Analysis of Tetrahydrocannabinol and its Metabolite, 11-Nor-∆⁹-Tetrahydrocannabinol-9-Carboxylic Acid, in Oral Fluid using Liquid Chromatography with Tandem Mass Spectrometry

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This paper describes the determination of tetrahydrocannabinol (THC) and its metabolite, 11-nor-∆⁹-tetrahydrocannabinol-9-carboxylic acid (THC-COOH) in oral fluid using solid-phase extraction and liquid chromatography with tandem mass spectrometry (LC–MS-MS) and its application to proficiency specimens. The method employs collection of oral fluid with the Quantisal™ device, base hydrolysis, solid-phase extraction and LC–MS-MS in positive ion electrospray mode. Because the concentration of the metabolite in oral fluid is quite low, extremely sensitive analytical methods are necessary. The requisite sensitivity was achieved by a simple, rapid derivatization of the compound after extraction. The derivatization conditions did not affect parent THC. The method was fully validated using standard parameters including linearity, sensitivity, accuracy, intra-day and inter-day imprecision, drug recovery from the collection pad, limit of quantitation, limit of detection and matrix effects. The procedure was applied to oral fluid proficiency specimens previously analyzed to assess the stability of THC-COOH.

Introduction
Marijuana is a widely used illicit drug generally administered orally or by smoking, resulting in euphoria and hallucinations. In ingesting marijuana, tetrahydrocannabinol (THC) is deposited into the oral cavity, and this may be the primary source of THC collected and measured in oral fluid analysis, rather than the drug that has circulated through the body. Recently, the presence of THC was reported in oral fluid after subjects were exposed passively to marijuana for approximately 3 hours (1), which has the potential of identifying innocent subjects as marijuana users in a workplace drug testing situation. The detection in oral fluid of a metabolite such as 11-nor-∆⁹-tetrahydrocannabinol-9-carboxylic acid (THC-COOH) would minimize this defense because its presence is difficult to explain as contamination. This has also been the conclusion of other researchers (2, 3).

In 2006, two publications described the determination of THC-COOH in oral fluid: one used two dimensional gas chromatography with mass spectrometry (GC × GC–MS) (4); the other used GC with tandem mass spectrometric detection (5). Such techniques are necessary because the concentration of THC-COOH in the oral fluid of marijuana smokers is often below 0.1 ng/mL. Because neither of these techniques appears to be routine in workplace testing, and with liquid chromatography–tandem mass spectrometric instruments (LC–MS-MS) providing greatly increased capabilities regarding sensitivity, the utility of bench-top LC–MS-MS systems for the analysis of THC-COOH in oral fluid was investigated. Very recently, Lee et al. (6) reported the derivatization of THC and THC-COOH with dansyl chloride to produce the sensitivity required for the detection of THC-COOH in oral fluid using LC–MS-MS for the first time. However, their procedure derived one MS transition from the dansyl chloride itself, so it is not scientifically useful as a monitoring transition. A new publication on the simultaneous detection of THC and THC-COOH in oral fluid using LC–MS-MS reported a limit of quantitation for the metabolite of 0.2 ng/mL, which is inadequate for the detection of THC-COOH in most authentic specimens. Such a high limit of quantitation (LOQ) would prevent many marijuana smokers from being identified via detection of the metabolite (7). In 2010, Higashi et al. (8) described the simple derivatization of carboxylic acids to enhance the detection response in electrospray LC–MS-MS systems. Our approach was to adapt the Higashi derivatization method to determine THC-COOH in oral fluid, and to ensure that two transitions characteristic of the molecule were available for monitoring.

Because oral fluid is being considered as an allowable specimen for workplace drug testing, the ability to detect THC and THC-COOH simultaneously using LC–MS-MS is timely. In this study, oral fluid specimens were collected with the Quantisal™ device.

Materials and Methods
Supplies and reagents
Quantisal™ devices for the collection of oral fluid specimens were obtained from Immunoanalysis Corporation (Pomona, CA). The devices contain a collection pad with a volume adequacy indicator, which turns blue when one milliliter of oral fluid (∓10%) has been collected. The pad is then placed into transport buffer (3 mL), allowing a total specimen volume available for analysis of 4 mL (3 mL buffer + 1 mL oral fluid). Because the oral fluid concentration is diluted 1:3 when using Quantisal™ collection devices, detected drug concentrations were adjusted accordingly. Deuterated d3-THC (internal standard) and the unlabelled drug standards were obtained from Cerilliant (Round Rock, TX). Solid-phase extraction columns (Trace-N, TN-315) were obtained from SPEWare (Baldwin Park, CA). All solvents were pesticide grade and all chemicals were ACS grade. Triphenylphosphine (TPP), 2-picolylamine (PA) and 2,2′-dipyridyl disulfide (DPDS) were purchased from Sigma–Aldrich (St. Louis, MO).

