Journal of Chromatography, 235 (1982) 507–512
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 14,360

MARIJUANA METABOLITES IN URINE OF MAN

XI. DETECTION OF UNCONJUGATED AND CONJUGATED Δ^9-TETRAHYDROCANNABINOL-11-OIC ACID BY THIN-LAYER CHROMATOGRAPHY

SAUL L. KANTER*, LEO E. HOLLISTER and JOSE U. ZAMORA
Veterans Administration Medical Center, Palo Alto, CA 94304 (U.S.A.)
(Received September 11th, 1981)

SUMMARY

A method for separating unconjugated and conjugated Δ^9-tetrahydrocannabinol-11-oic acid, the major urinary metabolite of Δ^9-tetrahydrocannabinol in man, by liquid–liquid extraction and detection of both forms by thin-layer chromatography is described. The unconjugated form of the metabolite is extracted with hexane–diethylether (65:35), and the conjugated form (which remains in the aqueous phase) is extracted with ether after enzymic hydrolysis. The residue of each extract is chromatographed in an alkaline and an acidic solvent sequence, and the metabolites are detected with Fast Blue Salt B.

INTRODUCTION

The principal aim in studies of drug metabolism is to identify the pathways by which drugs are transformed in the body, and to ascertain quantitatively the importance of each pathway and intermediate[1]. In order to identify these pathways, recovery and identification of metabolites are usual early steps. Thus, analytical procedures that separate metabolites, either individually or into groups of compounds, are of primary importance in the technology of methods used for identification.

In our studies of the metabolism of Δ^9-tetrahydrocannabinol (THC) we have used a multi-step extraction procedure by which, after enzymic hydrolysis and liquid–liquid extraction with hexane and anhydrous diethyl ether (ether) under various pH conditions, urinary metabolites were seaparated into four fractions, *viz.*, non-polar and polar neutral compounds, and weakly polar and more polar acids[2–5]. Recently, we found that the conjugated neutral metabolites and conjugated Δ^9-tetrahydrocannabinol-11-oic acid (THC-11-oic acid) were extracted (with the unconjugated forms of these metabolites) into ether from unhydrolyzed urine when it was acidified[6]. Until this observation, the assumption had been that conjugated metabolites of THC were not extracted from unhydrolyzed urine with ether[7,8].

We now report a method for separating unconjugated and conjugated THC-

0021-9673/82/0000–0000/$02.75

11-oic acid, the major urinary metabolite of THC in man, by liquid–liquid extraction and detecting both forms by thin-layer chromatography (TLC).

EXPERIMENTAL

Method

Extraction of unconjugated THC-11-oic acid. A volume of urine containing up to 50 mg of creatinine is adjusted to pH 4.7 to 6.3 and concentrated by evaporation in a Buchi rotary evaporator[3]. The concentrate is transferred to a 25 × 150 mm screw-capped culture tube (scct) and diluted to 10 ml with water, and the tube is stoppered with a PTFE-lined screw-cap. The liquid is extracted twice with 15 ml hexane–anhydrous diethyl ether (65:35) by shaking vigorously at 225 rpm for 2 min each time on a reciprocating shaker with the long axis of the tube in the direction of shaking. After each extraction, the tube is centrifuged at 2000 RCF for 3 min, and the hexane–ether extract is transferred with a pipet and a 3-ball-valve pipet filler to another 25 × 150 mm scct; the aqueous phase is reserved to be processed for conjugated THC-11-oic acid. The combined hexane–ether extracts are evaporated to dryness in a stream of nitrogen in a water bath at 50°C, and the residue is dissolved in 15 ml of ether. The solution is washed once with 10 ml of 5% $NaHCO_3$ solution by shaking as described above for 1 min. The tube is centrifuged briefly to separate the two phases quickly, and the $NaHCO_3$ layer is removed with a pipet and pipet filler and discarded. Approximately 1 g of anhydrous granular Na_2SO_4 is added to the ether solution, and the tube is stoppered, inverted three or four times and centrifuged briefly. The dried ether solution is poured through a small glass funnel into a 20 × 125 mm scct in a manner such as to avoid transfer of any Na_2SO_4. The Na_2SO_4 is washed once with 5 ml of ether, which is added to the contents of the scct, and the ether solution is evaporated as previously described. The inner wall of the scct is washed down with 0.5 ml of ethanol, which is then evaporated, and the residue is dissolved in 30 μl of absolute ethanol and stored at freezer temperature until chromatographed as described below.

