ORIGINAL PAPER

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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Abstract The detection and confirmation of cannabinoids in oral fluid are important in forensic toxicology. Currently, the presence of Δ^9-tetrahydrocannabinol (THC) is used for the detection of cannabis in oral fluid. A low concentration of 11-nor-9-carboxy-Δ^9-tetrahydrocannabinol (THC-COOH) is found in oral fluid, which suggested a convenient and low-sensitivity confirmation assay can be used in a routine forensic laboratory. In this study, a highly sensitive isotope dilution liquid chromatography–tandem mass spectrometry method following dansylation was successfully developed for simultaneous determination of THC and THC-COOH in oral fluid. The dansylated derivatives dramatically demonstrated and enhanced the sensitivity of THC and THC-COOH. To avoid signal influenced by the matrix, a 5-min liquid chromatography gradient program was evaluated and optimized, which reduced the sample diffusion and caused sharp peaks (less than 12 s) and thus helped to achieve detection at a low level. The sensitivity, accuracy, and precision were also evaluated, and high quantitative accuracy and precision were obtained. The limit of quantitation of this approach was 25 pg/mL for THC and 10 pg/mL for THC-COOH in oral fluid. Finally, the method was successfully applied to eight suspected cannabis users. Among them, in six oral fluid samples THC-COOH was determined at a concentration from 13.1 to 47.2 pg/mL.

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Introduction

Drug tests provide unbiased information. Oral fluid has been promoted as an alternative specimen to blood and urine in clinical and forensic toxicology [1–4]. The advantages of using oral fluid compared with blood are the noninvasive nature and the convenience. Furthermore, monitoring is not required to detect adulteration, substitution, and dilution of the sample. In addition, oral fluid is relatively clean and has lower protein and matrix content.

Cannabis (marijuana) is the most widely used illicit drug in the world [5]. Δ^9-Tetrahydrocannabinol (THC) is the major psychoactive agent in over 60 components of *Cannabis sativa*. Cannabis is commonly administered orally or by inhalation. THC is further metabolized to 11-nor-9-carboxy-Δ^9-tetrahydrocannabinol (THC-COOH) [6].

Generally, THC is the main compound found in biological samples following cannabis intake, but the detection of the metabolite THC-COOH would exclude the passive exposure issue [7, 8]. Therefore, the detection and confirmation of THC-COOH in urine samples have long been standard in forensic toxicology [9, 10].

Although little is known about the composition of cannabinoid compounds in oral fluid samples after cannabis intake, the concentration of THC-COOH is very low [11]. Because THC is activated and deposited in the oral cavity, it is the primary target which is ultimately collected and measured for drug tests using oral fluid [12]. The regulatory criterion for the detection of cannabis in oral fluid suggested by the Substance Abuse and Mental Health Services Administration (SAMHSA) is 2 ng/mL THC [13, 14]. However, for oral fluid, passive exposure must be considered [15]. Consequently, this often requires simultaneous collection of urine and the target THC-COOH to discriminate the individual's use or exposure, but this is inconvenient.

The detection of THC-COOH in oral fluid is a great analytical challenge since the concentration is relatively low (picograms per milliliter). As far as we know, the utilization of gas chromatography (GC) with tandem mass spectrometry (MS/MS) and two-dimensional GC–mass spectrometry (MS) has been reported, and provided limits of quantitation (LOQ) for THC-COOH in oral fluid of 10 and 2 pg/mL, respectively [16, 17], but those techniques are not popular in forensic and clinical laboratories.

Use of liquid chromatography (LC)–MS/MS with electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI) has been powerfully demonstrated in forensic toxicology for identification and quantitation of a wide range of drugs and metabolites in biological samples [18]. Owing to the high sensitivity, selectivity, and robustness of LC-MS/MS systems, like the triple-quadrupole MS/MS systems operated in multiple reaction monitoring (MRM) mode, which showed the convenience and sensitivity for drug tests using oral fluid [4, 19, 20]. It has been shown that LC-MS/MS is useful for detection of cannabinoids and their metabolites in urine and plasma [21, 22] but there have been few reports for its use for detection in oral fluid. Only two LC-MS/MS methods have been published for this purpose. In 2007, Quintela et al. [23] presented data on the detection of THC and THC-COOH in oral fluid using LC-MS/MS and the LOQ was 0.5 ng/mL. This method was applied to 27 samples from the ROSITA project, but no THC-COOH was detected in any of these samples that tested positive for THC, which further proves the necessity of assays with low picogram per milliliter sensitivity. In 2009, Sergi et al. [24] presented multiclass analysis of illicit drugs in oral fluids by LC-MS/MS, but the required sensitivity for the detection of THC

and THC-COOH in oral fluid was not achieved as the LOQ were only 3.7 and 3.5 ng/mL, respectively.

