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COMBINATION OF LIQUID CHROMATOGRAPHY WITH ULTRAVIOLET DETECTION AND GAS CHROMATOGRAPHY WITH ELECTRON-CAPTURE DETECTION FOR THE DETERMINATION OF Δ^9 -TETRAHYDROCANNABINOL-11-OIC ACID IN URINE

LENNART KARLSSON and CLAES ROOS^{*,*}

National Laboratory of Forensic Chemistry, Department of Toxicology, University Hospital, S-581 85 Linköping (Sweden)

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SUMMARY

A method is described for the determination of Δ^9 -tetrahydrocannabinol-11-oic acid (Δ^9 -THC-11-oic acid) in urine by using a combination of liquid chromatography and glass capillary gas chromatography with electron-capture detection.

Prior to extraction, the glucuronide conjugate of Δ^9 -THC-11-oic acid was enzymatically converted to the free acid and Δ^9 -THC-11-oic acid added as an internal standard. An aliquot of the extract was separated by liquid chromatography and one eluate fraction containing Δ^8 -THC-11-oic and Δ^9 -THC-11-oic acids was collected. Before gas chromatographic analysis the acids were converted to their pentafluoropropyl-pentafluoropropionyl derivatives. Authentic urine samples, positive with the EMIT[®] cannabinoid assay, were analysed and the results compared with those obtained from an earlier described gas chromatographic—mass spectrometric method. The detection limit for the overall method was approximately 20 ng/ml. The precision was 8% for a sample concentration of 90 ng/ml of Δ^9 -THC-11-oic acid in urine.

INTRODUCTION

To detect the abuse of cannabis, sensitive and selective or specific analytical methods are needed for the determination of tetrahydrocannabinol (THC) and its metabolites in body fluids. Most widely used is the EMIT[®] cannabinoid assay [1], which is rapid but cross-reacts with a variety of THC metabolites; thus, positive results need verification by an independent method. For this purpose analysis has been directed towards the determination of the acid metabolite, Δ^9 -tetrahydrocannabinol-11-oic acid (Δ^9 -THC-11-oic acid), present in urine after cannabis smoking. A number of gas chromatographic—mass

*Present address: AB Draco, P.O. Box 1707, S-221 01 Lund, Sweden.

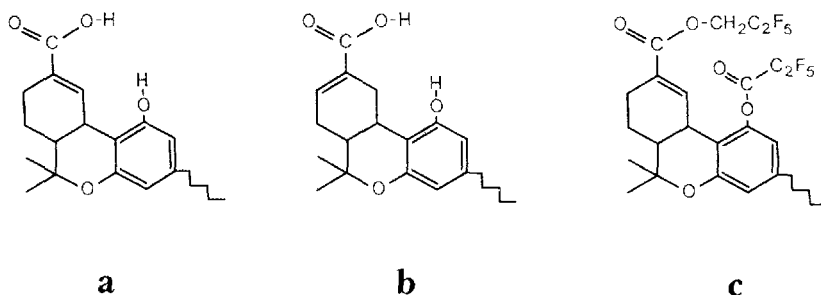


Fig. 1. Structure of (a) Δ^9 -THC-11-oic acid, (b) Δ^8 -THC-11-oic acid, and (c) pentafluoropropyl-pentafluoropropionyl derivative of Δ^9 -THC-11-oic acid.

spectrometric (GC-MS) methods are available [2-7], but to date only a few techniques other than GC-MS have been used for this purpose. One gas chromatographic (GC) method has been presented [8] where the THC-11-oic acid was converted to its methyl ester, methyl ether derivative, prior to analysis with flame ionization detection, resulting in a comparatively high detection limit. Although suitable only for fairly high concentrations, thin-layer chromatography (TLC) has also been used [9]. High-performance liquid chromatography (HPLC) has been used in combination with radioimmunoassay for the determination of cannabinoids in human urine [10] and as a clean-up step for GC-MS analysis of cannabinoid metabolites [4]. Methods for the determination of both THC and its metabolites have been extensively reviewed elsewhere [11].

