Journal of Chromatography B, 905 (2012) 85–95

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Fast liquid chromatography/tandem mass spectrometry determination of cannabinoids in micro volume blood samples after dabsyl derivatization

C. Lacroix, E. Saussereau*

Pharmacokinetic and Toxicology Laboratory, J. Monod Hospital, F-76083 Le Havre Cedex, France

ARTICLE INFO

Article history:
Received 1 June 2012
Accepted 1 August 2012
Available online 13 August 2012

Keywords:
Cannabinoids
Dabsyl derivatization
LC/MS-MS
Micro volume

ABSTRACT

Due to the non-polar nature and the absence of an ionizable group on the cannabinoids, the ionization efficiency in electrospray is low and leads to poor limits of detection (LOD). The reaction of chloride dabsyl with the phenolic OH group of cannabinoids results in a product containing a tertiary amine, which is easily protonated in positive electrospray mode and can significantly improve the cannabinoids LOD. A rapid, selective and sensitive LC/MS-MS method was developed for quantitative determination of Δ^9-tetrahydrocannabinol (THC), 11-hydroxy-Δ^9-tetrahydrocannabinol (11-OH–THC), 11-nor-9-carboxy-Δ^9-tetrahydrocannabinol (THC–COOH), cannabinol (CBN) and cannabidiol (CBD), in micro volume blood samples following dabsyl derivatization to enhance signal intensity. The method comprised protein precipitation followed by derivatization with dabsyl chloride and subsequent analysis using liquid chromatography–tandem mass spectrometry (LC/MS-MS). Chromatographic separation was achieved using a 150 mm × 2.1 mm C18 analytical column maintained at 65 °C and eluted with a gradient of water and acetonitrile, both containing 0.2% formic acid. The run time was 8 min. The assay was successfully validated using the approach based on the accuracy profile. Lower limits of quantification (LOQ) were 0.25 ng/mL for THC and THC–COOH, 0.30 ng/mL for 11-OH–THC, 0.40 ng/mL for CBN and 0.80 ng/mL for CBD. A comparative study of cannabinoids in blood and plasma, as determined by the developed LC/MS-MS method or the in-house GC/MS-MS technique, demonstrated an excellent correlation between the two methods. Dabsylation was also tested on-line with a spiral of peek tubing placed in the LC/MS-MS column heater at 65 °C before the analytical column. The results obtained with on-line dabsyl derivatization were similar to those observed off-line.

1. Introduction

Cannabis is the most commonly used illicit drug in France and the most prevalent illegal drug seen in traffic offences [1,2]. Smoked cannabis markedly affects cognitive and psychomotor skills [3,4]. Δ^9-Tetrahydrocannabinol (THC) is the primary psychoactive constituent of cannabis and is rapidly and extensively metabolized [5]. The major metabolic pathway in humans involves the initial reaction of hydroxylation at the C11 position, forming 11-hydroxy-Δ^9-tetrahydrocannabinol (11-OH–THC). This hydroxylation is followed by further oxidation to 11-nor-9-carboxy-Δ^9-tetrahydrocannabinol (THC–COOH). THC and its hydroxylated and carboxylated metabolites undergo phase II metabolism with glucuronide; little is known about the relative proportions of ether and ester glucuronide conjugates [6,7]. Glucuronic acid conjugates of THC–COOH and THC have been identified in human urine [8]. THC etherglucuronide involves addition of glucuronide acid on the phenolic hydroxyl (OH) group. The major THC–COOH glucuronide conjugate is the ester-linked-β-glucuronide via the 11-COOH group. The phenolic OH group may be a target as well. It is also possible to have two glucuronic acid moieties attached to THC–COOH [9–11]. 11-OH–THC is eliminated in urine as a di-etherglucuronide at C1 and C11, representing only 2% of a dose [12]. THC and its metabolites can occur in physiological fluids at very low concentrations, particularly if cannabis was consumed several hours before its analysis. Cannabis determination requires a methodology providing selective detection of trace amounts of cannabinoids in biological samples. There are several reported methods for the estimation of THC and/or its metabolites by GC/mass spectrometry (GC/MS) after liquid/liquid extraction (LLE) or solid-phase extraction (SPE) and derivatization [13–17]. Extraction recovery can be less than 60% for THC because of its lipophilic property and strong protein binding, but up to 80% for hydrophilic cannabinoids and the acidic metabolite THC–COOH [11,15,18–20]. GC/MS methods appear selective and specific, but reported sensitivities require 0.5–1 mL of blood or plasma. Recently, LC/MS-MS methods have been developed for the determination of cannabinoids in biological matrices to improve

* Corresponding author. Tel.: +33 2 32 73 32 18; fax: +33 2 32 73 32 38.
E-mail address: esaussereau@ch-havre.fr (E. Saussereau).

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http://dx.doi.org/10.1016/j.jchromb.2012.08.006

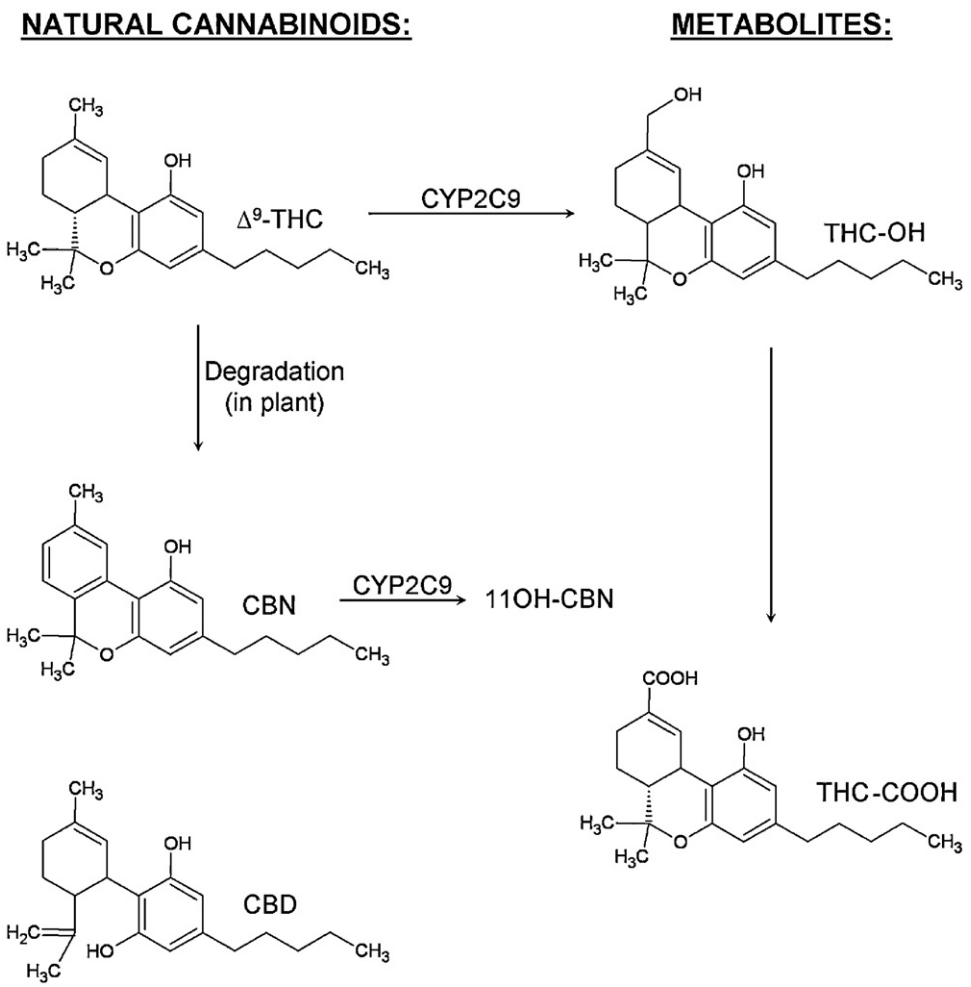

Fig. 1. Chemical structures of natural cannabinoids and their metabolites.

