Tetrahydrocannabinol and Two of its Metabolites in Whole Blood Using Liquid Chromatography–Tandem Mass Spectrometry

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Abstract
An analytical procedure for the determination of Δ²-tetrahydrocannabinol (THC), 11-nor-9-carboxy-Δ²-tetrahydrocannabinol (THCA), and 11-hydroxy-Δ²-tetrahydrocannabinol (11-OH-THC) in whole blood has been developed and validated using liquid chromatography with tandem mass spectral detection (MS). Cannabinoids present in the blood samples were quantified using solid-phase extraction followed by MS detection in positive electrospray ionization mode. For confirmation, two transitions were monitored and one ratio determined. Samples being reported as positive were required to have both transitions present, the ratio of quantifying transition to qualifying transition being within 20% of that determined from known calibration standards. The monitoring of the qualifying transition and requirement for its presence within a specific ratio to the primary ion has the potential of limiting the sensitivity of the assay; however, the additional confidence in the final result as well as forensic defensibility were considered to be of greater importance. The limit of quantitation was 0.5 ng/mL for THC, 5 ng/mL for THCA, and 2 ng/mL for 11-OH-THC. The limit of detection was 0.5 ng/mL for THC, 4 ng/mL for THCA, and 1 ng/mL for 11-OH-THC. The percentage recovery of the cannabinoids from whole blood at a concentration of 5 ng/mL was 71.5% for THC, 64.5% for 11-OH-THC, and 61.2% for THCA (n = 3).

Introduction
Marijuana is the most commonly used illicit drug in the U.S. and is frequently encountered in cases of driving under the influence of drugs. In a recent manuscript recommending cut-off concentrations for the analysis of drugs in blood and urine for driving cases, the majority of forensic laboratories reported cannabis as the most frequently encountered drug (1). There are various published procedures for the determination of THC and its metabolites in whole blood, which generally incorporate gas chromatography–mass spectrometry instrumentation (GC–MS) (2–4). However, the use of liquid chromatography with tandem mass spectrometry (LC–MS–MS) provides adequate sensitivity for the determination of these compounds in whole blood, without the need for derivatization, and usually a much shorter run-time. Many reported procedures monitor only one transition in the multiple-reaction monitoring (MRM) mode, which is inadequate for forensic defensibility of the result. Recently, several authors have focused on the need to monitor a second transition, allowing the ratio between the abundance of the primary and secondary transitions to be calculated, and establishing more confidence in the final result (5). Maralikova and Weinmann (6) noted that guidelines for confirmatory analysis using LC–MS–MS have not yet been established, and they suggested that the monitoring of at least two transitions is required to provide sufficient identification of drugs. They applied their recommendation to the detection of Δ²-tetrahydrocannabinol (THC), 11-nor-9-carboxy-Δ²-tetrahydrocannabinol (THCA), and 11-hydroxy-Δ²-tetrahydrocannabinol (11-OH-THC) in plasma as well as THCA-glucuronide using LC–MS–MS and monitoring two transitions.

In this procedure, two transitions are monitored for the cannabinoids providing excellent sensitivity for their determination in whole blood. The extraction efficiency of the cannabinoids from blood, the degree of ion suppression caused by the blood matrix, and the stability of the extracted specimens over a 72-h period were also investigated. The method is useful in routine testing for the determination of cannabinoids in whole blood, providing forensic defensibility by the inclusion of two monitored transitions.

Materials
Standards and reagents
Tri-deuterated internal standards of THC, THCA, and 11-OH-THC as well as unlabelled drug standards were obtained from Cerilliant (Round Rock, TX). Solid-phase extraction columns (Cerex® Polychrom™ THC 682-0353) were purchased from SPEWare (Baldwin Park, CA). All solvents were HPLC grade or better and obtained from Spectrum Chemicals (Gardena, CA). All chemicals were ACS grade. Quality control (QC)
samples for whole blood at concentrations of 7.5 ng/mL (low QC) and 50 ng/mL (high QC) were purchased from UTAK Laboratories (Valencia, CA).

**Calibrators**

For the chromatographic calibration standards, a working solution for the deuterated internal standard was prepared in methanol at a concentration of 1000 ng/mL. Unlabelled drug standard was prepared in methanol at the same concentration. All the working solutions were stored at –20°C when not in use. For each batch, seven calibration standards were prepared in authentic human blood (1 mL), which had been previously analyzed and found to be negative for cannabinoids. Drug concentrations of 0.5, 1, 2.5, 5, 10, 20, and 100 ng/mL of whole blood were prepared (internal standard concentration: 10 ng/mL) for the calibration. For limit of quantitation and limit of detection experiments, standards at 0.125, 0.25, 2, 3, and 4 ng/mL were also included.

