DEVELOPMENT AND VALIDATION OF A LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY METHOD FOR THE DETERMINATION OF CANNABINOIDS AND PHASE I AND II METABOLITES IN MECONIUM

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Abstract

A liquid chromatography–tandem mass spectrometry (LC–MSMS) method was developed and fully validated for the determination of Δ^9-tetrahydrocannabinol (THC), 11-hydroxyTHC (OHTHC), 11-nor-9-carboxyTHC (THCCOOH), 8-β-11-dihydroxyTHC (diOHTHC), cannabinol, cannabidiol, and THC and THCCOOH glucuronides in 0.25±0.02 g meconium. Samples were homogenized in methanol and subjected to cation exchange solid-phase extraction. Chromatographic separation was performed on a Kinetex C18 column (50 mm × 2.1 mm, 2.6 μm) at 35 ºC, with a gradient of 0.1% formic acid in water and acetonitrile at a flow rate of 0.3 mL/min; total run time was 10 min. Two transitions per analyte were monitored in MRM mode. The method was specific and sensitive; LOD was from 1 to 2ng/g, and LOQ from 4 to 10 ng/g; linearity ranged from 4 to 400 ng/g for all the analytes, except for THC glucuronide (10 to 400 ng/g); intra-assay, inter-assay and total imprecision were <11.2%, <13.45% and <15.6%, respectively; accuracy ranged from 93.9% to 109.0% of the target concentration; matrix effect, extraction and process efficiency ranged from -26.4% to -71.4%, 49.9% to 69.5% and 14.3% to 45.0%, respectively. The inclusion of THC and THCCOOH glucuronides avoided the need for the hydrolysis process, thus facilitating sample pretreatment. Application of the method to 19 authentic meconium specimens from uncontrolled pregnancies or women suspicious of drug consumption revealed fetal cannabis exposure in 4 newborns. THCCOOH (24.1-288.8 ng/g), diOHTHC (53.2-332.4 ng/g), THC (4.2-7.7 ng/g), CBN (30.7-93.3 ng/g) and CBD (7.1-251.5 ng/g) were detected in all cases; THCCOOH glucuronide (190.2-306.8 ng/g) in 3 cases; and OHTHC (11.9 ng/g) in the remaining one; however, THC glucuronide was not identified in any specimen.

Keywords: cannabis, metabolite, glucuronide, meconium, LC-MS/MS

Highlights

- A LC-MS/MS method was developed for the determination of cannabinoids in meconium.
- Main THC metabolites, including CarboxyTHC and THC glucuronides are quantified.
- Detection of THCCOOH glucuronide avoids hidrolysis to increase method sensitivity.
- The method was applied to 19 meconium specimens from uncontrolled pregnancies.

1. Introduction

According to the 2014 World Drug Report, 5.2% of the world population aged 15-64 had used an illicit drug in 2012. Among them, cannabis is the most widely consumed worldwide, with estimated consumption prevalence between 2.7% and 4.9% in this age range [1].

Drug use during pregnancy is related to a higher rate of fetal and medical obstetric complications [2-4]. There is still no definite consensus on the effects of prenatal exposure to cannabis, mainly due to the common association to other drugs. However, some studies reported that prenatal exposure to marijuana might have developmental consequences, shorter gestation length, decreased birth weight and deficit in other growth measures [5-8]. Other authors described sleep disturbances or high-pitched cry [9, 10], among other detrimental effects.

Data on drug use during pregnancy are very scarce. Prevalence of drug use among pregnant women is usually made by indirect estimation according to the information available on drug use surveys for women on childbearing age (15 to 44 years). The National Survey on Drug Use and Health is the only report available about prevalence of illicit drug users among pregnant women [11]. A direct method to obtain data about drug consumption during pregnancy is the maternal interview; however, drug use is usually underestimated due to maternal fear of legal repercussions and/or social stigmatization [12]. The analysis of biological matrices from the mother, the newborn, or tissues developed during pregnancy (placenta, umbilical cord, amniotic fluid) provides an objective determination of drug use during pregnancy, as reported by several authors [13-16]. Lendoiro et al. [13] determined by the analysis of hair segments that 15.4% of randomly selected pregnant women had consumed illicit drugs (12.4% cocaine, 3.8% cannabis, 1% opiates and 1% ketamine), 22.5% medicines (3.3% methadone, 11% benzodiazepines, 9.1% antidepressants, 1% zopiclone and 1.4% fentanyl) and 3.9% alcohol; these results were much higher than those provided by the maternal interview in that study (1.4% cocaine, 2.9% cannabis, 1% opiates, 1.9% methadone, 1.9% benzodiazepines, 0.5% antidepressants, and 13.7% alcohol) [13]. Falcon et al. [14], identified in serum and hair specimens illicit drug consumption in 30% pregnant women who voluntarily interrupted their pregnancy during the first trimester (20.4% were positive for cannabis, 14.1% for cocaine and 4.2% for opiates). García-Algar et al. [15] analyzed meconium specimens from a low socioeconomic population and detected 10.9% positivity for drugs of abuse (4.7% heroin, 2.6% cocaine and 5.3% cannabis); maternal interview from the same population identified heroin, cocaine and cannabis consumption in

0.3%, 1.2% and 1,5% of the pregnant women, respectively. Finally, Lozano et al. [16] detected the prenatal exposure to cannabis in 5.3% newborns.

Among the different matrices available, meconium, the first fecal matter from the newborn, is currently considered the sample of choice to detect prenatal exposure to drugs, as this matrix provides more complete information than other neonatal matrices such as urine or cord blood. Meconium starts its formation between the 12-16th week of pregnancy, and accumulates until birth [17,18]; therefore, it provides direct information about fetal drug exposure from the second or, more likely, the third trimester of pregnancy [19]. In addition, meconium is easily and noninvasively obtained directly from the diaper between 1 and 5 days after delivery. Moreover, meconium specimens remain stable when storage at ≤-20 °C and, under these conditions, cannabinoids remain unchanged for a period of at least six months [20].