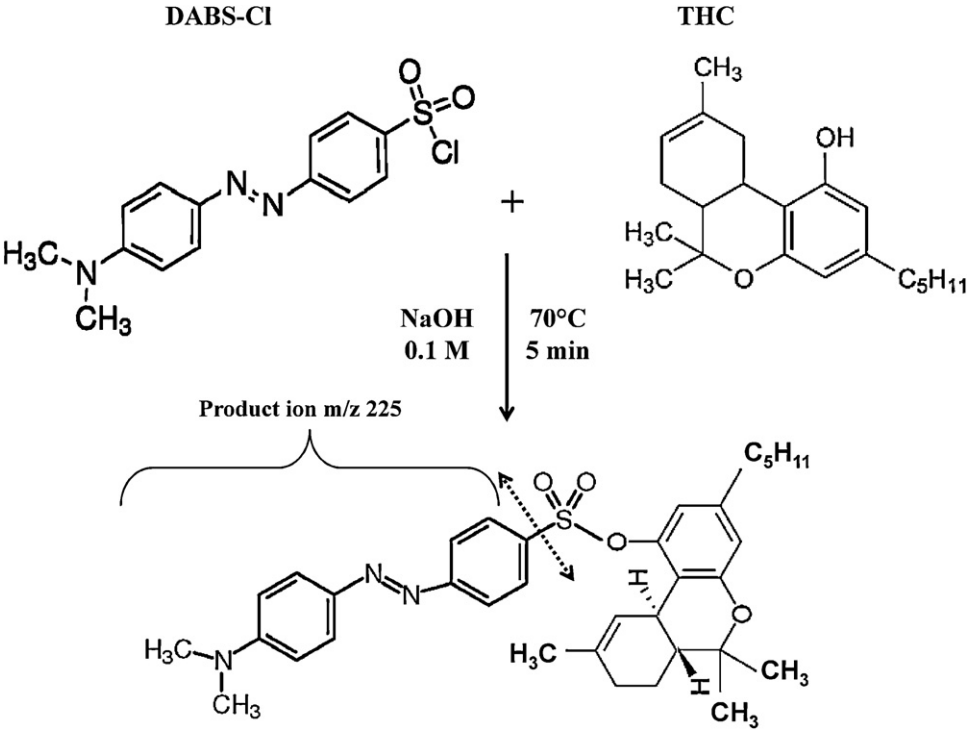

Fig. 2. Δ^9-Tetrahydrocannabinol (THC) dabsyl derivatization and its MS-MS fragmentation on dabsyl sulfur leading to the main *m/z* 225 product ion.

the selectivity and lower limits of quantification. Analysis times are usually reduced with LC/MS-MS methods because no derivatization step is required. However, the major disadvantage of these methods is the requirement of an extraction step before chromatographic analysis in order to detect the very low concentrations of THC and its metabolites [21–24]. Due to the non-polar nature and the absence of an ionizable group on the cannabinoid molecules, the ionization efficiency in electrospray (ESI), as well as in atmospheric pressure chemical mode (APCI), is low and leads to poor detection limits. Atmospheric pressure photoionization (APPI) mode has been applied for THC and its metabolites and leads to an increase in signal intensity [25], but tandem mass spectrometers equipped with an APPI interface are rarely used in toxicology laboratories owing to their fairly limited applicability. For these reasons, the sensitivities obtained in LC/MS-MS are similar as these observed in GC/MS, and thus the sample volume for both techniques is relatively important and typically ranges from 0.5 to 1 mL. Valiveti and Stinchcomb [20] described a LC/MS method requiring only 50 μL of plasma, but the limits of quantification and detection for THC, 11-OH–THC and THC–COOH were 5 ng/mL and 2 ng/mL, respectively. These LOQ and LOD are not sufficient in the context of drug testing on suspected cases of driving under the influence of illicit drugs as cannabis. The sample volume required for illicit drugs testing is a crucial parameter in forensic toxicology. In fact, the volume of whole blood obtained from a driver suspected of driving under the influence of illegal drugs could be small. This sample volume has to be sufficient for the detection and quantitative determination of a number of drug classes including the cannabinoids, opiates, cocainics and amphetamines. Previously we have described a LC/MS-MS method coupled to on-line extraction allows a simultaneous quantification of opiates, cocainics and amphetamines using only 100 μL of plasma or whole blood [26]. This on-line LC/MS-MS method has been successfully applied to dried blood spots using 30 μL of whole blood [27]. This analytical technique illustrates perfectly the main objectives to reach for an acceptable LC/MS-MS method in forensic science i.e. small sample volume, sensitivity similar or better than GC/MS, a reduced analytical time and without the need for any time-consuming off-line extraction step. As mentioned, sensitivity of THC and its metabolites, 11-OH–THC and THC–COOH, as well as cannabinol (CBN) and cannabidiol (CBD), is compromised owing to poor ionization. However, the presence of the phenolic OH group at the C1 position of all cannabinoids (Fig. 1) may present an opportunity for derivatization which ultimately could lead to improvement in the ESI process, particularly in acidic phase. Previous methods have used a dansyl derivatization step for amino acids [28,29], opiates [30] and other drugs [31], a dansyl derivatization could be use for cannabinoids. Forrest et al. described the dansyl derivatization of cannabinoids in 1971, coupled to a thin layer chromatographic (TLC) separation [32]. The same group later used normal phase HPLC to separate the dansyl derivatives and coupled this with fluorimetric detection [33]. However, this HPLC method was only applied to standard mixtures. Moreover, the dansyl derivatives appeared very unstable to light. For this reason, Vinson et al. developed a TLC method using an alternative derivatization agent 2-*p*-chlorosulfophenyl-3-phenylindone (DIS-Cl) [34]. In addition, another chromophoric labeling reagent i.e. the 4-dimethylaminoazobenzene-4′-sulfonyl chloride (DABS-Cl, dabsyl chloride), was found to be an active end-group reagent for amino acids [35–41], peptides and proteins. The sulfonyl group of DABS-Cl readily reacts with the primary and secondary amino groups, thiols, imidazoles, phenols and aliphatic OH groups. The detection of amino acid-dabsyl derivatives can be carried out in the visible region (λ: 436–460 nm) [35–40] or by tandem mass spectrometry [41], after a chromatographic analysis by TLC or HPLC. Dabsyl derivatization was also applied to amphetamine and its metabolite 4-hydroxyamphetamine, with detection at 436 nm [42]. The determination of THC and CBN dabsyl derivatives extracted from *cannabis* was first described in 1983 [43] (Fig. 2). Maseda et al. also applied dabsyl derivatization to cannabinoids for their quantitative determination in blood (1 mL) and urine (5 mL) after *n*-hexane extraction and evaporation to dryness. The extracts from urine and plasma were initially dabsylated, then analysed by reversed-phase HPLC coupled with detector wavelength at 450 nm. In this current work, cannabinoids dabsylation has been coupled to LC/MS-MS which presents a chromatographic method that is more sensitive and specific than those described above. The main objective was to develop a method using a small sample volume, without time-consuming extraction step and allowing a simultaneous quantitative determination of major cannabinoids (THC, 11-OH–THC, THC–COOH, CBD and CBN) with a sensitivity at least similar than GC/MS.

2. Experimental

2.1. Chemicals and reagents

Methanol and acetonitrile, both HPLC gradient grade, were purchased from Carlo Erba (Italy). Formic acid (99%) was from Panreac, Barcelona, Spain. Sodium hydroxide and tetrahydrofuran (THF) were both of analytical grade and from Merck, Darmstadt, Germany. Deionized water was purified using the Milli-Q-system from Millipore Corporation (Bedford, MA, USA). Dabsyl chloride was purchased from Sigma Chemical Co (St. Louis, MO, USA). Dabsyl chloride reagent was prepared by dissolving 4 mg of dabsyl chloride in 200 μL of THF. Eight-hundred microliters of acetonitrile were added to the dabsyl chloride-THF solution. Next, the mixture was ultrasonicated for 10 min. After centrifugation, the supernatant should be stored at −20 °C and no more than 7 days. Drug free whole blood was purchased from LGC Standards (Molsheim, France). Individual stock solutions for the following cannabinoids and deuterated analogs were from LGC Standards: THC, THC-d_3, 11-OH–THC, 11-OH–THC-d_3, THC–COOH, THC–COOH-d_3, CBD-d_3 and THC–COOH glucuronide. CBD and CBN were from Sigma Chemical Co and CBN-d_3 from Lipomed (Arlesheim, Switzerland). A mixed working solution of all of the non-deuterated compounds was prepared at 10 μg/mL in acetonitrile and stored at −20 °C. Standard solutions were prepared from this working solution diluted with drug-free whole blood at the concentrations of 0.25–0.50–1–5–10–50–100 ng/mL; an additional 250 ng/mL standard solution was prepared only for THC–COOH. A mixed internal deuterated standards (IS) working solution (acetonitrile-d_3 solution) was prepared in acetonitrile at 5 ng/mL, except for THC–COOH-d_3 (25 ng/mL) and CBN-d_3 (0.5 ng/mL).

