Journal of Chromatography, 617 (1993) 265–270
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 6912

Gas chromatographic–mass spectrometric quantitation of urinary 11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid after derivatization by direct extractive alkylation

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(Received February 23rd, 1993)

ABSTRACT

A gas chromatographic–mass spectrometric procedure for the quantitation of urinary 11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid (THC-COOH) has been developed in which the THC-COOH was derivatized to its corresponding methyl ester–methyl ether derivative by direct extractive alkylation using tetrahexylammonium (THA^+) counter-ion and iodomethane dissolved in toluene. The procedure utilised a sample volume of 2 ml and gave a detection limit of 0.25 ng/ml. The inter-run and intra-run coefficients of variation were 7.0% and 4.8%, respectively. The inter-day standard curves were linear in the concentration range 0–300 ng/ml with a mean r = 0.9997 (n = 4).

INTRODUCTION

Δ^9-Tetrahydrocannabinol (THC) is the major psychoactive component of cannabis. In humans THC (Fig. 1A) is metabolised and excreted primarily as the glucuronic acid conjugate of 11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid (THC-COOH). Bronner and Xu [1] have reviewed the application of gas chromatography–mass spectrometry (GC–MS) for the analysis of THC-COOH (Fig. 1B) in biological matrices. Isolation of the analyte from urine and its derivatization has been carried out by a number of procedures. Parry *et al.* [2] isolated it by solid phase extraction before generating the trimethylsilyl derivative. Karlsson *et al.* [3] extracted it by liquid–liquid partitioning with a hexane–diethyl ether mixture before generating the pentafluoropropyl–pentafluoropropionyl derivative. Paul *et al.* [4] and Whiting and Manders [5] methylated the THC-COOH after isolating it by ion exchange and liquid–liquid partitioning, respectively. These and similar techniques that are commonly used require long preparation times involving separate hydrolysis, extraction and derivatization steps before the GC analysis of the sample.

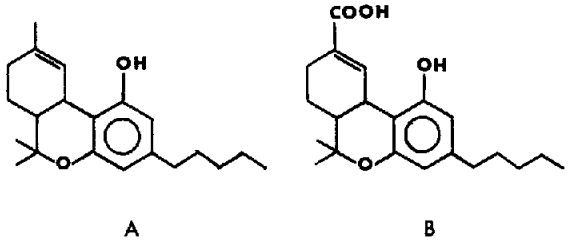

Fig. 1. Structure of (A) Δ^9-tetrahydrocannabinol and (B) 11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid.

This communication describes the development of a reliable, sensitive, precise and accurate extractive alkylation (EA) method for the GC–

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0378-4347/93/$06.00

MS determination of urinary THC-COOH in which the hydrolysis, extraction and derivatization of the analyte are performed in the same test tube, thus minimising analyte losses and sample preparation time. The optimized conditions for the hydrolysis and EA reaction are presented.

EXPERIMENTAL

Reagents and chemicals

Authentic L-11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid glucuronide (THC-COOH-glu) and 11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid (THC-COOH) were purchased from Alltech Associates (Sydney, Australia). Trideuterated THC-COOH (d_3-THC-COOH) was purchased from Radian (Austin, TX, USA). Freeze-dried human urine standard reference material (SRM) 1507 with a certified THC-COOH concentration of 20 ± 2 ng/ml when reconstituted was purchased from the National Institute of Standards and Technology (Gaithersburg, MD, USA). Iodomethane and nanograde toluene were purchased from Rhone-Poulenc (Melbourne, Australia), tetrahexylammonium hydrogen sulphate (THA^+) from Fluka (Buchs, Switzerland) and analytical grade 200–400 mesh SM-7 resin from Bio-Rad Labs. (Sydney, Australia).

Instrumentation

Urine densities were measured on a Paar DMA-48 density meter. Gas chromatographic–mass spectrometric measurements were performed on a Hewlett-Packard 5890 Series II gas chromatograph equipped with a Hewlett-Packard 5970B electron-impact mass selective detector via a capillary direct interface. The capillary column consisted of fused-silica coated with 95% methyl and 5% phenyl silicone to a thickness of 0.1 μm (HP ultra 2) and had dimensions of 12 m × 0.22 mm I.D. The carrier gas was helium at a flow-rate of 1 ml/min. The sample was introduced into the gas chromatograph in split mode with a split ratio of approximately 10:1. The injector and detector were maintained at a temperature of 290°C. The initial and final column temperatures were 138 and 300°C, respectively, and programmed to increase at 10°C/min. The mass selective detector was operated in the selected-ion mode (SIM) monitoring the fragment ions listed in Table I. The dwell time for each ion was 45 ms.

