Gas Chromatography–Mass Spectrometry–Single Ion Monitoring Measurement of 11-Nor-Δ^9-Tetrahydrocannabinol-Carboxylic Acid in Urine

O. Boldis, Gy. Kocsis, A. Gachályi, and **J. Fűrész**
Health Protection Institute of the Hungarian Defense Forces, H-1555, Budapest, P.O. Box 68, Hungary

Abstract

Gas chromatography combined with mass spectrometry is used to determine the urinary elimination of 11-nor-Δ^9-tetrahydrocannabinol-carboxylic acid. Single ion monitoring of both 11-nor-Δ^9-tetrahydrocannabinol-carboxylic acid and the internal standard (11-nor-Δ^9-tetrahydrocannabinol-carboxylic-D9) offers selective and sensitive measurement. In order to check the method and the results, we employ 11-nor-Δ^9-tetrahydrocannabinol-carboxylic acid-glucuronide as an external standard. Our study also demonstrates that the wall of the glass liner (glass tube) in the injector retards the active compound, which makes it imperative to replace the glass insert after each run. Otherwise, the value of the drug measured in subsequent runs will decrease because more of this compound will adhere to the glass walls.

Introduction

Currently, one of the drugs of abuse is marijuana, which contains 1–3% Δ^9-tetrahydrocannabinol (THC). The illicit use of marijuana can be proven by the determination of the urinary elimination of 11-nor-Δ^9-tetrahydrocannabinol-carboxylic acid (11-nor-Δ^9-THC-COOH). The standard method is the hydrolysis of 11-nor-Δ^9-THC-COOH with a base, followed by a wash-up with solid-phase extraction (SPE), and then gas chromatography–mass spectrometry (GC–MS) analysis of the material in the standard mode (1). The results we obtained were checked by running an external standard, which was a certified reference material of 11-nor-Δ^9-THC-COOH that we obtained from the LGC Promochem (Wesel, Germany). The tests were carried out on this known standard using both GC–MS and the internationally recognized AxSym system (polarization fluorescent immunoassay) (Abbot Diagnostics, Abbott Park, IL) in order to correlate these two methods. We then repeated this battery of tests, but this time adding known concentrations of the THC metabolite to contaminant-free urine. As an internal standard, we employed 11-nor-Δ^9-THC-COOH-D9 (11-nor-Δ^9-THC-COOH ninefold substituted hydrogen with deuterium) obtained from the LGC Promochem.

The increasing danger of drug abuse in the Hungarian Defense Forces since 1990 has made it necessary to look for a fast, selective, and reliable test allowing the detection of the active substance Δ^9-THC. The measuring of blood level can prove that a person is under the effect of the drug.

In this paper, our aim is to prove that the results of a urine test have as much validity as those of the blood test. The advantage of a urine test is that the collection of a urine specimen is not an invasive procedure: the sample is easier to collect, there is usually an adequate supply available, the presence of skilled personnel is not required, and not as many legal questions are raised. Another significant feature of the urine test is that it can detect whether the drug was used days before because the drug concentration is higher in the urine than in the blood.

Experimental

Equipment

An Agilent 6890 gas chromatograph with an Agilent 5973 mass spectrometer was used (Agilent, Wilmington, DE).

The analyzer operated with an installed automatic sampler. The results were analyzed by an HP Chemstation A3.01 (Hewlett-Packard, Palo Alto, CA), which used a program called B.E.N. (a program designed to run on the Windows Excel program that determinates the analytical parameters of the GC–MS measurement) (4). Before each run, a new glass liner was inserted into the injector. The inside surface of this tube was coated with Sylon CT from Supelco (Bellefonte, PA)

(5% dimethyldichlorosilane in toluene) in order to minimize adsorption. The supplier called these "silanized inserts". The analytical parameters during the GC–MS analysis are shown in Tables I and II.

Analytical conditions

For the gas chromatograph, the conditions were as follows: carrier gas, helium (> 99,99995%); flow rate, 1 mL/min; injection volume, 1 µL (splitless injection); purge on, 1.0 min; injector temperature, 250°C; column, HP-5MS (30-m × 0.25-mm i.d., 0.25-µm film thickness); transfer line temperature, 280°C; and oven program, 1 min isothermal at 60°C, then programmed at 10°C/min to 280°C, and then programmed at 20°C/min to 300°C with a run time of 29 min.

For the mass spectrometer, the conditions were as follows: source temperature, 200°C; quad temperature, 150°C; measuring mode, single-ion monitoring (SIM); solvent delay, 5 min; and detector off, 23 min.

