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LETTER TO THE EDITOR

Letter to the Editor regarding "Simultaneous determination of Δ^9-tetrahydrocannabinol and 11-nor-9-carboxy-Δ^9-tetrahydrocannabinol in oral fluid using isotope dilution liquid chromatography tandem mass spectrometry"

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Dear Sir,

We read with great interest the article entitled "Simultaneous determination of Δ^9-tetrahydrocannabinol and 11-nor-9-carboxy-Δ^9-tetrahydrocannabinol in oral fluid using isotope dilution liquid chromatography tandem mass spectrometry" by Lee et al. [1]. The authors describe a fast and very sensitive method to analyse dansyl derivatives of Δ^9-tetrahydrocannabinol (THC) and 11-nor-9-carboxy-Δ^9-tetrahydrocannabinol (THCCOOH) in oral fluid with limits of quantification of 25 and 5 pg/mL, respectively. Generally, liquid chromatography (LC)–mass spectrometry (MS) is preferred over gas chromatography–MS because it does not require prior derivatization and extensive sample clean-up, making this approach much less tedious and time consuming. However, this common rule has several exceptions. In this regard, there are some substances that do not show a signal sufficiently intense with the classic ion sources commonly used in LC-MS. Typically, the phytocannabinoids belong to this family. To overcome this problem, derivatization can be used to improve MS/MS detectability [2]. In this respect, Lee et al. reported the use of a chemical derivatization approach using isotope dilution LC-MS/MS with electrospray ionization in positive mode to detect and quantify THC and THCCOOH. Derivatization of the phenolic group was done with dansyl chloride (DC) at basic pH (10.5) at 70 °C for 5 min.

This method was tested in our laboratory with oral fluid samples spiked with a broader range of cannabinoids: THC, THCCOOH, 11-hydroxy-THC (11-OH-THC), cannabinol, cannabidiol, the two glucuronide conjugates THC–glucuronide (THC-gluc) and THCCOOH–glucuronide (THCCOOH-gluc) and the plant precursor of THC, Δ^9-tetrahydrocannabinolic acid A (THC-A). All except 11-OH-THC are known to be present in oral fluid, a few at high concentrations (THC, THC-A, cannabidiol), others in only tiny amounts (mainly the THC metabolites and their conjugates). Since this method involving derivatization followed by LC-MS/MS analysis has been advocated with other biological matrices, its evaluation with a more expanded range of cannabinoids is even more warranted.

Pure and dansylated standards were analysed first to determine the ion transitions and the best MS/MS parameters. Then, drug-free oral fluid samples spiked at different concentrations (5, 25, 50, 100, 250, 500 and 1,000 pg/mL) with the whole range of cannabinoids were pretreated, derivatized and extracted according to the protocol developed by Lee et al. [1]. These samples were also extracted according to our own procedure, which does not include a derivatization step [3]. Briefly, our method is as follows. To 250 μL of oral fluid, we added 10 μL of an internal standard solution of 5 ng/mL THC-d_3, 11-OH-THC-d_3 and THCCOOH-d_9. Then, the volume was made up to 1 mL with ammonium formate buffer (10 mM, pH 6.5) and the mixture was vortexed for 5 s. Oasis HLB extraction columns (Waters, Baden, Switzerland) were conditioned with 2 mL of methanol, 2 mL of deionized water and 2 mL of ammonium formate buffer (10 mM, pH 6.5). The samples were loaded by gravity. Cannabinoids were eluted with 3 mL of methanol. The eluates were evaporated to dryness and reconstituted in 150 μL of ammonium formate buffer (5 mM, pH 6.8)–acetonitrile (70:30, v/v). For both procedures, the LC parameters and the MS/MS equipment were the same. The separation was done on a Kinetex™ C_{18} 100-Å column (150 mm×2.1 mm inner diameter, 2.6 μm), (Phenomenex, Brechbühler, Echallens, Switzerland). Gradient elution was performed using a mixture of ammonium formate buffer at 5 mM, pH 6.8 (solvent A) and acetonitrile (solvent B)

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at a flow rate of 400 μL/min. The initial gradient conditions were as follows: 30 % solvent B, hold for 30 s, and then linearly increase to reach 90 % solvent B at 7 min. The conditions using 90 % solvent B were maintained for 2 min. Then, the gradient was decreased to 30 % solvent B over 30 s and held for 2.5 min. Then, 10 μL of the sample was injected into the LC-MS/MS system. Analytes were detected using an Applied Biosystems SCIEX API 5000 triple-quadrupole mass spectrometer with a TurboIonSpray interface. As described by Lee et al., the dansylated derivates were registered in positive electrospray ionization mode with the same multiple reaction monitoring transitions. In our procedure, MS transitions were recorded using a scheduled multiple reaction monitoring mode with three separate time windows. The first time window (from the start to 5.1 min) was operated in negative ionization mode, whereas the second (from 5.1 to 6.4 min) and the third (from 6.4 to 7.5 min) windows were operated in the positive mode. THC-A, THCCOOH and the glucuronides were analysed in the negative mode to achieve better sensitivity.