**Methods**

**Sample preparation for chromatographic analysis**

In a similar extraction procedure to that described by Scurlock et al. (2), deuterated internal standards were added to the calibrators, controls, and blood samples (1 mL) to achieve a final concentration of 10 ng/mL. Cold acetonitrile (2 mL) was added, the tube was capped, mixed for 10 min, and centrifuged (4000 rpm; 15 min). The supernatant was removed, and deionized water (4 mL) was added to each calibrator, control, or blood specimen. Polymeric extraction columns (Cerex) were placed into a positive pressure manifold. The samples were directly applied to the column because no conditioning is required when polymeric columns are used. The samples were allowed to flow through the columns, and then the columns were washed with a fresh solution of deionized water/acetonitrile/ammonium hydroxide (90:10:1, v/v, 1 mL). The columns were dried under pressure (30 psi/15 min), then THC was eluted with ethyl acetate (2 mL). The columns were dried for 10 min, then THCA and 11-OH-THC were eluted with hexane/ethyl acetate/glacial acetic acid (88:10:2, v/v, 2 mL) into the same elution tube, and evaporated to dryness under nitrogen at 40°C. The extracts were reconstituted in 20 mM ammonium formate (pH 8.6)/methanol (50:50, v/v, 40 µL), transferred into autosampler vials, capped, and analyzed using LC–MS–MS.

**LC–MS–MS**

An Agilent Technologies 1200 series LC pump coupled to a 6410 triple-quadrupole MS operating in positive electrospray mode (ESI) was used for analysis. The LC column was a narrow-bore Zorbax Extend C18 threaded system (2.1 x 50 mm x 1.8 µm), designed for high-pressure operation. The double endcapping of the stationary phase protected the silica support from dissolution at high pH extending the allowable pH range of operation from 2 to 11.5. The column temperature was held at 40°C, and the injection volume was 5 µL. The mobile phase consisted of 20 mM ammonium formate (pH 8.6, Solvent A) and methanol (Solvent B). Initially, the assay was operated with the pH of the buffer at 6.4; however, the response of the THCA was significantly reduced. At the beginning of the run, the mobile phase composition was 30% A/70% B at a constant flow rate of 0.3 mL/min. After 5 min, the percentage of solvent B was 90%, remaining there for 3 min; finally at 10 min, the percentage of B returned to 70%. The equilibration time was 4 min. The gas temperature was 350°C, the gas flow was 8 L/min, and the nebulizer pressure was 35 psi. Nitrogen was used as the collision gas, and the capillary voltage was 3500 V.

Two transitions were selected and optimized for each drug using flow injection analysis. The transitions and fragment voltages are shown in Table I. Each subsequent analysis required the ratio between the quantitative transition and the qualifying transition to be within ± 20% of that established by calibration standards, in order to meet the criterion for a positive result. The transition ratio was determined at the concentration of 5 ng/mL and was a mean of values taken over five days.

**Validation**

**Data analysis**

Calibration of the assay, slope, intercept, and correlation coefficients were calculated using linear regression analysis.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Precursor Ion</th>
<th>Fragment Ion</th>
<th>Fragment Voltage (V)</th>
<th>Collision Energy (V)</th>
</tr>
</thead>
<tbody>
<tr>
<td>THCA-d₃*</td>
<td>348.3</td>
<td>330.4</td>
<td>80</td>
<td>10</td>
</tr>
<tr>
<td>THCA</td>
<td>345.3</td>
<td>327.1</td>
<td>100</td>
<td>10</td>
</tr>
<tr>
<td>11-OH-THC-d₃</td>
<td>334.3</td>
<td>316.4</td>
<td>80</td>
<td>10</td>
</tr>
<tr>
<td>11-OH-THC</td>
<td>331.3</td>
<td>313.1</td>
<td>80</td>
<td>10</td>
</tr>
<tr>
<td>THC</td>
<td>315.4</td>
<td>193.3</td>
<td>150</td>
<td>20</td>
</tr>
</tbody>
</table>

* Abbreviations: THCA, 11-norΔ⁹-tetrahydrocannabinol-9-carboxylic acid; 11-OH-THC, 11-hydroxy-tetrahydrocannabinol; and THC, tetrahydrocannabinol.
over the concentration range from the limit of quantitation of each drug to 100 ng/mL. 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 was not forced through the origin.