TABLE I

SELECTED IONS MONITORED FOR THE METHYL ESTER–METHYL ETHER DERIVATIVE OF THC-COOH AND d_3-THC-COOH

THC-$COOCH_3$ (*m/z*)	d_3-THC-$COOCH_3$ (*m/z*)	Fragment ion
372	375	M^+
357	360	$M^+ - CH_3$
313	316	$M^+ - COOCH_3$

Preparation of the SM-7 resin columns

The fines were removed from the commercially available 200–400 mesh SM-7 sorbent by suspending it in methanol and decanting the supernatant. This was repeated until the supernatant was clear. The suspended SM-7 sorbent was packed into 2.5–3.0 cm columns using disposable glass pipettes (6 mm I.D.) fitted with small plugs of silanised glass wool to act as bed supports. Before use the methanol was removed from each column by conditioning with 2 ml of toluene.

Hydrolysis

Aliquots of urine (2 ml) were added to 16 mm × 150 mm PTFE-lined screw-capped test tubes, fortified with 4 μl of 10 μg/ml d_3-THC-COOH (internal standard), made alkaline with 100 μl of 6 *M* NaOH and allowed to stand at 25°C for at least 15 min with occasional shaking.

Direct extractive alkylation

To each of the hydrolysed samples were added 25 μl of 0.2 *M* THA^+ (prepared by dissolving 4.5 g of the salt in 50 ml of 0.5 *M* NaOH) and 5 ml of 0.2 *M* iodomethane in toluene. The urine and toluene phases were mixed at 25°C for 30 min and then centrifuged at 1500 *g* for 5 min. The toluene phases were passed through the pre-prepared SM-7 resin columns, collected in disposable test tubes and evaporated to dryness under a stream

of nitrogen at 35°C. The residues were reconstituted in 100 μl of toluene before injection of 2-μl aliquots into the GC–MS system.

RESULTS AND DISCUSSION

Procedure

The urinary glucuronic acid conjugate of THC-COOH was first hydrolysed under alkaline conditions at 25°C for at least 15 min and the liberated THC-COOH was methylated after extraction into the toluene phase as ion pairs with the THA^+ counter ion. The disadvantage of applying EA directly to a urinary matrix is the co-extraction of the chloride and uric acid salts of the counter ion, resulting in rapid capillary column deterioration and high background signals if they are not removed before GC analysis. The efficient removal of these salts was achieved by passing the toluene through a short column of acrylic copolymer beads (SM-7 resin), which absorb the polar THA^+ salts and allow the less polar methyl ester–methyl ether derivative of THC-COOH to pass through the column unretained [6].

Hydrolysis

THC-COOH is excreted into the urine in the unconjugated form and as the ester conjugate of glucuronic acid. Hydrolysis of the glucuronic ester is therefore required in order to liberate the conjugated THC-COOH. The effect of temperature and the optimum time for the alkaline hydrolysis of THC-COOH-glu were determined by fortifying urine samples with authentic THC-COOH-glu to give an unconjugated THC-COOH concentration equivalent to 50 ng/ml. Each urine sample was also fortified to 50 ng/ml with unconjugated d_3-THC-COOH (internal standard) and made alkaline with 100 μl of 6 *M* NaOH before hydrolysis at 25 or 50°C. The liberated THC-COOH was extracted with two 5-ml aliquots of toluene after making the urine to pH 5–6 with acetate buffer. The combined volume of toluene was reduced to 5 ml before the addition of 25 μl of 0.2 *M* THA^+, 75 μl of iodomethane and mixing for 30 min at 25°C. The time course

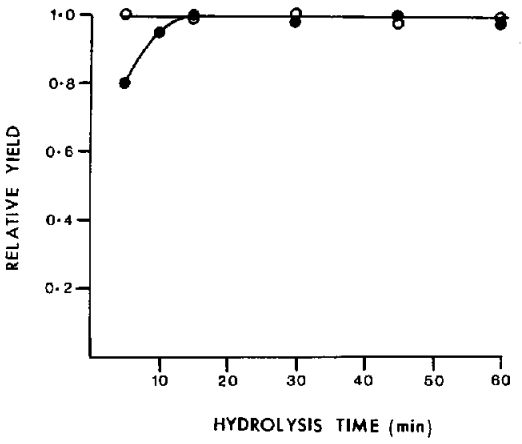

Fig. 2. Time-course for the alkaline hydrolysis of the glucuronic acid conjugate of THC-COOH in urine. Concentration of THC-COOH-glu: 75.8 ng/ml (equivalent to a concentration of 50 ng/ml unconjugated THC-COOH. Hydrolysis temperature: ● = 25°C; ○ = 50°C.

for the hydrolysis of THC-COOH-glu at 25 and 50°C, illustrated in Fig. 2, shows that the reaction is rapid with optimum yields of THC-COOH been achieved with a hydrolysis time of 15 min at 25°C and 5 min at 50°C.