In the work reported here, a chemical derivatization approach using isotope dilution LC-MS/MS (IDMS) with ESI in positive mode was developed and validated for simultaneously detecting THC and THC-COOH in oral fluid. It involved a fast derivatization with dansyl chloride (DC), which dramatically improves the sensitivity and separation efficiency of THC and THC-COOH. To avoid signal that is influenced by the matrix, a 5-min fast gradient chromatographic separation was evaluated and optimized and this reduced the sample diffusion and reduced the peak width sharply (less than 12 s) and thus helped to achieve lower-level detection. To the best of our knowledge, this is the first time that LC-MS/MS has had enough sensitivity to detect trace levels of THC-COOH (picograms per milliliter) in oral fluid specimens. Finally, the method has been successfully applied for the simultaneous detection of THC and low-concentration THC-COOH in authentic specimens.

Experimental

Chemicals and reagents

All solvents and chemicals were of analytical grade. Methanol, formic acid, hexane, tetrahydrofuran (THF), glacial acetic acid, hydrochloric acid, ethyl acetate ,and potassium hydroxide were purchased from Merck (Darmstadt, Germany). DC [5-(dimethylamino)-1-naphthalenesulfonyl chloride] and sodium bicarbonate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Drug standards of THC and THC-COOH and internal standards of THC-d_3 and THC-COOH-d_3 were purchased from Cerilliant (Austin, TX, USA). Each compound was dissolved in methanol to make a stock solution with a concentration of 1 μg/mL, and was used after dilution with methanol to the required concentration. Drug standard and internal standard solutions were stored at 4 °C.

Sample pretreatment

A 250-μL sample volume for positive control, negative control, and authentic specimens was mixed with the internal standards THC-d_3 and THC-COOH-d_3 at 100 pg/mL and 25 pg/mL, respectively. Then the sample was vortexed for 10 s. After addition of 500 μL of acetonitrile, the sample was vortexed for 30 s and centrifuged at 14,000 rpm for 10 min. The supernatant was evaporated to dryness under nitrogen at 45 °C. [19]

The residue was dissolved in 100 μL of sodium bicarbonate buffer (100 mM, pH adjusted to 10.5 with NaOH) followed by vortex-mixing for 1 min. For each

sample, 100 μL of DC solution (1.0 mg/mL in acetone) was added and the resulting solution was vortex-mixed for 1 min. The samples were placed in an oven at 70 °C for 5 min and then cooled to room temperature. Then, the derivatized mixture was extracted twice with 500 μL of hexane for 3 min and then evaporated to dryness. Finally, the dried sample was reconstituted in 50 μL of methanol and 20 μL was injected into the LC-MS/MS system.

Drug-free oral fluid was obtained from laboratory volunteers and added to 100 pg/mL and 20 pg/mL (50 μL and 10 μL of 2 pg/μL standard solution) THC and THC-COOH as samples used for method development and positive control.

Liquid chromatography mass spectrometry

The high-performance liquid chromatography (HPLC) system consisted of a Shimadzu SCL-10Avp controller, an SIL-10ADvp autosampler, a CTO-10ACvp column oven, and two Shimadzu LC-10 AD binary pumps from Gentech (Arcade, NY, USA). The gradient chromatographic separation was conducted by reverse-phase HPLC under the following conditions: column, Gemini C_{18} (3-μm particle size, 50 mm×2.0 mm; Phenomenex, Torrance, CA, USA); flow rate, 0.25 mL/min; injection volume, 20 μL; column oven temperature, 50 °C; mobile phase A, of water with 0.5% formic acid; and mobile phase B, THF with 0.5% formic acid. The gradient program was as follows: 0–0.5 min, 30% mobile phase B; 0.5-2.5 min, from 30 to 90% mobile phase B; 2.5–3.5 min, 90% mobile phase A. The autosampler cooling unit was maintained at 10 °C.

Analytes were detected using an Applied Biosystems SCIEX API 3000 triple-quadrupole mass spectrometer equipped with a TurboIonSpray interface (ESI) and a linearly accelerating high-pressure collision cell (Gentech, Arcade, NY, USA). All analyses were performed in MRM mode and positive ESI mode, the peak width settings for both the first quadrupole and the third quadrupole were unit resolution. Ionization were performed under the following conditions: nebulizer gas and curtain gas, 13 arbitrary units;

collision gas, 10 arbitrary units; ions spray voltage, 5,000 V; temperature, 300 °C; dwell time, 80 ms. To obtain optimal ion transmission conditions, ion signals were ramped by a semiautomated ramp procedure to maximize the signal. The conditions for particular MRM transitions are summarized in Table 1.