Selectivity

Commonly encountered drugs were added to drug-free blood specimens obtained from volunteers (n = 3). The specimens were subjected to the same extraction and analysis procedures described. The following drugs were included at a concentration of 10,000 ng/mL: cannabinol, cannabidiol, cocaine, benzoylcegonine, cocaethylene, norcocaine, morphine, 6-acetylmorphine, codeine, hydrocodone, hydromorphone, oxycodone, oxymorphone, tramadol, fentanyl, gamma-hydroxybutyrate (GHB), phencyclidine (PCP), amphetamine, methamphetamine, methylenedioxyamphetamine (MDMA), methylenedioxyamphetamine (MDA), methylenedioxyethylamphetamine (MDEA), carisoprodol, methadone, diazepam, nordiazepam, oxazepam, alprazolam, chlordiazepoxide, bromazepam, temazepam, lorazepam, flurazepam, nitrazepam, triazolam, amitryptiline, nortriptyline, imipramine, protriptyline, doxepin, nordoxepin, trimipramine, secobarbital, pentobarbital, butalbital, and phenobarbital.

Linearity and sensitivity

The linearity of the assays was established with seven calibration points, excluding the drug free matrix. The sensitivity of the method was determined by establishing the limit of quantitation (LOQ) defined as the lowest concentration detectable with a signal-to-noise ratio (S/N) of at least 10 for the quantifying ion and retention time within 0.2 min of the calibration standard. The limit of detection (LOD) was defined as the lowest concentration detectable with an S/N of at least 3 for the quantifying ion. In both cases, the qualifying ion was required to be present within the range established (± 20%), with acceptable chromatography. The limits were determined by the method of serial dilution, whereby blood samples fortified with decreasing cannabinoid concentration were analyzed, until the specified criteria were no longer met.

Accuracy, precision, and extract stability

The accuracy of the procedure was determined using six replicates at two concentrations (7.5 and 50 ng/mL). Accuracy was calculated as (mean measured concentration – fortified concentration) divided by the fortified concentration x 100%. Interday and intraday precision were determined at the calibration points of 7.5 and 50 ng/mL. Intraday data were obtained from six analyses performed on one day; interday data were obtained by analyzing six specimens on each of five days (n = 30).

In the event that specimens needed to be re-injected, the stability of the drug extracts at a concentration of 5 ng/mL was determined by allowing the autosampler vials to remain in the LC chamber for 24 and 72 h, after which time they were reanalyzed. The unit was maintained at 7°C. The responses were compared to those achieved on the first day of analysis; quantification was performed using the freshly prepared calibration curve each day.

Extraction efficiency and ion suppression

Using a 2007 publication by Chambers et al. (7) that discussed a systematic and comprehensive strategy for reducing matrix effects in LC–MS–MS as a template, we determined the percentage recovery of the cannabinoids from the blood matrix by first assessing the response of the extracted samples (n = 3) at a concentration of 5 ng/mL (RRES). Next, whole blood was extracted, and drug was added post-extraction at a concentration of 5 ng/mL (n = 3) (RPPES). The percentage recovery was then calculated from the equation (RRES/RPPES) × 100. Again, using the Chambers template, we determined the percentage reduction or improvement in response due to matrix effects (ion suppression or ion enhancement) by assessing the peak area response of a non-extracted neat drug standard (n = 3) at a concentration of 5 ng/mL (RREGS). The unextracted solution was analyzed in the same reconstitution solvent as the extracted specimens. The % matrix effect was then calculated using the equation [(RPES/RNES) – 1] × 100. Chambers et al. (7) modified the original equation by applying the subtraction of 1 to the quotient so that a negative result indicated ion suppression and a positive result indicated ion enhancement of the signal.

Results and Discussion

The development of a simple LC–MS–MS assay for the detection of THC, 11-OH-THC, and THCA in blood is reported. While these drugs have been detected in blood, the increasing utility of LC–MS–MS in laboratories makes development of confirmatory procedures necessary and timely. The monitoring of a second qualifying transition is reported for the first time for the determination of these cannabinoids in whole blood, and is necessary for confidence in the identification of the analyte, even though sensitivity may be lost.

The pH of the mobile phase (8.6) is slightly above the recommended limit for the usual LC columns (pH 8), so the Extend C18 column was chosen, allowing a pH range of 2 to 11.5 to be employed.