2.2. Sample preparation

A total of 50 μL of sample (blood, plasma or serum) was mixed with 100 μL of acetonitrile-d_3 solution in a 1.5 mL eppendorf conical centrifuge tube. The mixture was vigorously vortex-mixed and then ultrasonicated for 10 min. Samples were centrifuged at approximately 11,000 rpm for 5 min.

2.2.1. Off-line dabsylation

One-hundred microliters of the deproteinized supernatant were transferred in an eppendorf centrifuge tube. Twenty microliters of dabsyl chloride solution and 20 μL of 0.1 M NaOH were added and mixed. The samples were then heated at 70 °C for 5 min in a heating block. After 5 min the derivatization reaction was stopped by placing the bottom of the eppendorf vials under fresh water for 1 min. Thirty microliters of dabsyl derivatives were injected in the LC/MS-MS system.

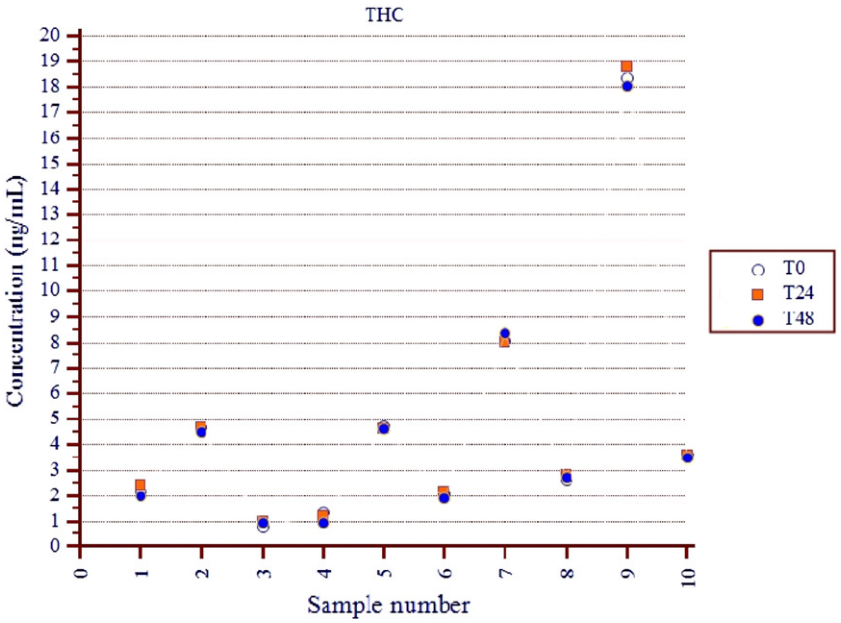

Fig. 3. THC dabsyl derivative stability tested in 10 whole blood samples at 4 °C for 3 days (T0, T24 and T48).

2.2.2. On-line dabsylation

For on-line dabsylation, the LC/MS-MS system was slightly modified to include a spiral peek tubing (Tube peek "telephone", 1/16″ × 0.5 mm ID, length 6930 mm, Interchim, France) before the analytical column and placed in the HPLC column heater. The dabsyl derivatization took place within this peek at 65 °C which was the maximum column oven temperature. A LC/MS-MS glass vial containing 100 μL of the deproteinized supernatant, 20 μL of dabsyl chloride solution and 20 μL of 0.1 M NaOH were placed in the LC/MS-MS; samples being kept at 30 °C before injection. Thirty microliters of the mixture were injected in the spiral peek tubing. Spiral peek tubing volume being 1.5 mL and mobile phase flow rate being 0.3 mL/min, 5 min were required to reach the end of the peek and thus the head of the analytical column.

2.3. Instrumentation and methods

The LC/MS-MS system consisted of an Alliance 2795 HPLC pump and a Quattro Micro™ tandem mass spectrometer (Waters, Milford, MA) controlled by computer using MassLynx software (Version 4.1). Analytes were separated at 65 °C on an Atlantis C18 column (2.1 mm × 150 mm; 3 μm, Waters) using gradient elution with water (*A*) and acetonitrile (*B*), both with 0.2% of formic acid. The flow rate was 0.3 mL/min and the gradient was as follows: 0.0–1.0 min: linear from 70 to 100% *B*; 1.0–6.0 min: 100% *B*; 6.0–6.1 min: linear from 100 to 70% *B*; 6.1–8 min: 70% *B*. For on-line dabsylation, exactly the same gradient was used but the last part at 70% *B* was prolonged until 13 min. Consequently, the retention times for the cannabinoids were delayed by 5 min in comparison with off-line approach, owing to the sample transfer through the spiral peek tubing. Positive electrospray ionization was used and all analysis were performed in the multiple reaction monitoring mode (MRM). The mass spectrometer conditions for the quantitative analysis were as follows: probe capillary voltage was 3.2 kV; the source block and desolvation temperatures were 120 °C and 450 °C, respectively. Two MRM transitions were selected for each analyte, the most intense being used for quantification and the other for confirmation.

2.4. Assay validation

Off-line cannabinoids dabsylation coupled to LC/MS-MS analysis was fully validated using total error approach [44–47]. The e-noval software 3.0 (Arlenda, Liège, Belgium) was used to compute all validation data and to build the accuracy profiles. In order to validate the presented method, two kinds of samples for calibration and validation were prepared in an independent way. The calibration standards consisted of spiked whole blood samples with known concentrations of the each cannabinoid. The samples were only used for calibration and they were prepared according to the protocol that will be applied routinely. Three sets of calibration standards were independently prepared, each calibration series comprised 6 different concentration levels (0.5, 1, 5, 10, 50, 100 ng/mL) with each concentration performed in triplicate. The 100 ng/mL calibration standard was not tested for CBN and CBD, and a seventh calibration standard at 250 ng/mL was prepared only for THC–COOH. The most appropriate response function was selected according to the accuracy profile approach in order to guarantee a reliable quantification. The validation standards were also matrix samples containing known concentrations of cannabinoids, prepared independently in the matrix simulating as much as possible the future routine analysis. For the validation phase, three validation standards series were independently prepared, each comprising 7 concentration levels (0.25, 0.5, 1, 5, 10, 50, 100 ng/mL) with each concentration performed in triplicate. The 100 ng/mL validation standard was not performed for CBN and CBD. One additional 250 ng/mL validation standard was prepared only for THC–COOH.

To assess the selectivity, a blank whole blood was prepared for each run. Moreover, authentic blood samples containing other illicit drugs than cannabinoids (amphetamines, opiates, cocainics) were analyzed to check interferences.

The recovery of analytes was determined by comparing the peak area of deproteinized whole blood sample to this of undeproteinized one. Five replicate spiked samples at 5 ng/mL for THC, 2.5 ng/mL for 11-OH–THC and 225 ng/mL for THC–COOH were examined. Samples are defined as: *A*, samples with no matrix and no deproteinization (compounds spiked in acetonitrile); *B*, samples with matrix, compounds being spiked after blank whole

blood deproteinization; and *C*, samples with matrix, compounds being spiked in blank whole blood before entire procedure. Then, $C/B \times 100$ gives the extraction recovery while $C/A \times 100$ represents overall method recovery. Moreover, the matrix effect will be given by $(B/A - 1) \times 100$.

Stability of the cannabinoid dabsyl derivatives was tested on 10 real samples obtained initially from drivers suspected driving under the influence of illicit drugs, under Justice's authority for forensic expertise. Stability was tested over 48 h; dabsyl derivatives being conserved at 4 °C between injections.

The extent of alkaline hydrolysis of THC–COOH ester-linked ß glucuronide occurring concurrently during the sample preparation was evaluated with blank whole blood fortified to 10, 20, 50, 100 and 500 ng/mL with THC–COOH glucuronide. Quantifying THC–COOH formed in this hydrolysis control allowed the calculation of percent hydrolysis for THC–COOH ester glucuronide.