Specific analyses for THC-A and glucuronide conjugates were also conducted. Drug-free oral fluid samples were spiked with THC-A, THC-gluc or THCCOOH-gluc at a concentration of 100 ng/mL and were analysed with the method of Lee et al. As deuterated homologues of these compounds were not commercially available, THC-d_3 was used as the internal standard for THC-gluc and THCCOOH-d_9 was selected for THC-A and THCCOOH-gluc. Calibration curves were obtained with six calibration levels (0.1, 0.5, 5, 10, 25 and 50 ng/mL) for THC, THCCOOH, cannabidiol, cannabinol and 11-OH-THC.

Therefore, two different methods in the analysis of cannabinoids in oral fluid were used and compared. What differentiates these two methods is the dansylation step used by Lee et al. as it combines a simple liquid–liquid extraction, whereas our method does not require any derivatization even though it involves a longer purification on solid-phase cartridges. This latter step was necessary to extract the underivatized glucuronide conjugates from oral fluid. Derivatization with DC was done at basic pH (10.5) and 70 °C for 5 min. With the method developed in our laboratory, samples were analysed straightforwardly at almost neutral pH (6.8) and without heating. The other parts of the methods were similar and rely upon LC-MS/MS analysis with electrospray ionization and reverse-phase chromatographic separation using a C_{18} column.

Our results confirm and extend those of Lee et al.: the sensitivity for THC and THCCOOH detection was improved, as was that for cannabinol, cannabidiol and 11-OH-THC detection. In this respect, the limits of detection (LOD) were lower for these five cannabinoids using the method developed by Lee et al.: the LOD of THCCOOH, 11-OH-THC and cannabinol decreased from 100 to 5 pg/mL, the LOD of THC decreased from 50 to 25 pg/mL and the LOD of cannabidiol was 50 pg/mL with DC derivatization, whereas it was 300 pg/mL with our method. However, we failed to detect the dansylated THC-A, THC-gluc and THCCOOH-gluc. The reasons of their non-detection were investigated, and one of the most important factors could be the derivatization conditions. It has been proved that THC-A degrades into THC at high temperature [4]. Incubation of DC with cannabinoids at 70 °C for several minutes could lead at least to partial thermal conversion of THC-A into THC. Furthermore, the basic conditions (obtained with 100 mM sodium bicarbonate buffer at pH 10.5) have been proved to be sufficient to hydrolyse THCCOOH-gluc [5] and could explain the non-detection of the glucuronide conjugates.

To test these hypotheses, the following experiments were performed to assess whether THC-A is converted into THC and whether the glucuronides are hydrolysed to their corresponding free cannabinoids during the dansylation step. Drug-free oral fluid samples were spiked with THC-A, THC-gluc or THCCOOH-gluc at a concentration of 100 ng/mL. Samples were extracted and analysed according to the protocol developed by Lee et al. [1]. For the oral fluid specimens spiked with THCCOOH-gluc, DC-THCCOOH was measured at a concentration of 20 ng/mL. Taking into account their molar differences, we found that more than 30 % of THCCOOH-gluc was hydrolysed to THCCOOH during the derivatization process. For samples spiked with THC-A, DC-THC was detected at a concentration of 9 ng/mL. This level corresponds to 10 % of the initial concentration of THC-A. For THC-gluc samples, only 2 ng/mL DC-THC was detected (4 %). As expected, our analyses revealed that dansylation resulted in significant hydrolysis of THCCOOH-gluc to THCCOOH. For THC-A and THC-gluc, THC was detected in low concentrations only. These results indicate that the glucuronide conjugates of THC and THC-A are partly converted into THC when dansylated. Therefore, quantitative results of dansylated cannabinoids must be interpreted with great caution because they represent a partial sum of free and conjugated metabolites of THC and THC-A. However, if qualitative results only are required, the method using dansylation should be preferred because it has better sensitivity. For blood, this method is not recommended because the concentrations of free cannabinoids could be wrongly increased.

More than 80 metabolites of THC have been identified [6]. In 2007, Jung et al. [7], who studied the metabolism of THC-A, reported 12 THC-A metabolites. We suggest that THC-gluc, THCCOOH-gluc, THC-A and other metabolites are also degraded, possibly into polymers, oxidized compounds and other cannabinoids that could not be detected with our analytical method. Recently, Schwilke et al. [8] suggested that the ether–glucuronide bond in THC is stabler than the ester–glucuronide bond in THCCOOH. This difference could explain the greater hydrolysis of THCCOOH-gluc compared with THC-gluc.

In the experimental design of Lee et al. [1], oral fluid samples of suspected cannabis users were analysed. They

were not concerned with the fact that after a user has smoked a joint, a significant amount of THC-A can be found in oral fluid [3, 9]. In this respect, THC-A was detected at a concentration of 1 ng/mL by Moore et al. [9] until 8 h after a user had smoked a joint. In another study, we detected THC-A until the end of the experiment, this being 4 h after the first puff of the joint had been inhaled. THCCOOH-gluc in very low concentrations was also detected.

As a conclusion, to obtain accurate measurements and a better comparison of results between laboratories, a method able to quantify separately each cannabinoid should be preferred. Furthermore, our results show that some minor cannabinoids and their metabolites which could be used as biomarkers to substantiate use [10] and/or to estimate the last time of use cannot be measured separately after derivatization with DC. Very recently, another approach was suggested by Coulter et al. [11], who advocate the use of picolylamine to derivatize the carboxylic function of THCCOOH in order to achieve the requisite sensitivity.

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