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Identification in human urine of Δ^9-tetrahydrocannabinol-11-oic acid glucuronide: a tetrahydrocannabinol metabolite

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A Δ^9-THC metabolite has been identified in human urine as an ester linked glucuronide of Δ^9-THC-11-oic acid. Its identity was established by a comparison of mass spectra from the metabolite extracted from human urine and synthetically prepared material. Δ^9-THC-11-oic acid glucuronide was found to be responsible for the major part of RIA cross-reactivity in urine with the Guildhay cannabinoid antiserum used in this study.

The general pattern of cannabinoid metabolism is gradually being established both in animals and man. This has significance for the development of methods for cannabinoid detection in body fluids as well as for a fuller understanding of the mechanisms of cannabinoid activity. It might be anticipated that an important pathway for the elimination of cannabinoid compounds from the body would be conjugate formation and there is much evidence for this. A major part of urinary Δ^9-tetrahydrocannabinol (Δ^9-THC) metabolites in animals and man are conjugates capable of hydrolysis with mixed β-glucuronidase/aryl-sulphatase enzymes (Christiansen & Rafaelsen 1969; Lemberger et al 1970; Wall et al 1976; Kelley & Arnold 1976) or with β-glucuronidase alone (Agurell et al 1972; Mechoulam et al 1973). For individual cannabinoids direct evidence has been presented for the formation of glucuronide conjugates of Δ^8-THC, cannabinol, cannabidiol, cannabichromene and some of their metabolites in mouse liver (Levy et al 1978; Harvey et al 1976, 1977). Some support for the existence of a conjugate of Δ^9-THC-11-oic acid has also been provided by Green et al (1979) who found that more of this acid metabolite could be detected in human urine after hydrolysis with mixed β-glucuronidase/aryl-sulphatase enzymes.

In a previous study (Williams et al 1979), the major cross-reacting peak present in high performance liquid chromatography—radioimmunoassay (h.p.l.c.-RIA) chromatograms, from the urines of three subjects who had smoked Δ^9-THC, was found to hydrolyse almost completely with methanolic sodium hydroxide to two other cross-reacting components. These were identified as Δ^9-THC-11-oic acid and its methyl ester. It was concluded that the major RIA cross-reacting cannabinoid in urine from Δ^9-THC smokers was a single unidentified conjugate of Δ^9-THC-11-oic acid. By a comparison of the mass spectral properties of the conjugate with synthetically prepared material, this compound has now been identified as an ester glucuronide of Δ^9-THC-11-oic acid.

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MATERIALS AND METHODS

Δ^9-THC and Δ^9-THC-11-oic acid were generously provided by the National Institute on Drug Abuse, Rockville, Md, U.S.A., antiserum (133Y/22/5) was obtained from Guildhay Antisera, University of Surrey, U.K.; β-glucuronidase type B1, glucuronyl transferase, and uridine-5′-diphosphoglucuronic acid (UDPGA) were purchased from Sigma Ltd., London, U.K.; *N,O*-bis-trimethylsilyl-trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane from Phase Separations Ltd., Queensferry, U.K. and h.p.l.c. grade acetonitrile was obtained from Fisons Loughborough, U.K.

H.p.l.c. was conducted with a pump (M-6000A, Waters Assoc., Milford, Mass., USA) used to deliver an acetonitrile-water eluent (adjusted to either pH 2 or pH 3 with hydrochloric acid) to a column (10 cm × 4·6 mm i.d.) packed with ODS-Hypersil (Phase Separations Ltd). Various acetonitrile concentrations and pH values were necessary for the different separations required and the details are included later. Samples were injected on to the column with a six port injection valve (Rheodyne, Model 7120, Phase Separations Ltd). The column eluate was monitored for u.v. absorption at 210 nm (Cecil CE 212) and for RIA cross-reactivity by a method described previously (Williams et al 1979). Gas chromatography-mass spectrometry (g.c.–m.s.)

was conducted using a Pye 104 GC, equipped with a 3% OV-17 column (0·3 m × 2 mm i.d., Gas-Chrom Q, 80–100 mesh) interfaced by a jet separator to a VG Micromass 16F Mass Spectrometer. Accurate mass measurements were made by probe on an Associated Electrical Industries MS 50.