Method validation

The sensitivity was estimated by determination of the limit of detection (LOD) and the LOQ. The LOD is the lowest concentration of an analyte that after the bioanalytical procedure can be reliably differentiated from background noise (signal-to-noise ratio greater than 3), and the two independent ion intensity ratios of the three ions monitored are within ±20% of the ratio observed in the calibration standard. The LOQ is the lowest concentration that can be quantitated (signal-to-noise ratio greater than 10) and matched the requirement of the LOD. The observed concentration is also within ±20% of the actual value.

Linearity of sample response was investigated in the range 0.2-20 ng/mL for THC and 5–500 pg/mL for THC-COOH. Calibration curves were obtained with six calibration levels from low to high concentration for THC by adding 0.05, 0.1, 0.2, 0.4, 2, and 5 ng of standards and for THC-COOH by adding 1.25, 2.5, 5, 10, 50, and 125 pg of standards, as well as 50 and 25 pg of their deuterated internal standards to 250 μL of drug-free oral fluid; the mixtures were then subjected to the analysis described. The linearity of the method was investigated by calculation of the regression line by the method of least squares and expressed by the squared correlation coefficient (R^2). A correlation analysis was then performed using the peak area ratios of target analytes and their respective internal standards for each concentration. R^2 for the detector response versus analyte concentration was reported.

Analytical recovery was evaluated across the linear range with fortified samples at target oral fluid concentrations. Dansylated drug standards (DC-THC and DC-THC-COOH) and dansylated deuterated internal standards (DC-THC-d_3

Table 1 Optimized multiple reaction monitoring parameters for analytes and internal standards

Compound	Precursor ion (m/z)	Product ion (m/z)	DP (V)	FP (V)	EP (V)	CE (eV)	CXP (V)
DC-THC	548	171	38	177	10	52	15
	548	299	38	177	10	42	18
DC-THC-d_3	551	171	38	177	10	52	15
DC-THC-COOH	578	171	37	189	10	43	11
	578	532	37	189	10	27	8
DC-THC-COOH-d_3	581	171	37	189	10	43	11

DC dansyl chloride, *THC* Δ^9-tetrahydrocannabinol, *THC-COOH* 11-nor-9-carboxy-Δ^9-tetrahydrocannabinol, *DP* declustering potential, *FP* focusing potential, *EP* entrance potential, *CE* collision energy, *CXP* collision cell exit potential

and DC-THC-COOH-d_3) were prepared from 100 ng of each original internal standard. The internal standards were dansylated and extracted with hexane, and then evaporated to dryness. The dried derivates were reconstituted in 1 mL of methanol and were stored at 4 °C until to use.

The recovery for each analyte was also determined at the low (0.4 ng/mL for DC-THC and 20 pg/mL for DC-THC-COOH), medium (2 ng/mL for DC-THC and 100 pg/mL for DC-THC-COOH), and high (10 ng/mL for DC-THC and 500 pg/mL for DC-THC-COOH) control concentrations. For determination of recovery, dansylated standard solution was added prior to or following liquid–liquid extraction (LLE). Recovery was expressed as the mean analyte area of samples with control solution added before LLE ($n=6$) divided by the mean analyte area of samples with control solution added after LLE ($n=6$).

The intraday precision and accuracy were evaluated by analyzing six oral fluid specimens containing THC and THC-COOH at three different concentrations of 0.4 ng/mL, 2 ng/mL, and 10 ng/mL for DC-THC and 20 pg/mL, 100 pg/mL, and 500 pg/mL for DC-THC-COOH on the same day. The interday precision and accuracy were evaluated on five separate days at the same concentrations. To determine the precision, the coefficients of variations were calculated for replicate measurements and were expected to be less than 15% for all concentrations, except for the LOQ, for which 20% is acceptable. The accuracy was expressed as the relative error from the expected value. It was calculated from the degree of agreement between the measured and nominal concentrations of the fortified samples, which has to be less than 15% of the theoretical values at each concentration except for the LOQ, should not be more than 20%.