Several methods for the determination of few cannabinoids in meconium have been published to date using GC or LC-MS [20-26]. Although identification of cannabinoides in meconium might be possible without hydrolyzing the sample in some cases, this process was performed in all reported methods as it was proved that hydrolysis increases the positivity identification rate. This process allows glucuronides cleavage, with the subsequent increase of the free analytes concentration and, therefore, the sensitivity of the method [21, 25]. A way to avoid sample hydrolysis without a negative impact on fetal cannabinoids exposure detection through meconium analysis could be the identification of the main glucuronides present in this matrix with their direct inclusion in the analytical method.

The aim of this work was the development and validation of a LC-MS/MS method for the determination of CBN, cannabidiol (CBD), and Δ^9-tetrahydrocannabinol (THC) and their main metabolites in meconium, including THC and 11-nor-9-carboxyTHC (THCCOOH) glucuronides, thus avoiding the hydrolysis step needed for the determination of the free analytes.

2. Materials and methods

2.1. Chemicals

THC, 11-hydroxyTHC (OHTHC), THCCOOH, CBN and CBD standars at 1 mg/mL, and THCCOOH glucuronide, and the deuterated internal standards (IStd) THC-d3, OHTHC-d3, THCCOOH-d3, CBN-d3 and CBD-d3 at 0.1 mg/mL in methanol were purchased from Cerilliant™ (Round Rock, TX, USA). 8-β-11-dihydroxyTHC (diOHTHC) and diOHTHC-d6 standards at 0.1 mg/mL in methanol, and THC glucuronide standard at 0.01 mg/mL in

methanol were from ElSohly Laboratories (Oxford, MS, USA). Water was purified with a Milli-Q water system (Millipore, Le-Mont-sur-Lausanne, Switzerland). Chromasolv® gradient grade methanol and reagent grade dichloromethane were from Sigma-Aldrich (Steinheim, Germany). Chromasolv® LC-MS grade 2-propanol was from Fluka. Reagent grade formic acid 98-100%, ammonium hydroxide 32% and hydrochloric acid 37%, and LC-MS grade acetonitrile were from Scharlau Chemie (Sentmenat, Spain). Solid phase extraction Oasis MCX cartridges (3 cc, 60 mg) were purchased from Waters Corp. (Milford, MA, USA).

2.2. Blank meconium specimens

For the preparation of the calibration curves and quality control (QC) samples we employed meconium specimens sent to the laboratory for toxicological analysis in which cannabinoids absence had been previously confirmed.

2.3. Preparation of calibration and QC solutions

For the preparation of the calibration curve, a working solution containing all the compounds, except diOHTHC and the glucuronides, at 10 µg/mL was generated in methanol from the individual ampoules. This solution was diluted to obtain 0.1 µg/mL working solution, to which diOHTHC and the glucuronides were added at the same concentration. Finally, this solution was further diluted to obtained 0.01 µg/mL working solution. Six to seven calibrators at 4, 10, 20, 40, 100, 200 and 400 ng/g were elaborated by addition of the appropriate volume of the described working solutions to blank meconium samples.

Different working solutions at 0.05, 0.1 and 1 µg/mL in methanol were prepared for the generation of low, medium and high QC samples (6, 30 and 300 ng/g, respectively).

A working solution containing all the IStds at 1 µg/mL was prepared by dilution of the original individual ampoules in methanol.

2.4. Sample pretreatment

0.25±0.01 g meconium were weighed into Pyrex® glass tubes. The tubes were centrifuged for a few seconds to push the sample to the bottom of the tube, and 25 µL of the IStd at 1 µg/mL and 1 mL of methanol were subsequently added for sample homogenization. After mechanical shaking for 30 min, the sample was centrifuged for 10 minutes at 5000 rpm. The supernatant was subsequently evaporated in a water bath at 40 °C with a stream of nitrogen gas in order to remove the methanol and reconditioned the sample at acid pH to performed the solid phase extraction (SPE). The extract was reconstituted in 200 µL methanol to increase cannabinoids solubility, vortexed, and 2 mL of 1% formic acid in water were added.

2.5. SPE procedure

Oasis MCX cartridges (3cc, 60 mg) were employed for cation exchange solid-phase extraction. The samples were directly loaded without a preconditioning step, and the cartridges was subsequently washed with 2 mL of acetonitrile:water (15:85, v/v). After 5 min cartridge drying, the analytes of interest were eluted using 2 mL of dichloromethane:2-propanol (50:50, v/v). The eluates were evaporated using a TurboVap LV evaporator (Zymark, Hopkinton, MA, USA) and reconstituted in 50 µL 0.1% formic acid in water:acetonitrile (60:40, v/v). The reconstituted extracts were transferred to Eppendorf tubes inside glass insert vials for centrifugation 10 minutes at 14000 rpm. Finally, the glass inserts were transferred into injection vials for LC-MS/MS analysis.

2.6. LC-MS/MS

The HPLC system was an Alliance 2795 Separation Module with an Alliance series column heater/cooler (Waters Corp.). Chromatographic separation was performed with a Kinetex C18 (50 mm x 2.1 mm, 2.6 µm) reversed-phase analytical column (Phenomenex®, Torrance, CA, USA), maintained at 35 ºC. A C18 security guard column for 2.1 mm internal diameter analytical columns (Phenomenex®) was employed. Formic acid 0.1% in water (A) and acetonitrile (B) were used as mobile phase at a flow rate of 0.3 mL/min. Gradient was programmed as follow: 40% B from 0 to 0.2 min, linearly increased to 100% until min 6, to return to initial conditions at min 6.8. A divert valve was set to direct the flow to the MS from 1.5 to 7.5 min, and to waste the remaining time. The autosampler was maintained at 6 ºC.

