-11-oic acid in order to have a high ratio of background to analyte were purified by shaking vigorously on the reciprocal shaker with 8 ml of 2% $NaHCO_3$ solution for various periods. The results showed that three extractions for 2 min each gave chromatograms as clean as those obtained after extraction for longer periods, and that three extractions for 30 sec each gave chromatograms that were almost as

good. Shaking the ether extracts with 10 ml of 5% $NaHCO_3$ solution for 1 min twice effected an adequate and slightly better clean-up than three extractions with 2% $NaHCO_3$ solution for 30 sec each, as evidenced by more intense coloring and slightly better defined spots in the chromatograms, and was still quite rapid.

After each extraction, the tube was centrifuged briefly to separate the two phases quickly. The aqueous layer was removed easily by inserting a 10-ml pipette through the ether layer, ejecting the small volume of ether in the pipette by hand-warming the pipette and drawing up the $NaHCO_3$ solution using a 3-ball valve pipette-filler.

To ensure that THC-11-oic acid was not taken up by 5% $NaHCO_3$ solution as reported for $\Delta^6$-THC-7-oic acid[14] (monoterpenoid numbering for $\Delta^8$-THC-11-oic acid), an ether solution of 200 ng of THC-11-oic acid was extracted with 5% $NaHCO_3$ solution, and the extract was treated with anhydrous $Na_2SO_4$ and chromatographed as described in the test procedure. The chromatogram was compared with that of an equivalent ether solution that had not been treated with $NaHCO_3$ and $Na_2SO_4$. There was no difference in the intensity of the spots, thus indicating that 5% $NaHCO_3$ solution did not extract THC-11-oic acid from ether. This experiment also demonstrated that no loss occurred due to the use of $Na_2SO_4$.

*Drying the purified ether extract*

Approximately 1 g of anhydrous $Na_2SO_4$ was found to be an adequate amount to dry the purified ether extract. After it has been added, the tube is capped, inverted several times and centrifuged briefly, and the ether extract is poured through a small glass funnel into a 20 × 125 mm scct. The $Na_2SO_4$ is washed once with 5 ml of ether, and the combined ether solutions are evaporated as previously described. The inner wall of the scct is washed down with 0.5 ml of absolute ethanol, which is also evaporated.

*Thin-layer chromatography*

A blank urine that, after hydrolysis, had a great deal of chromogenic material and other substances that gave non-cannabinoid color reactions with Fast Blue Salt B was selected for this phase of the work to ensure that optimum TLC conditions would be devised. Different chromatographic conditions were tested with residues of ether extracts of this urine "spiked" with 500 ng of THC-11-oic acid before chromatography. A single-development procedure for distances of both 15 and 10 cm using mixtures of acetone, chloroform and triethylamine in proportions ranging from 80:10:1 to 50:50:1 were tried. Mono- and dihydroxy derivatives of THC migrate at this range of polarity and specific alkalinity and have $R_F$ values greater than that of THC-11-oic acid, which remains at the origin uncontaminated by non-acidic hydroxy compounds[15]. Background material made it difficult to evaluate results in single-development procedures with samples containing less than 1 $\mu$g of added THC-11-oic acid. Additionally, the single-development procedure was tried with acetone–chloroform (80:20) containing 0.5 or 2% triethylamine. The separation of THC-11-oic acid from endogenous material was poor with either concentration of triethylamine; with 0.5% of the amine, the mobility of THC-11-oic acid increased, and with 2% the mobility of the endogenous material was suppressed.

The two-development procedure overcame the disadvantages of the single-

development procedure. A definitive band of appropriate $R_F$ value and truer color was obtained, and, with a 10-cm development each time, the total time was only slightly greater than for one 15-cm development. An acetic acid concentration of 1.5% in the second solvent system was essential; 1% acetic acid produced little movement of the THC-11-oic acid, and 2% caused the THC-11-oic acid to move into a zone of endogenous material.