Extraction of conjugated THC-11-oic acid. Without further adjustment of pH, the aqueous phase from the extraction of the unconjugated metabolite is incubated with 0.1 ml of β-glucuronidase–arylsulphatase at 55–60°C for 30 min[9]. The hydrolysate is cooled to room temperature and extracted with ether (15 ml × 2) by shaking vigorously as previously described for 1 min. After each extraction, the tube is centrifuged at approximately 2000 RCF for 3 min to separate the two phases quickly, and the ether extracts are transferred to another 25 × 150 mm scct. Between extractions, the ether of of the first extract is evaporated in a stream of nitrogen in a water bath at 50°C. The ether extract is washed twice with 5% $NaHCO_3$ as previously described, then the processing is continued as described above.

The residues of the unconjugated and conjugated fractions are transferred, as a streak, to a pre-coated silica gel G TLC plate (Analtech, 250 μm) with 30 and 20 μl of absolute ethanol. The origin is 2.5 cm from the bottom of the plate, and the solvent path is 10 cm. Chromatography is carried out in two tanks each saturated with its solvent mixture[9]. The first development is with acetone–chloroform–triethylamine (80:20:1) and the second is with light petroleum (b.p. 30–60°C)–diethyl ether–glacial acetic acid (50:50:1.5). After the first development, the TLC plate is placed in a fume

hood for 5 min, and approximately 5 min after the second development, it is sprayed with a 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 0.1 or corresponding to a reference standard of THC-11-oic acid. The reference standard, in absolute ethanol, is stored at freezer temperature.

Experiments

Extraction of unconjugated THC-11-oic acid. Although hexane did not extract conjugated THC-11-oic acid from acidified unhydrolyzed urine, it did extract unconjugated THC-11-oic acid, but not quantitatively. Thus, we examined the possibility of finding a hexane–ether mixture that would extract unconjugated THC-11-oic acid completely from acidified unhydrolyzed urine and not extract any conjugated THC-11-oic acid.

We first determined the minimum concentration of ether in a hexane–ether mixture that would completely extract unconjugated THC-11-oic acid from urine. A concentrate of a urine (from a user of cannabis) containing THC-11-oic acid was enzymically hydrolyzed at pH 5.5 in order to have a large amount of the metabolite in the unconjugated form. Aliquots were extracted with various hexane–ether mixtures as described for extracting unconjugated THC-11-oic acid. The aqueous phases were then checked for residual THC-11-oic acid by the procedure described for extraction of conjugated THC-11-oic acid, except that hydrolysis was not repeated. TLC showed that a hexane–ether mixture containing at least 30% of ether was needed for complete extraction of unconjugated THC-11-oic acid.

We then determined the maximum concentration of ether in a hexane–ether mixture that would not extract conjugated THC-11-oic acid from unhydrolyzed urine at pH 4. A concentrate of the same urine previously used was adjusted to pH 7.5 and the unconjugated THC-11-oic acid (as well as a small amount of conjugated THC-11-oic acid) was completely extracted with ether. The aqueous phase was adjusted to pH 4, and aliquots of it were extracted with various hexane–ether mixtures as described for extracting unconjugated THC-11-oic acid. The hexane–ether extracts were evaporated, and each residue was mixed with 10 ml of a "blank" urine adjusted to pH 5.5. Enzyme was added, and all mixtures were processed as described for conjugated THC-11-oic acid; TLC showed that a hexane–ether mixture containing 40% ether was the maximum concentration of ether that did not extract conjugated THC-11-oic acid from unhydrolyzed urine at pH 4.