The chromatographic procedure developed for THC and metabolites was validated by determining accuracy, precision, linearity, limits of detection and quantitation, selectivity, and extract stability. The limit of quantitation was 0.5 ng/mL for THC, 2 ng/mL for 11-OH-THC, and 4 ng/mL for THCA. These limits meet or surpass the recommended concentrations for the analysis of cannabinoids in whole blood published by Farrell et al. (1) who indicated a confirmatory cut-off concentration of 2 ng/mL for THC and 11-OH-THC; 5 ng/mL for THCA, be employed in cases of driving under the influence of drugs. In 2006, Khiabani et al. (8) reported on the relationship between THC blood concentrations and impairment in apprehended drivers. They showed that impaired drivers had a higher THC blood concentration (median 2.5 ng/mL) compared to non-impaired (median 1.9 ng/mL). Further, drivers with a THC blood concentration above 3 ng/mL were considered to be of considerable risk of
being judged as impaired (8). The importance of including 11-OH-THC in the assay was underscored by the observation that the sum of THC and 11-OH-THC blood concentrations provided a better estimate of impairment than THC alone, following known doses of dronabinol (9).

**Precision, accuracy, and extract stability**

The accuracy and precision of the assay were determined as described, and the data are presented in Table II. The procedure was very accurate, with a minimum variation of –0.13% for THC at 50 ng/mL and a maximum variation of –13.5% from the fortified level for 11-OH-THC at 7.5 ng/mL. Interday (between day) and intraday (same day) precision of the assay was determined using replicate analyses. The maximum interday precision was 11% at a concentration of 7.5 ng/mL for THCA; the maximum interday precision was 5.7% at a concentration of 7.5 ng/mL for THCA.

Quality controls at 7.5 ng/mL and 50 ng/mL were included with each batch. In order for the batch to be accepted, the controls were required to be within 20% of their stated value.

External QC controls were purchased from UTAK, and internal QC controls were prepared using separate lot numbers from the calibration standards.

The stability of cannabinoids in whole blood has been reported showing cannabinoids in glass containers to be stable when refrigerated for at least four months (10). The extracted specimens were stable for at least three days when kept in the instrument rack inside the autosampler, which was maintained at 7°C. There was less than a 5% difference in the quantitation of the extracts after 24 and 72 h.

Linearity was obtained with an average correlation coefficient for all the drugs of > 0.99 over the range from 0.5 to 100 ng/mL of blood for THC, 2 to 100 ng/mL for 11-OH-THC, and 5 to 100 ng/mL for THCA. The mean correlation coefficient and equation of the calibration curve for the cannabinoids based on five replicate analyses on five separate days were

\[ r^2 = 0.9973, \quad y = 0.1121x \quad (SD \ 0.0094) - 0.014 \quad (SD \ 0.0041) \] (THC); \[ r^2 = 0.997, \quad y = 0.0883x \quad (SD \ 0.0031) + 0.0248 \quad (SD \ 0.0092) \] (THCA); and \[ r^2 = 0.9987, \quad y = 0.1021x \quad (SD \ 0.0084) + 0.0021 \quad (SD \ 0.023) \] (11-OH-THC). The allowable qualifying ratio for the intensity of the second transition for THC was 67.9 to 101%; for 11-OH-THC; 12.6 to 19.0% and for THCA: 48.0 to 72.0%. The ranges were an average of five replicates over five days at the 5 ng/mL concentration.

### Table II. Cannabinoids in Blood using LC–MS–MS

<table>
<thead>
<tr>
<th>Target Concentration</th>
<th>THC</th>
<th>11-OH-THC</th>
<th>THCA</th>
<th>THC</th>
<th>11-OH-THC</th>
<th>THCA</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.5 ng/mL</td>
<td>7.5</td>
<td>6.2</td>
<td>7.6</td>
<td>48.6</td>
<td>47.0</td>
<td>43.9</td>
</tr>
<tr>
<td>50 ng/mL</td>
<td>7.3</td>
<td>6.4</td>
<td>6.8</td>
<td>49.8</td>
<td>46.2</td>
<td>47.4</td>
</tr>
<tr>
<td></td>
<td>7.0</td>
<td>6.7</td>
<td>7.2</td>
<td>50.7</td>
<td>44.3</td>
<td>45.6</td>
</tr>
<tr>
<td></td>
<td>7.6</td>
<td>6.4</td>
<td>6.6</td>
<td>50.8</td>
<td>44.6</td>
<td>43.8</td>
</tr>
<tr>
<td></td>
<td>7.2</td>
<td>6.7</td>
<td>7.6</td>
<td>49.3</td>
<td>45.3</td>
<td>44.0</td>
</tr>
<tr>
<td></td>
<td>6.9</td>
<td>6.5</td>
<td>7.0</td>
<td>50.4</td>
<td>44.2</td>
<td>44.8</td>
</tr>
<tr>
<td>Mean</td>
<td>7.2</td>
<td>6.4</td>
<td>7.1</td>
<td>49.9</td>
<td>45.2</td>
<td>44.9</td>
</tr>
<tr>
<td>CV (%)</td>
<td>3.7</td>
<td>2.9</td>
<td>5.7</td>
<td>1.74</td>
<td>2.50</td>
<td>3.11</td>
</tr>
<tr>
<td>Deviation from target</td>
<td>–3.3</td>
<td>2.9</td>
<td>–4.8</td>
<td>0.13</td>
<td>9.47</td>
<td>–10.1</td>
</tr>
</tbody>
</table>