Reagents

The solvents and reagents used were of the highest quality available. The active drug metabolites (100 µg/mL) 11-nor-Δ^9-THC-COOH and 11-nor-Δ^9-THC-COOH-D9 were obtained from the Cerilliant Company (Radian International, Austin, TX). The reagent *N*,*O*-bis-trimethylsilyl-trifluoroacetamide (BSTFA) and a trimethylchlorosilane-based reagent were obtained from the Supelco (supplied by Sigma-Aldrich Hungary Kft., Budapest, Hungary). The 130-mg SPE column was obtained from Varian B.V. (Middelburg, the Netherlands.) This was a mixed-mode solvent bed containing octyl (C8)–benzenesulfonic acid (SCX) certified as "Bond Elut Certify". For external control, 11-nor-Δ^9-THC-COOH in the glucuronide form, which was supplied by Bio-Rad Laboratories (Hercules, CA), was added. Its certified concentration was 150 µg/mL. This drug was added in various concentrations to the control urine.

Hydrolysis procedure

The urine specimen was hydrolyzed by a method closely correlated to the method proposed by Baker et al (5). Portions of the sample of 1.5 mL were hydrolyzed in a 4-mL silanized air-tight vial. A 4-µL solution containing 10 µg/mL of 11-nor-Δ^9-THC-COOH-D9 were added both to the control solution and to the sample so that each urine specimen would have a concentration of 40 ng/mL. The hydrolysis was carried out by placing 1.5 mL of the sample in a sample dish. An amount of 100 µL of 10N KOH was added and the temperature was held at 60°C for 10 min. After 10 min, the mixture was cooled to room temperature and 1500 µL of 50 mmol phosphoric acid was added along with 135 µL glacial acetic acid. The solution was mixed with a Super-Mixer (LAB-LINE Instruments, Melrose Park, IL).

Extraction procedure

The extraction was carried out according to the recommendations of Varian Company (6). The pH of the solution was adjusted to 4.2 by the addition of glacial acetic acid (~ 135 µL) and then pipetting 1500 µL of 50 mmol H_3PO_4 into the solution.

The extraction columns were filled with 6-mL reservoirs, and they were prewashed first with a 3-mL solution of methanol and then 3 mL of 50-mmol H_3PO_4. Next, the test solutions were injected into the column. The vacuum in the column was adjusted to between 2- and 4-mm Hg in order to have the solution drip through the column at a rate of 1 mL/min. After the entire sample had passed through the column, the vacuum was increased to approximately 10 mm Hg, and the column was washed in one of three different ways. In the first method, 5 mL of 50-mmol phosphoric acid was run through, followed by a 2-mL mixture of methanol–50 mmol phosphoric acid (20:80). In the second method, 5 mL of 50-mmol phosphoric acid was run through, followed by a 2-mL mixture of methanol–50 mmol phosphoric acid (10:9). In the third method, only 5 mL of 50-mmol phosphoric acid was run through. Following this step, the columns were dried by applying a vacuum of 20-mm Hg for 5 min, which allowed air to flow freely through the columns. The last drying step consisted of injecting 150 µL *n*-hexane into the column and the column was allowed to dry for an additional 10 min. In the last step, the THC-metabolites were eluted from the columns by adding 1.5 mL of a mixture of *n*-hexane–ethyl acetate (80:20), and this time we allowed the solution to drip slowly through the column, driven by gravity. It ended up in 2-mL vials with walls previously treated to minimize the adsorption of the compound. The

Table I. SIM Parameters

Group	Name	Dwell time	Resolution	Start time	Fragment mass (amu)		
1	11-nor-Δ^9-THC-COOH	30 ms*	Low	15.00 min	371	398	473 488
2	11-nor-Δ^9-THC-COOH-D9	30 ms	Low	15.00 min	380	407	482 497

* ms, millisecond.

Table II. Compounds Information for the Database

Compound	t_R (min)	Tgt	Q1 (%-Resp.)	Q2 (%-Resp.)	Q3 (%-Resp.)
11-nor-Δ^9-THC-COOH	20.61	371	398 (35)	473 (25)	488 (5)
11-nor-Δ^9-THC-COOH-D9	20.58	380	407 (38)	482 (27)	497 (6)

* Abbreviations: t_R, retention time; Tgt, target ion; Q1, qualifier ion 1; Q2, qualifier ion 2; and Q3, qualifier ion 3.

resulting solution was desiccated under a stream of dry nitrogen at 40°C. The extraction procedure is detailed in Table III.

Derivatization procedure

To the desiccated dried residue, 100 µL BSTFA was added. The vials were capped and heated to a temperature of 70ºC for 10 min. After the vials had cooled to room temperature, the contents were transferred to 100-µL microvolume glass containers, and placed into the 12- × 32-mm 2-mL autosampler vials. The analysis was carried out within 24 h. If there was a cause for delay, the specimen was stored at a temperature between 0°C and 5°C.