Results and discussion

The detection and confirmation of cannabinoids in oral fluid samples are important in forensic toxicology. However, the current oral fluid test for cannabis is targeted to detect THC, and THC-COOH has still not been detected in routine toxicological analysis because of the lack of a convenient and low-sensitivity assay. Therefore, regulatory criterion was proposed by the SAMHSA requiring the simultaneous collection of urine to be used by targeting the metabolite THC-COOH for the confirmation of cannabis use [13]. Recently, LC-MS/MS has had increased significance in the field of forensic toxicology owing to its specificity and versatility. Although THC-COOH can be detected using LC-MS/MS in both positive and negative ion modes, and better sensitivity can be achieved in negative ion mode [25, 26], the sensitivity is still far from the required sensitivity for drug tests using oral fluid. To overcome this, an approach using chemical derivatization,

that is, conversion of a poorly ionized compound into derivatives easily detectable by LC-MS/MS, was developed and assessed in this study.

## Characterization of dansylated derivatives by ESI-MS/MS

Chemical derivatization plays an important role in drug analysis of biological samples. Derivatization in GC-MS is often needed to enhance the volatility of the analyte, and alter its ionization efficiency and fragmentation characteristics. Derivatization is generally not needed in LC-MS, for which soft ionization techniques such as ESI and APCI are applied. However, for analytes that are not efficiently ionized by ESI or APCI, derivatization can still be performed to improve ionization efficiency. In addition, derivatization can improve the fragmentation characteristics and enhance the sensitivity when using MS/MS.

Dansylation is an old fluorescent derivatizing method that is commonly used in the analysis of proteins and primary or secondary amines [27]. Recently, dansylation has been applied to form derivatives of phenolic compounds, followed by LC-MS/MS analysis, for the detection of estradiols and estrone [28–30], 1-hydroxypyrene [31], and propofol [32]. Because of the presence of a dimethylamino group in this compound, it is amenable to protonation under acidic conditions. In addition, a simplified and high-intensity fragment ion was often obtained; therefore, the dansylation-enhanced sensitivity was often impressive. THC-COOH is commonly classified as a carboxylic acid compound. However, the advantage of the hydroxyl group in the aromatic ring (phenolic group), which is an active functional group for dansylation, is seldom considered. In addition, the phenolic group is contained in the structure of most cannabinoids and their metabolites, including both THC and THC-COOH. Therefore, simultaneous determination of THC and THC-COOH using LC-MS/MS following dansylation was proposed in this study.

It is clear that derivatization is often time-consuming and it is generally not needed in LC-MS, but dansylation of the phenolic group is quick, with high reaction yield [28]. Figure 1 shows the derivatization reaction scheme. After derivatization and LLE with hexane, the full-scan MS spectra in positive ESI mode for DC-THC and DC-THC-COOH were obtained by direct infusion. The reaction yields of DC-THC and DC-THC-COOH were studied using LC-ESI-MS/MS. More than 98% yield was found within 5 min of reaction time at 70 °C. The full-scan spectra of DC-THC and DC-THC-COOH are shown in Fig. 2. As expected, DC-THC produced an intense molecular ion peak at m/z 548, and DC-THC-COOH produced an intense molecular ion peak at m/z 578.

Product ion spectra of DC-THC and DC-THC-COOH were obtained and are shown in Fig. 3. Both DC-THC and DC-THC-COOH show a major special fragment at m/z 171
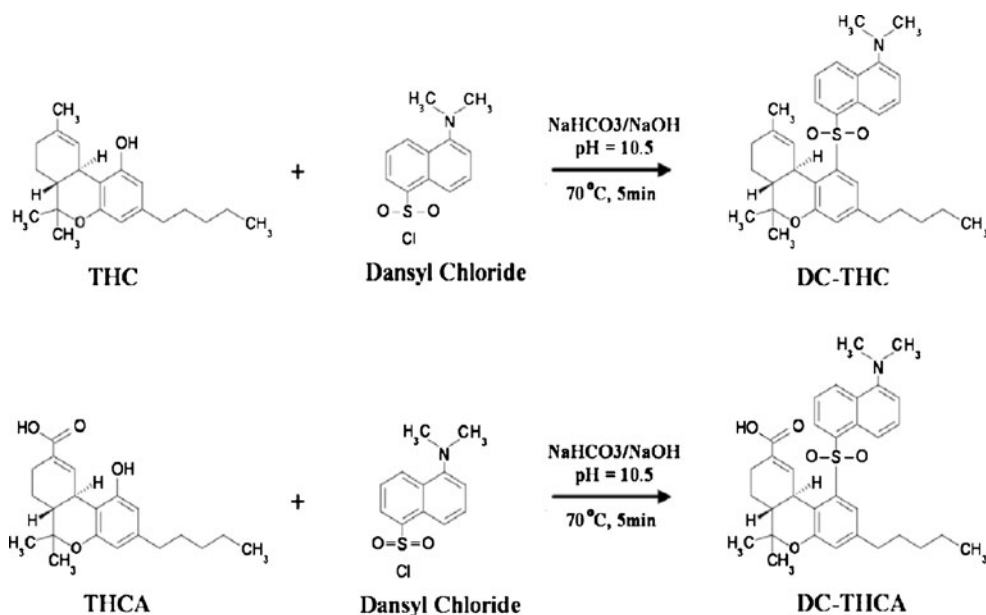
corresponding to the 5-(dimethylamino)naphthalene moiety. Once the primary ion for each of DC-THC, DC-THC-COOH, and their internal standards had been selected, the instrument settings were optimized for maximum signal using the semiautomated ramp procedure of the instrument. The two most intense product ions for DC-THC (*m/z* 171, 299) and DC-THC-COOH (*m/z* 171, 532) were used for quantitation and identification, and the proposed fragmentation of DC-THC and DC-THC-COOH is shown in Fig. 4.