The mass spectrometer was a Quattro Micro™ API ESCI triple quadrupole (Waters Corp.). The instrument was operated in electrospray in the positive mode (ESI+) to produce protonated molecules of the analytes with the following optimized settings: capillary voltage 3.0 kV; source block and desolvation gas (nitrogen) temperature 150 ºC and 400 ºC, respectively; desolvation and cone gas (nitrogen) flow rate 800 L/h and 80 L/h, respectively. Data were recorded on multiple reaction monitoring (MRM) mode. A post-column infusion of each individual analyte (1 or 10 µg/mL, depending on the analyte sensitivity) at 10 µL/min connected with a "T" valve to the chromatographic effluent (formic acid 0.1% in water:ACN, 50:50, v/v) was employed to select MRM transitions, cone voltages and collision energies for the target analytes and IStds. Data acquisition was controlled with MassLynx 4.0 software and processed with QuanLynx 4.1 software (Waters Corp.).

2.7. Method validation

The following parameters were studied for method validation: linearity, selectivity, limits of detection (LOD) and quantification (LOQ), intra-assay, inter-assay and total imprecision,

accuracy, potential glucuronide hydrolysis, matrix effect, extraction and process efficiency [27, 28].

Linearity was evaluated by the analysis of calibration curves from 4 or 10 to 400 ng/g on four different days, using 6-7 calibration levels. The straight-line fit was performed by linear regression, and a weighting factor of 1/x was applied. Acceptable linearity was achieved if the coefficient of determination (r^2) was ≥0.99, and residuals were <20% at the LOQ, and <15% at the remaining concentrations.

Selectivity was evaluated by assessment of endogenous and exogenous interferences. Potential endogenous interferences were assessed by the analysis of blank meconium samples collected from 10 different sources, and fortified with the IStd. Exogenous interferences were assessed by the analysis of blank meconium samples fortified with 43 common drugs of abuse and medicines at 0.5 µg/g The following drugs were tested: morphine, codeine, 6-acetylmorphine, methadone, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP), amphetamine, methamphetamine, 3,4-methylendioxyamphetamine (MDA), 3,4-methylendioxymethamphetamine (MDMA), 3,4-methylendioxyethylamphetamine (MDEA), cocaine, benzoylecgonine, ecgonine methylester, cocaethylene, lysergic acid diethylamide (LSD), ketamine, norketamine, gammahydroxybutyric acid (GHB), nicotine, cotinine, fentanyl, amitriptyline, paroxetine, zolpidem, zopiclone, ibuprofen, omeprazole, acetaminophen, diclofenac, naproxen, alprazolam, temazepam, lormetazepam, lorazepam, flunitrazepam, 7-aminoflunitrazepam, clonazepam, diazepam, nordiazepam, oxazepam, triazolam, nitrazepam, bromazepam.

The LOD was defined as the lowest concentration at which the two MRM transitions monitored for each analyte could be identified with a signal-to-noise >3 and appropriate ion ratio, and within ±0.2 min of the mean calibrators retention time. LOD was determined by the analysis of fortified blank mecomium samples at decreasing concentrations.

LOQ was defined as the lowest concentration that could be quantified with adequate precision (%CV <20%) and accuracy (% of target concentration ±20%), and with a signal-to-noise >10. The LOQ was calculated by the analysis of five replicates at the lowest concentration of the calibration curve.

Imprecision and accuracy were assessed at low, medium and high QC concentrations for THC, OHTHC, THCCOOH, diOHTHC, CBN, CBD and THCCOOH glucuronide, and at medium and high QC concentrations for THC glucuronide. These parameters were evaluated by the analysis of five replicates for each concentration on four different days (n=20). Intra and inter-assay, and total imprecision was determined by calculation of the coefficient of

variation (%CV) following Krouwer and Rabinowitz' recommendations [29], and using SPSS v. 20.0 statistical software. %CV was required to be less than 15%. Accuracy was expressed as the percentage of the nominal concentration, and was required to be within 85-115%.

Possible hydrolysis of THC and THCCOOH glucuronides was evaluated by the analysis of meconium fortified only with THC glucuronide at 300 ng/g and the IStd (n=3), and meconium fortified only with THCCOOH glucuronide at 300 ng/g and the IStd (n=3).

Matrix effect, extraction and process efficiency were calculated at 30 and 300 ng/g following Matuszewski et al. recommendations [30]. Matrix effect was assessed by comparing average analyte peak area in blank meconium samples from 10 different sources fortified after extraction, with average peak area when the analytes were prepared at the same concentration in formic acid 0.1% in water:ACN (60:40, v/v) (n=5). Extraction efficiency was calculated by comparing average analyte peak area in samples fortified with the analytes before extraction (n=5) with average peak area obtained in blank samples fortified after extraction (n=5). Process efficiency was evaluated by comparing average analyte peak area in samples fortified with the analytes before extraction (n=5) with average peak area when the analytes were prepared at the same concentration in formic acid 0.1% in water:ACN (60:40, v/v) (n=5).

2.8. Application to authentic cases

As a proof of the method, 19 authentic meconium specimens received in our laboratory for toxicological analysis during 2015 were analyzed using the described LC-MS/MS method.

3. Results

3.1. Method development and validation

Chromatographic elution of all the analytes was achieved in 6.5 min, and the total chromatographic run was 10 min. Quantification was based on the most prominent MRM transition. A second transition was monitored for qualitative purposes to fulfill the European Commission Decision 2002/657/EC identification criteria using mass spectrometric techniques [31]. Table 1 shows quantification and qualification transitions, cone voltages, collision energies, retention time and selected IStd for each analyte.