Results and Discussion

Table IV shows the results of our analysis carried out on samples containing five different concentrations of drug in urine. The statistical analysis of each determination was performed according to the German Industrial Standards (DIN) 32645 rules (7) and calculated with the B.E.N. (4) software. The results demonstrated a relative standard deviation (RSD) between 5.6% and 10%. The samples containing the psychoactive metabolite (11-nor-Δ^9-THC-COOH) showed a variation much like the reference samples, and there were no observable differences between the peaks of the samples and those of the controls. The correlation coefficient showed a linear response between 15 and 150 ng/mL.

The B.E.N. program showed that in our samples the smallest peak's confidence limit had an uncertainty of 33% (8) at the lowest concentration we analyzed. The RSD diminished with increasing concentration: 15 ng/mL the RSD was 2.30%, which diminished to 0.26% when we reached a concentration of 150 ng/mL. All of the exact measurements are displayed in Table V.

Recovered percentages of the analyzed material were determined in each of the three different types of extraction. The difference between the measured and the mean values were calculated for each concentration (9). There was a significant difference at the lower concentrations (namely at the concentrations between 15 and 25 ng/mL), and the difference tended to become smaller at the higher concentrations. The significance of the difference was calculated using the Student's *t*-test to a confidence limit of the following equation (10):

$$t_{(99\%,n-1)} = \frac{(X - X_{mean}) \div \sqrt{n}}{RSD} \qquad \text{Eq. 1}$$

where X is the reference value, X_{mean} is the mean value, and n is the number of measurements.

Table III. Extraction Procedure

	Operational steps	Substance	Volume	Speed of aspiration*	End point
1	Pretreatment 1	methanol	3 mL	10	not analyzed
2	Pretreatment 2	50mM phosphoric acid	3 mL	10	not analyzed
3	Sample injection	sample, pH ≈ 3.5	entire hydrolized mixture	1	not analyzed
4	One of the three types of wash (see below)				not analyzed
5	Drying cycle I	air flow		5 min	not analyzed
6	Drying cycle II	*n*-hexane	150 µL	10	not analyzed
7	Agent elution	*n*-hexane–ethyl acetate, 80:20 (v/v)	1.5 mL	1	collection of analysis

	Operational steps	Substance	Volume	Speed of aspiration*
Type 1	wash 1	50 mmol phosphoric acid	5 mL	10
	wash 2	methanol–50 mmol phosphoric acid (20:80)	2 mL	10
Type 2	wash 1	50 mmol phosphoric acid	5 mL	10
	wash 2	methanol–50 mmol phosphoric acid (10:90)	2 mL	10
Type 3	wash 1	50 mmol phosphoric acid	5 mL	10

* mL/min.

Table VI demonstrates recovered percentages of the analysis. The assay was linear for the THC* metabolite in urine from 15 to 150 ng/mL.

Conclusion

Urine drug testing has advantages over other types of tests. One can usually obtain a urine specimen without a problem, there is usually an adequate supply available, and finally, the drug can be successfully detected in the urine even several days after exposure, but the blood levels of the same compound rapidly diminish after a few hours.

Today's analysis requires a selective, exact, and specific measurement of samples. The THC measurement described in this paper fulfills requirements with the additional advantage that it minimizes analytical error. This is especially important in legal cases. The procedure is not complicated, and it can be carried out easily. Therefore, it is recommended for use in laboratories in which routine tests have to be carried out. It is important that the test is in accordance with DIN 32645 rules. The B.E.N. program has to be used in order to carry out the analysis of the analytical results.

The essential part of the laboratory test is the analytical method used, which was applied to both the sample and the control (11).

The chemical analysis consisted of four major parts: (*a*) preparation of the sample and the reference standard, (*b*) chemical reaction, (*c*) analytical measurement, and (*d*) interpretation of the results.

The procedure did not require the sample and the standard to be run separately. The calibration material was run through the process along with the samples. Finally, in case of any error during measurement, the results of both the sample and the standard were changed in the same degree, thereby minimizing any error in the results (13).

Table IV. The Characteristic of the Measured Values

Standard*	Mean relative response†	Standard Deviation	RSD
15	0.348	0.035	10.07
25	0.531	0.042	9.98
50	1.073	0.076	7.11
75	1.585	0.090	5.66
150	3.156	0.233	7.38
LOD	2.448	0.516	21.09
LOI	4.894	1.030	21.05
LOQ	9.481	1.974	20.83

* ng/mL.
† Average of $n = 7$. Relative response, unlabeled signal/labeled signal.
‡ Abbreviations: LOD, limit of detection; LOI, limit of identification; and LOQ, limit of quantitation.