Chromatography

The gradient chromatographic separation was conducted using reverse-phase HPLC. At the beginning, a common mobile phase system with water–methanol for separation was used. However, owing to the highly hydrophobic property of DC-THC and DC-THC-COOH, the total analysis time was more than 20 min, and resulted in the unacceptably broadened or distorted peaks (data not shown). To improve separation efficiency, mobile phase B was changed to THF with 0.5% formic acid. As a result, the following gradient elution profile was successful in separating DC-THC-COOH and DC-THC: 0–0.5 min, 40% mobile phase B; 0.5-1.5 min, from 40 to 90% mobile phase B; 1.5-2 min, 90% mobile phase A (Fig. 5a). The water–THF mobile phase system dramatically altered the chromatographic elution behavior and improved the separation efficiency. The total analysis time was successfully reduced to 2 min and a sharp peak was obtained for both DC-THC-COOH and DC-THC (below 0.2 min).

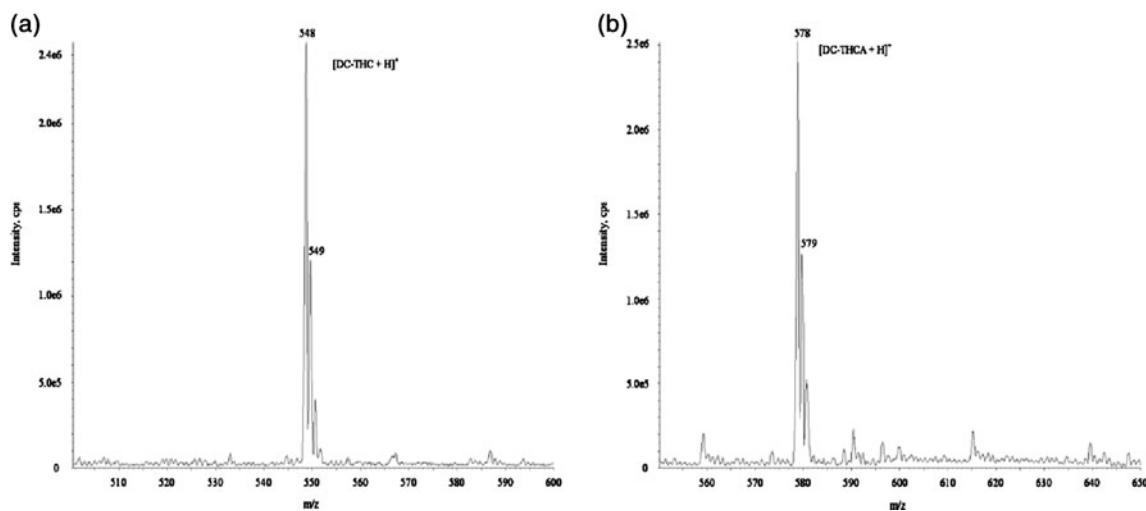To obtain a robust HPLC–ESI–MS/MS method, the matrix effect must be removed or minimized. In particular,