Collection of samples

Urine containing a high concentration of cannabinoid RIA cross-reacting material was obtained from a rabbit (New Zealand white) after the daily oral administration of 50 mg of Δ^9-THC (in a 200 mg ml^{-1} ethanol solution) over 10 days.

Human urine samples containing Δ^9-THC metabolites were obtained from two volunteers who smoked, over 10 min, tobacco cigarettes impregnated with 10 mg of Δ^9-THC. Urine samples were collected in silanized glass containers during the following 24 h and were stored at −20 °C.

Enzymic synthesis of Δ^9-THC-11-oic acid glucuronide

The synthesized glucuronide was prepared by a method similar to that of Parikh et al (1976), Lyle et al (1977) and Pallante et al (1978) except that commercially available glucuronyl transferase was used and the concentration of incubate components and pH were optimized for this reaction.

A buffered solution (pH 7·4, 0·025 M phosphate) containing glucuronyl transferase (40 mg ml^{-1}), UDPGA (40 mg ml^{-1}) and Δ^9-THC-11-oic acid (100 μg ml^{-1} added as a 25 mg ml^{-1} methanol solution) was incubated at 37 °C for 4 h. Three volumes of methanol were then mixed with the incubate and this was centrifuged. A methanol wash of the centrifugal pellet together with the supernatant was evaporated in a stream of nitrogen. Methanol (50 μl for a 1 ml incubate) was added to the dry residue and the resulting solution chromatographed with h.p.l.c. using a 45% acetonitrile–55% water eluent (pH 3) with a flow rate of 2 ml min^{-1}. The reaction yield was estimated by a comparison of the glucuronide peak area (retention volume 4 ml) with that of the unreacted Δ^9-THC-11-oic acid (retention volume 18 ml). The method produced approximately 50% of glucuronide. Δ^9-THC-11-oic acid glucuronide was purified by collecting h.p.l.c. elutate at the appropriate retention volume, evaporating this with nitrogen and dissolving the dry residue in methanol.

Extraction of metabolite from urine

Urine was adjusted to pH 3 with phosphoric acid and extracted once with a half volume of 80% ether–20% n-propanol. The organic phase was evaporated with a stream of nitrogen and the residue dissolved in methanol (100 μl of methanol for an extract from 200 ml of urine). This was chromatographed with h.p.l.c. using a 35% acetonitrile–65% water (pH 3) eluent at a flow rate of 4 ml min^{-1}. The h.p.l.c. eluate was monitored for u.v. absorption at 210 nm and for cross-reactivity by assaying 1 ml fractions with the cannabinoid RIA. The major urinary cross-reacting component was isolated by repeatedly chromatographing aliquots of the extract and collecting the h.p.l.c. eluate at the appropriate retention volume (29 ml). The eluate was evaporated with nitrogen and the residue dissolved in methanol.

Gas-chromatography—mass spectrometry

Metabolite solutions were derivatized for gas-chromatography by removing solvent with a stream of nitrogen and the addition of BSTFA (50 μl) to the dry residue. Samples were heated at 90 °C for 10 min and chromatographed at 270 °C with a helium flow rate of 30 ml min^{-1}. The m.s. source temperature was 250 °C and the accelerating voltage 3 kV.

Metabolite hydrolysis

Samples were hydrolysed with sodium hydroxide using a procedure similar to that described previously (Williams et al 1979). To a sample in methanol an equal volume of aqueous sodium hydroxide (1 M) was added and after 0, 2, 10, 30 and 60 min at room temperature (20 °C), hydrolysis was stopped by the addition of glacial acetic acid (2 μl to 10 μl of hydrolysis mixture). Changes in the reaction constituents were observed by h.p.l.c. of the reaction mixture using 50% acetonitrile - 50% water (pH 3) and a flow rate of 4 ml min^{-1}.

Samples were enzymically hydrolysed by incubating a buffered solution (pH 5, 0·075 M phosphate) of the extract (10 μl in 500 μl of buffer) or synthesized metabolite from which the solvent had been removed, with β-glucuronidase (2000 units ml^{-1}) at 37 °C for 2 h. The progress of the hydrolysis reaction was monitored by h.p.l.c. of the incubate using an eluent of 45% acetonitrile-55% water (pH 2) and a flow rate of 2 ml min^{-1}. The activity of the β-glucuronidase under these conditions was checked by hydrolysis of samples of phenolphthalein glucuronide and measurement of absorption at 550 nm after adjustment of the pH of the incubate to 10·5.