For sample extraction, we assayed liquid-liquid extraction in acid conditions employing hexane as extraction solvent, and SPE using Strata Drug, Strata X (Phenomenex®) and Oasis MAX cartridges (Waters Corp.). For Strata Drug we employed a similar protocol to that proposed by Phenomenex for the extraction of cannabis and metabolites in different matrices [32-34]. In our case, after meconium homogenization, the sample was acidified, and washing

and elution steps were performed with acetonitrile/water (15/85) and ethyl acetate/hexane (85/15), respectively. For MAX cartridges we applyed a similar protocol after sample alkalinization, using the same solvents for the washing and elution steps in basic and acidic conditions, respectively. Finally, for the Strata X reversed phase cartridges we assayed two different protocols, conditioning the sample at pH 4 and pH 9. Given the different nature of the analytes included in the methodology (from very polar analytes such as diOHTHC or the glucuronides, to quite apolar ones such as CBD or THC), we needed to get to a compromise between the sensitivity of each analyte and the recovery achieved with each extraction procedure. Overall best results were obtained for the mixed mode columns, probably due to a more intense clean-up achieved with the Strata Drug cartridges. The elution solvent was subsequently optimized, selecting dichlorometane/2-propanol (50:50). However, cartridges were sometimes clogged with the meconium sample due to the low particle size of the cartridge. Therefore, the selected protocol was applied to Oasis MCX cartridges, which have higher particle size to Strata Drug columns, obtaining similar results in terms of sensitivity. Thus, Oasis MCX cartridges were finally selected.

The method proved to be specific as no quantifiable interferences were detected in blank meconium specimens from 10 different newborns or in blank samples fortified with common drugs of abuse and medicines at 0.5 μg/g concentration. LOD and LOQ ranged from 1 to 2 ng/g, and from 4 to 10 ng/g, respectively (Table 2). Fig. 1A and B shows MRM chromatograms of the quantifier transitions for all the analytes in a blank specimen, and in a meconium sample fortified at the LOQ, respectively.

Linearity of the compound-to-IStd ratio versus the theoretical concentration was verified through 4 calibration curves elaborated on four different days with 1/x weighting factor. For all the analytes the curves were fitted to a linear regression model, except for THCCOOH glucuronide, for which data better fitted assuming a quadratic model. Coefficients of determination were >0.99 for all the analytes, and residuals within ±20% of the target concentration at the LOQ, and ±15% at the remaining concentrations. Table 2 includes calibration parameters for all the analytes.

Results for imprecision and accuracy in meconium at low, medium and high QC concentrations are shown in Table 3. Intra-assay, inter-assay and total imprecision were <11.2%, <13.5% and <15.6%, respectively. Accuracy was satisfactory in all cases, ranging from 93.9% to 110.6% of the target concentration. For THC glucuronide, these parameters were only studied at 30 and 300 ng/g as the LOQ for this analyte is higher than the lower QC concentration.

Hydrolysis of THCCOOH glucuronide was ruled out as no THCCOOH was identified in the meconium samples fortified only with the glucuronide and the IStd. However, 1.1 ng/g THC was determined in the meconium samples fortified with THC glucuronide at 300 ng/g, which represents a 0.35% THC formation.

Signal suppression ranged from -26.4% to -71.4%, and the %CV obtained for the 10 meconiums used for the assessment of the matrix effect ranged from 20.9% to 48.0%. Extraction efficiency ranged from 49.9% to 69.5% depending on the analyte (Table 4), and %CV values for the 5 replicates analyzed to calculate this parameter were between 7.5-41.5%. As shown in Table 4, similar results for the matrix effect and the extraction efficiency were observed for the deuterated analogues. For the glucuronides, labeled IStd were not available when developing the method and, therefore, diOHTHC-d6 was used due to the similar retention time, achieving good results for linearity, precision and accuracy for all the analytes at all tested concentrations, including the LOQ. To evaluate the correct matrix effect compensation by the corresponding IStd, we calculated the %CV of the ratio analyte peak area/IStd peak area among the 10 meconium specimens used to evaluate the matrix effect (Table 5). These %CVs were ≤15% in all cases, showing that the ratio analyte peak area/IStd peak area are kept constant within different matrices (n=10), and therefore, proving that the IStd employed in each case compensated the analytes' matrix effect observed in each matrix. Finally, although process efficiency was somehow low, ranging from 14.3% to 45.0%, low LOD and LOQ were achieved for all the analytes as compared to previously published methods.

3.2. Application to real specimens

Application of the present method to 19 authentic meconium specimens from uncontrolled pregnancies or women suspicious of drug consumption revealed fetal cannabis exposure in 4 newborns. THCCOOH, diOHTHC and THC were detected in all the positive cases at concentrations ranging from 24.1-288.8 ng/g, 53.2-332.4 ng/g, and 4.2-7.7 ng/g), respectively, and CBD and CBN in concentrations ranging from 7.1-251.5 ng/g and 30.7-93.3 ng/g, respectively. In addition, THCCOOH glucuronide was detected in 3 cases (190.2-306.8 ng/g), and OHTHC (11.9 ng/g) in the remaining one. THC glucuronide was not identified in any specimen. Fig. 2 shows the chromatogram of the two MRM transitions of the analytes identified in one of the positive specimens. Results for the 4 positive specimens are shown in Table 6.

4. Discussion

THCCOOH, followed by OHTHC, are the main metabolites included in the analytical methods developed for the identification of fetal cannabis exposure through meconium analysis [23, 24, 26]. In addition, some authors also included THC [20], diOHTHC [22] or both [21, 25]. The present analytical method allows the simultaneous quantification of THC and its main metabolites OHTHC, THCCOOH, diOHTHC, and THCCOOH and THC glucuronides in meconium using LC-MS/MS. In addition, other main components of the cannabis plant such as CBN and CBD have been included in the method, the latter for the first time.