On the basis of these experiments, hexane–ether (65:35) was selected as optimum for the procedure.

We determined that residues of hexane–ether extracts that were prepared with as little as 5% ether could not be chromatographed without being washed with $NaHCO_3$ solutions, as, after chromatography, the THC-11-oic acid could not be distinguished from background material. We also determined that THC-11-oic acid was extracted by $NaHCO_3$ solution from hexane–ether extracts containing more than 20% hexane. Thus, the hexane–ether extract containing unconjugated THC-11-oic acid was replaced with ether before the treatment with $NaHCO_3$.

We also established that the presence of either residual ether or residual hexane had no evident effect on enzyme activity.

RESULTS

Clinical

Two subjects, each of whom smoked cannabis as well as having THC administered intravenously, provided the urine samples analyzed in Fig. 1 and 2. Fig. 1 shows the pattern of conjugated to unconjugated metabolites in these subjects after smoking. Subject BU showed an early predominance of conjugated THC-11-oic acid, with a later predominance of unconjugated metabolite. Subject WF showed no conjugated metabolite early, but a majority of conjugated THC-11-oic acid later. Fig. 2 shows that these same inter-individual differences in the pattern of metabolite excretion obtained following intravenous administration of THC. A third subject differed from either of these two in that his urine showed both an early and a later predominance of conjugated metabolite.

We have applied these analyses to eight additional experimental subjects and to a number of suspected users of cannabis. From the results, some tentative observations can be made. (1) In spite of the difference in the excretion patterns noted between ingestion by smoking and intravenous administration for subject WF, the excretion patterns within a subject tended to be similar for both modes of ingestion. (2) If unconjugated THC-11-oic acid was present, conjugated THC-11-oic acid was almost always present also; the reverse was noted far less often. (3) Based on the