RESULTS AND DISCUSSION

Rabbit urine obtained after the oral administration of Δ^9-THC contained a high concentration of cannabinoid RIA cross-reacting material (20 μg ml^{-1};

Δ^9-THC-11-oic acid and its glucuronide conjugate cross-react in the RIA to approximately the same extent as the Δ^9-THC used to calibrate the assay, Williams & Moffat unpublished) and was used as a source of large quantities of metabolite initially. Human urine samples were obtained from two subjects by combining samples obtained over 24 h after tobacco cigarettes impregnated with Δ^9-THC had been smoked. The RIA of the separate samples has been described (Williams et al 1979).

The synthetic method used for the production of the Δ^9-THC-11-oic acid glucuronide produced approximately 50% of glucuronide. Of the two types of glucuronide which could have been formed by this in vitro method, only one was produced and this was found to be the same as that formed by in vivo metabolism (see below).

The major RIA cross-reacting cannabinoid was extracted from urine with an ether–propanol mixture. One extraction with this solvent removed 90% of the metabolite; a second extraction increased the level of endogenous interfering material. Both the urine extracts and the synthesized material were chromatographed with h.p.l.c. and the eluate monitored for u.v. absorption and cannabinoid RIA cross-reactivity (Fig. 1). A u.v. absorbing and RIA cross-reacting peak was present on the chromatograms at retention volume 29 ml for both the urine extract and synthesized glucuronide. The ratio of the u.v. absorption to RIA cross-reaction for this peak was the same for all samples. Synthesized Δ^8-THC-11-oic acid glucuronide (retention volume 26 ml) was well separated from the Δ^9-THC-11-oic acid metabolite with this h.p.l.c. system.

H.p.l.c. was used to isolate the urinary cross-reacting metabolite and to purify the synthesized metabolite. The samples were trimethylsilyl (TMS) derivatized before g.c.–m.s. and all the derivatized samples gave a peak at a retention time of 2 min. The mass spectra of these peaks from all the urinary and synthesized samples were the same (Fig. 2). The spectra had parent ions at *m/z* 880 with major

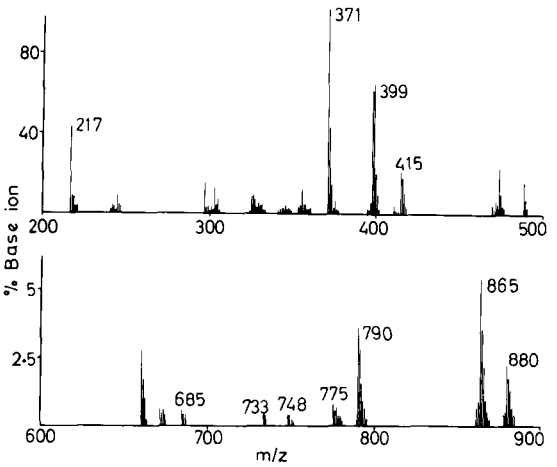

FIG. 2. Mass spectrum of the TMS derivative of Δ^9-THC-11-oic acid glucuronide. No ion fragments were present between 500–600 *m/z* and this part of the spectrum has been omitted.

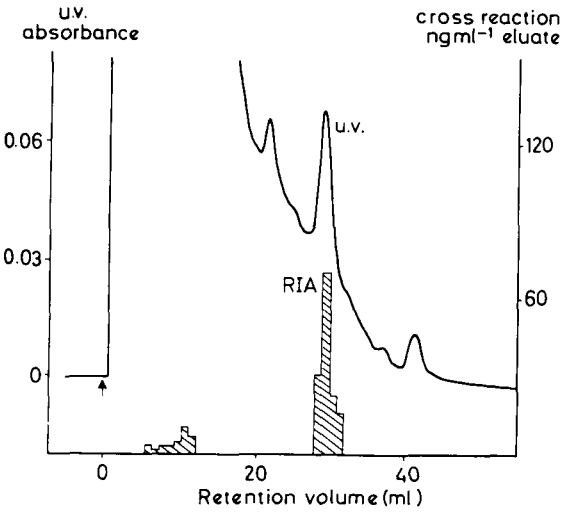

FIG. 1. Chromatogram of a urine extract from a subject who had smoked 10 mg THC. Both u.v. detection (u.v.) and RIA were used to monitor the eluate.

