Identification of 11-Nor-Δ^9-Tetrahydrocannabinol-9-Carboxylic Acid in Urine by Ion Trap GC–MS–MS in the Context of Doping Analysis

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Abstract

The purpose of this study is to develop a sensitive and specific alternative to current gas chromatography (GC)–mass spectrometry (MS) selected ion monitoring confirmation methods of 11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid (cTHC) in human urine samples, in the context of doping analysis. An identification procedure based on the comparison, among suspicious and control samples, of the relative abundances of cTHC selected product ions obtained by GC–tandem MS in an ion trap is presented. The method complies with the identification criteria for qualitative assays established by sports authorities; the comparison procedure is precise, reproducible, specific, and sensitive, thus indicating that it is fit for the purpose of identification accordingly to World Antidoping Agency requirements.

Introduction

The International Olympic Comitee (IOC) and the World Anti-Doping Agency (WADA) include cannabinoids in the class of substances prohibited in sports under certain circumstances, stating that a concentration in urine of 11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid (cTHC) higher than 15 ng/mL is considered to be doping (1,2). cTHC is the main metabolite excreted in urine of Δ^9-tetrahydrocannabinol (3), existing primarily as the ester-linked β-glucuronide (4). Current methods of detection and confirmation of cTHC involve enzymatic hydrolysis of the metabolite glucuronide, extraction with organic solvent, derivatization, and gas chromatography (GC)–mass spectrometry (MS) analysis of the derivatives in single ion monitoring (SIM) mode (5,6). For confirmation purposes, the relative abundance of the monitored ions is compared among the suspicious urine sample and a positive control urine. The absolute and relative difference in relative abundance of all the selected ions should be within established limits to confirm the presence of the metabolite (5,7).

In the past decade, GC–tandem MS (MS–MS) using a low resolution quadrupole ion trap was presented as a valuable tool to determining trace levels of compounds in complex matrices (8). Since then, the storage of selected precursor ions in an ion trap followed by their fragmentation by collision induced dissociation (CID) has become a powerful technique for both the determination of ion structures and the analytical determination of compounds of forensic and environmental interest (9). In the field of forensic science, a GC–MS–MS method for the detection of cTHC in biological samples has been published that employs basic hydrolysis of the sample and ion *m/z* 371 as the precursor ion (10). However, in this study only spiked samples were employed, no comparison among suspicious and control samples was made, and no explanation of the origin of the products ions was proposed.

Presented here is an alternative procedure for the identification of cTHC in urine based on the comparison, among suspicious and control samples, of the relative abundances of cTHC selected product ions obtained by GC–MS–MS in an ion trap, obtained from the molecular ion *m/z* 488 as the precursor ion. This procedure is more convenient in the context of doping analysis than the cited method (10) because sample preparation does not involve an additional protocol to current doping control screening procedures because it is analogous to that employed for the screening of anabolic steroids.

Experimental

Equipment

GC–MS and GC–MS–MS chromatograms and spectra were obtained with a Varian CP-3800 GC coupled to a Varian Saturn 2000 ion trap MS (Varian, Walnut Creek, CA), equiped with a Varian CP-8400 autosampler, and a CP-Sil 5 CB Lowbleed/MS (30 m × 0.25 mm × 0.1 µm) Chrompack capillary column (Varian). The injector temperature was set at 280°C, and 1 µL of sample was injected in splitless mode. The oven temperature program started at 130°C and increased to 270°C in 14 min

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(10°C/min), then increased to 300°C in 0.6 min (50°C/min) and held for 2 min at 300°C. The constant flow of carrier gas (He) was 0.8 mL/min, the transfer line temperature was 270°C, and the trap temperature was 170°C.

The ion trap was operated under the following conditions: electron ionization mode (70 eV, emision current 90µA); electromultiplier set by autotuning at 1500 V; maximum number of 5000 ions in the trap; for cTHC the precursor ion was *m/z* 488 isolated with a 3 *m/z* window and dissociated under nonresonant excitation conditions at 80 V for 20 ms; excitation storage level at *m/z* 150; product ions acquired in the *m/z* 200–500 range at 0.75 s/scan (4 microscans); for methyltestosterone the precursor ion was *m/z* 356 isolated with a 3 *m/z* window and dissociated under nonresonant excitation conditions at 85 V for 20 ms; excitation storage level at *m/z* 120; and product ions acquired in the *m/z* 180–380 range at 0.75 s/scan (4 microscans).