Most of the analytical methods described in the literature for the determination of cannabinoids in meconium have been developed by GC-MS and, therefore, a derivatization step is needed to achieve enough sensitivity [20, 22-25]. The present method was developed using LC-MS/MS, allowing getting rid of the analytes derivatization process and thus, simplifying the analysis. In addition, this method allows identification of the highest number of cannabinoids with a shorter chromatographic run time (10 min) than that described in the previous published methods, where chromatographic separation required between 10 (including just 2 analytes) to 30 min. Thus, the method described by Gray et al. allows identification of the same analytes than those described in the present method, except for the glucuronides and CBD, but required around 30 min for their separation.

This method demonstrated to be very sensitive, with LODs and LOQs from 1-2 ng/g and 4-10 ng/g, respectively, similar to or lower than those reported in previously published methods (1-20 ng/g and 10-20 ng/g, respectively) [20-26]. In addition, just 0.25 g meconium were employed, when the amount usually reported by other authors ranged from 0.5 to 2 g. Only Gray et al. [25] used 0.25 g meconium, and reported LODs from 5 to 10 ng/g.

Several authors identified the presence of cannabinoids glucuronide metabolites through enzymatic and/or alkaline hydrolysis of positive meconium specimens [21, 23, 25]. ElSohly et al. [21] reported a significant increase of diOHTHC, OHTHC and THCCOOH concentrations when meconium positive specimens were enzymatically hydrolyzed. Coles et al. [23] also reported an increase of OHTHC concentrations after enzymatic hydrolysis of screen positive specimens. Finally, Gray et al. [25] applied their analytical method to 56 authentic specimens and observed that alkaline hydrolysis increased THCCOOH concentrations, and enzymatic hydrolysis OHTHC and CBN concentrations. Moreover, the positivity rate was almost doubled with alkaline hydrolysis compared to the results obtained without hydrolysis, and THCCOOH was the main metabolite in that conditions. Another way to increase cannabinoids sensitivity in meconium would be identification of the

glucuronides themselves. LC-MS/MS was employed in the present method, which allows the chromatographic separation and detection of the polar glucuronides of THCCOOH and THC for the first time, making it possible to increase the detection of in utero cannabis exposure through meconium analysis without performing the hydrolysis step needed to get the free conjugated metabolites.

Glucuronides fragmentation was observed in the LC-MS/MS interface and, therefore, the selected transitions for these compounds were the same as those for the free analytes. However, all the analytes could be correctly identified due to the different retention times (3.2 min for THCCOOH glucuronide and 4.82 min for THCCOOH, and 3.97 min for THC glucuronide and 6.4 min for THC).

The method was applied to 19 meconium specimens sent to our laboratory, and cannabis exposure was verified in 4 specimens. As reported by Gray et al. [25] when performing the basic or tandem enzymatic-basic hydrolysis, THCCOOH and diOHTHC were the main detected metabolites. Coles et al. [23] and Marchei et al. [20] also identified THCCOOH in a higher number of specimens than OHTHC following enzymatic hydrolysis; however, in both cases, a strong basic pH was employed during the extraction procedure. Finally, Feng et al. [22] and ElSohly et al. [21] performed enzymatic hydrolysis and reported OHTHC as the main THC metabolite, followed by THCCOOH and diOHTHC. As previously reported [21, 25], THC was detected at low concentrations compared to the other analytes. With regard to the other cannabinoid components, according to Gray et al. [25], CBN was the second most prevalent analyte following THCCOOH when applying no-hydrolysis or alkaline hydrolysis, and the third when using enzymatic hydrolysis. In the specimens analyzed with the present method, CBN and CBD were identified in the three positive meconium specimens.

The main limitation of the method described in this manuscript is the absence of OHTHC and CBN glucuronides, which are present in high amount in the meconium from newborns exposed to cannabis [21-23, 25]. Unfortunately, these two analytes are not commercially available. However, detection of THCCOOH and its glucuronide will ensure the identification of fetal exposure to cannabis without the need to hydrolyzed meconium specimens [25].

4. Conclusion

An analytical method that allows simultaneous quantification of THC, THCCOOH, OHTHC, diOHTHC, CBN, CBD, and THC and THCCOOH glucuronides was developed, obtaining better sensitivity than reported in previous published methods, using low amount

of meconium. The method was successfully validated, achieving good results for all the studied parameters. Moreover, the use of LC-MS/MS avoided the derivatization of analytes prior to analysis. The main innovative aspect of this work is the inclusion of THCCOOH and THC glucuronides, which avoids the need of a hydrolysis step for the determination of the free analytes, thus reducing complexity and time required for sample analysis. Application of the method to authentic specimens sent to our laboratory confirmed the usefulness of this method to objectively identify fetal exposure to cannabis during pregnancy, and allow us to evaluate the disposition of CBD in positive cannabis meconium specimens for the first time.

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Tables

Table 1. MRM transitions, cone voltage (CV), collision energy (CE), retention time (Rt) and internal standard (IStd) selected for each analyte.

Analyte	MRM transition	CV (V)	CE (eV)	Rt (min)	IStd
THC	<u>315.3>193.4</u> 315.3>135.2	27	24	6.40	THC-d3
OHTHC	<u>331.3>313.5</u> 331.3>193.4	25	14	4.70	OH-THC-d3
diOHTHC	<u>347.3>329.4</u> 347.3>311.5	25	12	2.85	diOHTHC-d6
THCCOOH	<u>345.2>327.4</u> 345.2>299.4	40	16	4.82	THCCOOH-d3
CBN	<u>311.3>223.4</u> 311.3>293.4	30	22	6.28	CBN-d3
CBD	<u>315.3>193.4</u> 315.3>135.2	27	24	5.74	CBD-d3
THC glucuronide	<u>315.3>193.4</u> 315.3>123.1	50	24	3.97	diOHTHC-d6
THCCOOH glucuronide	<u>345.2>327.4</u> 345.2>299.4	40	20	3.20	diOHTHC-d6
Underlined transitions were used for quantification. THC: tetrahydrocannabinol; OH-THC: hydroxy-tetrahydrocannabinol; diOH-THC: di-hydroxy-tetrahydrocannabinol; THCCOOH: carboxy-tetrahydrocannabinol; CBN: cannabidiol; CBD: cannabidiol					

Table 2. Calibration parameters, limit of detection (LOD) and quantification (LOQ) for all the analytes.