The present paper describes a method for the determination of Δ^9 -THC-11-oic acid in urine which utilizes a combination of liquid chromatography (LC) with ultraviolet (UV) detection and GC with electron-capture detection. The isomer, Δ^8 -THC-11-oic acid, was used as an internal standard, and the THC-11-oic acids were converted to their pentafluoropropyl-pentafluoropropionyl derivatives prior to GC. Structures of Δ^8 -THC-11-oic acid, Δ^9 -THC-11-oic acid, and the derivative of Δ^9 -THC-11-oic acid are shown in Fig. 1. Qualitative and quantitative results obtained for a number of authentic urine samples were compared with those obtained with an independent GC-MS method [7].

EXPERIMENTAL

Chemicals

Δ^9 -Tetrahydrocannabinol-11-oic acid was purchased from the Faculty of Pharmacy (University of Uppsala, Sweden), and Δ^8 -THC-11-oic acid from the National Institute of Drug Abuse (Research Triangle Institute, NC, U.S.A.). Mirex (1,1a,2,2,3,3a,4,5,5,5a,5b,6-dodecachloro-octahydro-1,3,4-metheno-1H-cyclobuta [cd] pentalene) from Poly Science (U.S.A.) was used as an alternative internal standard. Pentafluoropropionic anhydride (PFPA) was purchased from Reagenta (Uppsala, Sweden) and pentafluoropropanol (PFPOH) from ICN Pharmaceuticals (Plainview, NY, U.S.A.). The enzyme β -glucuronidase was purchased from Sigma (lot No. G-0251) (St. Louis, MO, U.S.A.). Acetonitrile, far-UV grade and HPLC grade, from Fisons (Loughborough, U.K.) was used in the mobile phase. Other organic solvents

used throughout were *n*-hexane p.a. (pro analysi) and cyclohexane p.a. from Merck (Darmstadt, F.R.G.), diethyl ether analytical reagent grade from Mallinckrodt (Paris, KY, U.S.A.), and methanol p.a. from May and Baker (Dagenham, U.K.). All other chemicals were of analytical reagent grade.

Sample preparation

One millilitre of urine was transferred to a 16 × 100 mm glass tube with PTFE-lined screw cap, adjusted to pH 4–5 with 0.8 ml of 0.5 M acetate buffer, pH 4.5, and incubated with β -glucuronidase (2300 U) at 56°C for 30 min [8]. Thereafter, 130 ng of Δ^8 -THC-11-oic acid were added as an internal standard and the sample was shaken with 4.0 ml of diethyl ether–*n*-hexane (1:1) for 10 min, centrifuged, and 3 ml of the organic phase were evaporated to dryness under a stream of nitrogen. The residue was dissolved in 200 μ l of acetonitrile–water (1:1) and an aliquot of 100 μ l was further used for LC analysis (for details see below). One eluate fraction from the LC analysis containing both the THC acids was collected and reduced from 2 ml to 200–300 μ l by evaporation before being shaken with 1 ml of diethyl ether–*n*-hexane (1:1), which alternatively contained Mirex (42 ng/ml) as an internal standard. After centrifugation, the organic phase was transferred to a 1.0-ml silanized Reacti-vial (Pierce) with a PTFE-lined screw cap and evaporated to dryness under a stream of nitrogen. The residue was dissolved in 100 μ l of PFPA–PFPOH (4:1) and heated at 70°C for 15 min. Before GC analysis the reagent mixture was evaporated and the residue dissolved in 50 μ l of cyclohexane.

Liquid chromatography

Liquid chromatography was performed with an SP 8000 liquid chromatograph (Spectra Physics) equipped with a variable UV detector (Model 770, Schoeffel). Separation was accomplished on a Hibar[®] column (Merck), 125 × 4 mm, packed with LiChrosorb (5 μ m) RP-8. The mobile phase was acetonitrile–0.05 M ammonium dihydrogen phosphate (50:50, v/v) with a flow-rate of 1.0 ml/min. The UV detector was operated at 220 or 225 nm depending on the UV cut-off value of the acetonitrile used.

Gas chromatography

GC analyses were made on a Fractovap 4160 gas chromatograph (Carlo Erba) equipped with an electron-capture detector. The GC conditions used were as follows: fused-silica column, 25 m × 0.3 mm I.D., with SE-54 as stationary phase (Hewlett-Packard); helium carrier gas flow-rate approximately 40 cm/sec. Nitrogen was used as make up gas to the electron-capture detector with a flow-rate of 46 ml/min; GC temperatures are given in the figure captions. One microlitre of the cyclohexane extract was injected with a preset split ratio of 1:30.

