Validation of a Cannabinoid Quantitation Method Using an Agilent 6430 LC/MS/MS

Application Note

Forensics

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Abstract

A method was developed and validated for the quantitation of cannabinoids with LC/MS/MS using an Agilent 6430 Triple Quadrupole LC/MS system. Validation showed that the LC/MS/MS method provides reliable results for the quantitation of THC, OH-THC, and carboxy-THC that meet predetermined acceptance criteria [1]. The concentration range of target compounds used in this validation was chosen to fit the commonly encountered range of analyte concentrations seen in casework. The method displays excellent accuracy and precision for the detection of cannabinoids in blood. Other aspects evaluated during validation include interference, stability, dilution integrity, suppression/enhancement, and recovery for all target compounds.

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Introduction

Cannabinoids are analyzed in urine, oral fluid, and blood in many forensic toxicology laboratories. Quantitative analysis of cannabinoids in blood, specifically THC, is necessary for meaningful toxicological interpretation in the investigation of DUID cases. The quantitation and confirmation of cannabinoids for DUID cases constitutes a significant portion of the workload for many forensic toxicology laboratories worldwide. Cannabinoid analysis in blood has been driven by advances in GC/MS technology, notably 2D-GC/MS [2] and GC/MS/MS with column backflushing [3]. During the last decade, the role and progress of liquid chromatography triple quadrupole mass spectrometry (LC/MS/MS) in forensic and clinical toxicology has been assessed several times by leading experts in the field. This technique is becoming increasingly important in routine toxicological analysis [4].

This application addresses the development and validation of an LC/MS/MS method on an Agilent 6430 Triple Quadrupole LC/MS system for the quantitation of THC and its metabolites. This LC/MS/MS method has the advantage of simpler sample preparation, without derivatization, compared to standard GC/MS and GC/MS/MS methods. It uses liquid-liquid extraction at acidic pH to eliminate artifactual elevation of carboxy-THC concentration from hydrolysis of carboxy-THC glucuronide. Validation studies included linearity and calibration model fits, precision and accuracy, sensitivity measured by the limit of detection (LOD) and limit of quantitation (LOQ), and interferences. In addition, the method was also evaluated and validated for robustness, carryover, dilution integrity, stability, and ion suppression/enhancement. The method met all criteria for data integrity, and was found to be a reliable method for routine cannabis analysis in toxicology studies.

Experimental

The method was validated for the target compounds shown in Table 1.

Table 1. Target Compounds and Internal Standards

Target	Internal Standard
THC	THC-d3
Carboxy-THC	Carboxy-THC-d3
OH-THC	OH-THC-d3

The method includes an acidic liquid-liquid extraction with quantitation and confirmation by an Agilent 6430 Triple Quadrupole LC/MS system, using MassHunter Quantitative Analysis (B.0.4) software for data acquisition and analysis. Whole blood samples were extracted using 9:1 hexane:ethyl acetate with 10 % acetic acid in accordance with the Virginia Department of Forensic Science's Procedures Manual [1]. A more detailed explanation of the method, including sample preparation, and instrument parameters, is detailed in "Cannabinoid Quantitation Using an Agilent 6430 LC/MS/MS" [5].

Sample preparation

Validation studies were performed using pooled and spiked standards. Samples were extracted using the procedure outlined in "Cannabinoid Quantitation Using an Agilent 6430 LC/MS/MS" [5]. Pooled standards were prepared spiking a large volume of blank blood with respective concentrations of THC/carboxy-THC. The samples were rotated for 1 hour and then stored between 4–8 °C for 72 hours prior to use. One-milliliter aliquots were taken from the pooled samples when required for validation experiments. Table 2 describes the preparation of pooled samples.

Table 2. Preparation of Pooled Samples

Concentration (ng/mL)	Volume 1/5 µg/mL Working Standard (µL)	Volume Blank Blood (mL)
1/5	50	50
3/15	150	50
10/50	500	50
75/375	3.75	50
120/600	6.0	50

Spiked standards were prepared by pipetting appropriate volumes of working standard solutions into clean test tubes.

Working standard solution (1/5 µg/mL): Pipette 25 µL/125 µL of the 1.0 mg/mL stock solution standards (THC, OH-THC/Carboxy-THC) into a 25-mL volumetric flask and qs to volume with methanol.

Working standard solution (0.1/0.5 µg/mL): Pipette 1 mL of the 1/5 µg/mL working standard solution into a 10-mL volumetric flask and qs to volume with methanol.

