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Note

Extraction of glucuronide metabolites of Δ^9-tetrahydrocannabinol by diethyl ether

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Conjugation of drugs and their metabolites converts them into highly polar compounds that are excreted rapidly by the kidney. This increase in polarity is exploited analytically for studying the metabolism of drugs as it is generally assumed that solvents such as ether extract the relatively non-polar free compounds leaving the polar conjugates in the aqueous phase. Thus diethyl ether extraction of conjugated metabolites is thought to occur only after hydrolysis to their free form[1].

In contrast, we have found that conjugated neutral metabolites and conjugated weakly polar acids including Δ^9-tetrahydrocannabinol-11-oic (THC-11-oic) acid, the major urinary metabolite of THC, are extracted along with unconjugated metabolites from acidified, unhydrolyzed human urine with anydrous diethyl ether. This result challenges the generally held assumption that conjugated metabolites are not extracted by ether, and suggests that previous work on the metabolism of THC, especially those using radioactivity or gas chromatography–mass spectrometry (GC–MS) as the analytical detection technique, be reevaluated.

EXPERIMENTAL

Extraction of conjugated Δ^9-THC-11-oic acid from acidified, unhydrolyzed urine

A volume of urine containing metabolites of THC was concentrated by evaporation to 10 ml, adjusted to pH 8 and shaken with anydrous ethyl ether (ether), 3 × 15 ml, to extract unconjugated THC-11-oic acid[2,3]. The combined ether extracts were evaporated to approximately 15 ml in a stream of nitrogen with heat (not over 50°C), washed once with 5% $NaCHO_3$ and dried with anhydrous, granular Na_2SO_4. The dried ether was evaporated and the residue chromatographed as described below (Fig. 1, chromatogram A).

The aqueous phase was adjusted to pH 4 and extracted with ether as above. The extracts were combined and the ether evaporated. The dried residue was dissolved in 10 ml of absolute ethanol and divided in two. The ethanol was evaporated and each residue was mixed with 10 ml of a blank urine adjusted to pH 5.5. Both were incubated at 55–60°C for 45 min, one without enzyme (Fig. 1, chromatogram B), the other with 0.1 ml of Boehringer-Mannheim β-glucuronidase–arylsulphatase (Fig. 1, chromatogram C). Both were extracted with ether and the ether extract of each was washed twice with $NaCHO_3$, then processed as previously described.

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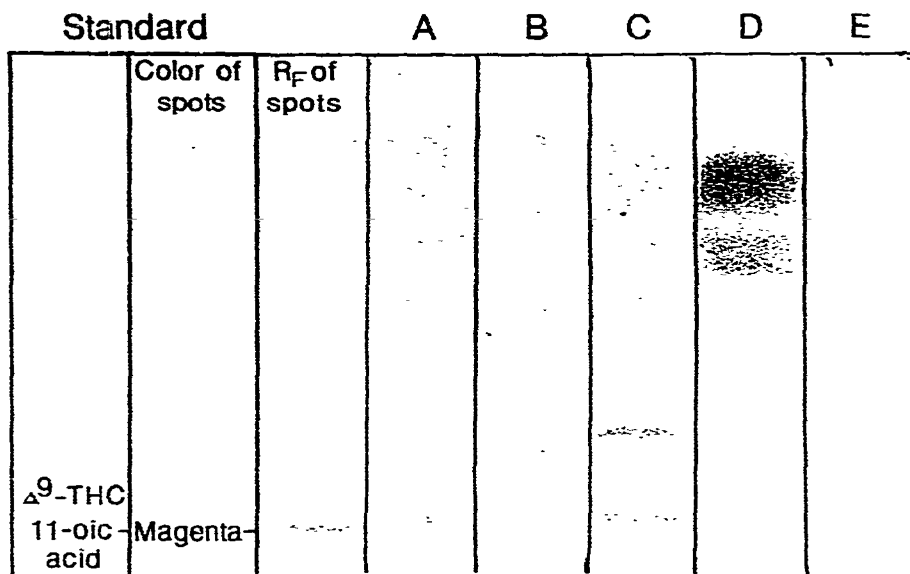