Gas chromatography–mass spectrometry

GC–MS analyses were carried out as described in an earlier reported method for the determination of Δ^9 -THC-11-oic acid in urine [7]. By this method Δ^9 -THC-11-oic acid was extracted from enzymatically hydrolysed urine with

hexane—ether, followed by evaporation of the organic solvent and derivatization with a mixture of PFPA and PFPOH as described in the present paper. The d_3 -labelled Δ^9 -THC-11-oic acid was used as internal standard. GC—MS was carried out by chemical ionization with methane as reactant gas and by selective detection of negative ions.

RESULTS

Combined HPLC and GC

Fig. 2a and b shows an example from analysis of a pool of authentic urine samples which were found positive by EMIT assay. The concentration of

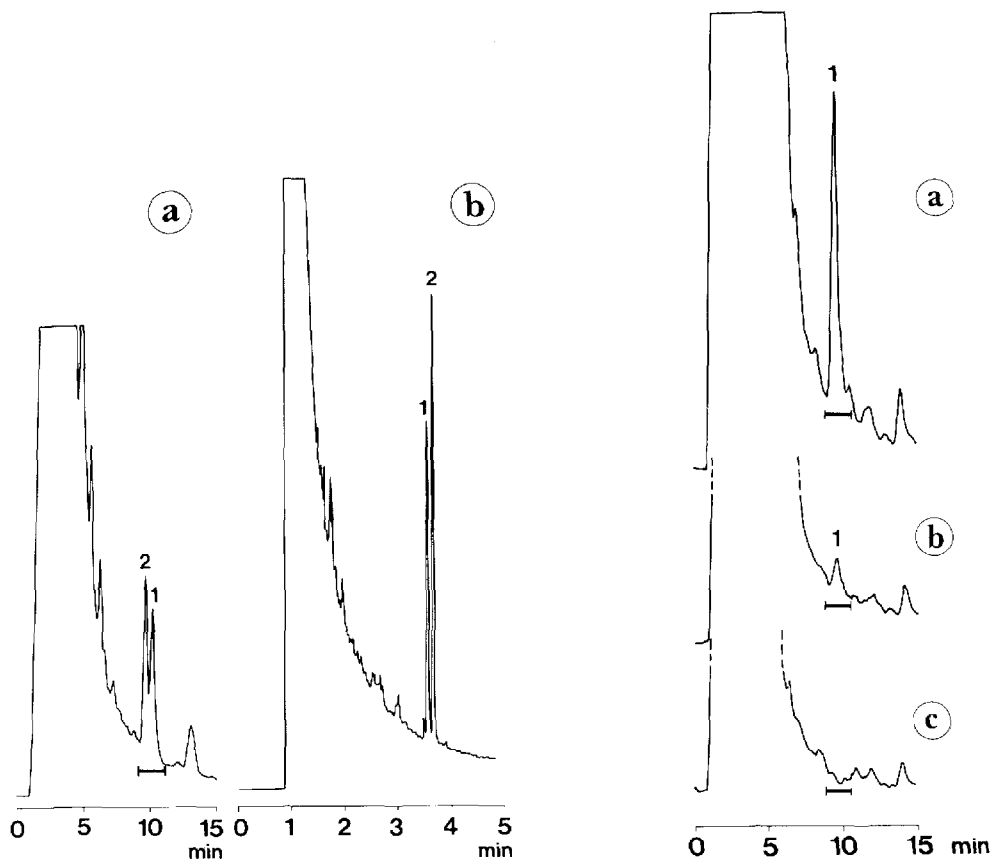


Fig. 2. Analysis of an authentic pool of urine, which was found positive with the EMIT cannabinoid assay, containing 1 = Δ^9 -THC-11-oic acid (90 ng/ml) and 2 = Δ^8 -THC-11-oic acid (130 ng/ml) used as internal standard. (a) LC chromatogram. UV conditions: 220 nm, 0.04 a.u.f.s. (b) GC chromatogram of derivatized LC fraction (see Experimental). Conditions: oven temperature 220–280°C, 5°C/min; injection temperature 250°C; detector temperature 300°C; helium carrier gas flow-rate 45 cm/sec; attenuation \times 128; chart-speed 2 cm/min.

