Detection of THCA in Oral Fluid by GC–MS–MS

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Abstract

A major marijuana metabolite, 11-nor-9-carboxy-tetrahydrocannabinol (THCA), has been identified in oral fluids from donors that previously tested positive for Δ^9-tetrahydrocannabinol (THC). The method consisted of solid-phase extraction of the oral fluid samples followed by gas chromatography–tandem mass spectrometry analysis of the extracts. Testing for THCA was performed on 223 oral fluid samples previously analyzed for THC. The THCA assay was linear from 10 to 240 pg/mL. The mean recovery of spiked THCA in oral fluid was 104%, and precision was 4% at 20 pg/mL using fortified negative samples. This method was rugged and robust, providing detection and quantification of THCA in oral fluids at levels not previously reported. Results of this study showed that THCA was detectable in 21 of 26 oral fluid samples previously reported positive for THC. The range of concentrations from these samples was from 10 pg/mL up to 142 pg/mL THCA.

Introduction

Using oral fluid for the detection of drugs, including marijuana, has become popular for employment drug testing for several reasons: 1. oral fluid collection is less intrusive than urine collection; 2. this collection can be witnessed to minimize adulteration and sample switching or substitution; and 3. no special collection facilities are required. The current oral fluid analysis to detect marijuana use is to analyze for Δ^9-tetrahydrocannabinol (THC). The notice of proposed rule making (NPRM) by the Substance Abuse and Mental Health Services Administration (SAMHSA) for alternate matrices (Fed Register Notice #FR Doc 04-7984) requires the simultaneous collection of urine to be used for the detection of marijuana use, by targeting the metabolite, 11-nor-9-carboxy-tetrahydrocannabinol (THCA). This is because of a perception that testing for THC alone in oral fluid does not preclude possible passive contamination. Using highly sensitive gas chromatography–tandem mass spectrometry (GC–MS–MS) methodology, this laboratory tested previously analyzed oral fluid samples to determine if THCA could be detected. The purpose of this study was to determine if THCA can be quantitatively measured in oral fluids from marijuana users and thereby eliminate the requirement for collection and analysis of urine samples.

Urine testing for THC is currently performed on a routine basis. THC is the major metabolite of THC and can be detected in urine from hours to days after marijuana is administered (1). High-volume methodologies have been developed for detection of THC in urine (2). Urine THC levels are used to indicate marijuana use; however, urine is not the only biological matrix available for detection of THC.

The most useful biological matrices for detection of marijuana use are blood, hair, urine, sweat, and oral fluid. Mechanisms of mass transfer of THC from blood to urine are well understood. Kidney filtration is the most obvious of these transfer routes. Additionally, transfer of THC from the blood to the hair has also been investigated and theorized (3). Although drug transport from blood to saliva is well understood (4,5), the mechanisms of THC transfer from blood to saliva require further investigation. Schramm et al. (6) purported to identify THC in a single saliva specimen using HPLC and mass spectrometry.

In addition to abused drug detection, oral fluid testing is used as a diagnostic tool for viral infections and monitoring disease states (7). Moreover, oral fluid testing remains a useful analytical tool in forensic toxicology, including for workplace drug testing.

This article describes a useful oral fluid test for THCA at low picogram-per-milliliter levels. Testing for THCA in oral fluid samples, that were shown to contain THC, was performed on authentic donor samples previously tested by two laboratories.

Materials and Methods

Oral fluid samples

Oral fluid samples were collected from 223 subjects with the Intercept® oral fluid collection device manufactured by Ora-
Sure Technologies, Inc. (Bethlehem, PA), and were screened for THC using the manufacturer's oral fluid reagents following instructions described in the package insert. The Intercept device collects oral fluid onto a cotton pad and the pad is transferred to a polypropylene tube with a buffer. Oral fluid from drug-free volunteers was collected, pooled, and used as a negative control. The negative oral fluid was certified free from THC and THCA by GC–MS–MS. Standards and controls were prepared in ethanol and diluted for testing into negative oral fluid. THCA, THC, THC-d3, and THCA-d3 were purchased from Cerilliant (Austin, TX). Each standard material was independently verified to be 99.9% pure. 1,1,1,3,3,3-Hexafluoropropanol (HFIP) was purchased from Alfa Aesar (Ward Hill, MA), and pentafluoropropionic anhydride (PFPA) was purchased from Pierce (Rockford, IL).