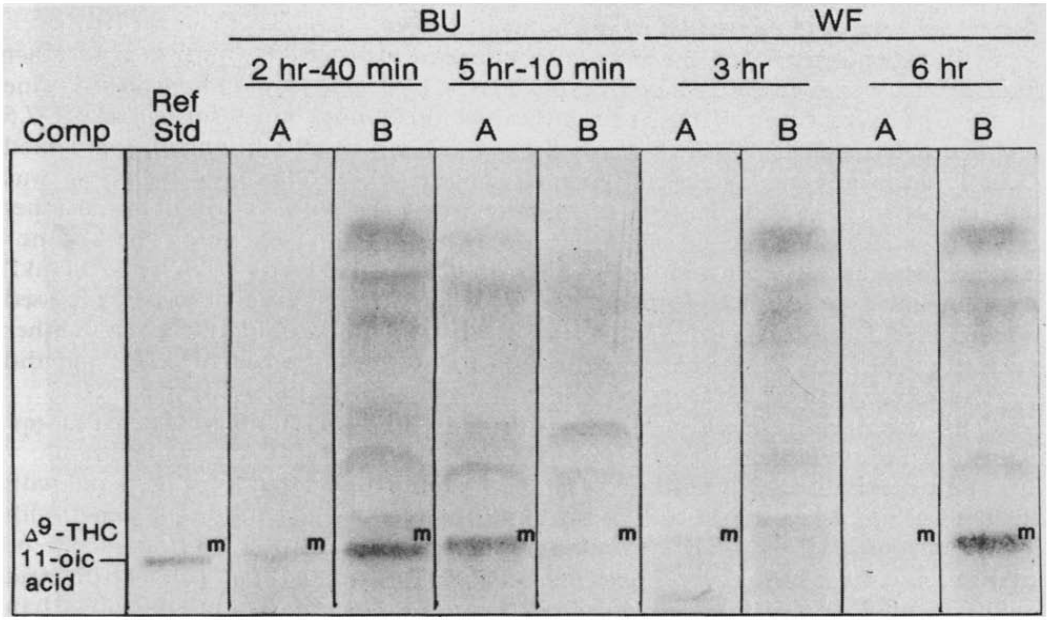

Fig. 1. Detection of THC-11-oic acid in urine after ingestion of THC by smoking: chromatograms of post-THC urines. Collection times noted by numbers at top of each chromatogram. A, unconjugated, extracted with hexane–ether (65:35) from unhydrolyzed urine; B, conjugated, extracted with ether after enzymic hydrolysis; m = magenta; Only the spot of THC-11-oic acid is identified. The other spots are either not characteristic of the reaction between cannabinoids and Fast Blue Salt B or are not identifiable. For details of TLC, sample size, see text.

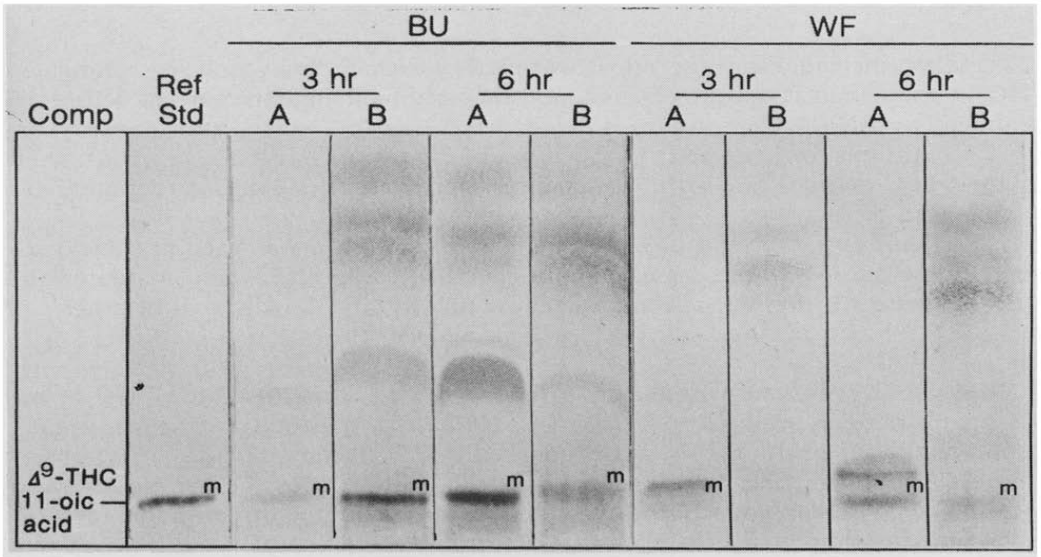

Fig. 2. Detection of THC-11-oic acid in urine after intravenous administration of THC; see Fig. 1 for details. Sample size, volume of urine containing 30 mg of creatinine.

results obtained after intravenous administration (by which procedure the amount of THC ingested between subjects varies least), individual metabolism appears to be a factor. (4) As the interval between ingestion of THC and collection of urine increased, the conjugated form tended to increase or persist, while the unconjugated form tended to decrease and become undetectable. Additionally, of twelve positives among the group of suspected users, urine from seven contained both forms of the metabolite.

DISCUSSION

Experimental

A volume of urine containing 50 mg of creatinine approximates the maximum that can be used for analysis of conjugated THC-11-oic acid. Larger amounts frequently yield emulsions during extraction and "overloading" on the TLC plate.

The concentration of ether in the hexane–ether mixture that was required for complete extraction of unconjugated THC-11-oic acid was established at the pH of hydrolysis to avoid, if possible, a second pH adjustment. At this pH, a greater concentration of ether would be required to extract unconjugated THC-11-oic acid quantitatively than at a more acid pH.