Authentic whole blood and plasma samples were tested with two chromatographic methods: the developed LC/MS-MS method and our routine in-house GC/MS-MS method. Briefly, this GC/MS-MS method used a liquid–liquid extraction (LLE) step, carried out in 10 mL glass tubes. Twenty microliters of a solution of deuterated internal standards (THC-d_3, 11-OH–THC-d_3, THC–COOH-d_3, CBD-d_3, CBN-d_3, each at 10 μg/mL) and 200 μL of acetic acid 0.1 M were added to 1 mL of whole blood. After vortexing, 5 mL organic phase (hexane:ethylacetate 90%, v/v) were added. After mechanical shaking (15 min) and centrifugation (10 min at 3000 × *g*), 3 mL of the organic phase were transferred to a 6 mL glass test tube and then evaporated to dryness at ambient temperature under nitrogen for 45 min. The extracts were reconstituted with 50 μL of BSTFA [N,O-bis(trimethylsilyl) trifluoroacetamide] with 1% TMCS (trimethylchlorosilane) [15]; the derivatization step was carried out at 80 °C for 30 min. Total GC/MS-MS run time was 20 min. The samples used for the comparison test were initially obtained from drivers suspected of driving under the influence of drugs, under Justice's authority for forensic expertise. Medcalc statistical package was used for statistical analysis. A paired *t*-test was performed to test whether there was any difference between the mean cannabinoids concentrations as determined by these two analytical methods; the threshold chosen for statistical significance was 0.05. Agreement of the two methods was further assessed by Passing–Bablok regression analysis and the Bland–Altman method [48,49]. A second comparison test was performed between off-line and on-line dabsyl derivatization with 23 real blood samples; Medcalc statistical package was also used for the Bland–Altman statistical analysis.

3. Results and discussion

3.1. Sample pre-treatment

3.1.1. Deproteinization

For whole blood samples, the protein precipitation approach was the simplest method available for preparation. However one drawback of this deproteinization step was micro coagulum formation which was addressed by an additional ultrasonication step for 10 min for an efficient cannabinoids extraction. Without the ultrasonication step, there was a significant decrease (ranging from 25 to 50%) in measured cannabinoids concentrations in comparison with these obtained from GC/MS-MS: vortex agitation was not sufficiently powerful to extract cannabinoids from blood. With plasma or serum samples, no micro aggregate was observed even with the same organic solvent i.e. acetonitrile. Acetonitrile and acetone were both investigated for the deproteinization and dabsylation steps; overall results were comparable. Because of its better protein precipitation efficiency, and despite the better dabsyl chloride solubility in acetone, acetonitrile was used owing to its better compatibility with the mobile phases used in the subsequent chromatographic analysis.

3.2. Dabsyl chloride derivatization

For this step an excess of dabsyl chloride was used, thus the reaction was not affected by any slight increases in solubility owing to the organic solvent. Moreover, the excess of dabsyl chloride achieved the stability of the di-dabsyl cannabidiol. To deproteinize, one blood sample volume with two volumes of acetonitrile still maintained a good solubility of dabsyl chloride present in excess, as this of cannabinoids derivatives, which have higher lipid solubility and higher risk of precipitation in aqueous phase. Regardless of the compound, dabsyl derivatization requires alkaline conditions. Derivatization of primary and second amine functions has been shown to reach an optimal plateau between pH 8.5 and 9 [36,38]. Maseda et al. demonstrated an optimum dabsylation at pH 10.5 for THC and CBD; no dabsyl-product appeared below pH 6 and above pH 13 [43]. Some authors have used carbonate-bicarbonate buffer to adjust pH, but this buffer could induce a significant background noise, not observed with NaOH. Dabsylation reaction time was not found to be very critical and was commonly around 10 min depending on the application; according to our own findings, 5 min appeared sufficient for maximum dabsylation of the cannabinoids. Increasing the incubation time more than 15 min did show any further gain in the recovery of dabsyl derivatives but led to the partial hydrolysis of dabsyl cannabinoids [43]. The same authors also demonstrated that the optimum temperature for cannabinoids dabsylation was 70 °C. Derivatization was shown to occur even at 25 °C but for adequate recovery, an extended incubation time i.e. 30 min, was required and an occurrent formation of by-product could be increased. The dabsyl derivatization reaction has been stopped by adjusting the pH to below 6 with buffer. The main consequences were a dilution of cannabinoids dabsyl derivatives and a probable alteration of their solubility in the mixture. As shown in the developed method, the dabsylation could be stopped by placing the bottom of the eppendorf vials under fresh water resulting in the drop in temp. In our hands, the micro volume of cannabinoids dabsyl derivatives (140 μL) did not require more than one minute under fresh water to stop dabsylation. An alternative method to stop the dabsyl derivatization was investigated; vials were simply placed at −20 °C for 10 min. The reactional mixture was composed of two phases, one aqueous (40%) and one organic (60%); the lipid soluble cannabinoids derivatives being concentrated in the organic phase. After cooling in freezer, these two phases were separated and the upper organic phase was transferred to a vial and injected in the LC/MS-MS with a response twice higher than without sample keeping at −20 °C. For routine practice, dabsylation is stopped with fresh water because more simple and rapid, with a sufficient sensitivity for our applications.

3.3. Dabsyl cannabinoids derivatives stability

The LC/MS-MS signal of 10 derivatized authentic blood samples was monitored initially and then each 24 h for 48 h; derivatives solution being kept at 4 °C between injections. The stability of the dabsyl cannabinoids derivatives at 4 °C was good for at least 48 h (Fig. 3). Stability for at least for 7 days has also been observed for dabsyl amino acid derivatives [38]. Furthermore it has been demonstrated that THC and CBN, when crystallized by dabsylation, were unchanged at least for one year [43].

3.4. THC–COOH glucuronide hydrolysis

The THC–COOH metabolite predominately forms an ester bond between the glucuronide and the carboxy moiety at C-11 (ester glucuronide). THC–COOH may form a C-1 ether bond as well, between the glucuronide and OH moiety of the phenolic ring (ether and ester diglucuronide). Free THC–COOH and its glucuronide metabolites are present in blood and urine. The ester-linked glucuronide of THC–COOH is easily hydrolyzed using base or β-glucuronidase, but the ether bond is not susceptible to cleavage under alkaline conditions [8]. Ester bond alkaline hydrolysis can be obtained with NaOH 2 M at 60 °C for 15 min [8], or with NaOH 10 M at 60 °C for 20 min [11]. In the described method, the dabsylation process required NaOH 0.1 M, and was performed at 70 °C for 5 min. Under these alkaline and temperature conditions, hydrolysis recoveries assessed with blank whole blood fortified to 10, 20, 50, 100 and 500 ng/mL with THC–COOH glucuronide, were found to be 106, 91, 79, 81, and 83% respectively, leading to a mean THC–COOH glucuronide hydrolysis of 88%. The THC–COOH concentration determined in blood samples consisted of the sum of free THC–COOH and its ester-linked glucuronide. However, as the ether-glucuronide was not hydrolyzed under these alkaline conditions, it could not be derivatized with dabsyl chloride. On the contrary, GC/MS-MS currently used in routine for cannabinoids analysis allowed only free THC–COOH quantification. When GC/MS-MS and LC/MS-MS comparison was performed with authentic samples, THC–COOH was determined with both techniques. The difference between LC/MS-MS and GC/MS-MS concentrations of THC–COOH reflected ester-linked glucuronide hydrolysis. The mean percentage of hydrolysis obtained from 50 authentic whole blood samples tested with both methods, was 83.9% (RSD = 8.0%), which was in close agreement with results observed with spiked blood samples (88%). The total THC–COOH quantitative determination in blood, as the excellent LC/MS-MS method sensitivity, may improve our ability to detect cannabinoids a long time after cannabis consumption.

4. LC/MS-MS assay development

4.1. Liquid chromatographic separation

Owing to the high liposolubility of the cannabinoids, a highly organic mobile phase was required for their chromatographic elution (100% ACN containing 0.2% formic acid). Thus the initial chromatographic mobile phase composition i.e. water (containing 0.2% formic acid)/acetonitrile (containing 0.2% formic acid) (30/70, v/v), allowed the pre-concentration of the analytes onto the head of the column before the start of the gradient. An Atlantis C18 column was used in these studies and was the same as the one used for the quantitative analysis of other illicit drugs (opiates, amphetamines, cocainics) quantitative analysis in our laboratory [26,27]. Similar results were obtained with different octadecyl columns. Chromatography was carried out at the maximum HPLC column heater temperature (65 °C) for off-line and on-line derivatization, and provided a rapid elution of cannabinoids derivatives with sharp and symmetrical chromatographic peaks. No significant adverse effect on the column was observed at this elevated chromatographic temperature. The applied chromatographic method coupled to off-line dabsylation ensured the elution of all cannabinoids within 7 min (with retention times from 5.11 to 6.73 min), dabsyl derivatives being well separated (Fig. 4). Injection to injection time was 8 min. For on-line dabsylation, the derivatized cannabinoids were also well separated, with retention times recorded between 9.56 and 11.01 min (Fig. 4); injection to injection time was 13 min.