Fig. 1. Extraction of conjugated Δ^9-THC-11-oic acid by ether from acidified, unhydrolyzed urine. Chromatograms of a single aliquot of post-THC urine extracted with ether sequentially. A, unconjugated Δ^9-THC-11-oic acid extracted in ether from unhydrolyzed urine at pH 8. B and C, aqueous phase treated as described. B, incubated without enzyme. C, with enzyme. Only C shows reaction to THC-11-oic acid. D, extract of aqueous phase hydrolyzed at pH 5.5. Absence of reaction at R_F corresponding to THC-11-oic acid indicates all TH-11-oic acid had been extracted during the previous treatment. E, extract of hydrolyzed blank urine. Color of spots due to reaction with FBSB. Chromatographic solvent systems: acetone–chloroform–triethylamine (80:20:1), first pass; light petroleum–diethyl ether–glacial acetic acid (50:50:1.5), second pass. See text for more detail. Spots not identified by color are not characteristic of reaction between cannabinoids and FBSB.

To ensure that most, if not all, the conjugated THC-11-oic acid was extracted from the unhydrolyzed urine, at pH 4, the aqueous phase remaining was adjusted to pH 5.5, incubated with 0.1 ml enzyme at 55–60°C for 45 min, then extracted with ether. The ether extract, after washing twice with $NaCHO_3$, was processed as previously described. A 10-ml amount of the blank urine was also analyzed to ensure that it did not contribute contamination of interference (Fig. 1, chromatograms D and E).

Ethanolic solutions of the residues of the ether extracts were quantitatively transferred in a streak to an Analtech pre-coated 250-μm silica gel G thin-layer plate and chromatographed 10 cm in two saturated tanks using two solvent mixtures; the first was acetone–chloroform–triethylamine (80:20:1) and the second was light petroleum (b.p. 35–60°C)–diethyl ether–glacial acetic acid (50:50:1.5). Between solvent passes, the thin-layer plate was placed in a fume hood for 5 min. Approximately 5 min after the second pass, the thin-layer plate was sprayed with a cold solution of freshly prepared 0.1% Fast Blue Salt B (FBSB) in 2 N NaOH. A reference standard of THC-11-oic acid was chromatographed at the same time[4].

Extraction of conjugated neutral metabolites of Δ^9-THC by ether from acidified, unhydrolyzed urine

Four 10-ml aliquots of a concentrate of a urine containing neutral metabolites

were adjusted to pH 2.5, 2.5, 7.0 and 12.4, respectively, and extracted with ether, 3 × 15 ml. The combined ether extracts of each aliquot were evaporated and the dry residues were mixed with 10 ml of the blank urine at pH 5.5. All were incubated at 55–60°C for 45 min, one, of the two that were extracted at pH 2.5, without enzyme, all the others with 0.1 ml of enzyme. After cooling, all were adjusted to pH 12.4 and extracted with ether as above[5]. The combined ether extracts of each were evaporated and the residues chromatographed as described below. The aqueous phases that remained after the initial ether extraction were adjusted to pH 5.5 and incubated at 55–60°C for 45 min, one as before, without enzyme, the others with enzyme. After cooling, all were adjusted to pH 12.4 and processed as described. These eight extracts were chromatographed sequentially as previously reported[5] with three minor modifications; only one pass was made with the first solvent system, glacial acetic acid was eliminated from the latter and FBSB was prepared in 2 *N* NaOH.

RESULTS

THC-11-oic acid

The extraction of unhydrolyzed urine at pH 8 removed all but a minimum amount of the unconjugated THC-11-oic acid (Fig. 1, chromatograms A and B). The latter plus the conjugated THC-11-oic acid were extracted at pH 4 (Fig. 1, chromatograms B and C). The intense reaction in chromatogram C corresponding to THC-11-oic acid is due to the reaction between hydrolyzed conjugated THC-11-oic acid and FBSB. The faint reaction in chromatogram B at the same R_F value indicates very little FBSB-reactive THC-11-oic acid. This faint reaction was most likely due to a small amount of unconjugated THC-11-oic acid that was not initially extracted at pH 8. The absence of a reaction at the R_F value of THC-11-oic acid in chromatogram D shows that all the THC-11-oic acid had been extracted from the urine by the previous treatment. The data from this chromatogram and chromatogram C prove that conjugated THC-11-oic acid is extractable from acidified, unhydrolyzed urine with ether.