Extractive alkylation reaction

Fig. 3 shows the SIM GC–MS profile obtained after performing alkaline hydrolysis and EA on a urine sample with a THC-COOH concentration of 28 ng/ml. The yield of methyl ester–methyl ether derivative of THC-COOH during EA was found to be dependent on the urinary concentration of THA^+, the concentration of iodomethane in the toluene phase and the mixing time of the two phases.

In a urinary matrix THC-COOH must compete with chloride ions and uric acid for the available THA^+ counter-ion. To determine the optimum urinary THA^+ concentration, a urine with a density of 1.030 g/ml was used as a model sample. The time course for the methylation of THC-COOH by EA at 25°C and with urinary THA^+ concentrations of 0.5, 1.0 and 2.5 m*M* is illustrated in Fig. 4. The optimum yields were achieved at a THA^+ concentration of 2.5 m*M* and a mixing time of 30 min. For THA^+ concentrations greater than 2.5 m*M* there was no im-

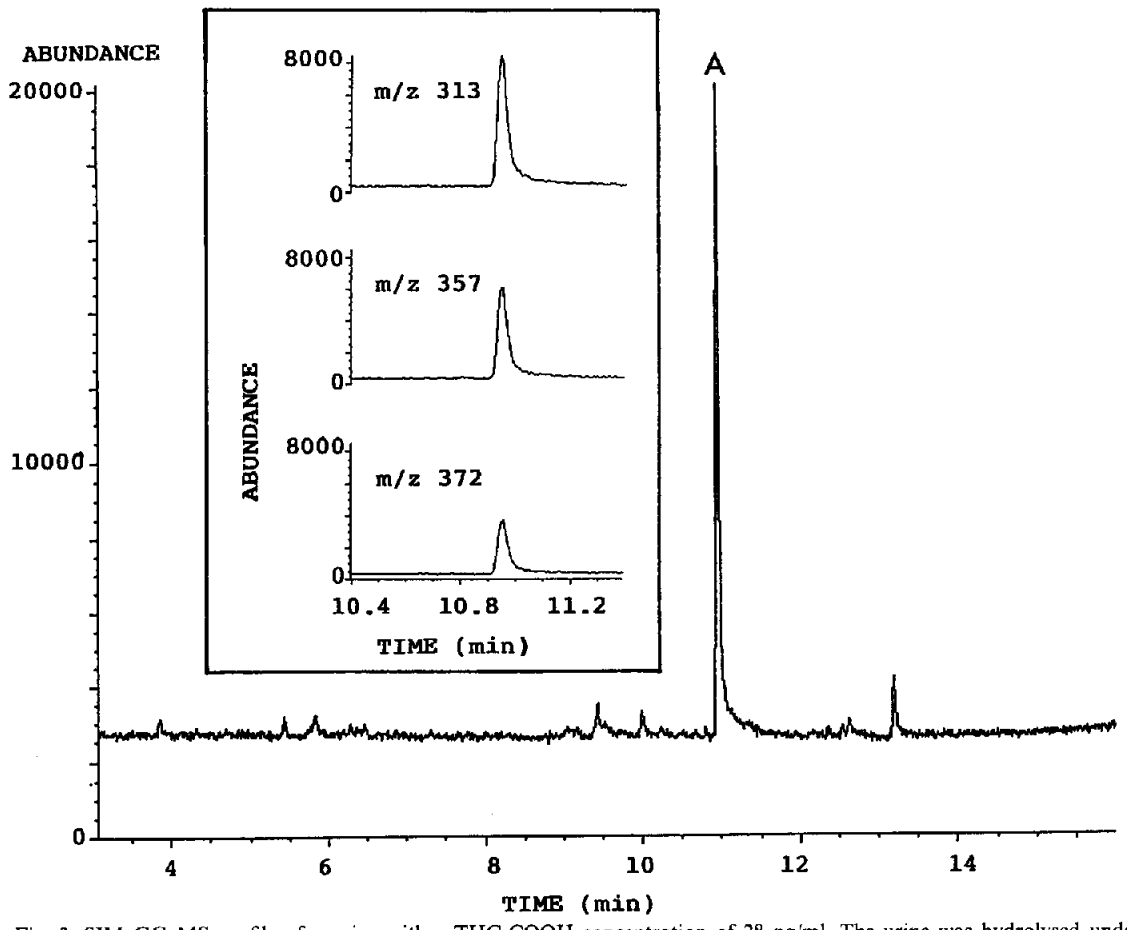

Fig. 3. SIM GC–MS profile of a urine with a THC-COOH concentration of 28 ng/ml. The urine was hydrolysed under alkaline conditions at 25°C before direct EA. The insert displays the *m/z* 313, 357 and 372 ion traces for the methyl ester–methyl ether derivative of THC-COOH. Peak A = methyl ester–methyl ether derivative of THC-COOH.

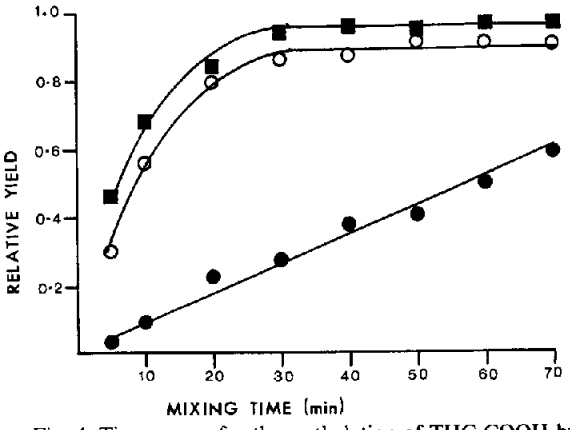

Fig. 4. Time-course for the methylation of THC-COOH by direct EA. Aqueous phase: 2 ml of urine with a density of 1.030 g/ml. Organic phase: 5 ml of 0.2 *M* iodomethane in toluene. Concentration of THC-COOH: 50 ng/ml. Urinary concentration of THA^+: ● = 0.5 m*M*; ○ = 1.0 m*M*; ■ = 2.5 m*M*. Temperature: 25°C.

provement in the yield of the methyl ester–methyl ether derivative of THC-COOH.