The oral fluid samples, obtained from two drug-testing laboratories, were separated into three groups. Samples in Group A were screened negative by Enzyme Linked Immunoassay (ELISA) for THC (cutoff 1.0 ng/mL, cross-reactivity with THCA at 0.33 ng/mL) and consisted of 144 samples. Group B contained 53 samples that had screened positive for THCA by GC–MS–MS. All samples in these groups were averaged and the relative deviation was calculated and reported as the between-run precision at each concentration. Group C contained 26 samples that screened positive for THC by ELISA and confirmed positive for THC by GC–MS–MS (Table I). All samples in these groups were tested for THCA by GC–MS–MS.

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<th>Table I. Between-Run Precision (23 Replicates)</th>
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THCA production from smoke or oral fluid bioconversion

Production of THCA as a pyrolysis product of cannabis smoke or as a result of bioconversion in the oral fluid was briefly investigated. Metabolism of THC in oral fluid was briefly investigated to rule out conversion of THC to THCA in the oral fluid. This was done by incubating THC at 1.0 mg/mL in oral fluid at 37°C for 24 h and then testing for the formation of THCA. Production of THCA from cannabis smoke was also briefly investigated. A volunteer participating in a cannabis smoking study inhaled smoke from a lit marijuana cigarette and exhaled this smoke into an aqueous solution. This solution was tested for THC and THCA by GC–MS–MS.

Accuracy

Accuracy of the analytical method was determined at 10, 20, 40, 60, and 120 pg/mL by extracting five samples at each concentration. The average response was calculated as a percent of the expected response and reported with the relative standard deviation. Precision was calculated as the relative standard deviation of the sample mean of these replicates across this range. Between-run precision was calculated at 10 and 60 pg/mL by collecting data from 23 batch runs performed on separate days. The 10 and 60 pg/mL control from each batch of 23 were averaged and the relative deviation was calculated and reported as the between-run precision at each concentration.

Linearity

Linearity of sample response was evaluated in a range below 250 pg/mL. This was performed by spiking known amounts of THCA in oral fluid and subjecting these samples to the analysis described. A correlation analysis was then performed, and the $r^2$ for detector response versus analyte concentration was reported. Repeated batches were plotted together in the low range of 10 to 60 pg/mL.

Recovery

Analytical recovery or extraction efficiency was performed at 10, 20, and 60 pg/mL. Analytical recovery experiments were performed by first extracting the THCA drug then adding internal standard after the extraction.

THCA-glucuronide liberation

Additional studies were performed to determine if THCA-glucuronide was hydrolyzed to liberate free THCA using the alkaline extraction procedure. THCA-glucuronide was purchased from Altech Applied Science (State College, PA) and was determined to be greater than 99% pure when assayed for THCA by the GC–MS–MS described here. A sample of the glucuronide was dried and derivatized without the hydrolysis step in the following protocol to determine the free THCA content. THCA-glucuronide was also subjected to hydrolysis and extraction to verify recovery of free THCA.

Sample preparation

Solid-phase extractions were performed without column conditioning. One milliliter of 1.0M NaOH was added to CEREX® Polychrome extraction columns (CERA, Baldwin Park, CA). Then 0.1 mL of the oral fluid, calibrator, or control
was added, followed by THCA-d$_3$ internal standard. These mixtures were then passed through the extraction columns at a low flow rate with positive pressure using nitrogen gas. Before allowing the column to dry, the column was washed with 1.0 mL of a mixture of water/methanol/ammonium hydroxide (85:15:1) followed by 0.5 mL methanol. The columns were dried for 15–30 min with nitrogen gas using positive pressure.

Elution of column bound contents was performed using a solvent mixture of hexane/ethyl acetate/acetic acid (80:20:2). Following this elution, the eluate was dried completely under a stream of nitrogen at 40°C. Fifty microliters of HFIP and PFPA were added, and the vial was capped and heated at 75°C for 15 min. Derivative was dried again using nitrogen, and 50 µL toluene was added to the residue. After brief mixing, the toluene was then transferred to a 0.7-mL amber glass autosample vial for injection.