fragmentation ions at 865 (loss of $-CH_3$), 790 (loss of TMSOH), 775 (loss of TMSOH and $-CH_3$), 415 (loss of $(TMS)_4$-glucuronide), 399 (loss of $-O-(TMS)_4$-glucuronide), 398 (loss of $HO-(TMS)_4$-glucuronide), 217 (a possible fragmentation ion of $(TMS)_4$-glucuronide) and a base peak at 371 (loss of $-CO_2-(TMS)_4$-glucuronide). This base peak could only be produced by the fragmentation of the glucuronide ester at position 11 and not the glucuronide ether metabolite at position 1. The accurate mass value of the parent ion of the metabolite was found to be 880·4268 equivalent to $C_{42}H_{76}O_{10}Si_5$ (theoretical mass = 880·4251) ie. the TMS derivative of THC-11-oic acid glucuronide. When small quantities of the metabolite were analysed by g.c.–m.s., additional ions at 822 (M-58) and 807 (M-73) could also be observed. These ions did not appear to be associated with the main glucuronide spectrum and did not occur until the intensity of the main spectrum was decreasing. They may result from

thermal decomposition of the glucuronide. Similar behaviour was also found by Lyle et al (1977) with m.s. of derivatized cannabinoid glucuronides.

Hydrolysis of the synthesized glucuronide with a 50% aqueous methanolic solution of sodium hydroxide (0·5 M) resulted in an immediate conversion of the glucuronide to a compound with the h.p.l.c. retention volume of the methyl ester of Δ^9-THC-11-oic acid (retention volume 26 ml with 50% acetonitrile–water solvent). Over a period of 30 min, half of this ester was hydrolysed further to a compound with the retention volume (11 ml) of Δ^9-THC-11-oic acid. This behaviour had previously been observed with the Δ^9-THC-11-oic acid conjugate detected in human and rabbit urine (Williams et al 1979). Formation of the methyl ester suggests that a transesterification reaction has occurred at the carboxylic acid group on Δ^9-THC-11-oic acid and gives support to the conclusion from the mass spectral data made above that the conjugate is an ester linked glucuronide.

Synthesized glucuronide and human urine extracts were treated with β-glucuronidase and the reaction products monitored by chromatographing the incubate with h.p.l.c. using 45% acetonitrile–55% water eluent (pH 2). For both types of sample the metabolite (retention volume 4·5 ml) was completely converted to a compound with the retention volume of Δ^9-THC-11-oic acid (16 ml) within 2 h at 37 °C but urine extracts had to be diluted with sufficient buffer in order to avoid inhibition of β-glucuronidase activity. In a previous study (Williams et al 1979) no hydrolysis of this metabolite was observed with β-glucuronidase. However no measurement of enzyme activity was made with phenolphthalein glucuronide under the incubation conditions used and it is probable that the urine added to the incubate inhibited enzyme activity. No indication of the presence of unconjugated Δ^9-THC-11-oic acid before hydrolysis with β-glucuronidase could be observed by h.p.l.c. in the human urine extracts.

The major RIA cross-reacting cannabinoid in human urine using Guildhay antiserum is, therefore the Δ^9-tetrahydrocannabinol-11-oic acid glucuronide (Fig. 3). Over the past three years the RIA and h.p.l.c.-RIA methods have been used for the analysis of urine samples submitted for forensic examination. The mean value of the 103 samples found to be positive by RIA was 35 ng ml^{-1} with a range of 3 ng ml^{-1} to 2 μg ml^{-1}. All the 43 samples submitted to the h.p.l.c.-RIA procedure had the Δ^9-tetrahydrocannabinol-11-oic acid glucuronide, identified in this work, as the major cross-reacting component.

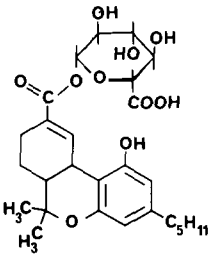

FIG. 3. Δ^9-THC-11-oic acid glucuronide.

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