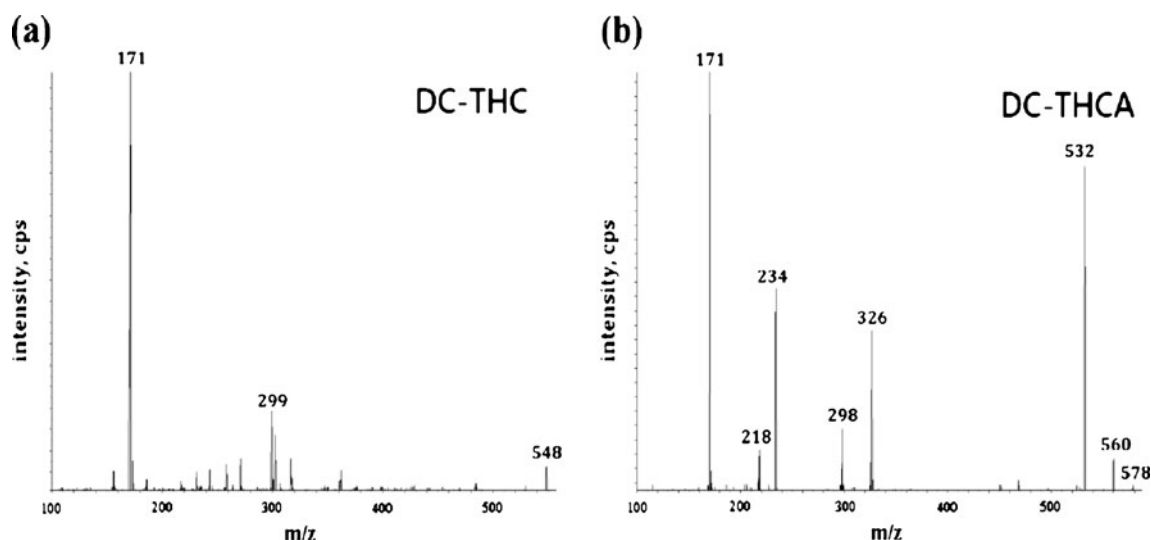**Fig. 2** Full-scan mass spectra of **a** DC-THC and **b** DC-THCA

Fig. 3 Product ion spectra of a DC-THC and b DC-THCA

a shorter separation time often causes a severe matrix effect when appropriate sample preparation and adequate chromatography are lacking. Therefore, the matrix effect was taken into account in this study. The matrix effect was evaluated by postcolumn infusion of DC-THC and DC-THC-COOH during LC-MS analysis of different solutions (methanol and matrix extracted from the blank of oral fluid). It was found that the ion suppression by coeluted species affected the ESI signal stability of DC-THC-COOH and DC-THC. Therefore, a slower gradient elution was used to reduce the influence of the matrix effect (Fig. 5b).

The postcolumn infusion chromatogram showed the ion suppression (Fig. S1). Two obvious suppression zones (0.5-

1 min and 3.5-3.8 min) were observed. Both DC-THC (3.05 min) and DC-THC-COOH (2.81 min) at the particular retention time (shown as arrows in Fig. S1) were separated from the suppression zone and caused less than 5% suppression (DC-THC caused 3.45% suppression and DC-THC-COOH caused 3.95% suppression). Moreover, the use of the deuterated internal standards allowed us to further compensate for small variations, thus resulting in improved quantitation.