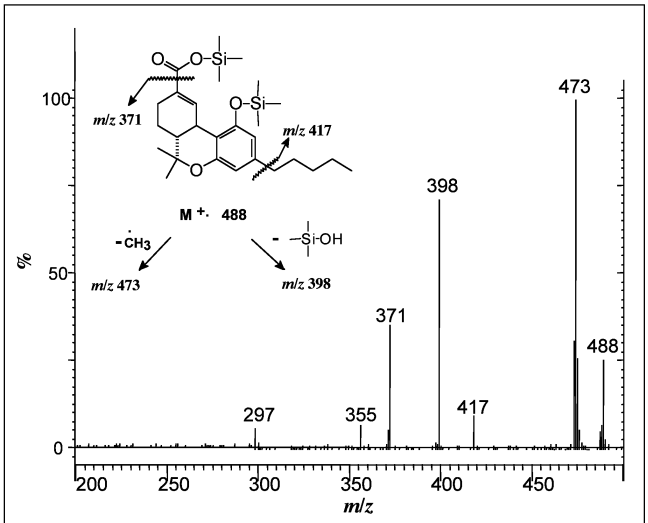

Figure 1. cTHC–TMS product ion MS–MS spectrum of molecular ion *m/z* 488 and fragmentation pathways of diagnostic ions (insert).

Evaporation under a nitrogen stream was performed on a TurboVap LV evaporator (Zymark, Hopkinton, MA). Enzymatic hydrolysis and trimethylsilyl derivatization were carried out on a Type 16500 Dri-Bath heat block (Thermolyne, Dubuque, IA).

Materials

cTHC (Sigma, St. Louis, MO) and 17α-methyltestosterone were used as pure standards. β-glucuronidase (Roche Diagnostics, Manheim, Germany) was from *E. coli* K12. *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA) (Avocado, Research Chemicals Ltd., Heysham, U.K.) was 98% pure. Methanol and *t*-butylmethyl ether were of high-performance liquid chromatography (HPLC) grade. Other chemicals were of analytical or HPLC grade.

Standard stock solutions

cTHC and methyltestosterone stock solutions were prepared in methanol at 200 µg/mL and 1 mg/mL, respectively, and stored at –18°C. Working solutions were freshly obtained by dilutions with methanol using volumetric materials.

Samples

cTHC true positive samples containing 11, 26, 49, 75, and 91 ng/mL were selected. In these samples the presence of cTHC had been previously confirmed and quantitated by an independent method similar to that of Mareck-Engelke et al. (5). Spiked urine samples at levels of 5, 10, 30, 50, 75, and 100 ng/mL were prepared by addition of 10, 20, 60, 100, 150, and 200 µL, respectively, of a 1 µg/mL cTHC working solution to 2-mL aliquots of negative control samples.

Sample preparation

Spiked urine samples and positive samples were processed following a protocol analogous to that employed for the screening of anabolic steroids (11). Urine samples (2 mL) were treated with 100 µL of methyltestosterone (1 µg/mL), 1 mL of phosphate buffer (1M, pH 7.0), and 50 µL of *E. coli* β-glucuronidase, and they were heated to 55°C for 1 h. The hydrolytic reaction was stopped by addition of 0.5 mL of 20% K_2CO_3–$KHCO_3$ (1:1) solution, and extracted with *t*-buthylmethyl ether (5 mL). The mixture was mechanically shaked for 5 min and then centrifuged at 2000 rpm for 10 min. The organic layer was collected, evaporated to dry-

Table I. Average Relative Abundance and Average Difference* in Relative Abundance of cTHC–TMS MS–MS Spectra Diagnostic Ions†

Concentration level (ng/mL)	Sample and difference type	Average relative abundance (% of base peak) and average difference (%)		
		m/z 417	*m/z* 398	*m/z* 371
10	Positive sample	12.3 (2.2)	78.0 (5.7)	41.1 (7.0)
	Spiked control	11.7 (2.3)	77.4 (8.2)	35.9 (6.3)
	Absolute difference	2.5 (1.4)	4.4 (3.1)	5.6 (2.2)
	Relative difference			16.0 (6.4)
30	Positive sample	11.1 (1.3)	75.0 (5.5)	39.3 (4.1)
	Spiked control	11.1 (2.0)	75.4 (4.9)	38.0(4.7)
	Absolute difference	2.1 (1.3)	3.0 (1.7)	2.4 (1.5)
	Relative difference			6.6 (4.9)
50	Positive sample	12.3 (1.6)	81.0 (2.9)	37.4 (4.2)
	Spiked control	11.4 (1.1)	76.9 (2.4)	39.0 (4.8)
	Absolute difference	1.9 (1.4)	3.2 (3.3)	2.9 (1.2)
	Relative difference			7.6 (3.4)
75	Positive sample	11.5 (1.2)	75.6 (5.6)	38.3 (3.9)
	Spiked control	11.4 (1.5)	74.1 (4.6)	40.1 (4.4)
	Absolute difference	1.2 (1.3)	2.5 (1.3)	2.0 (2.1)
	Relative difference			5.0 (5.2)
100	Positive sample	12.2 (1.2)	77.4 (4.1)	37.1 (2.9)
	Spiked control	11.3 (1.0)	75.6 (2.6)	40.1 (2.0)
	Absolute difference	1.3 (1.1)	3.3 (2.1)	3.9 (1.9)
	Relative difference			9.6 (4.3)

* Absolute values.
† *N* = 9. Standard deviation shown in parentheses.

ness under a nitrogen stream, and stored at 30 min in a desiccator under vacuum. The residue was then treated with 100 µL of MSTFA–NH_4I–ethanetiol (1000:6:18) and heated to 70ºC for 30 min to obtain the sample to be injected into the GC–MS–MS system.