Working internal standard solution (1 µg/mL): Pipette 100 µL of the 0.1 mg/mL (or 10 µL of 1.0 mg/mL) stock solution of deuterated standards into a 10-mL volumetric flask and qs to volume with methanol.

Working internal standard solution (0.1 µg/mL): Pipette 1 mL of the 1 µg/mL working internal standard solution of deuterated standards into a 10-mL volumetric flask and qs to volume with methanol.

Results and Discussion

Chromatography

Figure 1 shows an example chromatogram of an extracted sample illustrating the chromatographic separation achieved with this method. As demonstrated in Figure 1, separation of the three targets is satisfactory with a run time of less than 12.5 minutes. Peak shape is excellent with no significant tailing or other chromatographic abnormalities.

Linearity and calibration Model Fit

Seven calibrators were analyzed with every batch and used to assess the linearity of the instrumental response. To establish the calibration model, the origin was ignored, the correlation coefficient (R^2) could not be ≥ 0.985, and the back calculated concentration had to be within ± 20% of the target. Upon assessment of the calibration curves, it was determined that a weighted linear fit was the most appropriate calibration model for the three targets [6].

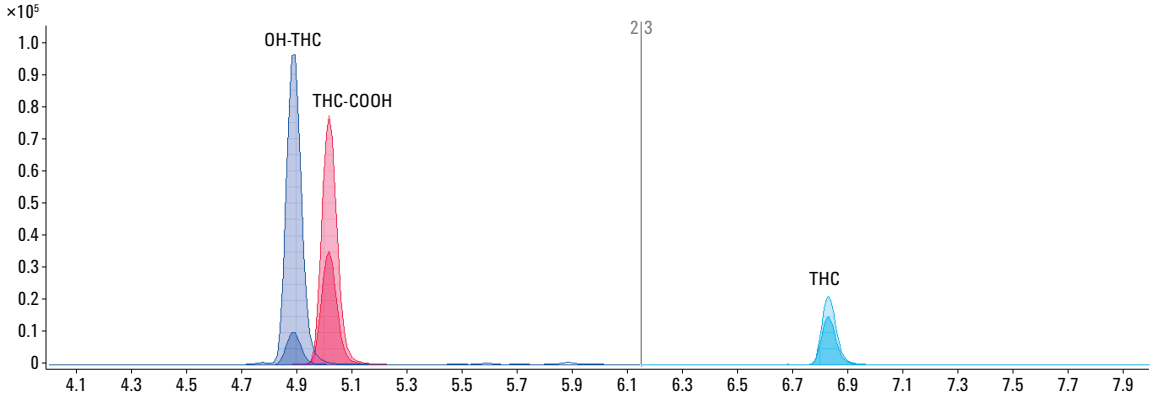

Figure 1. Chromatogram of target analytes.

The R^2 coefficients for all batches in this study were greater than 0.992 ± 0.007 for all target compounds, which meets the acceptance criteria of ≥ 0.985 (Table 3). The average slope was determined over 14 batches for THC and carboxy-THC with an average slope of 0.1031 ± 0.002 and 0.0618 ± 0.001 respectively. The slope was, therefore, determined to be stable over multiple days of analysis. The average slope and correlation coefficient for OH-THC were evaluated over a total of seven batches on different days. The average slope was also consistent for this target over a period of batches per days.

The linear dynamic range for THC and OH-THC in this study was 1.0–100.0 ng/mL while the dynamic range for carboxy-THC was 5.0–500.0 ng/mL.

Accuracy

Accuracy studies were conducted with both spiked and pooled samples. Spiked samples were fortified each time prior to extraction. Pooled samples were fortified into 50 mL of blank blood and a 1-mL aliquot was taken and extracted.

The acceptance criterion for the spiked accuracy was ± 20% for all three concentration levels. The pooled accuracy acceptance criterion was also ± 20% for all concentrations except at the LOQ. The acceptance criterion at the LOQ was ± 30%. All extractions were used to determine the overall accuracy for the method.

Table 4 represents the accuracy of the spiked blood samples. The *n* was 15 for the 5.0/2.5 ng/mL concentration and 21 for the 2.5/12.5 ng/mL and 10.0/50.0 ng/mL concentrations.

The spiked accuracy ranged from 106 ± 6% to 93 ± 7% for all analytes. All targets are within the acceptance criteria of ± 20%.

Table 5 details the accuracy results of the pooled blood samples. THC and carboxy-THC were the only targets investigated during the pooled accuracy analysis and are the only targets quantitatively assessed in the final standard operating procedure. All extractions were used to establish an *n* of 18.