Method validation

The LC-MS/MS assay developed in this study was then validated for the following parameters: sensitivity (LOD
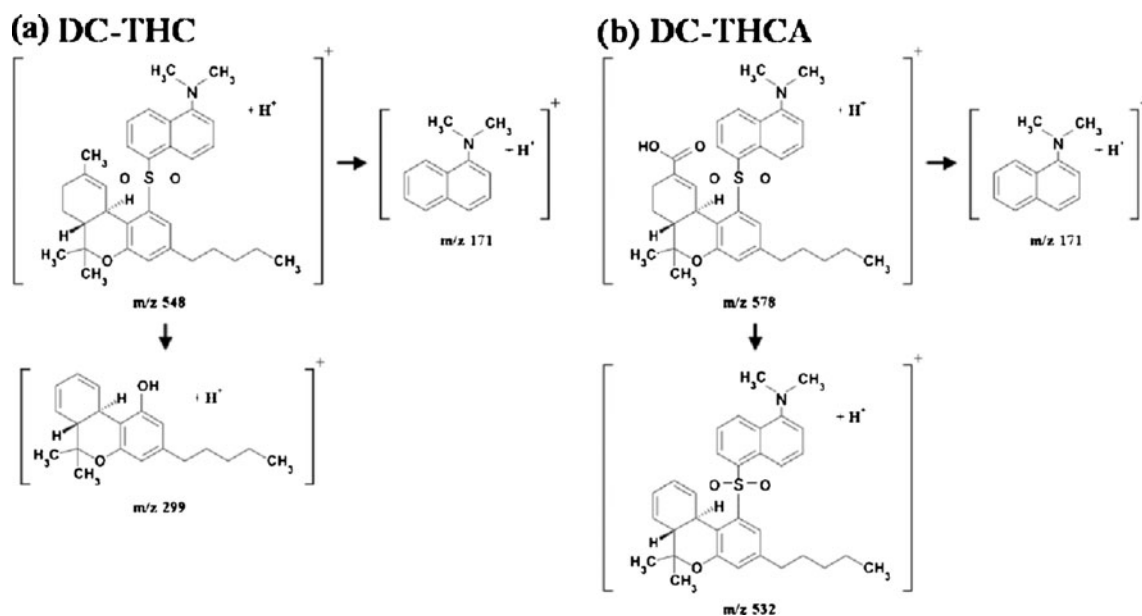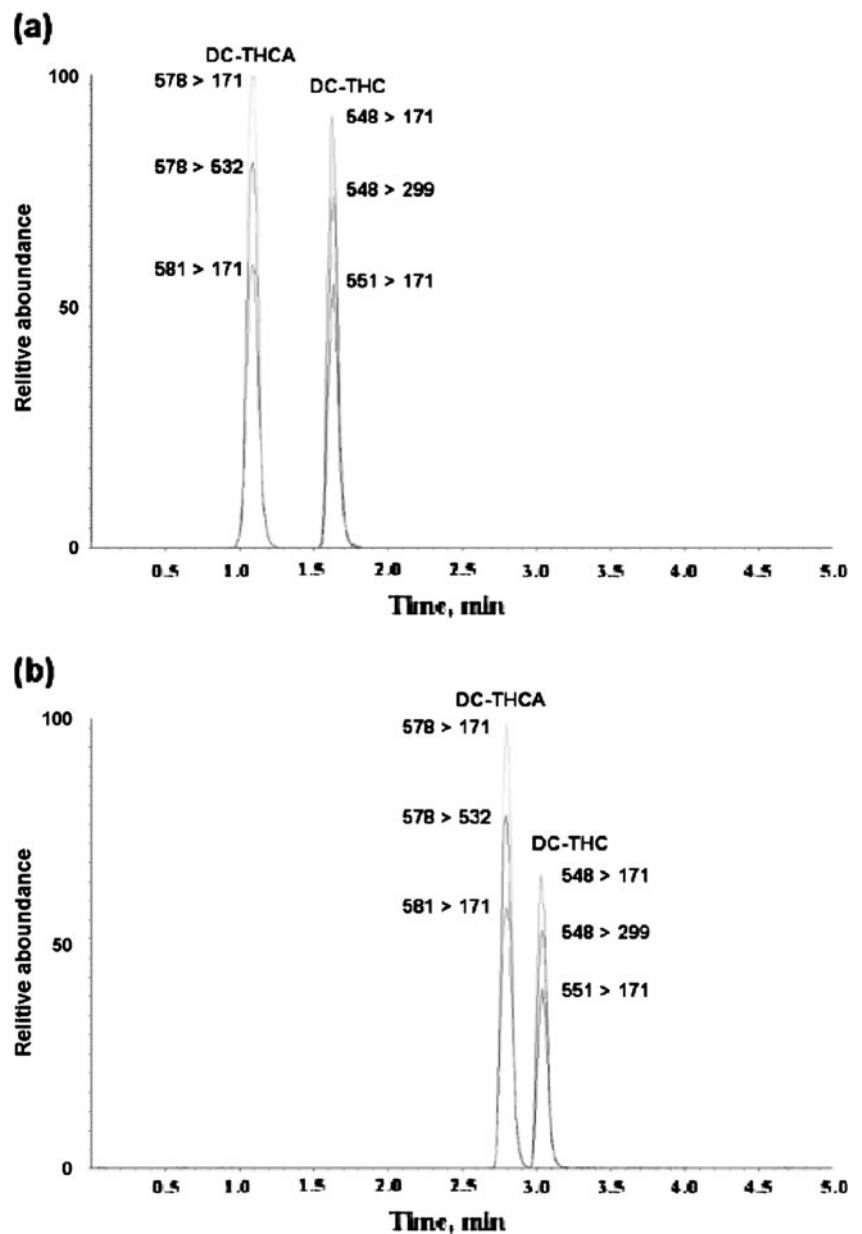

Fig. 4 Proposed fragmentation of a DC-THC and b DC-THCA

Fig. 5 Multiple reaction monitoring chromatograms obtained with a single injection of 250 μL of extracted preserved oral fluid

and LOQ), linearity, precision, accuracy, recovery, and matrix effect. DC-THC-d_3 (50 pg) and DC-THC-COOH-d_3 (25 pg) were added to 250 μL of drug-free oral fluid samples. The LOD was defined as the lowest concentration of the drug resulting in a signal-to-noise ratio of 3 or greater. The LOQ was defined as the lowest concentration of the drug resulting in a signal-to-noise ratio of 10 or greater. The LOD and LOQ obtained were 5 and 15 pg/mL for DC-THC and 2 and 5 pg/mL for DC-THC-COOH when 250 μL of oral fluid was used.