The internal standard solution contained deuterated d3-THC at a concentration of 25 ng/mL. Deuterated THC-COOH was deliberately not chosen as an internal standard for this assay.
due to the extreme sensitivity required. It is possible that small percentages of unlabelled THC-COOH in the deuterated standard could lead to a false positive result; therefore, d3-THC was used to quantitate both compounds. Calibration curves were prepared in the transport buffer associated with the Quantisal™ device. The concentrations were equivalent to neat oral fluid volumes of 0.01, 0.02, 0.05, 0.1, 0.5 and 1 ng/mL of THC-COOH; 1, 5, 10, 20, 50 and 100 ng/mL of THC.

Sample preparation
Because THC-COOH is conjugated to the glucuronide in oral fluid (9), it was necessary to hydrolyze the specimen before extraction. Sodium hydroxide (1N; 0.2 mL) was added to 1 mL of each specimen (oral fluid + buffer). The samples were heated at 60°C for 15 min, and then allowed to cool; glacial acetic acid (0.5 mL) was then added along with internal standard (50 µL). Solid-phase extraction columns were conditioned with methanol (0.5 mL) and 0.1M acetic acid (0.1 mL). The samples were loaded onto the respective columns and allowed to dry. Columns were washed with deionized water–acetic acid (80:20 v/v; 1 mL), then deionized water–methanol (40:60 v/v; 1 mL) and allowed to dry for 5 min. The drugs were eluted with hexane–acetic acid (98:2, 1 mL) into reaction vials. The eluent was evaporated to dryness under nitrogen at 40°C.

Derivatization
A freshly prepared solution of TPP (10 mM) in acetonitrile (10 µL) was added to the dried extract. DPDS (10 mM) in acetonitrile (10 µL) and 10 µL of PA (10 µg in acetonitrile) were successively added and the mixture was incubated at 60°C for 15 min; evaporated to dryness under nitrogen at 40°C and reconstituted in acetonitrile–deionized water (50:50; 25 µL) for LC–MS-MS analysis.

LC–MS-MS
An Agilent Technologies 1290 pump connected to a 6490 triple quadrupole mass spectrometer, operating in positive electrospray mode (ESI) with Jet Stream technology was used for analysis. The liquid chromatographic column was a Stable Bond-C18 (2.1 × 50 mm × 1.8 µm). The column temperature was 35°C and the injection volume was 20 µL. The mobile phase consisted of 5 mM ammonium formate (pH 6.4, Solvent A) and 0.5% formic acid in acetonitrile (Solvent B). At the beginning of the run, the mobile phase composition was 30% A–70% B at a constant flow rate of 0.2 mL/min; this was held for 2 min. After 4 min, the percentage of solvent B was 90%, and stop time was 7 min. The equilibration time was 1.5 min. The gas temperature was 200°C, the gas flow was 18 L/min and the nebulizer pressure was 30 psi. The sheath gas was heated to 400°C and the sheath gas flow was 10 L/min. Nitrogen was used as the collision gas and the capillary voltage was 3,500 V. The fragment voltage on this instrument could not be changed and was set at 380 V. For d3-THC, the precursor ion 318.3 was fragmented to 196.3 at optimized collision energy of 20 V; cell accelerator 5 V. Two transitions were selected and optimized for THC and derivatized THC-COOH (quantifying transition is bold): THC: 315.3 > 193.3, 315.3 > 123.3 (qualitative transition). THC-COOH - 2PA: 435.3 > 327.0, 435.3 > 299.0 at collision energies of 20 V; cell accelerator voltage 5 V. The fragmentation pattern for the derivative is shown in Figure 1. Each subsequent analysis required the ratio between the quantitative transition and the qualifying transition to be within ±20% of that established by calibration standards to meet the criterion.
for a positive result. For THC, the acceptable range for the qualifying transition was 47–71%, THC-COOH FA 41.6–62.4%. The retention times were 1.8 min for derivatized THC-COOH and 4.6 min for THC.

Method Validation

Linearity and sensitivity
Calibration using deuterated d3-THC as an internal standard was calculated using linear regression analysis over a concentration range of 0.01–1 ng/mL for THC-COOH and 1–100 ng/mL for THC. Peak area ratios of the target analyte and the internal standard were calculated using Mass Hunter software (Agilent). The data were fit to a linear least-squares regression curve with a 1/x weighting and not forced through the origin. The linearity of the assays was established with six calibration points, excluding the drug-free matrix. The limit of quantitation (LOQ) was determined using serial dilutions to the lowest point at which the acceptable criteria for the quantitation of a compound were met, i.e., the chromatographic peak shape, retention time (within 2% of calibration standard) and qualifier transition ratio (± 20%) compared to a calibration standard were acceptable. The quantitative value of the LOQ had to be within ± 20% of the target concentration and replicate analyses were required to have low variation in response \[ n = 5; \ \text{coefficient of variation (CV), 15\%}. \] The limit of detection (LOD) was estimated using a linear calibration curve. Three calibration curves were constructed across the working range of the analytical method. The LOD was estimated from the standard deviation of the y intercept (\( \sigma_y \)) and the average slope \( (m_{\text{avg}}) \) as: \[ \text{LOD} = \left( 3.3 \ \sigma_y / m_{\text{avg}} \right). \]

Drug recovery from the pad, accuracy and imprecision
Recovery: the efficiency of extraction for both drugs from the collection device has previously been reported.