Fig. 3. LC chromatograms obtained from the analysis of three urine samples (no internal standard added). 1 = Δ^9 -THC-11-oic acid. (a) 530 ng/ml, (b) 35 ng/ml, (c) negative. UV conditions: 225 nm, 0.04 a.u.f.s.

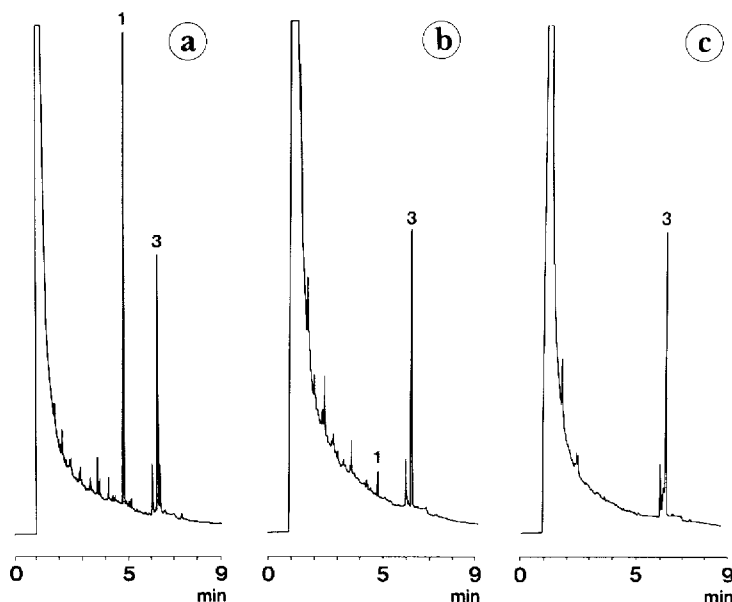


Fig. 4. GC chromatograms of derivatized LC fractions (see Experimental) from the samples in Fig. 3. 1 = Δ^9 -THC-11-oic acid, 3 = Mirex (internal standard). (a) 530 ng/ml, (b) 35 ng/ml (c) negative. Conditions: oven temperature 200–280°C, 10°C/min; injection temperature 250°C; detector temperature 300°C; helium carrier gas flow-rate 40 cm/sec; attenuation \times 256; chart-speed 1 cm/min.

Δ^9 -THC-11-oic acid was 90 ng/ml. Both the liquid chromatogram and the gas chromatogram are shown. The latter was obtained by collecting the eluate fraction (as marked in the chromatogram) containing both the THC acids and further treating it as described in the experimental section.

As an alternative to Δ^8 -THC-11-oic acid as internal standard, Mirex (useful only in the GC step) was used, and two examples from analysis of authentic urine samples and one blank urine are presented. The liquid chromatograms Fig. 3a–c and gas chromatograms 4a–c are shown in each case. The three examples represent concentrations of 530 ng/ml (a), 35 ng/ml (b) and a blank urine (c). Chromatographic data are given in the figure captions and in the experimental section.

Linearity, precision, recovery, and detection limit

The linearity for the overall method was determined by analysing blank urines to which known amounts of Δ^9 -THC-11-oic acid had been added. Peak heights in the gas chromatograms were measured and related to the peak height of the internal standard (Mirex). The resulting calibration plot was linear from 16 ng/ml up to 1020 ng/ml with a correlation coefficient of 0.998.

The precision for the method using Δ^8 -THC-11-oic acid as internal standard was determined by analysing a pool of authentic urine samples with a measured Δ^9 -THC-11-oic acid concentration of 90 ng/ml. The relative standard deviation (R.S.D) obtained was thus calculated as 8.0% ($n = 5$). The precision for the method by using Mirex as internal standard was similarly determined by analysing a pool of urine samples with a measured concentration of 235 ng/ml which resulted in an R.S.D of 10.3% ($n = 5$).

The recovery through the entire method was measured after analysis of blank urines to which known amounts of Δ^9 -THC-11-oic acid (68 ng) had been added. After adjusting for aliquots this result was compared with GC analysis of the same amount of Δ^9 -THC-11-oic acid subjected only to the derivatization and the GC step with Mirex as internal standard. The recovery was measured on two different occasions as 77% and 79%, respectively.

The detection limit for the overall method was approximately 20 ng/ml.