Neutral metabolites

All the chromatograms of Fig. 2 are of ether extracts prepared at pH 12.4. Each pair, A and B, etc., is from one aliquot. A, C, E and G are of residues of unhydrolyzed urine initially extracted at pH 2.5, 2.5, 7.0 and 12.4, respectively, then mixed with blank urine at pH 5.5 and incubated, A, without enzyme and C, E and G with enzyme. B, D, F and H are of the aqueous phases remaining after the initial extractions, incubated at pH 5.5, B, without enzyme D, F, and H with enzyme. Thus, any magenta colored spots in chromatograms A and B are due to unconjugated metabolites of THC and similarly colored spots in the other chromatograms are due to unconjugated and hydrolyzed conjugated metabolites. Chromatograms A and B show that only a minimum amount of the neutral metabolites are excreted unconjugated and that all of them were extracted at pH 2.5. Chromatogram C, except for hydrolysis, is equivalent to chromatogram A. It shows numerous and intensely colored spots due to the reaction between both unconjugated and hydrolyzed conjugated metabolites and FBSB. This clearly shows that conjugated neutral metabolites are extracted by ether from acidified, unhydrolyzed urine. Chromatogram D, except for hydrolysis, is equivalent to chromatogram B. It shows some colored spots due to the

reaction between hydrolyzed conjugated metabolites and FBSB. This indicates that not all of the conjugated metabolites were extracted from the unhydrolyzed urine at pH 2.5. Chromatograms E and G, except that the extractions of the unhydrolyzed urine were at pH 7.0 and 12.4, respectively, instead of 2.5, are equivalent to chromatogram C. Each shows a single spot. In chromatogram E the spot may be due to a combination of unconjugated and hydrolyzed conjugated metabolites. In chromatogram G the spot is probably due to unconjugated metabolite only. Chromatograms F and H, except that the extractions of the unhydrolyzed urine were at pH 7.0 and 12.4, respectively, instead of 2.5, are equivalent to chromatogram D. One of the more polar spots in F and H is not present in D. The metabolite(s) responsible for this spot was completely extracted from the acidified, unhydrolyzed urine (chromatogram C). Another difference among these chromatograms is the greater intensity of the spots in F. Between F and D the difference appears to be quantitative. The intensity of the spots in D are less because much of the metabolites were extracted at pH 2.5 from the unhydrolyzed urine. Between F and H the difference appears to be a loss of FBSB reacting material perhaps due to the longer exposure to the alkaline pH. The data in these chromatograms show that the neutral urinary metabolites of THC are excreted as conjugates, primarily, and that these conjugated metabolites are extractable with ether from acidified, unhydrolyzed urine.