### Table III. Matrix Effects for Δ⁹-Tetrahydrocannabinol (THC), 11-nor-9-Carboxy-Δ⁹-tetrahydrocannabinol (THCA), and 11-Hydroxy-Δ⁹-tetrahydrocannabinol (11-OH-THC) in Whole Blood at a Concentration of 5 ng/mL

<table>
<thead>
<tr>
<th>Drug</th>
<th>Extracted blood (R_E)</th>
<th>Post-extracted blood (R_PES)</th>
<th>Non-extracted blood (R NES)</th>
<th>% SPE Recovery (R_ES/R_PES × 100)</th>
<th>% Matrix Effect (R_PES/R NES – 1 × 100)</th>
</tr>
</thead>
<tbody>
<tr>
<td>THC</td>
<td>974</td>
<td>1589</td>
<td>3185</td>
<td>61.2</td>
<td>–50.1</td>
</tr>
<tr>
<td>THCA</td>
<td>1579</td>
<td>2208</td>
<td>19,293</td>
<td>71.5</td>
<td>–88.5</td>
</tr>
<tr>
<td>11-OH-THC</td>
<td>4769</td>
<td>7391</td>
<td>18,920</td>
<td>64.5</td>
<td>–60.9</td>
</tr>
</tbody>
</table>
Interference

Blood specimens collected from drug-free individuals (n = 3) showed no interference with any of the assays. All authentic research blood samples received into the laboratory for analysis were collected in gray-topped tubes containing sodium fluoride and potassium oxalate. For exogenous interferences, commonly encountered drugs of abuse, cannabinol, and cannabidiol were studied as described in the Validation section. The absence of interference was assessed by the observation that no chromatographic interference was observed in the channels of the monitored transitions. The quantitation of the cannabinoids was not affected by the presence of the other drugs. Matrix effects were reduced, though not eliminated, through the use of sample dilution and a specific solid-phase extraction. Samples were free from residue upon drying which provided a clean baseline. Window groupings reduced the baseline noise and provided a higher signal for weaker qualifying peaks.

Extraction recovery, ion suppression, and matrix effects

Chambers et al. (7) stated that the solid-phase extraction of drugs from complex matrices reduced ion suppression effects. Accordingly our specimens were subjected to extraction using CereX PolyChrom THC columns specifically intended for the extraction of cannabinoids from whole blood. Also, using the Chambers et al. (7) paper as a template, we determined the percentage recovery of the cannabinoids from the blood matrix and assessed matrix effects. The results are shown in Table III.

The extraction itself was relatively efficient for all three drugs (over 60% at 5 ng/mL, over 70% at 10 ng/mL), but ion suppression even after protein precipitation and solid-phase extraction was significant for all drugs (Table III). Figure 1 shows the response for the primary transition for the extracted sample (RES), the post-extracted sample (RRes), and the non-extracted sample (RRes) at a concentration of 5 ng/mL of THC. Ion signal was suppressed for all the cannabinoids, but the use of deuterated internal standards allowed accurate quantitation. The sensitivity of the instrument was adequate for the determination of cannabinoids in whole blood even when significant suppression of the signal was occurring, and two transitions were monitored for each drug.

Authentic specimens

The described procedure is in routine use. Figure 2 shows the extracted transitions for an authentic negative blood sample, a calibration standard at a concentration of 5 ng/mL, and an authentic positive specimen. The authentic specimen was confirmed, having screened positively for cannabinoids, and was found to contain 20 ng/mL THCA and 1.8 ng/mL THC. The specimen did not show the presence of 11-OH-THC.

Conclusions

The determination of THC, THCA, and 11-OH-THC in whole blood has been described. The LC–MS–MS procedure was reproducible, robust, and precise. The assay included monitoring a qualifying transition and calculation of a ratio required to be within 20% of that of a known calibration standard in order for definitive identification to be made.
References