The pooled accuracy for both targets are within the predetermined acceptance criteria. THC at a concentration of 1.0 ng/mL had an accuracy of 127 ± 18%. The accuracy at 1.0 ng/mL is within the acceptance criteria of ± 30% since 1.0 ng/mL is the LOQ for THC. All other concentrations are within the acceptance criteria of ± 20%. The accuracy ranges for all other concentrations not at the LOQ are between 109 ± 10% and 103 ± 8%. Both THC and carboxy-THC can accurately be quantitated throughout the calibration curve. They were also successfully quantitated at the upper LOQ after being diluted by one half.

Table 3. Calibration Reproducibility of Cannabinoids Using LC/MS/MS

Target	Dynamic range (ng/mL)	Calibration model	Average R^2	Average slope
THC	1.0–100.0	Linear	0.998 ± 0.013	0.1031 ± 0.002
Carboxy-THC	5.0–500.0	Linear	0.999 ± 0.006	0.0618 ± 0.001
OH-THC	1.0–100.0	Linear	0.998 ± 0.006	0.0959 ± 0.001

Table 4. Accuracy of the Spiked Blood Samples

	% Accuracy (SD)		
	2.5/12.5 ng/mL* *n* = 21	5.0/25.0 ng/mL* *n* = 15	10.0/50.0 ng/mL* *n* = 21
THC	101 (15)	100 (11)	105 (10)
Carboxy-THC	103 (13)	106 (6)	105 (5)
OH-THC	94 (11)	93 (7)	99 (8)

* The concentrations represent the THC, OH-THC, and carboxy-THC.

Table 5. Percent Accuracy/Bias for Pooled Cannabinoids Quantitated by LC/MS/MS

	% Accuracy (SD); n = 18				
	1.0/5.0 ng/mL*	3.0/15.0 ng/mL*	10.0/50.0 ng/mL*	75/375 ng/mL*	120/600 ng/mL*
THC	127 (18)	108 (10)	105 (8)	106 (12)	109 (10)
Carboxy-THC	97 (15)	104 (10)	108 (9)	103 (8)	104 (9)

* The concentrations represent THC/carboxy-THC.

Precision

The inter-run and intra-run precisions were also assessed for both spiked and pooled blood samples. Spiked samples were fortified each time prior to extraction. Pooled samples were fortified into 50 mL of blank blood and a 1-mL aliquot was taken and extracted. Results of these analyses are shown in Tables 6–9.

The precision of the samples was measured and evaluated as the coefficient of variance (% CV) for the inter-run and intra-run analyses. The standard acceptance criteria for inter-run and intra-run precision was ± 20% at each concentration level.

The inter-run precision, as shown in Table 6, ranged from 15% to 5% CV, and was within the acceptance criteria for all targets.

The intra-run precision is described in Table 7 for spiked cannabinoids. The % CV range for the intra-run precision was between 6% and 0.4%, within the predetermined acceptance criterion of ± 20%. The method meets the predetermined acceptance criteria for both inter-run and intra-run precision of spiked samples.

The inter-run precision for the pooled blood samples ranged from 16% to 8% CV, while the intra-run precision for the pooled blood samples ranged from 6% to 1% as shown in Tables 8 and 9. All targets are within the predetermined acceptance criterion of ± 20% (% CV). The precision of the pooled blood samples indicates that the analysis of both THC and carboxy-THC are precise at all points on the calibration curve including the LOQ and ULOQ.

The interpretation of the accuracy and precision for both spiked and pooled samples indicates that the method is both accurate and precise for both THC and carboxy-THC. The targets are well within the acceptance criteria.

Table 6. Inter-run Precision of Spiked Cannabinoids Quantitated by LC/MS/MS

	Mean ± SD ng/mL (% CV); n = 15		
	2.5/12.5 ng/mL*	**5.0/25.0 ng/mL***	**10.0/50.0 ng/mL***
THC	2.54 ± 0.38 (15)	5.00 ± 0.57 (11)	10.49 ± 0.95 (9)
Carboxy-THC	12.81 ± 1.60 (13)	26.40 ± 1.49 (6)	52.68 ± 2.44 (5)
OH-THC	2.3 ± 0.3 (11)	4.7 ± 0.3 (7)	9.9 ± 0.8 (8)

* The concentrations represent the THC, OH-THC, Carboxy-THC.