Results and Discussion

Method development

cTHC bis-trimethylsilyl (TMS) derivative ion trap full scan MS spectra are similar to conventional quadrupole spectra (5): three main ions are, in order of increasing relative abundance, *m/z* 488 (molecular ion), 473, and 371 (base peak). These three were selected to perform the CID experiments. The molecular ion was finally chosen as the precursor ion for further method development because it provided the highest yield of diagnostic ions. Chromatographic and CID parameters were adjusted in order to optimize the quality and reproducibility of the product ion spectra. Some improvements were achieved since our initial communication (12). First, aiming to reduce the analytical run time (25 min), we changed GC columns from 0.25- to 0.10-µm film thickness and optimized the chromatography temperature program. The analytical run time was thus shortened by 8 min, and the separation efficiency was sligthly diminished [relative retention time (RRT) 0.9741 vs. 0.9889]. Carrier gas constant flow rate was varied in the range 0.2–2.0 mL/min, not only to improve separation efficiency but also to find the optimum helium pressure in the ion trap for CID. At low flow rates, precursor ion dissociation was poor. At high flow rates, product ion spectra were not reproducible. The best results were obtained with 0.8 mL/min. The second major change was the choice of nonresonant excitation CID instead of our original resonant CID method. This was done in order to avoid the observed changes in product ion relative abundances with cTHC concentration (12). Most of the MS–MS experiments described in the literature use resonant excitation mode. It is suggested that the nonresonant mode is suitable for single-bond cleavage, and that it suffers the loss of ions by ejection when slow fragmentation reactions (rearrangements) are monitored (13). This could explain the loss in signal intensity we observed when changing the excitation method. However, the relative abundance of ion *m/z* 398, whose formation occurs via the breakage of multiple chemical bonds (Figure 1), was highly reproducible throughout this study, as discussed later. Improved product ion yields under nonresonant CID were achieved setting the excitation radio frequency storage level (in *m/z*) at 150 (assayed range 48–210). Excitation amplitude was then optimized using the automated method development capability of the software with few injections, and the excitation time, assayed in the range 5–40 ms, was set at 20 ms.

Aliquots of spiked urine samples and true positive samples in the levels 10, 30, 50, 75, and 100 ng/mL were studied under the established optimized conditions. Figure 1 presents a typical product ions MS–MS spectrum of cTHC–TMS molecular ion *m/z* 488, in which the probable origin of diagnostic ions *m/z* 473 (base peak), 417, 398, and 371 is postulated (minor ions like *m/z* 355 and 297, whose relative abundances were in the range 5–10 %, though reproducible, were not included in the comparison). From the reconstructed ion chromatogram (RIC) traces for *m/z* 473 and 398, the peak apex was chosen to obtain the absolute abundance for each diagnostic ion.