Accuracy: the accuracy of the procedure was determined over six replicates at 0.012 and 0.075 ng/mL for THC-COOH; 12 and 75 ng/mL for THC. Accuracy was calculated as mean measured concentration divided by the fortified concentration × 100%.

Imprecision: inter-day and intra-day imprecision of the assays was determined at two concentrations: 0.012 and 0.075 ng/mL for THC-COOH; 12 and 75 ng/mL for THC. Intra-day data were obtained from six analyses performed on one day \( (n = 6) \); inter-day data were obtained by analyzing six specimens each day for 5 days \( (n = 30) \).

Matrix effects
Oral fluid specimens were obtained from drug-free volunteers, extracted and analyzed according to the described procedures to assess interference from extraction or matrix, or potential ion suppression. The protocol from Matuszewski (10) was used to assess matrix effects and process efficiency. To perform experiments according to these protocols, a non-extracted drug standard at a concentration of 0.02 ng/mL (THC-COOH) of 20 ng/mL (THC) was prepared, in addition to drug-free matrix extracts and negative controls (extracts containing only internal standard).

The recovery of the compounds from oral fluid was determined by first assessing the response of the extracted samples \( (n = 6) \) at a concentration of 0.02 or 20 ng/mL \( [\text{RES}] \). Next, oral fluid was extracted and drug was added post-extraction at a concentration of 0.02 or 20 ng/mL \( (n = 6) \) \( [\text{RRES}] \). The percentage recovery was then calculated from the equation \( \text{RES} / \text{RRES} \times 100 \).

The percentage reduction and improvement in response due to matrix effects (ion suppression and ion enhancement) was determined by assessing the peak area response of a non-extracted neat drug standard \( (n = 6) \) at a concentration of 0.02 or 20 ng/mL \( [\text{RNES}] \). The non-extracted solution was analyzed in the same reconstitution solvent as the extracted specimens. The percent matrix effect was then calculated using the equation \( \text{RRES} / \text{RNES} - 1 \times 100 \). A negative result indicated ion suppression and a positive result indicated ion enhancement of the signal. The overall efficiency of the process was calculated as \( \text{RES} / \text{RNES} \times 100 \). Reduction of matrix effects is best achieved by utilization of deuterated internal standards where possible, extensive matrix clean-up before injection and optimal chromatographic and mass spectral conditions.

Exogenous interference
To assess potential problems arising from exogenous sources, commonly encountered drugs were added to the drug-free oral fluid specimens and subjected to the same extraction and analysis procedures. The following drugs were analyzed at a concentration of 10,000 ng/mL: morphine, codeine, 6-acetylmorphine, oxycodone, oxymorphone, hydrocodone, hydromorphone, buprenorphine, norbuprenorphine, cocaine, benzoylcegonine, cocaethylene, norcocaine, tramadol, fentanyl, amphetamine, methamphetamine, methylenedioxyxymethamphetamine (MDMA), methylenedioxymethylamphetamine (MDEA), nortriptyline, amitriptyline, methadone, dazepam, nordiazepam, oxazepam, alprazolam, chlordiazepoxide, bromazepam, temazepam, lorazepam, flurazepam, nitrazepam, triazolam, tramadol, secobarbital, pentobarbital, butalbital and phenobarbital.

Stability of drugs in the extracts
Because almost the entire extract is injected into the instrument, the stability of the extracts was assessed by injecting a second and third extract that remained in the auto-sampler overnight maintained at 7°C. The extracts were re-analyzed after 24 h and after 48 h against a fresh calibration curve.

Results and Discussion

Method validation

Linearity and Sensitivity
The developed LC–MS-MS procedure was validated according to accepted protocols. The LOQ was 1 ng/mL for THC; LOD 0.6 ng/mL. The LOQ for THC-COOH was 0.01 ng/mL; LOD 0.006 ng/mL. Linearity over the tested ranges was established with an average correlation coefficient of 0.998.