Analyte	LOD (ng/g)	LOQ (ng/g)	Linearity (ng/g)	Intercept ± SD (n = 4)	Slope 1 (x) ± SD (n = 4)	Slope 2 (x^2) ± SD (n = 4)	r^2 ± SD (n=4)
THC	1	4	4-400	0.5777 ± 0.4031	0.5405 ± 0.0055	-	0.9985 ± 0.0012
OHTHC	1	4	4-400	0.0686 ± 0.0274	0.0640 ± 0.0059	-	0.9981 ± 0.0004
diOHTHC	2	4	4-400	0.2786 ± 0.2709	0.3305 ± 0.0082	-	0.9984 ± 0.0005
THCCOOH	1	4	4-400	0.2442 ± 0.3144	0.6628 ± 0.0312	-	0.9980 ± 0.0005
CBN	1	4	4-400	0.1939 ± 0.0350	0.4509 ± 0.0796	-	0.9990 ± 0.0007
CBD	2	4	4-400	0.3194 ± 0.3198	0.4902 ± 0.0312	-	0.9986 ± 0.0011
THC glucuronide	2	10	10-400	16.7870 ± 12.0963	2.5879 ± 1.0326	-	0.9919 ± 0.0044
THCCOOH glucuronide	2	4	4-400	2.3998 ± 1.2693	1.9935 ± 1.6865	-0.0015 ± 0.0024	0.9971 ± 0.0010

THC: tetrahydrocannabinol; OH-THC: hydroxy-tetrahydrocannabinol; diOH-THC: di-hydroxy-tetrahydrocannabinol; THCCOOH: carboxy-tetrahydrocannabinol; CBN: cannabidiol; CBD: cannabidiol

Table 3. Results for imprecision and accuracy in meconium at low, medium and high QC concentrations.

Analyte	Intra-assay imprecision (n =20; %CV)			Inter-assay imprecision (n =20; %CV)			Total imprecision (n = 0; %CV)			Accuracy (n =20; % target concentration)		
	6 ng/g	30 ng/g	300 ng/g	6 ng/g	30 ng/g	300 ng/g	6 ng/g	30 ng/g	300 ng/g	6 ng/g	30 ng/g	300 ng/g
THC	5.7	4.0	1.7	7.7	4.8	4.1	9.6	6.3	4.4	97.8	106.7	106.8
OHTHC	7.9	4.4	3.2	6.2	6.8	5.1	10.0	8.1	6.0	99.7	107.5	107.4
diOHTHC	0.1	6.3	5.0	0.1	0.0	5.1	0.2	6.3	7.2	96.6	99.0	96.6
THCCOOH	6.3	2.4	2.9	3.3	4.3	2.5	7.1	4.9	3.8	101.8	108.4	109.0
CBN	4.2	2.9	2.2	0.0	3.3	2.6	4.2	4.4	3.4	93.9	105.2	108.8
CBD	6.3	3.6	2.8	7.4	2.6	1.1	9.7	4.4	3.0	100.8	108.7	110.6
THC glucuronide	-	7.0	11.2	-	13.5	10.9	-	15.2	15.6	-	100.6	99.2
THCCOOH glucuronide	7.8	7.4	8.1	0.0	3.2	3.8	7.8	8.1	9.0	105.1	96.5	99.4

THC: tetrahydrocannabinol; OH-THC: hydroxy-tetrahydrocannabinol; diOH-THC: di-hydroxy-tetrahydrocannabinol; THCCOOH: carboxy-tetrahydrocannabinol; CBN: cannabidiol; CBD: cannabidiol

Table 4. Matrix effect, extraction and process efficiency results at medium (30 ng/g) and high QC (300 ng/g) concentrations.

Analyte	Matrix effect (%CV) (n=10)		Extraction efficiency (%) (%CV) (n=5)		Process efficiency (%) (n=5)	
	30 ng/g	300 ng/g	30 ng/g	300 ng/g	30 ng/g	300 ng/g
THC	-35.5 (33.1)	-26.4 (35.6)	50.2 (19.1)	61.1 (29.0)	32.4	45.0
THC-d3	-38.8 (32.1)	-30.4 (35.5)	51.5 (15.7)	60.9 (27.8)	30.0	42.4
OHTHC	-51.1 (42.0)	-59.4 (48.0)	55.2 (37.9)	63.1 (41.5)	27.1	25.6
OHTHC-d3	-53.5 (46.5)	61.4 (49.6)	51.4 (41.4)	62.3 (42.9)	29.3	24.0
diOHTHC	-53.2 (26.9)	-61.5 (24.5)	67.9 (7.6)	69.5 (7.5)	30.7	26.7
diOHTHC-d6	-54.5 (21.7)	-54.1 (20.4)	62.3 (11.3)	68.4 (7.7)	28.3	31.4
THCCOOH	-62.4 (34.6)	-61.6 (43.3)	51.8 (26.1)	56.7 (31.2)	19.5	21.8
THCCOOH-d3	-62.5 (35.5)	-60.4 (42.5)	62.3 (11.3)	68.4 (7.7)	28.3	31.4
CBN	-57.6 (36.8)	-52.5 (36.6)	50.0 (10.7)	63.5 (22.8)	21.2	30.0
CBN-d3	-58.4 (35.6)	-54.0 (36.4)	49.1 (10.0)	62.5 (21.7)	20.4	28.8
CBD	-52.3 (31.6)	-57.5 (38.0)	49.9 (16.2)	67.2 (17.1)	23.8	28.5
CBD-d3	-53.7 (32.1)	-58.6 (39.1)	48.2 (14.4)	66.8 (17.3)	22.3	27.7
THC glucuronide	-71.3 (25.7)	-67.8 (26.5)	61.7 (18.8)	66.8 (17.7)	17.7	21.5
THCCOOH glucuronide	-71.4 (30.8)	-71.3 (20.9)	50.1 (18.0)	64.3 (12.3)	14.3	18.5
THC: tetrahydrocannabinol; OH-THC: hydroxy-tetrahydrocannabinol; diOH-THC: di-hydroxy-tetrahydrocannabinol; THCCOOH: carboxy-tetrahydrocannabinol; CBN: cannabidiol; CBD: cannabidiol						

Table 5. Coefficient of determination (%CV) for the ratio analyte peak area/IStd peak area obtained in the specimens employed to evaluate the matrix effect (n=10), at 30 and 300 ng/g.