*Standard*

A 1-$\mu$g amount of pure THC-11-oic acid, dissolved in absolute ethanol, is mixed with the dried ether extract of a blank urine. This adjusts for the slight increase of mobility of THC-11-oic acid due to the effect of background material on the binding sites of the silica gel.

*Recovery and sensitivity*

Ethanolic solutions of THC-11-oic acid added to 50-ml aliquots of the highly chromogenic blank urine containing 50 mg of creatinine were analyzed. The intensity of the reaction of a 500-ng spot of THC-11-oic acid and Fast Blue Salt B was faint, but definitely positive, and approximately half of that obtained by adding 500 ng of THC-11-oic acid to the final residue of the extract of an aliquot of the same urine.

Evaluation of the effect of each step in the procedure showed that none contributed disproportionately to the apparent low recovery. Background material had a large effect on sensitivity. We found that 250 ng of THC-11-oic acid were required to give a detectable spot in the presence of urinary background material. This was approximately equal to the intensity of 80 ng applied directly to the TLC plate.

Duplicate samples of a urine "spiked" with THC-11-oic acid were refrigerated overnight, then frozen for 5 days in silylated and in untreated glass bottles before being routinely processed. Silylated and untreated glassware, respectively, were used throughout. No difference could be detected between the results; thus, non-silylated glassware is used in the recommended procedure.

*Comparison between TLC and EMIT assay methods[8]*

For 39 of 43 samples analyzed by both methods, results were in agreement (see Table I); as 3 of the 4 samples that did not check were in the borderline range of the EMIT procedure, the correlation is excellent. The correlation was absolute among the 19 samples that were either zero or exceeded the EMIT detection limit (50 ng/ml), but was less among the 17 that were greater than zero but less than 20 ng/ml. In this range, results by EMIT are negative[16]. By the TLC procedure, 16 were negative and one was positive. The correlation was poorest among the 7 samples that were in the range 20–50 ng/ml. In this range, results by EMIT are positive[16]; by the TLC procedure, 3 were negative and 4 were positive. Such mixed results can be expected in the borderline range, as the TLC method measures only THC-11-oic acid, whereas the EMIT procedure measures total cannabinoids.

CLINICAL RESULTS

Fig. 1 shows the results of analysis for THC-11-oic acid in spontaneously voided urines from six subjects participating in a study for which "heavy" and "light"

TABLE I

COMPARISON OF RESULTS WITH THOSE OF EMIT ASSAY FOR TOTAL CANNABINOIDS IN URINE*

The EMIT assays were carried out as described in ref. 16.

| TLC result | EMIT test values | | | |
|---|---|---|---|---|
| | Negative | < 20 ng ml** | 20–50 ng ml*** | >50 ng ml |
| Negative | 11 | 16 | 3 | |
| Positive | | 1 | 4 | 8 |

* See ref. 8.
** Interpreted as negative[16].
*** Interpreted as positive[16].

users were recruited[17]. In this series. there were three positives and three negatives; thus. three were considered as being "heavy" users and three "light" users.

We have also used the method to assay urines from patients on several of our psychiatric wards to determine whether or not some of their unexpected aberrant behavior could possibly be attributed to marijuana[18-20]. Of 31 tests. 12 were negative and 16 positive (the 16 positives included four patients who were positive on each of two trials and one who was negative on one trial and positive on another).