4.2. Tandem mass spectrometry detection

Selectivity of the method was achieved by a combination of retention times, precursor and two product ions. The most prominent precursor–product transition was used for quantification and the next most abundant as qualifier (Table 1). MRM dwell times were adjusted to optimize sensitivity. The *m/z* 225 or 224 product ions were obtained for all cannabinoids, except 11-OH–THC, and emerged from dabsyl derivatives fragmentation on dabsyl sulfur (Fig. 2). Cannabidiol has two OH phenolic functions; dabsylation can occur on both OH allowing cannabidiol di-dabsyl derivative synthesis. Di-dabsyl CBD chromatographic signal represented about 30% of mono-dabsyl CBD signal. For this reason, di-dabsyl CBD was not used for quantitative CBD determination, but only for confirmation. In the presence of CBN-d_3 (601.3 → 225.3), a peak was also observed for *m/z* 602.4 → 225.1 at 6.10 min (Fig. 5). It is likely that this peak corresponds to the C13 isotope of the CBN. This MRM transition also corresponds to those used for THC and CBD, however the interference peak is chromatographically resolved from the other two analytes. However, in order to minimise interferences, the concentration of cannabinol-d_3 in the deuterated internal standards solution was used at 0.5 ng/mL, which was in correlation with cannabinol concentration in cannabis user whole blood sample.

5. Assay validation

5.1. Linearity, trueness, precision and uncertainty

The response function was within the range of the existing relationship between the response (area ratio) and the concentration of the analyte in the sample [46]. It was built from the calibration standards. The response function was a weighted ($1/X^2$) linear regression for all cannabinoids. The method presented a good linearity for each cannabinoid: from 0.25 to 100 ng/mL for THC, 0.30 to 100 ng/mL for 11-OH–THC, 0.25 to 250 ng/mL for THC–COOH, 0.40 to 50 ng/mL for CBN and 0.80 to 50 ng/mL for CBD. For THC–COOH, linearity was evaluated up to 250 ng/mL for several reasons: firstly, THC–COOH blood concentration is generally significantly higher than other cannabinoids. Moreover, the developed method, as mentioned above, allows for total THC–COOH (free and glucuronide) quantification because of alkaline and temperature conditions used. In urine, THC–COOH concentration can also be very high; however the use of an initial 5-fold dilution with water was found to be suitable and kept concentrations within the linearity range of the assay. Moreover, this initial dilution with water also minimised any additional effects that the pH of the urine sample (being lower than blood) might have on the final conditions for the dabsylation reaction. Trueness, expressed in terms of relative bias (systematic error), was acceptable for all cannabinoids, even excellent for THC (<5%) (Table 2). Precision was assessed by computing the relative standard deviations (RSDs) for repeatability and intermediate precision at each concentration of the validation standards. Results were acceptable for all cannabinoids, in particular for THC (RSD < 5% for repeatability; RSD < 6% for intermediate precision) (Table 2). The expanded uncertainty represents an interval around the results where the unknown true value can be observed with a confidence level of 95%. Dividing the expanded uncertainty with the corresponding introduced concentration gives the relative expanded uncertainty (%); results obtained are presented in Table 2.

5.2. Accuracy profile

The total error evaluates the ability of the method to produce accurate results. Thus, the total error estimation of a procedure

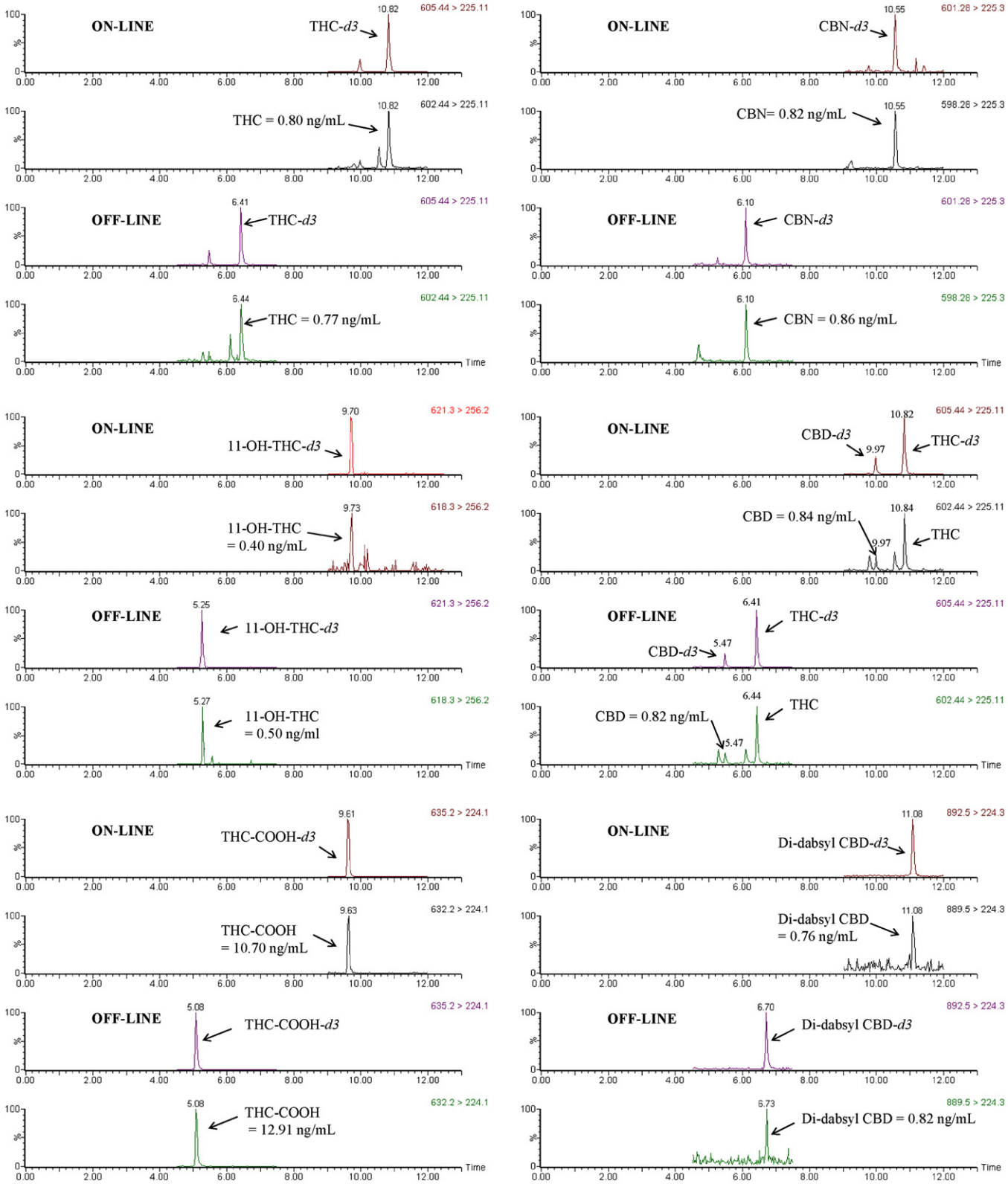

Fig. 4. Dabsyl cannabinoids chromatograms obtained from real whole blood samples, after off-line and on-line derivatization.

is fundamental to assess the validity of the method. Total error, which corresponds to the sum of trueness and precision, is represented from the accuracy profile. The concept of total error introduces upper limit of quantification (ULOQ) given by the intersection between the accuracy profile and the upper acceptance limit. The intersection with the lower limit defines the lower limit of quantification (LLOQ). The acceptance limits were set at ±40% for concentrations lower than 1 ng/mL and ±30% for concentrations higher than 1 ng/mL. The limit of detection (LOD) corresponds to one-third of the LLOQ. LOD, LLOQ, ULOQ for each cannabinoid are

Table 1
Dabsyl cannabinoids MRM conditions.

Compound	Parent ion (*m/z*)	Product ions[a] (*m/z*)	Cone voltage (V)	Collision energy (eV)
THC	602.4	**225.1**	50	40
		75.9	54	60
THC-d_3	605.4	**225.1**	50	40
		75.9	54	60
11-OH–THC	618.3	**256.2**	30	30
		312.5	30	30
11-OH–THC-d_3	621.3	**256.2**	30	30
		315.5	30	30
THC–COOH	632.2	**224.1**	40	30
		120.0	40	60
THC–COOH-d_3	635.2	**224.1**	40	30
		120.0	50	60
CBN	598.3	**225.3**	50	40
		120.4	54	60
CBN-d_3	601.3	**225.3**	50	40
		120.4	50	60
Mono-dabsyl-CBD	602.4	**225.1**	50	40
		75.9	54	60
Mono-dabsyl-CBD-d_3	605.4	**225.1**	50	40
		75.9	54	60
Di-dabsyl-CBD	889.5	**224.3**	40	40
		120.1	40	70
Di-dabsyl-CBD-d_3	892.5	**224.3**	40	40
		120.1	40	70

[a] Product ions underlined are used for quantitative determination.

presented in the Table 2. Results obtained for THC, the cannabinoid of major interest in forensic toxicology, particularly in DUID cases, were excellent as illustrated in Fig. 6, with ß-expectation tolerance limits within 15% in the dosing range, as the relative expended uncertainty (Table 2).