Table V. Accuracy Measurement

Standard matrix: urine*	Mean ($n = 7$)	Standard Deviation*	RSD
15	15.43	0.36	2.31
25	24.30	0.28	1.17
50	50.15	0.42	0.84
75	74.54	0.53	0.72
150	150.06	0.39	0.26

* ng/mL.

Table VI. Recovery

	11-nor-Δ^9-THC-COOH			11-nor-Δ^9-THC-COOH-D9		
SPE extraction	%Recovered mean†	%Standard deviation	CV‡	% Recovered mean†	%Standard deviation	CV
1st type	54.93	3.03	5.52	53.84	2.97	2.52
2nd type	63.68	1.12	1.77	64.34	0.83	0.29
3rd type	69.10	0.97	1.41	68.86	1.13	1.64

* Concentrations of compounds were 40 ng/mL in urine matrix.
† $n = 7$.
‡ CV, coefficient of variation.

References

1. P.M. Kemp, I.K. Abukhalaf, J.E. Manno, B.R. Manno, D.D. Alford, and G.A. Abusada. Cannabinoids in humans. I. Analysis of Δ^9-tetrahydrocannabinol and six metabolites in plasma and urine using GC–MS. *J. Anal. Toxicol.* **19:** 285–90 (1995).
2. P.M. Kemp, I.K. Abukhalaf, J.E. Manno, B.R. Manno, D.D. Alford, M.E. McWilliams, F.E. Nixon, M.J. Fitzgerald, R.R. Reeves, and M.J. Wood. Cannabinoids in humans. II. The influence of three methods of hydrolysis on the concentration of THC and two metabolites urine. *J. Anal. Toxicol.* **19:** 292–98 (1995).
3. M.A. Elsohly, T.L. Little, Jr., and D.F. Stanford. Hexadeutero-11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid: A superior, internal standard for the GC/MS analysis of Δ^9-THC acid metabolite in biological specimens. *J. Anal. Toxicol.* **16:** 188–91 (1992).
4. Michael Herbold and Georg Schmitt. *B.E.N. program.* Institut für Rechts und Verkehrsmedizin, Heidelberg, Germany, 1999.
5. T.S. Baker, J.V. Harry, J.W. Russell, and R.L. Myers. Rapid method for the GC/MS confirmation of 11-nor-9-carboxyl-Δ^9-tetrahydrocannibinol in urine. *J. Anal. Toxicol.* **8:** 255–59 (1984).
6. *Bond Elute Certify Instruction Manual.* Varian, Harbor City, CA,1992.
7. *DIN 32645: Nachweis-, Erfassung- und Bestimmungsgrenze.* Beuth Verlag, Berlin, Germany, 1994.
8. M. Herbold and G. Schmitt. Qualitätsansprüche an die Quantitative MS-Untersuchung. Toxichem. + *Krimtech.* **65:** 87–96 (1998).
9. J.E. Manno, B.R. Manno, P. Kemp, D.D. Alford, I.K. Abukhalaf, M.E. McWilliams, F.N. Hagaman, and M.J. Fitzgerald. Temporal indication of marijuana use can be estimated from plasma and urine con-

centrations of Δ^9-tetrahydrocannabinol, 11-hydroxy- Δ^9-tetrahydrocannabinol, and 11-nor- Δ^9-tetrahydrocannabinol-9-carboxylic acid. *J. Anal. Toxicol.* **25:** 538–49 (2001).
10. P.R. Stout, C.K. Horn, and K.L. Klette. Solid-phase extraction and GC–MS analysis of THC-COOH method optimized for a high-throughput forensic drug-testing laboratory. *J. Anal. Toxicol.* **25:** 550–54 (2001).
11. S. Feng, M.A. ElSohly, S. Salamone, and M.Y. Salem. Simultaneous analysis of D9-THC and its major metabolites in urine, plasma, and meconium by GC–MS using an immunoaffinity extraction procedure. *J. Anal. Toxicol.* **24:** 395–402 (2000).
12. D.K. Crockett, G. Nelson, P. Dimson, and F.M. Urry. Solid-phase extraction of 11-nor-Δ^9-tetrahydrocanabbinol-9-carboxilic acid from urine drug-testing specimens with the Cerex PolyCrom-THC column. *J. Anal. Toxicol.* **24:** 245–49 (2000).
13. J. Singh and L. Johnson. Solid-phase extraction of THC metabolite from urine using the Empore Disk cartridge prior to analysis by GC–MS. *J. Anal. Toxicol.* **21:** 384–87 (1997).
14. M.A. Huestis, J.M. Mitchell, and E.J. Cone. Urinary excretion profiles of 11-nor-9-carboxy-Δ^9-tetrahydrocannabinol in humans after single smoked doses of marijuana. *J. Anal. Toxicol.* **20:** 441–52 (1996).

Manuscript accepted March 12, 2003.