Sample analysis
Sample analysis was performed on a Finnigan TSQ-7000 triple-quadrupole MS coupled with a ThermoFinnigan Trace GC and a Leap Technologies CTC A200S autosampler. The instrument operated in negative ion mode, using ammonia gas at a pressure of approximately 7 Torr. Collision gas was argon with a pressure of 1.0 mTorr. Source temperature was held constant at 180°C. Injection volume was 3 µL. The temperature of the injector was 250°C, and the GC oven temperature program started at 130°C and ramped to 300°C using a gradient of 30°C/min. The column used was a J&W DB-5 (15 m × 0.25-mm i.d., 1.0-µm film thickness, Agilent Technologies, Palo Alto, CA). The transfer line temperature was set at 270°C. Tuning of the instrument was performed prior to each set of analysis.

Retention times for each sample peak were within ±2% of the retention time for the calibrator. Precursor ions monitored for THCA and THCA-d$_3$ were m/z 620.5 and 623.5, respectively. Product ions were m/z 492 and 383 for THCA and 495 and 386 for THCA-d$_3$. Quantitation was performed using the internal standard peak-area ratios of the m/z 495 peak with the m/z 492 peak. Calculations were performed using Finnigan Excaliber software and Microsoft Excel for ion ratio calculations. Acceptance of unknown samples required retention time and ion ratio match. Ion ratio acceptance of each unknown sample required the presence of a peak at the correct retention time for the 492 product ion and the 383 product ion; moreover, the ion ratio of 492/383 was within 30% of the ion ratio of the calibrator. Samples that contained both 492 and 383 peaks with greater than 3:1 signal to noise, but did not meet the 30% ion-ratio criteria were below the LOQ.

Specificity
Specificity experiments were performed to verify that no interference was detected by subjecting a known library of compounds to this assay. Compounds used for specificity experiments included THC, 11-hydroxy-THC, acetaminophen, meperidine, d-methamphetamine, carisoprodol, meprobamate, PPA, hydroxyxine, norcotyline, PCP, caffeine, amityriptyline, nicotine, pseudoephedrine, d-amphetamine, codeine, mor-

phine, methadone, lidocaine, naproxen, chlorpheniramime, erythromycin, diazepam, pentazocine, imipramine, phellathaline, doxepin, carbamazepine, quinine, methaqualone, diphenhydramine, cocaine, amoxapine, desipramine, benzoycgonrine, propoxyphene, trazodone, and phentermine. Concentration ranges were from 100 ng/mL to 10 µg/mL.

Stability
Short-term sample stability was briefly investigated, and additional experiments are currently underway. Authentic samples were placed on stability refrigerated stored in amber glass vials. THCA-spiked oral fluid samples stored in polypropylene tubes (Dependable Scientific, Midvale, UT) were evaluated at two temperature conditions: room temperature and refrigerated. THCA-glucuronide spiked oral fluid samples stored in polypropylene tubes were evaluated at two temperature conditions: room temperature and frozen. Derivatized analyte was studied for stability over a 72-h period stored at room temperature in amber glass vials to determine stability of the analyte after extraction and derivitization, and before analysis.

Results and Discussion
To our knowledge, this is the first published report of a method for quantitative determination for THCA in oral fluids from marijuana users. This method consists of alkaline hydrolysis followed by solid-phase extraction and GC–MS–MS analysis for the detection and quantitation of THCA. The assay is linear from 10 to 240 pg/mL with a sample volume of 100 µL oral fluid. This represents a low of 60 fg of derivatized THCA on column with a signal-to-noise ratio above three for the qualifier ions.

Oral fluid samples
THCA was detected in 81% of the oral fluid samples in Group C (screened and confirmed positive for THC) with the remaining samples containing THCA below the cutoff of 10 pg/mL. All samples in Group C were from long-term storage of donor samples that had positive THC results previously reported. Samples ranged from the lower limit of detection of 10 pg/mL to a high of 142 pg/mL. THCA was detected in 1 sample from Group A (screened negative) at 15.7 pg/mL, and 2 samples in Group B (screened positive) at 32.2 and 82.0 pg/mL. The data show that the current oral fluid cutoff used for THC correlates with THCA detection at 10 pg/mL. Table II shows the THC concentrations determined in the oral fluids and the corresponding THCA determined for those screened and confirmed positive for THC.