5.3. Selectivity, method recoveries and matrix effect

Under the assay conditions described above, no interfering signals were observed at the cannabinoids retention times after blank blood samples analysis, as well as with authentic blood samples containing other illicit drugs than cannabinoids (amphetamines, opiates and cocainics), demonstrating the method selectivity. The extraction recoveries were as follows: 90.9% for THC, 98.4% for 11-OH–THC and 112.4% for THC–COOH. The overall method recoveries for these cannabinoids ranged from 96.5 (THC) to 116.3% (THC–COOH). Calculated matrix effects were determined at 6.1% for THC, 6.8% for 11-OH–THC and −2.3% for THC–COOH, demonstrating moderate enhancement or suppression from matrix. Moreover, investigation according to Matuszewski et al. [50] demonstrated no ion suppression or enhancement at the analytes retention times.

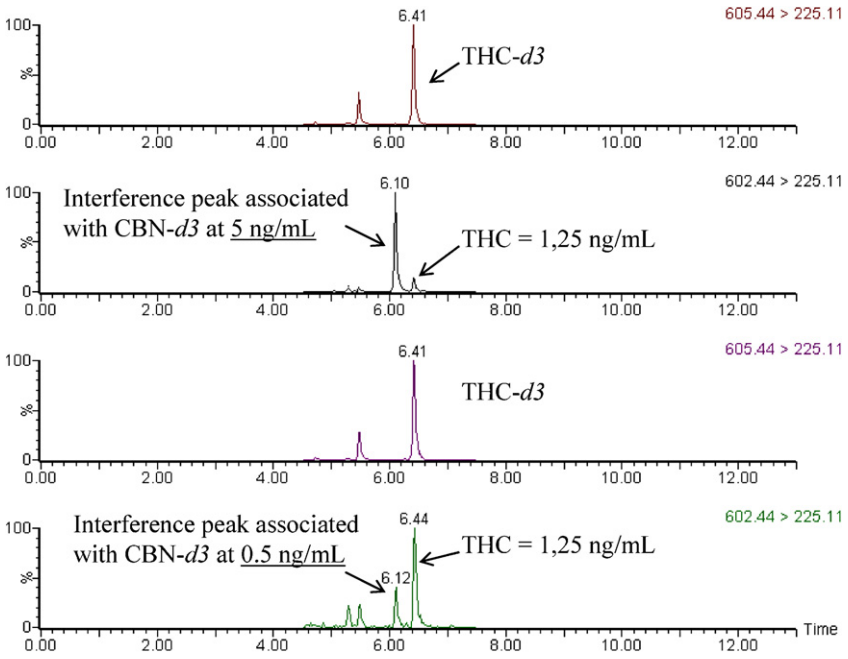

Fig. 5. Chromatogram of THC in whole blood (1.25 ng/mL) in the presence of CBN-d_3 set at 5 or 0.5 ng/mL. The peak observed at 6.10 min was an interference resulting from CBN-d_3 presence in the reactional mixture.

Table 2
Trueness, precision, uncertainty of measurement, limit of detection (LOD), lower and upper limits of quantification (LLOQ, ULOQ).

	Target concentration (ng/mL)	THC	11-OH–THC	THC–COOH	CBD	CBN
Trueness	0.25	−0.76	−15.80	11.29	−16.73	−55.43
Relative bias (%)	0.5	−3.13	−5.41	0.87	2.67	−8.40
	1.0	1.74	2.30	−4.95	12.17	13.00
	5.0	0.02	0.97	−2.61	8.62	19.05
	10	0.64	0.38	−4.58	4.79	10.75
	50	−1.66	1.19	3.99	−16.94	−33.96
	100	−4.47	−3.84	5.57	–	–
	250	–	–	13.11	–	–
Intraasssay precision	0.25	4.26	15.64	16.01	22.08	13.54
Repeatability (RSD %)						
	0.5	2.97	16.96	3.83	12.06	4.99
	1.0	3.62	9.89	16.5	7.08	5.89
	5.0	3.89	4.25	4.17	7.72	7.38
	10	2.43	4.14	2.46	3.28	3.29
	50	3.25	4.41	2.59	5.13	1.60
	100	3.51	4.54	4.07	–	–
	250	–	–	5.38	–	–
Interasssay precision	0.25	6.48	15.64	16.68	11.29	16.00
Intermediate precision (RSD %)						
	0.5	4.06	16.96	5.15	10.07	5.34
	1.0	3.74	10.85	17.23	1.69	5.89
	5.0	3.89	4.71	4.17	0.14	7.38
	10	2.78	6.16	4.19	3.28	5.38
	50	3.46	4.57	4.22	5.13	2.21
	100	4.17	4.97	4.07	–	–
	250	–	–	5.38	–	–
Uncertainty	0.25	14.36	32.66	35.04	48.87	34.37
Relative expended uncertainty (%)	0.5	8.90	35.31	11.26	45.35	11.27
	1.0	7.84	23.02	36.21	19.79	12.26
	5.0	8.09	10.01	8.67	16.07	15.42
	10	5.94	13.61	9.37	6.82	11.97
	50	7.30	9.58	9.39	10.68	4.84
	100	8.98	10.54	8.48	–	–
	250	–	–	11.20	–	–
LOD		0.08	0.10	0.08	0.24	0.12
LLOQ		0.25	0.30	0.25	0.80	0.40
ULOQ		100	100	250	50	50

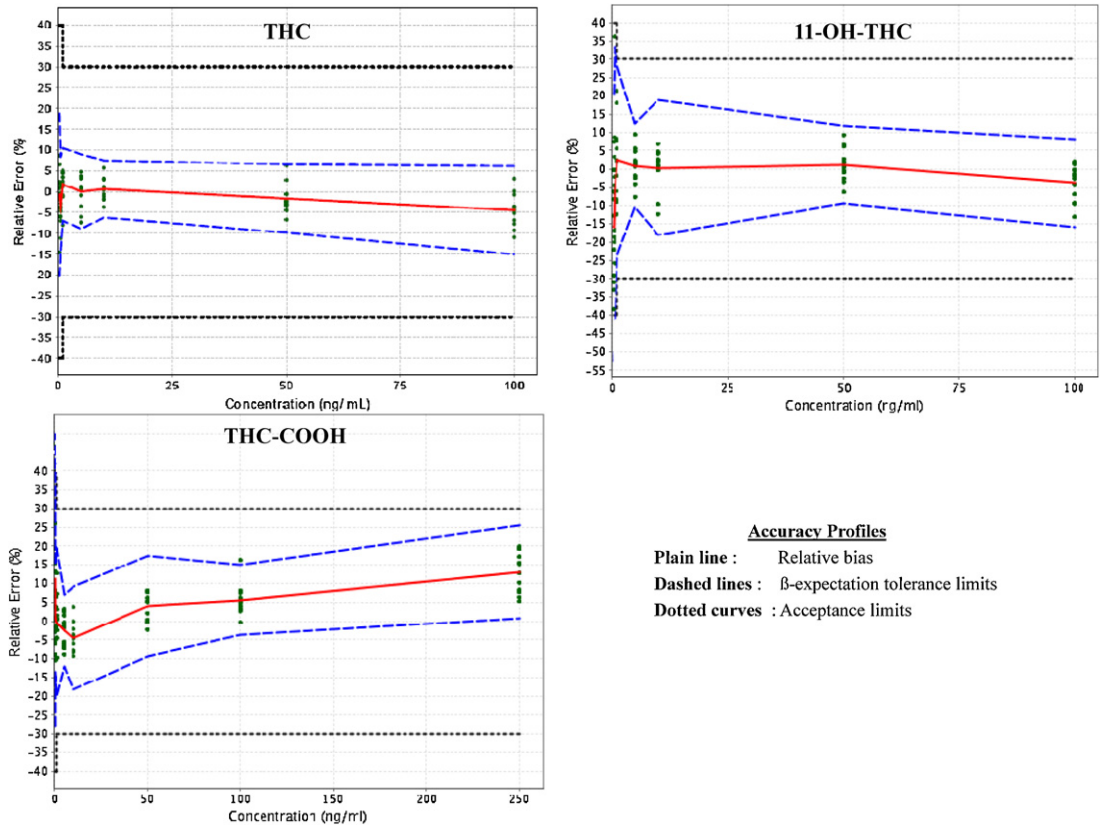

Fig. 6. THC, 11-OH–THC and THC–COOH dabsyl derivatives accuracy profiles using a weight $1/X^2$ linear regression model. The dots represent the relative back-calculated concentrations and are plotted with respect to their targeted concentration.