Currently. we are using the test routinely in conjunction with a hospital drug-abuse treatment program. Of the first 100 tests. 34 have been positive. In contrast,
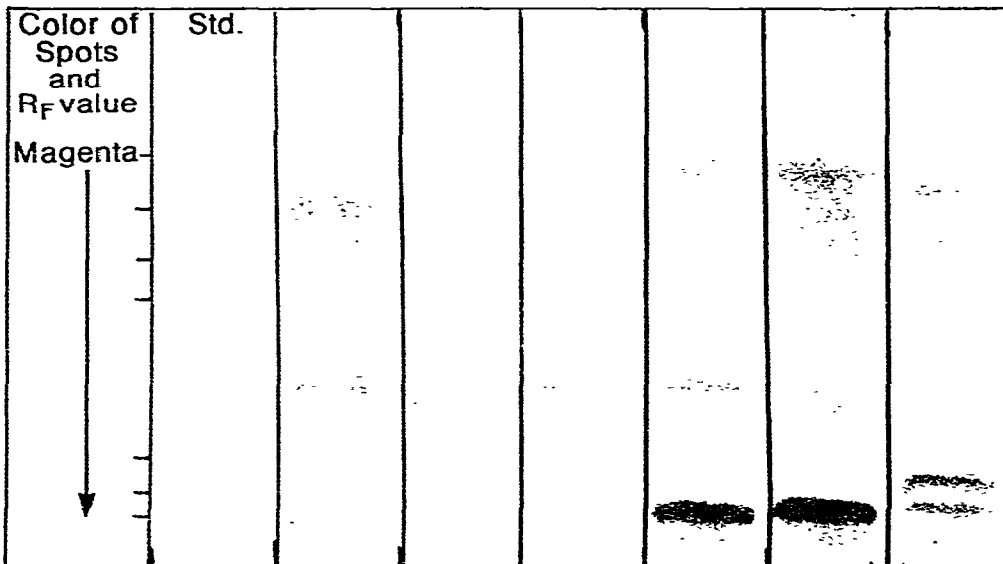


Fig. 1. Chromatograms of spontaneously voided urines from six volunteers for a study on "heavy" and "light" users of marihuana analyzed by the test procedure. Of these six, three are positive and three are negative. Only colors characteristic of the reaction of metabolites of THC and Fast Blue Salt B noted. Std. = $\Delta^9$-THC-11-oic acid.

screening for methadone, morphine, barbiturates, codeine, amphetamine and meth-amphetamine yielded less than 2 % positive samples. This may be due in part to use of urine as delivered, which, in many instances, is quite dilute.

Clinically, THC-11-oic acid can be detected after the smoking of one marijuana cigarette, depending on potency, smoking efficiency and metabolism. Among spontaneously voided urines tested after the smoking of one standard marijuana cigarette containing 16–18 mg of THC, THC-11-oic acid was detected as early as 1.5 h after smoking. Among the urines tested from the patients in the hospital drug-abuse treatment program cited above, we have obtained positive results as much as 1 week after admission and, in one instance, 28 days, from a self-admitted user of 20–30 marijuana cigarettes a day for approximately 3 months. This exceeds the 48 and 72-hour excretion periods previously described for this metabolite[1,5] and is most likely due to the amount ingested and prolonged use.

DISCUSSION

At present, immunoassays are the only means available for the relatively routine detection of urinary cannabinoids. The proposed procedure makes available an independent method that can be used in some laboratories routinely and in others as a simple confirmatory test.

Laboratories performing drug-abuse screening on a large scale would obviously find it more feasible to use a set volume and eliminate the determination of creatinine and the concentration of the urine. But such an approach reduces the effectiveness of this test, and of other tests involving use of spontaneously voided urine samples, because of the wide range of concentration. Among the samples tested for the drug-abuse treatment program we have used a wide range of sample volumes and have obtained a moderately strong positive result (250 ng) with a 250-ml sample.

A possible source of false positives might be due to the non-specificity of Fast Blue Salt B, which reacts with phenols and amines and with various herbs, such as rosemary, sage and thyme[21,22]. However, it is highly unlikely that these materials would be taken in quantities sufficient to produce urinary metabolites or that such metabolites would be extracted and chromatographed in the same way as cannabinoids. Additionally, of 150 urines tested by this procedure, none has shown interference due to metabolites of cannabidiol and cannabinol and only two have shown interference due to background.

The procedure has been used to detect current cannabis use among psychiatric patients and those being treated for drug abuse. A positive test allows physicians to confront patients with reliable support information. On the other hand, a negative test alleviates suspicion that patients are abusing privileges.

Some evidence suggests that use of cannabis may also aggravate the psychosis of schizophrenics, as well as the depressed mood of patients with affective disorders[18–20]. Thus, any measure that might discourage use of this drug in such patients might be clinically useful.

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