Analyte	%CV at 30 ng/g	%CV at 300 ng/g
THC	3.7	2.0
OHTHC	7.2	5.4
diOHTHC	13.2	7.2
THCCOOH	2.2	2.4
CBN	1.8	1.7
CBD	1.2	2.4
THC glucuronide	9.7	14.9
THCCOOH glucuronide	15.9	12.2
THC: tetrahydrocannabinol; OH-THC: hydroxy-tetrahydrocannabinol; diOH-THC: di-hydroxy-tetrahydrocannabinol; THCCOOH: carboxy-tetrahydrocannabinol; CBN: cannabidiol; CBD: cannabidiol		

Table 6. Cannabinoids concentrations in the 4 positive meconium specimens.

Case/year	Analyte concentrations (ng/g)							
	THC	OHTHC	diOHTHC	THCCOOH	CBN	CBD	THCCOOH glucuronide	THC glucuronide
1910/2015	5.6	0.0	53.2	45.0	70.5	112.1	190.2	0.0
1944/2015	7.4	0.0	53.2	288.8	92.2	251.5	291.4	0.0
2177/2015	4.2	11.9	236.2	24.1	30.7	7.1	0.0	0.0
2497/2015	7.7	0.0	332.4	53.8	93.3	76.4	306.8	0.0
THC: tetrahydrocannabinol; OH-THC: hydroxy-tetrahydrocannabinol; diOH-THC: di-hydroxy-tetrahydrocannabinol; THCCOOH: carboxy-tetrahydrocannabinol; CBN: cannabidiol; CBD: cannabidiol								

Figures

Fig. 1. MRM chromatograms of the quantifier transition of the analytes in a blank meconium sample (A) and a blank meconium sample fortified at the LOQ (B). THC: tetrahydrocannabinol; OHTHC: 11-hydroxyTHC; diOHTHC: 8-β-11-dihydroxyTHC; THCCOOH: 11-nor-9-carboxyTHC; CBN: cannabidiol; CBD: cannabidiol.