Extraction of conjugated THC-11-oic acid and conjugated neutral metabolites

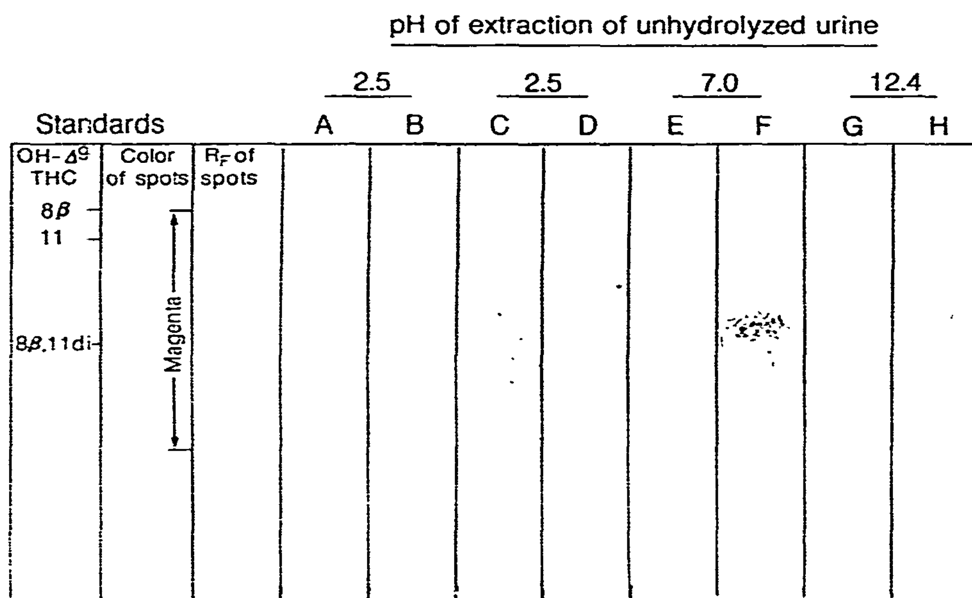

Fig. 2. Extraction of conjugated neutral metabolites of Δ^9-THC by ether from urine. Chromatograms of ether extracts prepared at pH 12.4 of four aliquots of a single post-THC urine. See text for details. Magenta colored spot in A is due to unconjugated metabolites; in C, to unconjugated as well as hydrolyzed, conjugated metabolites; in E and G to unconjugated metabolites; and in D, F and H to hydrolyzed, conjugated metabolites. Absence of spots in B indicates no FBSB reacting metabolites. Numerous and intensely colored spots in C demonstrate that conjugated, neutral metabolites of THC are extractable by ether from acidified, unhydrolyzed urine. Spots not identified by color are not characteristic of reaction between cannabinoids and FBSB.

from acidified, unhydrolyzed urine by ether was not due to hydrolysis. If it was, stronger reactions would have occurred at the appropriate R_F values in chromatograms B of Fig. 1 and A of Fig. 2.

DISCUSSION

Our results differ from those who found that conjugated and unconjugated metabolites of THC could be separated by ether extraction, the conjugated metabolites remaining in the aqueous phase[6]. This difference may be due to our using raw urine and their using Amberlite XAD-2 purified urine. Others, using raw urine as we have, interpreted the data of their studies of the metabolism of THC assuming that ether extracted only unconjugated metabolites of THC from acidified, unhydrolyzed urine[7–9]. Since our results yield a different interpretation to such data, our work may affect the understanding of the metabolism of THC.

The extraction of conjugated metabolites of THC along with the unconjugated metabolites from acidified, unhydrolyzed urine explains differently the data of Agurell *et al.*[7]. It explains the manifold increase in the amount of metabolites of THC extracted from unhydrolyzed urine at pH 3.8 compared to pH 7.8. At pH 7.8, the amount of conjugated metabolites extracted from unhydrolyzed urine by ether is minimal while the amount of unconjugated metabolites is almost complete. At pH 3.8, conjugated as well as unconjugated metabolites are almost completely extracted into the ether from unhydrolyzed urine. Thus our results do not support (1) the interpretation that only a small amount of labile conjugates (of THC and its metabolites) are present in rabbit urine after the administration of THC, and (2) the suggestion of the introduction of an acidic group in Δ^1-THC-^{3}H not affected by glucuronidase. Furthermore, interpreting their data using our results yields a ratio of conjugated to unconjugated metabolites of approximately 4:1 rather than the very small difference they evaluated.

Our results suggest reevaluating the interpretation of the results of Melikian *et al.*[8] and Green[9] as their conclusions of the effect of pH on the ether extraction of metabolites of THC from unhydrolyzed urine do not take into account the extraction of conjugated metabolites without hydrolysis. Their conclusions were based on radioactive and GC–MS methods, respectively. The former did not differentiate different forms of metabolites and the latter was so programmed that it would detect only the unconjugated form of THC-11-oic acid.

The conjugates of THC and its metabolites have been presumptively identified as glucuronides and/or sulphates based on measurement after treatment with glucuronidase and sulphatase in man, rabbit and rat[6,7,10]. Recently two groups have definitely identified glucuronides by MS. One, an O-glucuronide of THC, from rabbit urine, the other, an ester linked glucuronide of THC-11-oic acid from human urine[11,12]. Thus, we feel reasonably certain that the metabolites extracted by ether were truly glucuronides.

Residues of extracts were mixed with a blank urine instead of aqueous buffer because our previous experience indicates that the solute content of the aqueous phase significantly affects the extraction of THC-11-oic acid by ether[3].

A procedure for separating unconjugated and conjugated THC-11-oic acid by liquid–liquid extraction of raw urine will be presented separately.

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