Fig. 5 illustrates the effect of iodomethane concentration in the toluene phase on the yield of the methyl ester–methyl ether derivative of THC-COOH. Optimum yields were obtained for iodomethane concentrations in the range 0.1–0.4 *M*. Extractive alkylation is a solvent-dependent reaction, and the decline in yield at iodomethane concentrations greater than 0.4 *M* may be due to changes induced in the properties of the toluene solvent by the increasing iodomethane content.

Linearity

Urinary concentrations of d_3-THC-COOH (internal standard) equivalent to 20 ng/ml and THC-COOH equivalent to 10, 20, 50, 100, 150,

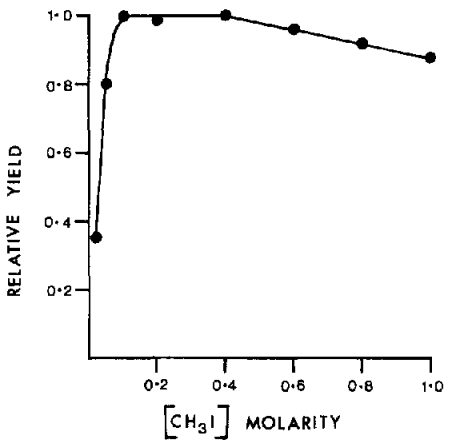

Fig. 5. Effect of iodomethane concentration in the toluene phase on the yield of methyl ester–methyl ether derivative of THC-COOH. Aqueous phase: 2 ml of urine. Concentration of THA^+: 2.5 m*M*. Concentration of THC-COOH: 50 ng/ml. Mixing time: 30 min. Temperature 25°C.

200, 250 and 300 ng/ml were taken to dryness to remove the methanol solvent before the addition of 2 ml of drug-free urine and derivatization by EA. The peak-area ratios of fragment ions *m/z* 357 for THC-COOH and *m/z* 360 for d_3-THC-COOH were subjected to least-squares regression analysis. The results in Table II indicate that the inter-day standard curves were reproducible with linear responses for peak-area ratios *versus* concentration.

Accuracy and precision

The method accuracy and intra-run precision were evaluated at a concentration of 20 ng/ml by analysing ten 2-ml aliquots of urine with a certified THC-COOH concentration of 20 ± 2 ng/ml (SRM 1507). The results shown in Table III indicate that the method is accurate, giving an intra-run coefficient of variation of 4.8%.