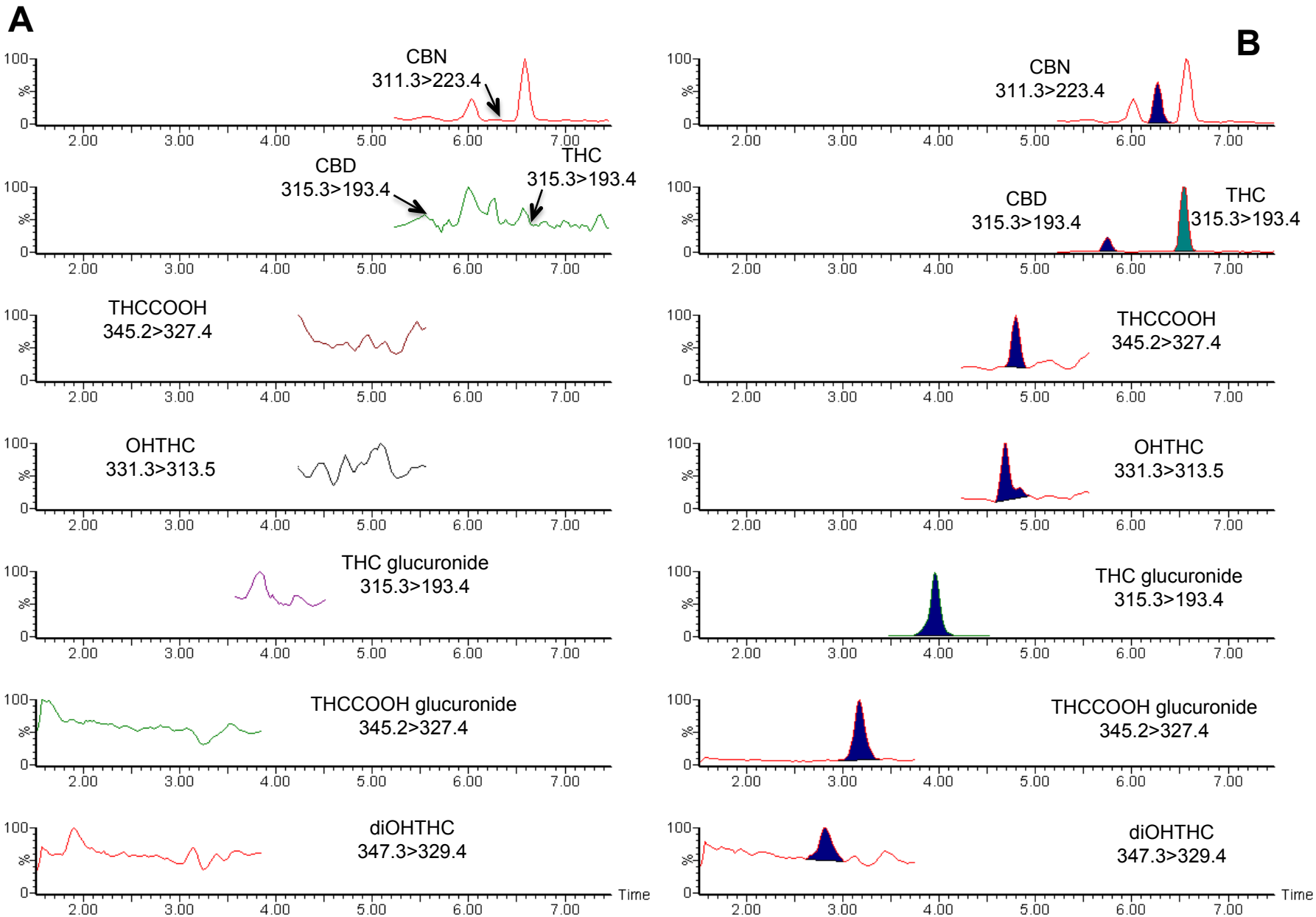
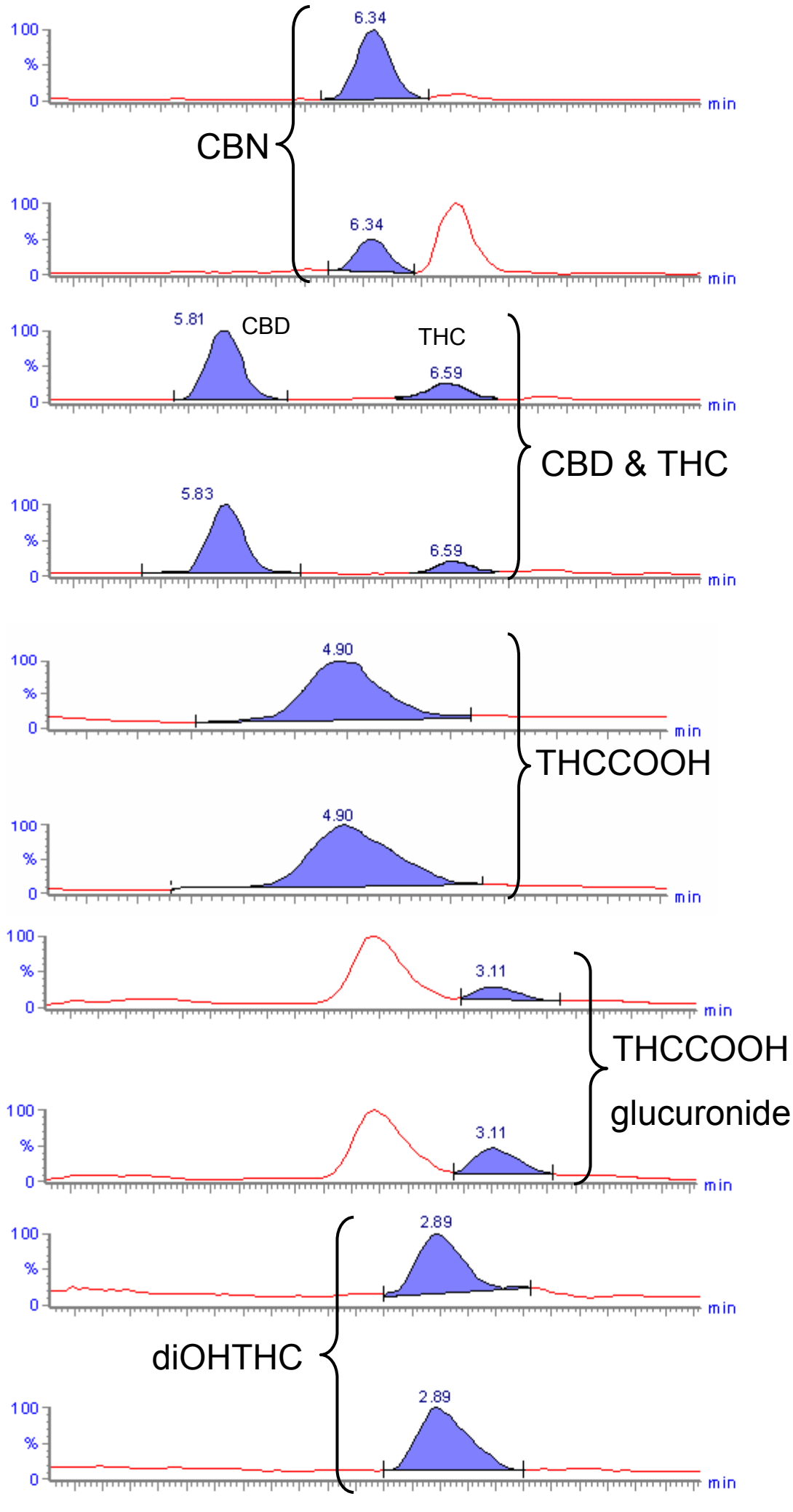

A
B
CBN
311.3>223.4
CBD
315.3>193.4
THC
315.3>193.4
THCCOOH
345.2>327.4
OHTHC
331.3>313.5
THC glucuronide
315.3>193.4
THCCOOH glucuronide
345.2>327.4
diOHTHC
347.3>329.4
100
%
0
2.00
3.00
4.00
5.00
6.00
7.00
Time

Fig. 2. Chromatograms of the two MRM transitions of the analytes identified in a real specimen from an uncontrolled pregnancy (case #: 2497/2015). THC: tetrahydrocannabinol; diOHTHC: 8-β-11-dihydroxyTHC; THCCOOH: 11-nor-9-carboxyTHC; CBN: cannabidiol; CBD: cannabidiol.


6.34
100
%
0
min
CBN
6.34
100
%
0
min
5.81
CBD
THC
6.59
100
%
0
min
CBD & THC
5.83
6.59
100
%
0
min
4.90
100
%
0
min
THCCOOH
4.90
100
%
0
min
3.11
100
%
0
min
THCCOOH
glucuronide
3.11
100
%
0
min
2.89
100
%
0
min
diOHTHC
2.89
100
%
0
min