Table 2 Calibration results, retention times, peak width, limits of detection (*LOD*), and limits of quantitation (*LOQ*)

Compound	Retention time (min)	Peak width (min)	LOD[a] (pg/mL)	LOQ[b] (pg/mL)	Linear range (pg/mL)	Linearity[c] (R^2)
DC-THC	3.05	0.20	5	25	200-20,000	0.9993
DC-THC-COOH	2.81	0.21	2	5	5-500	0.9994

[a] Determined as the concentration with a signal-to-noise ratio of at least 3

[b] Determined as the concentration with a signal-to-noise ratio of at least 10

[c] Described by the correlation coefficient for the calibration curve

Table 3 Validation data for the recovery and the intraday and interday precision and accuracy experiments for DC-THC and DC-THC-COOH

Compound	Nominal concentration (pg/mL)	Recovery (%)	Intraday (*n*=5)		Interday (*n*=6)	
			Precision (CV, %)	Accuracy (bias, %)	Precision(CV, %)	Accuracy(bias, %)
DC-THC	400	93.3±3.3	8.4	5.6	5.4	6.7
	2,000	93.4±2.4	5.7	5.9	3.8	6.1
	10,000	95.1±2.7	2.9	3.2	3.1	5.3
DC-THC-COOH	20	88.5±2.4	9.5	11.8	9.6	10.3
	100	89.2±3.1	4.6	7.4	4.8	4.2
	500	92.5±1.9	3.1	8.5	5.5	−3.2

CV coefficient of variation

The standard curve range was 0.2-20 ng/mL for THC and 5–500 pg/mL for THC-COOH when 250 μL of oral fluid was used. A simple linear regression analysis was performed. The LOD, LOQ, and calibration results are detailed in Table 2. The confidence parameters of the validated method (interday and intraday precision and accuracy) for the determination of the analyte studied are shown in Table 3. The precision and accuracy for the analyte under investigation at the reported concentrations satisfactorily met the internationally established acceptance criteria (less than 15%). The recoveries obtained are presented in Table 3. These results suggest that at a low control concentration, the mean recoveries are slightly lower than at a high control concentration. The analytical procedure proposed for the determination of THC and THC-COOH in oral fluid was shown to be highly precise with the use of the deuterated internal standard. Good sensitivity and linearity were also obtained for the analyte.

Oral fluid analysis

The proposed method was applied to the routine analysis of eight authentic oral fluid samples. They were collected from suspected cannabis abusers at the drug testing center of Chung Shan Medical University Hospital in Taiwan.

Table 4 Positive results of application to real suspected oral fluid samples

Subject	THC (ng/mL)	THC-COOH (pg/mL)
O1	2.6.	21.3
O3	5.8	47.2
O4	2.4	13.1
O5	2.1	<LOQ
O6	8.7	31.5
O8	10.2	31.8

Volumes of 250 μL of each sample were used for analysis following protein precipitation, dansylated derivatization, and LLE with hexane as described "Experimental." Table 4 shows that six subjects gave positive values (higher than 2 ng/mL) for THC. Positive values for THC-COOH were obtained in these THC-positive subjects, except for subject O5, for which the value was below the LOQ but higher than the LOD. The quantitation range was from 1.7 to 10.2 ng/mL for THC and from 13.1 to 47.2 pg/mL for THC-COOH. These results show that the method is suitable for simultaneous quantitative determination of THC and THC-COOH.

Conclusion

In this study, a high-sensitivity LC-MS/MS method was developed and fully validated for the simultaneous determination of THC and THC-COOH in oral fluid. To achieve the sensitivity for low concentrations (picograms per milliliter) of THC-COOH in oral fluid, a chemical derivatization strategy was applied to enhance the detection characteristics of THC-COOH. This validated IDMS method provides specific and accurate results over an analyte concentration range that is consistent with expected oral fluid concentrations following oral THC administration. In addition, the total LC analysis cycle time was under 5 min. Moreover, MS detection was in the positive ESI mode, which makes the method expandable to more drugs.

Although derivatization was necessary in this method, this process is quick and simple to perform. As far as we know, the sensitivity obtained is the best sensitivity of all the methods based on LC-MS/MS, and was high enough to detect trace levels of THC-COOH in oral fluid specimens. The application of the dansylation method to simultaneous determination of THC and THC-COOH in oral fluid was successful. In future investigations, we will apply this method for more challenging analysis in hair.

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