The inter-run precision was determined over a five-day period by analysing two 2-ml aliquots of urine from a known positive sample and injecting each aliquot into the GC–MS system in duplicate. The results in Table IV indicate that the method gives an inter-run precision with a coefficient of variation of 7.0% ($n = 5$).

TABLE II

INTER-RUN STANDARD CURVES OBTAINED BY LINEAR REGRESSION ANALYSIS FOR THE GC–MS QUANTITATION OF URINARY THC-COOH

Each standard curve was derived from eight points in the concentration range 0–300 ng/ml and the area ratio of ion 357 to ion 360.

	Slope (*m*)	*y*-Intercept (*b*)	Correlation coefficient (*r*)
	0.0537	−0.0266	0.9998
	0.0555	−0.0100	0.9999
	0.0513	+0.0852	0.9993
	0.0571	−0.0582	0.9996
Mean	0.0544	−0.0024	0.9997
S.D.	0.0025	0.0617	0.0003

Recovery and detection limit

The recovery of the method was determined by fortifying ten 2-ml aliquots of urine with THC-COOH-glu, to give an unconjugated THC-COOH concentration equivalent to 20 ng/ml, and analysing each aliquot as outlined in the Experimental section. The method has a recovery of

TABLE III

ACCURACY OF THE DETERMINATION OF THC-COOH IN HUMAN URINE STANDARD REFERENCE MATERIAL (SRM) 1507

Certified THC-COOH concentration: 20 ± 2 ng/ml.

Aliquot	[THC-COOH] (ng/ml)
1	21.88
2	21.15
3	20.72
4	22.28
5	19.49
6	22.11
7	20.63
8	21.17
9	19.77
10	19.72
Mean	20.89
S.D.	1.01
C.V.[a]	4.8%

[a] Coefficient of variation.

TABLE IV

INTER-RUN PRECISION FOR THE DETERMINATION OF URINARY THC-COOH

Sample volume, 2 ml; hydrolysis time, 20 min; urinary THA^+ concentration, 2.5 m*M*; organic phase, 0.2 *M* iodomethane in toluene; urine–toluene mixing time, 30 min.

Day	[THC-COOH] (ng/ml)	Mean [THC-COOH] (ng/ml)
1	31.20, 30.25, 30.48, 30.75	30.67
2	30.56, 31.34, 28.06, 29.04	29.75
3	26.84, 27.28, 30.01, 29.41	28.39
4	25.67, 25.80, 24.71, 25.24	25.36
5	29.28, 29.42, 27.88, 28.86	28.86
Mean		28.61
S.D.		2.01
C.V.[a]		7.0%

[a] Coefficient of variation.

97.5 ± 1.6% (n = 10). For maximum recovery of the analyte the hydrolysis step should be allowed to proceed to completion before commencing the EA procedure. When simultaneous hydrolysis and EA were attempted at 25°C the recovery was only 82%.

The detection limit, defined as the concentration of analyte that gave a signal-to-noise ratio of 3, was 0.25 ng/ml. The instrument noise level was measured from peak to peak. The limit of reporting in our laboratory is 20 ng/ml.

Interference

Traces of close-eluting substances with similar mass spectral properties to the methyl ester–methyl ether derivatives of THC-COOH and the trideuterated internal standard can interfere with the analysis [7–9]. With column ageing and reduced column efficiency some samples showed an interference with the m/z 316 fragment ion of the internal standard. The interference was the result of incomplete resolution of traces of an endogenous urinary compound. For this reason the more intense m/z 313 and 316 ions of THC-COOH and the trideuterated internal standard were not used for quantitation. On the occasions that this interference was encountered a slower temperature ramp of 5°C/min was able to separate it from the methyl ester–methyl ether derivatives of THC-COOH and d_3-THC-COOH. An alternative approach to overcoming the interference is to use the ethyl [10] or propyl [11] derivatives of THC-COOH for quantitation. Best yields for ethylation and propylation by direct EA require larger reaction times than methylation.

CONCLUSIONS

The GC–MS procedure outlined in this communication for the quantitation of urinary THC-COOH as its methyl ester–methyl ether derivative is reliable, accurate and reproducible, giving good linearity and detection limits. The advantage of the procedure is that the hydrolysis, extraction and derivatization of the analyte are carried out in the same test tube, resulting in reduced loss of analyte because of fewer transfer steps and reduced sample preparation time.

ACKNOWLEDGEMENTS

We thank Professor Graham Johnston, Department of Pharmacology, Sydney University, for his valuable discussion of the manuscript and the Australian Sports Drug Agency for their continuing financial support of the AGAL sports drug testing division.

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