Tests to establish the maximum concentration of ether in the hexane–ether mixture that would not extract any conjugated THC-11-oic acid were carried out at pH 4, as, at this pH, less ether is needed to extract conjugated THC-11-oic from unhydrolyzed urine than at higher pH values. Thus, the hexane–ether mixture developed was optimum. It completely extracted unconjugated THC-11-oic acid from unhydrolyzed urine at the pH needed for hydrolysis, without extracting conjugated THC-11-oic acid.

Ether, instead of hexane–ether, was used to extract the hydrolyzed conjugated THC-11-oic acid as it is more efficient, and the need to change the solvent before the treatment with $NaHCO_3$ solution is avoided.

Conditions for hydrolysis, purification of the extracts with 5% $NaHCO_3$ solution and TLC on one plate with two solvent systems were developed earlier and have been used in other work[9].

A blank urine instead of an aqueous buffer solution was used in the experimental work because our previous experience indicated that the solute content of the aqueous phase significantly affected the extraction of THC-11-oic acid by ether[5].

Clinical

Clinical results showing that both unconjugated and conjugated THC-11-oic acid are excreted in urine after ingestion of THC (with the conjugated form usually predominant) agree with the recently published work of Williams *et al.*[10]. The observations that the conjugated form increased proportionately as the interval between ingestion of THC and collection of urine increased could not be compared, as Williams *et al.* did not report such fractionation for successive samples, but it does agree with the work of Wall *et al.*[11].

A procedure that can be used to detect both unconjugated and conjugated THC-11-oic acid in urine by TLC has been described. It is sensitive to as little as one standard marijuana cigarette containing approximately 16 mg of THC or the intravenous administration of 5 mg of THC. Both forms of the metabolite have also been detected in spontaneously collected urines from psychiatric patients suspected of illicitly using marijuana.

ACKNOWLEDGEMENTS

This work was supported by a grant from the National Institute of Drug Abuse, No. DA 00424, and from the Research Services of the Veterans Administration. The THC, THC-11-oic acid and standard marijuana cigarettes were provided by the National Institute of Drug Abuse, Public Health Service, NIMH.

REFERENCES

1 A. Goldstein, L. Aronow and S. M. Kalman, *Principles of Drug Action: the Basis of Pharmacology*, Wiley, New York, 2nd ed., 1974, p. 230.
2 L. E. Hollister, S. L. Kanter, F. Moore and D. E. Green, *Clin. Pharmacol. Ther.*, 13 (1972) 849.
3 S. L. Kanter, L. E. Hollister, F. Moore and D. E. Green, *Res. Commun. Chem. Pathol. Pharmacol.*, 9 (1974) 205.
4 S. L. Kanter, L. E. Hollister and F. Moore, *Res. Commun. Chem. Pathol. Pharmacol.*, 10 (1975) 215.
5 S. L. Kanter and L. E. Hollister, *Res. Commun. Chem. Pathol. Pharmacol.*, 17 (1977) 421.
6 S. L. Kanter, L. E. Hollister and M. Williams, *J. Chromatogr.*, 234 (1982) 255.
7 D. E. Green, in R. E. Willette (Editor), *NIDA Res. Mon.*, No. 7, 1976, p. 70.
8 S. Agurell, I. M. Nilsson, A. Ohlsson and F. Sandberg, *Biochem. Pharmacol.*, 19 (1970) 1333.
9 S. L. Kanter, L. E. Hollister and M. Musumeci, *J. Chromatogr.*, 234 (1982) 201.
10 P. L. Williams, A. C. Moffat and L. J. King, *J. Chromatogr.*, 186 (1979) 595.
11 M. E. Wall, D. R. Brine and M. Perez-Reyes, in M. C. Braude and S. Szara (Editors), *Pharmacology of Marihuana*, Raven Press, New York (1976) p. 93.