Table 7. Intra-run Precision of Spiked Cannabinoids Quantitated by LC/MS/MS

	Mean ± SD ng/mL (% CV); n = 3		
	2.5/12.5 ng/mL*	**5.0/25.0 ng/mL***	**10.0/50.0 ng/mL***
THC	2.45 ± 0.01 (0.4)	4.46 ± 0.16 (4)	9.04 ± 0.16 (2)
Carboxy-THC	13.48 ± 0.043 (3)	25.66 ± 0.83 (3)	51.96 ± 1.27 (2)
OH-THC	2.5 ± 0.1 (4)	4.5 ± 0.2 (3)	9.3 ± 0.5 (6)

* The concentrations represent the THC, OH-THC, Carboxy-THC.

Table 8. Inter-run Precision of Pooled Cannabinoids Quantitated by LC/MS/MS

	Mean ± SD (% CV); n = 18				
	1.0/5.0 ng/mL*	**3.0/15.0 ng/mL***	**10.0/50.0 ng/mL***	**75/375 ng/mL***	**120/600 ng/mL***
THC	1.28 ± 0.17 (14)	3.24 ± 0.31 (10)	10.46 ± 0.84 (8)	79.63 ± 9 (12)	130.25 ± 11 (9)
Carboxy-THC	4.87 ± 0.75 (16)	15.57 ± 1.57 (10)	53.92 ± 4.40 (8)	384.76 ± 30 (8)	624.65 ± 54 (9)

* The concentrations represent THC/Carboxy-THC.

Table 9. Intra-run Precision of Pooled Cannabinoids Quantitated by LC/MS/MS

	Mean ± SD (% CV); n = 6				
	1.0/5.0 ng/mL*	**3.0/15.0 ng/mL***	**10.0/50.0 ng/mL***	**75/375 ng/mL***	**120/600 ng/mL***
THC	1.10 ± 0.04 (3)	2.97 ± 0.11 (4)	9.62 ± 0.17 (2)	73.35 ± 1.95 (3)	117.16 ± 1.29 (1)
Carboxy-THC	4.10 ± 0.11 (3)	14.13 ± 0.45 (3)	49.65 ± 0.96 (2)	362.74 ± 11 (3)	564.14 ± 33 (6)

* The concentrations represent THC/Carboxy-THC.

Sensitivity (LOD, LOQ)

The LOD and lower LOQ were evaluated with samples spiked at 1.0, 2.5, and 5.0 ng/mL along with the calibrators at 1.0–200 ng/mL. The standard identification criteria for LOD were ± 5% for retention time, ± 20% for qualifier ion ratio and a signal-to-noise level of at minimum 3:1. The retention time and qualifier ion ratio were compared to the average of the calibrators. The standard identification criteria for LOQ were ± 5% for retention time, ± 20% for qualifier ion ratio and a signal-to-noise level of at minimum 10:1. The retention time and qualifier ion ratio were compared to the average of the calibrators. The back calculated concentration should also be within ± 20% of the target concentration for LOQ. Samples were required to meet the acceptance criteria in ≥ 75% of the samples to be established as the target LOD and LOQ. Table 10 lists the LOD and LOQ for the target compounds.

THC and OH-THC both meet the criteria for LOD and LOQ at 1.0 ng/mL while carboxy-THC meets the LOD criteria at 2.5 ng/mL and the LOQ criteria at 5.0 ng/mL.

Recovery

Recovery was assessed with three different concentrations over a period of four batches. The extracted control response was compared to a double blank blood sample that was spiked with both internal standard and control after extraction. The raw instrumental response was used to calculate the average recovery for each concentration. Table 11 represents the average percent recovery for both THC and carboxy-THC at 5.0/25.0, 10.0/50.0, and 25.0/125 ng/mL respectively.

The average percent recovery was approximately 50% for THC and carboxy-THC at all three concentration levels. With the use of deuterated internal standard, the percent recovery does not have an effect on the overall calculated concentration after extraction.

Table 10. LOD and LOQ for Cannabinoids Using LC/MS/MS

Target Compound	LOD (ng/mL)	LOQ (ng/mL)
THC	1.0	1.0
Carboxy-THC	2.5	5.0
OH-THC	1.0	1.0

Table 11. Average Percent Recovery for THC and Carboxy-THC Using a Liquid/Liquid Extraction

	Recovery (%)		
	Recovery 5/25 ng/mL*	Recovery 10/50 ng/mL*	Recovery 25/125 ng/mL*
THC	47	55	54
Carboxy-THC	50	51	52

* The concentrations represent THC/Carboxy-THC.