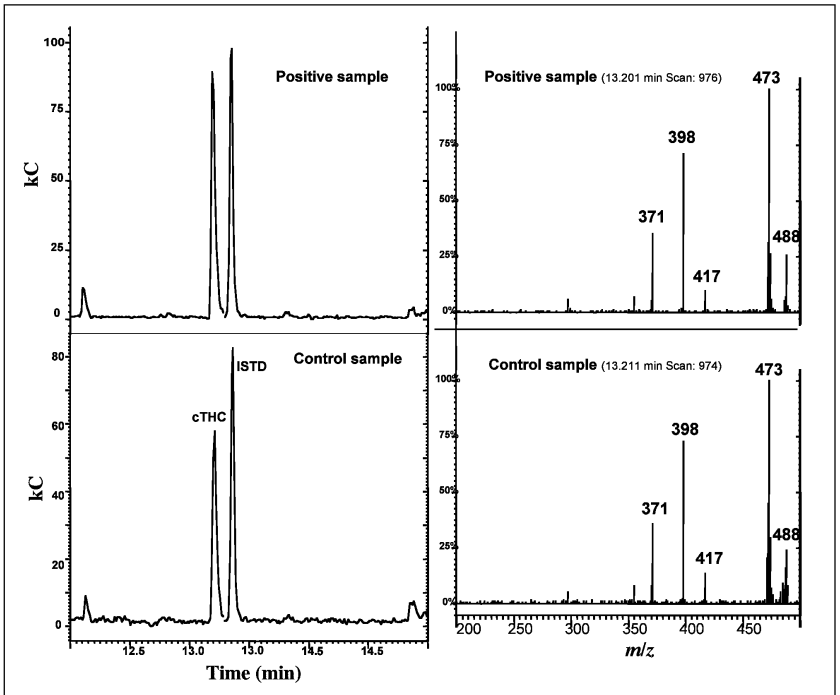

Figure 2. Identification of a cTHC positive sample at 30 ng/mL by MS–MS. Reconstructed ion (*m/z* 473, 398, and 341) MS–MS chromatograms (left) and MS–MS spectra (right).

Method validation

The results obtained indicate that the present method complies with most of the factors relevant to determining whether a method is fit for the purpose of identification, accordingly to WADA technical documents (7).

Robustness in chromatography and MS

Aliquots of true positive and spiked urine samples at the levels mentioned were prepared in three diferent days and injected at three diferent moments of each day. cTHC–TMS retention times obtained were reproducible and precise: 13.22 ± 0.02 min and RRT of 0.9889 ± 0.0005 ($N = 59$, positive and spiked urine samples). The values obtained fully satisfy WADA's tolerance limit of 1% for both cases (7).

In Table I the average relative abundances of cTHC–TMS MS–MS spectra diagnostic ions are presented (ion *m/z* 473 was the base peak in all cases). It can be observed that the diagnostic ions relative abundances of both suspicious and control samples are reproducible and precise (intraday and interday), and independent of analyte concentration. A comparison of the RIC for *m/z* 473 and 398 (cTHC) and 341 (internal standard base peak), and the corresponding MS–MS

spectra for both a true positive and a spiked control sample at 30 ng/mL concentration level is presented in Figure 2.

WADA's identification criteria for qualitative assays establish, for ions obtained by tandem MS detection, the following maximum tolerance windows for differences in relative ion intensities: ± 15% (absolute), ± 25% (relative), and ± 10% (absolute) for relative abundance (% of base peak) 50, 25, and < 25, respectively (7). The Table shows that this method fully complies with WADA's criteria in all the range of concentrations assayed. It can be seen that average absolute differences of ion *m/z* 417 (average relative abundance 11.6 %) and 398 (average relative abundance 76.6%) fall below the respective 10% and 15% limits. The tolerance limits are not trespassed upon even by adding three standard deviations to these average values. Similar considerations can be made for the average relative differences of ion *m/z* 317 (average relative abundance, 38.6%) that fall below the 25% limit. The only exception is that of the 10-ng/mL concentration level, where the addition of only one standard deviation to the average value does not trespass upon the tolerance limit. Overall, these data demonstrate the reliable repetition of the results at different times and concentrations.

Selectivity

Only positive and spiked samples containing cTHC gave chromatograms and spectra such as those shown in Figure 2. More than 40 negative control urine or reagent blank samples gave no signals at the expected cTHC retention time.

Carryover

Injecting samples in the IOC recomended order (i.e., negative control urine, sample confirmation, negative control urine, and positive control urine) allowed us to establish that the method has no carryover of cTHC from sample to sample even in the high concentration region (100 ng/mL).

Specificity

There were no interferences observed from the analysis of more than 60 samples at the cTHC or internal standard retention times, in spite of the different samples assayed containing varying endogenous steroids concentrations.

Linearity

An internal standard method was used to study this factor. Aliquots of cTHC spiked urine samples of 10, 30, 50, and 100 ng/mL were injected twice. Calibration curves were obtained by plotting the *m/z* 473/341 peak height ratio against cTHC concentration. There was good linearity in the range studied (R^2 = 0.9965).

Sensitivity

The method shows good limit of detection below the cut-off limit. Signal-to-noise values around 25 were usually obtained at the 10-ng/mL concentration level. This meant a 2-fold decrease in sensitivity compared with our original resonant CID method (11), with the loss compensated by the gain in precision as previously discussed. Additionally, we can estimate that the limit of detection, if defined as the concentration of cTHC that produces analytical signals equal to three times the deviation of the background signals, has to be in the range 1–5 ng/mL, which is similar to current GC–MS SIM analysis (6).

Conclusion

The identification of cTHC in urine samples, in the context of doping analysis, can be acomplished by ion trap GC–MS–MS. This study has shown that MS–MS detection is a sensitive, reproducible, and precise method for the identification of cTHC in human urine samples at concentration levels from 10 to 100 ng/mL. The method complies with the identification criteria for qualitative assays established by sports authorities thus indicating that it is fit for the purpose of identification of cTHC levels above the threshold in routine doping control samples, according to WADA requirements.

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