Correlation with the GC-MS method

The results obtained by the described method with Δ^8 -THC-11-oic acid or Mirex as internal standards were compared with the results obtained by the GC-MS method [7] for 27 authentic urine samples which were found positive by EMIT assay. The concentration of Δ^9 -THC-11-oic acid in these samples ranged from 8 to 850 ng/ml determined by GC-MS. By comparing the results for seventeen samples with Δ^8 -THC-11-oic acid as internal standard, a correlation coefficient of 0.990 was obtained. The corresponding value for ten samples with Mirex as internal standard was 0.969. When all 27 samples were included the correlation coefficient was 0.974 and the correlation plot is shown in Fig. 5. To visualize the correlation over the wide concentration range, logarithmic values were used in the plot shown Fig. 5.

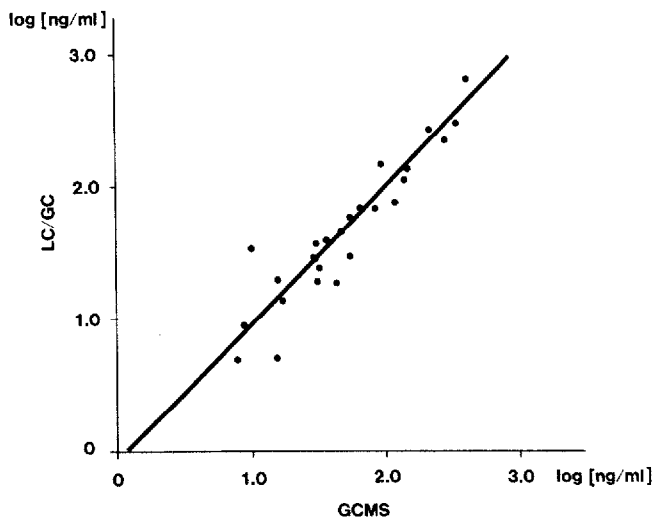


Fig. 5. Plot of the correlation (logarithmic values) between LC/GC and GC-MS for 27 samples.

DISCUSSION

The combination of LC and GC served two functions. First, the determination of the THC acid in high concentrations (> 100 ng/ml) could be made already in the LC step alone by monitoring the UV response of the liquid effluent which is shown in Figs. 2a and 3a. Second, a selective clean-up was accomplished for the GC analysis. Furthermore, a high resolution was achieved in the GC step and the high selective response to the derivative improved the detectability. An approach was made to use the GC technique alone but a

large number of interfering peaks resulted in unreliable results. Most of the urine samples which were found positive with the EMIT cannabinoid assay yielded an amount of THC acid which could be detected with the described method.

The UV detection of Δ^9 -THC-11-oic acid in the LC step was limited by the background from the urine sample and also by instrumental sensitivity. By monitoring the UV response, large variations in the urinary background were observed in different samples and, if the LC step was used alone, background peaks could interfere with the determination of Δ^9 -THC-11-oic acid. Although a comparatively sophisticated sample clean-up step was used before the GC analysis, the detectability was still limited by background peaks. The electron-capture detector showed high response to the derivative. The example shown in Fig. 4b corresponded to about 10 pg of derivative injected on the column.

In the raw material of cannabis, minute amounts of Δ^8 -THC relative to that of Δ^9 -THC may be found [12]. Therefore, one could expect small amounts of Δ^8 -THC-11-oic acid appearing as a metabolite in the urine. If so, this amount can be considered negligible compared to the amount of Δ^8 -THC-11-oic acid added as internal standard provided that the content of Δ^9 -THC-11-oic acid is not too high. We have, however, not been able to detect this metabolite in any of the samples analysed so far. The obtained results showed acceptable correlation with those from the GC-MS method. The use of Δ^8 -THC-11-oic acid as internal standard did not significantly improve the correlation of the quantitative results compared with those samples in which Mirex was used as internal standard. If the Δ^8 analogue is unavailable, Mirex can be used as internal standard, but it fails to compensate for losses during the extraction step or for incomplete derivatization. It is suitable as a GC internal standard to control the injected amount, also when the Δ^8 analogue is used as internal standard.

In conclusion, the described method offers a less expensive alternative to GC-MS and is useful for confirmation of positive findings made by EMIT assay. By combining LC and GC a high degree of selectivity was obtained.

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