Recovery, accuracy and imprecision
The recovery of the drugs from the Quantisal™ collection pad has been previously reported at ≥80% for both THC-COOH and THC (4, 11).
The developed assay showed an accuracy of >95% for both drugs at both concentrations. Intra-day imprecision for THC at 12 and 75 ng/mL was 0.9 and 1.4%, respectively; intra-day imprecision for THC-COOH at 0.012 and 0.075 ng/mL was 3.0 and 1.6%, respectively. For inter-day, THC imprecision was 4.0 and 4.8% at 12 and 75 ng/mL; 6.5 and 1.9% for THC-COOH at 0.012 and 0.075 ng/mL.

Matrix effects
Because the oral fluid is diluted during collection, deuterated internal standards are added, and specific solid phase procedures are employed. It was expected that there would be minimal ion suppression associated with the method. However, for THC-COOH, the matrix actually enhanced the response +35%; process efficiency was 46%; and recovery was 34%. For THC, the recovery was 92%; matrix effect was −65%; and process efficiency was 32%.

Although the use of a deuterated analogue will not reduce matrix effects, it will in many cases compensate for ion suppression or enhancement, making sure the quantification is not compromised. In this method, THC-d3 is used for the quantification of both analytes, so the quantification of THC-COOH may be particularly influenced because there was a large difference in recovery and matrix effect between the two compounds. Deuterated THC-COOH was not utilized due to concerns with unlabelled drugs that may be present, causing problems when the requisite sensitivity is so low. Future improvements will focus on the inclusion of a related internal standard and improved recovery of THC-COOH.

Interference
Oral fluid specimens collected from drug-free individuals showed no interference with the assay. For exogenous interferences, commonly encountered drugs of abuse were studied as described and no interference was observed.

Stability
The extracts were stable for at least two days when kept inside the auto-sampler, which was maintained at 7°C. Less than a 5% difference was observed in the quantitation after 24 and 48 hours.

<table>
<thead>
<tr>
<th>THC-COOH (ng/mL)</th>
<th>Initial analysis (2d-GC/MS)</th>
<th>Re-analysis (LC-MS/MS)</th>
<th>% loss</th>
</tr>
</thead>
<tbody>
<tr>
<td>12 month storage: 22-02</td>
<td>0.013</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>22-09</td>
<td>0.010</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>6 months storage: 23-09</td>
<td>0.099</td>
<td>0.040</td>
<td>54</td>
</tr>
<tr>
<td>3 months storage: 25-01</td>
<td>0.010</td>
<td>0.012</td>
<td>0</td>
</tr>
<tr>
<td>25-02</td>
<td>0.077</td>
<td>0.075</td>
<td>0</td>
</tr>
</tbody>
</table>

Figure 2. Proficiency specimen OF23-09 analyzed by two-dimensional GC–MS and then six months later by LC–MS-MS.
Application to proficiency specimens

In our laboratory, proficiency specimens are received from RTI International (Research Triangle Park, NC). Previously analyzed specimens were re-tested using the described method. In earlier methods, THC-COOH was analyzed using two-dimensional GC–MS according to a published procedure (4). The results are shown in Table I.

Oral fluid proficiency specimens are not stored in transportation buffers or other preservatives. The exact composition of the specimens is unknown, but presumably a synthetic mix is manufactured to resemble the characteristics of oral fluid. The specimens showed no loss while stored for three months at 4°C; an approximate 50% loss after six months; and the THC-COOH had completely degraded after one year of refrigerated storage. Figure 2 shows the chromatogram of specimen OF23-09 analyzed by two-dimensional GC–MS and 6 months later using the described method.

The procedure is currently applied as a confirmatory method for routine oral fluid specimens received into our laboratory that screen positively for THC using immunoassay. The Quantisal collection device provides an adequate consistent volume for the simultaneous analysis of THC and THC-COOH in oral fluid.

Limitations of the study

The procedure is not as sensitive as others described in the literature, which use either two-dimensional GC–MS or GC–MS-MS, however, LC–MS-MS instrumentation is far more widespread in testing laboratories and is therefore more likely to be adopted as an analytical procedure. In a 2006 study, 66.4% of oral fluid specimens that had screened positively for cannabinoids using immunoassay were positive for both THC and THC-COOH; 9.7% were positive for THC-COOH only, and 18.8% were positive for THC only (12). The concentration range for THC-COOH was 0.002–0.352 ng/mL (n = 109). The LOQ of the two-dimensional GC–MS procedure used was 0.002 ng/mL, and 36 samples (33%) would have been below the LOQ of the method described in this paper (0.01 ng/mL). However, only three of those 36 samples did not contain THC, so only three of 109 oral fluid specimens may have been missed using this LC–MS-MS method.

Summary

The described method has the sensitivity to detect both THC and THC-COOH in oral fluid specimens using standard LC–MS-MS instrumentation in a rapid, simple, one-step derivatization procedure. Identification of both THC and THC-COOH in oral fluid minimizes the potential for misidentifying individuals passively exposed to marijuana smoke.

References