Interference studies

Interferences from endogenous compounds, internal standard, target analytes, and commonly encountered analytes were evaluated. There should be no source of interference for the method to be accepted. The results showed that no interferences were detected for all targets. The commonly encountered analytes that were assessed for interference are tabulated in Table 12. At minimum, 10 different negative matrix samples were analyzed without the addition of internal standard for endogenous interferences. Also, a high concentration of internal standard with no target was analyzed for contribution of internal standard to target. A high concentration of target was also evaluated without internal standard to assess contribution from the target compound to internal standard.

Ion suppression and enhancement

Potential interference from ion suppression and enhancement was evaluated. These results are shown in Table 13 and illustrate that none of the target analytes produce interference from suppression or enhancement.

Carryover

Carryover was addressed by injecting progressively higher concentrations of target analytes followed by solvent blanks. The solvent blanks were monitored for any signs of carryover (such as contribution to the quantitation transition). The highest concentration injected was 500.0/2,500 ng/mL with no carryover detected.

Table 12. Interferents and Concentrations of Commonly Encountered Analytes

Drug Class	Drug	Concentration (µg/mL)
Opioids	Codeine, Morphine, Hydrocodone, Hydromorphone, Oxycodone, Oxymorphone	10
	6-Monoacetylmorphine	2.5
Cocaine	Cocaine, Cocaethylene, Benzoylecgonine	10
Amphetamine	Amphetamine, Methamphetamine Phentermine, MDA, MDMA	10
Benzodiazepines	Diazepam, Nordiazepam, Alprazolam, Midazolam, Clonazepam	10
Barbiturates	Butalbital, Secobarbital, Phenobarbital	100
Carisoprodol and Meprobamate	Carisoprodol, Meprobamate	100
Fentanyl	Fentanyl	1
Acetaminophen, Salicyclic acid	Acetaminophen, Salicylic acid	500
Base drugs	Clorpheniramine, Imipramine, Desipramine, Paroxetine, Trazodone	10
Acid/Neutral drugs	Ibuprofen, Butalbital, Acetaminophen, Meprobamate, Caffeine, Gluetheminde, Naproxen, Metaxolone, Carbamazepine, Diazepam	10

Table 13. Ion Suppression/Enhancement of Cannabinoids Using LC/MS/MS

Target	Average suppression/enhancement (%)
THC	92.9
Carboxy-THC	93.7
OH-THC	97.2

Note: Data from Batch 4 was not used due to fortifying inconsistencies; this data was deemed to be an outlier.

Stability

Processed/extracted sample stability

Processed/extracted sample stability was addressed through the daily injection of three control samples over a period of four days. The response ratio was averaged over the three samples and compared over the four day period. If the average response ratio deviated greater than ± 20%, the target was considered stable up to that time period. All targets analytes were stable over the four day period with deviations less than ± 10%.

Bench top stability

The stability of a sample at standard laboratory operating conditions was assessed by storing five concentrations of pooled blood samples on the bench top for over 24 hours. After 24 hours, the pooled samples were extracted in triplicate and analyzed. The concentration was compared to the mean calculated value of the pooled samples from time zero. The predetermined acceptance criterion was that the accuracy cannot exceed ± 20% of the previously determined mean. All samples at each concentration were within the acceptance criteria. This study indicates that the targets are stable in the blood matrix after being subjected to standard laboratory temperatures and humidity for at least 24 hours.

Dilution integrity

To address dilution integrity, a larger volume sample was fortified at approximately 50.0/250.0 ng/mL and samples were taken as undiluted (1.0 mL sample), 1:2 (0.5 mL sample), 1:4 (0.25 mL sample), and 1:5 (0.20 mL sample). The acceptance criteria for accuracy was ± 20% of the back calculated concentration. This study showed that the 1:2 and 1:4 dilutions met the acceptance criteria whereas the 1:5 dilution did not meet the designated criteria.

Previously analyzed or non-probative casework samples

Non-probative DUID casework samples were reanalyzed with the newly developed method. The results were then compared and used as an accuracy assessment. Minimum weight was placed on the result due to the age difference of the samples and the limited scope of testing when comparing the previous method to the newly developed method.

Conclusion

This method development and validation provides a rapid and sensitive technique for the detection and quantitation of cannabinoids by triple quadrupole LC/MS. THC, OH-THC, and carboxy-THC passed all criteria for the method validation, which proves that this method provides reliable quantitative results. The range used in this validation was chosen to fit the commonly encountered range of analyte concentrations seen in casework. It has been determined that this method is a valid means of analyzing cannabinoids in blood for routine drug analysis, providing quick, accurate, and reproducible results.

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