Table 3
Comparison of LC/MS-MS and GC/MS-MS in whole blood and plasma: coefficient of determination (r^2), paired *t*-test absolute values, Bland–Altman analysis results.

Compound	THC		11-OH–THC		CBD		CBN	
Matrices	Blood	Plasma	Blood	Plasma	Blood	Plasma	Blood	Plasma
Sample size	128	100	128	100	68	53	68	56
r^2	0.96	0.99	0.97	0.99	0.85	0.97	0.93	0.98
Paired *t*-test absolute value	1.33	1.22	0.30	1.70	1.63	0.96	0.88	1.44
Differences mean (ng/mL)	−0.103	0.046	0.013	−0.039	−0.120	−0.040	−0.012	−0.021
SD (ng/mL)	0.875	0.378	0.501	0.230	0.607	0.304	0.114	0.109
Lower limit (ng/mL)	−1.818	−0.694	−0.969	−0.490	−1.310	−0.636	−0.211	−0.234
Upper limit (ng/mL)	1.612	0.786	0.995	0.4119	1.070	0.556	0.235	0.192

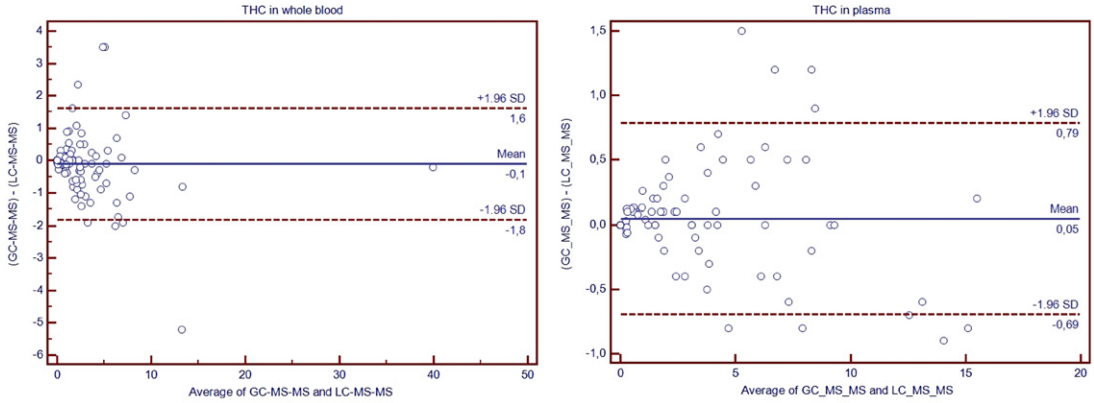

Fig. 7. Comparison LC/MS-MS vs GC/MS-MS: Bland–Altman analysis in whole blood and in plasma for THC. The solid lines illustrate the mean differences; and the dotted lines indicate the limits of agreements set to 1.96 SD.

5.4. Carryover

No carryover was observed in the analysis of whole blood blank samples spiked with IS and analyzed after the injection of the upper calibrator, neither after the analysis of highly concentrated authentic whole blood samples from cannabis users.

5.5. Comparison of the results determined from either LC/MS-MS or GC/MS-MS, in whole blood and plasma samples for THC, 11-OH–THC, CBN and CBD

THC–COOH alkaline hydrolysis in LC/MS-MS leads to the quantification of total (free and glucuronide) THC–COOH within the specimen. Thus, no comparison could be done with GC/MS-MS which quantifies only free THC–COOH. For the remaining cannabinoids, a paired *t*-test was used to determine whether there was a difference between mean values obtained from LC/MS-MS or GC/MS-MS: the absolute test values of each cannabinoid were below the critical value of 2.00 (Table 3). The difference between cannabinoids concentrations determined from LC/MS-MS or GC/MS-MS in whole blood and plasma samples were further analyzed using the Bland–Altman difference plots. Bland–Altman analysis demonstrated good agreement between the methods in whole blood and plasma (Fig. 7). The calculated SD of the mean differences as a degree of the random error were also likely to be satisfactory in whole blood as in plasma (Table 3), and indicated that individual measurements of cannabinoids with either LC/MS-MS or GC/MS-MS were in close-agreement. The LC/MS-MS data for each of the cannabinoids were also plotted against GC/MS-MS results, displaying the Passing–Bablok regression line together with its confidence limits and the identity line ($y = x$). Passing–Bablok analysis also demonstrated good correlation between methods for the two matrices (Fig. 8). In conclusion, there was no significant difference between cannabinoids concentrations measured

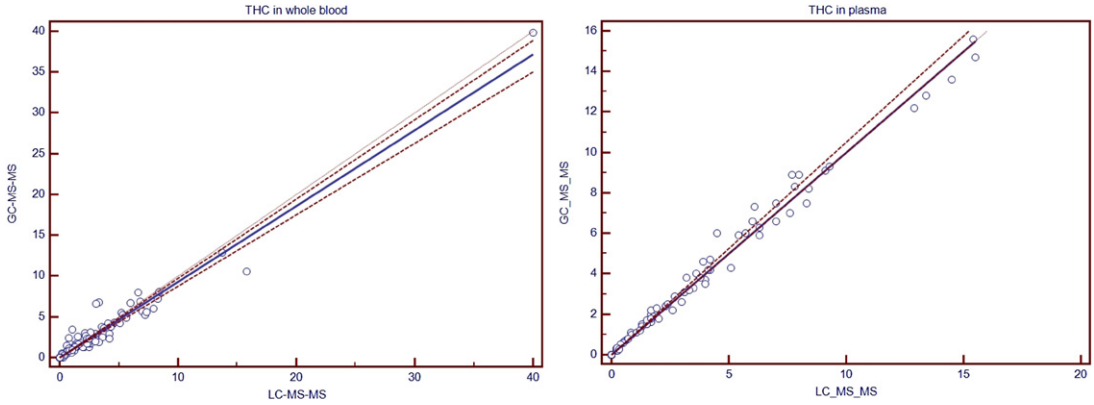

Fig. 8. Comparison LC/MS-MS vs GC/MS-MS: Passing Bablok regression in whole blood and in plasma for THC (solid line). The dashed and plotted lines represent the confidence interval and the identity line ($y = x$), respectively.

Table 4
Comparison of off-line and on-line dabsyl derivatization ($n = 23$): Bland–Altman analysis results.

Compound	THC	11-OH–THC	THC–COOH	CBD	CBN
Differences mean (ng/mL)	0.028	0.006	−6.665	0.027	0.030
SD (ng/mL)	0.238	0.213	16.388	0.233	0.142
Lower limit (ng/mL)	−0.438	−0.411	−38.786	−0.429	−0.248
Upper limit (ng/mL)	0.494	0.423	25.455	0.484	0.309

by LC/MS-MS requiring a 20 fold smaller sample volume than this used in GC/MS-MS; gas chromatographic method being moreover more time consuming than LC/MS-MS.

5.6. Comparison of off-line and on-line dabsyl derivatization in whole blood

Agreement between off-line and on-line derivatization methods was assessed using the Bland–Altman analysis; no significant bias was observed (Table 4). Satisfactory results obtained with on-line dabsylation using spiral peek tubing and demonstrated that manual sample preparation for cannabinoids quantitative determination could be limited to just a simple deproteinization.

6. Conclusion

Quantification of major cannabinoids (THC, 11-OH–THC, THC–COOH, CBD and CBN) in blood, plasma, serum or urine, using a dabsyl derivatization was successfully achieved by coupling the technique with LC/MS-MS. The importance of this dabsylation approach lies in the excellent sensitivity obtained with a micro volume sample (50 μL), and without extraction step which is significantly time-consuming and currently used for the cannabinoids quantitative determination in biological matrices. The assay was successfully validated using the approach based on the accuracy profile. The excellent correlation with our in-house GC/MS-MS method demonstrated the ability of the LC/MS-MS technology coupled to dabsylation to quantify cannabinoids in drivers suspected driving under the influence of illicit drugs (DUID). For urine, the method is exactly the same except for an initial sample dilution in water to 1:5. Moreover, dabsyl derivatization could be applied to other illicit drugs containing primary or secondary amine groups (e.g. amphetamines) or containing OH phenolic groups (e.g. opiates), thus allowing a simultaneous illicit drugs quantification. Cocainics, illicit drugs without amine or OH phenolic groups, do not react with dabsyl chloride, but after protein precipitation remain, within the acetonitrile phase not derivatizated. In the perspective of applying the developed method to simultaneous illicit drugs quantification in DUID cases, a part of deproteinized sample would be analyzed after dabsylation for cannabinoids, opiates, amphetamines, and the other underivatized part would be used for cocainics. In this context, the next step would be to apply the presented method to illicit drugs quantitative determination in dried blood spots [27].