THCA production from THC
Blown cannabis smoke did not contain detectable THCA. No formation of THCA was detected when incubation of THC in oral fluid was performed. Further, it is unlikely that any biotransformation in the oral mucosa could be responsible for converting THC to THCA. Several authors have reported that the enzymes responsible for this conversion, are hepatic re-
lated and require several microsomal enzyme pathways (8–12). Microsomal hydroxylation allylic to Δ²-THC double bond occurs to form 11-OH-Δ²-THC. The conversion to THCA is accomplished by alcohol dehydrogenase (12), which is then subsequently glucuronidated (11). None of these enzymes are known to exist in the oral mucosal. The presence of THCA in oral fluid may come from diffusion of THCA from the blood or transport by some other mechanism. The low levels of THCA detected in oral fluid are consistent with its high degree of plasma protein binding, and are below the levels of detection for validated methods previously reported in the literature.

Accuracy
Accuracy average in the range of 10 to 120 pg/mL was 108% with a relative standard deviation of 15%. Precision of repeated extractions on 23 different batch runs of the same concentration was 16.6% at the LOD (10 pg/mL) and 9.38% at 60 pg/mL. Precision within a batch run (n = 5) was 20% at 10 pg/mL, 4% at 20 pg/mL, and 7% at 60 pg/mL. Analytical recovery/extraction efficiency was 113% at 10 pg/mL, 92.5% at 20 pg/mL, and 105% at 60 pg/mL. Between-run accuracy and precision at 10 pg/mL and 60 pg/mL are shown in Table I.

Linearity
The correlation coefficient for assay linearity exceeded 0.995 between the LOQ of 10 pg/mL and the high concentration of 240 pg/mL. Chromatograms are shown in Figure 1. The linearity curve is shown in Figure 2.

Recovery
Analytical recovery of freshly spiked oral fluid is tabulated in Table III. At the LOQ of 10 pg/mL recovery was 113%
with a relative standard deviation of 16%. At 20 pg/mL, the concentration of the calibrator, the average recovery was 100% with a relative standard deviation of 2%. And at 60 pg/mL, the average recovery was 120% with a relative standard deviation of 15%. In this low range, the method meets current requirements listed in the Center for Drug Evaluation and Research (CDER) for recovery of analyte of interest (13).

**THCA-glucuronide liberation**

THCA recovery from the THCA-glucuronide spiked oral fluid was good. Three samples of THCA-glucuronide were spiked at 66 pg/mL THCA content. Recoveries of these three samples were 64.47, 70.80, and 52.65 pg/mL, or 94.9% on average. The THCA-glucuronide, if present in the oral fluid, was measured and reported as free THCA. Because of the limited supply of purified THCA-glucuronide, these recovery studies were only performed in triplicate. A larger sample size is desired before making any conclusion about recovery statistics of the glucuronide form of THCA using this method. THCA-glucuronide is known to be present in the blood and urine after cannabis use (14), but it is not known at this time if THCA-glucuronide can be found in oral fluid.

**Specificity**

Interference was not seen from specificity samples tested including a library of known compounds investigated, listed here. All negative samples of oral fluid collected from drug free volunteers showed no interference while subjected to this analysis.

**Stability**

Room temperature stability of THCA spiked in oral fluid is known to be poor when samples are stored at room temperature (15). Great care was taken to perform method validation with freshly prepared samples. Stability studies performed with spiked oral fluid confirmed that THCA is not stable in oral fluid at room temperature (Figure 3). Only 10% of the amount spiked could be recovered after four days storage. In contrast, stability of THCA-glucuronide does not show this same trend (Figure 4). For a period of 60 days THCA-glucuronide samples...
were stable. Authentic Samples stored in amber glass vials were also stable for a period of five months (data not shown). Authentic samples showed similar stability to the THCA-glucuronide samples, yet, because of limited supply of authentic oral fluid samples and limited supply of THCA-glucuronide, storage conditions were not identical and future stability studies are limited by this supply. However, data clearly show THCA in oral fluid of authentic samples well above the LOQ. Table II shows the level of THCA determined in authentic samples. Some of these authentic samples had been in storage for up to two years before this test was performed. Full conclusions about the stability of THCA and THCA-glucuronide in oral require further investigation.

Conclusions

THCA was detected in oral fluid of samples previously identified as positive for THC. A method is now available to investigate the appearance of the major metabolite of cannabis in oral fluid at low picogram-per-milliliter levels. The low levels detected in authentic samples indicate previous reports may have missed detection of THCA because of sensitivity.

Acknowledgments

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References


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