References

[1] B. Gadegbeku, E. Amoros, B. Laumon, Ann. Adv. Automot. Med. 55 (2011) 293.
[2] M.B. Biecheler, J.F. Peytavin, S.A.M. Group, F. Facy, H. Martineau, Traffic Inj. Prev. 9 (2008) 11.
[3] A. Liguori, C.P. Gatto, D.B. Jarrett, Psychopharmacology 163 (2002) 399.
[4] C.C. Hunault, T.T. Mensinga, K.B. Böcker, C.M. Schipper, M. Kruidenier, M.E. Leenders, I. de Vries, J. Meulenbelt, Psychopharmacology 204 (2009) 85.
[5] P. Kelly, R.T. Jones, J. Anal. Toxicol. 16 (1992) 228.
[6] E.W. Schwilke, D.M. Schwope, E.L. Karschner, R.H. Lowe, W.D. Darwin, D.L. Kelly, R.S. Goodwin, D.A. Gorelick, M.A. Huestis, Clin. Chem. 55 (2009) 2180.
[7] D.M. Schwope, K.B. Scheidweiler, M.A. Huestis, Anal. Bioanal. Chem. 401 (2011) 1273.
[8] P.M. Kemp, I.K. Abukhalaf, J.E. Manno, B.R. Manno, D.D. Alford, M.E. McWilliams, F.E. Nixon, M.J. Fitzgerald, R.R. Reeves, M.J. Wood, J. Anal. Toxicol. 19 (1995) 292.
[9] G. Skopp, L. Pötsch, Clin. Chem. 48 (2002) 301.
[10] G. Skopp, L. Pötsch, M. Mauden, B. Richter, Forensic Sci. Int. 126 (2002) 17.
[11] T.T. Abraham, R.H. Lowe, S.O. Pirnay, W.D. Darwin, M.A. Huestis, J. Anal. Toxicol. 31 (2007) 477.
[12] R.C. Baselt, Disposition of Toxic Drugs and Chemicals in Man, 9th ed., Biomedical Publications, Foster City, CA, 2011.
[13] P.M. Kemp, I.K. Abukhalaf, J.E. Manno, B.R. Manno, D.D. Alford, G.A. Abusada, J. Anal. Toxicol. 19 (1995) 285.
[14] R.A. Gustafson, E.T. Moolchan, A. Barnes, B. Levine, M.A. Huestis, J. Chromatogr. B 798 (2003) 145.
[15] T. Nadulski, F. Sporkert, M. Schnelle, A.M. Stadelmann, P. Roser, T. Schefter, F. Pragst, J. Anal. Toxicol. 29 (2005) 782.
[16] A. Thomas, C. Widmer, G. Hopfgartner, C. Staub, J. Pharm. Biomed. Anal. 45 (2007) 495.
[17] R.H. Lowe, T.T. Abraham, W.D. Darwin, R. Herning, J.L. Cadet, M.A. Huestis, Drug Alcohol Depend. 105 (2009) 24.
[18] S.B. Grauwiler, A. Scholer, J. Drewe, J. Chromatogr. B 850 (2007) 515.
[19] C. Jamey, E. Szwarc, A. Tracqui, B. Ludes, J. Anal. Toxicol. 32 (2008) 349.
[20] S. Valiveti, A.L. Stinchcomb, J. Chromatogr. B 803 (2004) 243.
[21] B. Maralikova, W. Weinmann, J. Mass Spectrom. 39 (2004) 526.
[22] C. Coulter, E. Miller, K. Crompton, C. Moore, J. Anal. Toxicol. 32 (2008) 653.
[23] M. del Mar Ramírez Fernández, S.M. Wille, N. Samyn, M. Wood, M. López-Rivadulla, G. De Boeck, J. Chromatogr. B 877 (2009) 2153.
[24] A.A. Elian, J. Hackett, J. Anal. Toxicol. 33 (2009) 461.
[25] R.L. Foltz, in: M.A. ElSohly (Ed.), Forensic Science and Medicine: Marijuana and the Cannabinoids, Humana Press Inc, Totowa, NJ, 2007, p. 179.
[26] C. Lacroix, E. Saussereau, G. Bodin, J.P. Goullé, Ann. Toxicol. Anal. 20 (2008) 25.
[27] E. Saussereau, C. Lacroix, J.M. Gaulier, J.P. Goullé, J. Chromatogr. B 885–886 (2012) 1.
[28] Y. Tapuhi, D.E. Schmidt, W. Lindner, B.L. Karger, Anal. Biochem. 115 (1981) 123.
[29] F.J. Márquez, A.R. Quesada, F. Sánchez-Jiménez, I. Núñez de Castro, J. Chromatogr. 380 (1986) 275.
[30] D.A. Barrett, P.N. Shaw, S.S. Davis, J. Chromatogr. 566 (1991) 135.
[31] F. Beaudry, S.A. Guénette, A. Winterborn, J.J. Marier, P. Vachon, J. Pharm. Biomed. Anal. 39 (2005) 411.
[32] I.S. Forrest, D.E. Green, S.D. Rose, G.C. Skinner, D.M. Torres, Res. Commun. Chem. Pathol. 2 (1971) 787.
[33] S.R. Abbott, A. Abu-Shumays, K.O. Loeffler, I.S. Forrest, Res. Commun. Chem. Pathol. 10 (1975) 9.
[34] J.A. Vinson, D.D. Patel, A.H. Patel, Anal. Chem. 49 (1977) 163.
[35] J.K. Lin, C.C. Lai, Anal. Chem. 52 (1980) 630.
[36] J.K. Lin, J.Y. Chang, Anal. Chem. 47 (1975) 1634.
[37] J.Y. Chang, E.H. Creaser, J. Chromatogr. 116 (1976) 215.
[38] E.H. Jansen, R.H. Van den Berg, R. Both-Miedema, L. Doorn, J. Chromatogr. 553 (1991) 123.
[39] I. Krause, A. Bockhardt, H. Neckermann, T. Henle, H. Klostermeyer, J. Chromatogr. A 715 (1995) 67.
[40] R. Sethuraman, T.L. Lee, S. Tachibana, Clin. Chem. 50 (2004) 665.
[41] Y.H. Chen, Y.P. Lin, S.E. Liou, C.C. Chen, Int. J. Food Sci. Technol. 42 (2007) 593.
[42] M.E. Soares, F. Carvalho, M. de Lourdes Bastos, Biomed. Chromatogr. 15 (2001) 452.
[43] C. Maseda, Y. Fukui, K. Kimura, K. Matsubara, J. Forensic Sci. 28 (1983) 911.
[44] P. Hubert, J.J. Nguyen-Huu, B. Boulanger, E. Chapuzet, P. Chiap, N. Cohen, P.A. Compagnon, W. Dewé, M. Feinberg, M. Laurentie, N. Mercier, G. Muzard, L. Valat, Rozet E, J. Pharm. Biomed. Anal. 36 (2004) 579.
[45] P. Hubert, J.J. Nguyen-Huu, B. Boulanger, E. Chapuzet, P. Chiap, N. Cohen, P.A. Compagnon, W. Dewé, M. Feinberg, M. Laurentie, N. Mercier, G. Muzard, L. Valat, E. Rozet, J. Pharm. Biomed. Anal. 45 (2007) 70.
[46] P. Hubert, J.J. Nguyen-Huu, B. Boulanger, E. Chapuzet, N. Cohen, P.A. Compagnon, W. Dewé, M. Feinberg, M. Laurentie, N. Mercier, G. Muzard, L. Valat, E. Rozet, J. Pharm. Biomed. Anal. 45 (2007) 82.
[47] N. Dubois, A.P. Paccou, B.G. De Backer, C.J. Charlier, J. Anal. Toxicol. 36 (2012) 25.
[48] H. Passing, W. Bablok, J. Clin. Chem. Clin. Biochem. 21 (1983) 709.
[49] J.M. Bland, J.G. Altman, Lancet 1 (1986) 307.
[50] B.K. Matuszewski, M.L. Constanzer, M. Chavez-Eng, Anal. Chem. 75 (2003) 3019.