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Journal of Chromatography B, 810 (2004) 319–324
JOURNAL OF CHROMATOGRAPHY B
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Short communication

Development and validation of a method for the quantitation of Δ^9tetrahydrocannabinol in oral fluid by liquid chromatography electrospray–mass-spectrometry

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Received 15 June 2004; accepted 9 August 2004

Abstract

Analysis of Δ^9tetrahydrocannabinol (Δ^9THC) and its metabolites in biological samples is of great relevance for forensic purposes. In the case of oral fluid (OF), the analysis should determine Δ^9THC, whereas in urine, it detects the inactive metabolite tetrahydrocannabinol carboxylic acid (THC-COOH). Most laboratories analyze Δ^9THC in such samples using GC–MS methods, but these procedures are time-consuming and involve unavoidable previous extraction and derivatization. No data is yet available on the application of liquid chromatography–mass-spectrometry to detect Δ^9THC in oral fluid. We report a validation method in which the Δ^9THC is isolated from oral fluid by a simple liquid–liquid extraction with hexane and subsequently analyzed by liquid chromatography–mass-spectrometry.

The method here reported for the determination of Δ^9THC in oral fluid only requires 200 μl of sample and achieves limits of detection of 2 ng/ml, and has been used to analyze oral fluid samples collected from current drug users.

Keywords: Δ^9Tetrahydrocannabinol; Oral fluid; LC–MS

1. Introduction

The oral fluid (OF) contains both saliva and other fluids and substances which are present in the oral cavity. Substances tend to be detectable in saliva for shorter periods than in urine, typically for the 12–24 h after consumption [1]. A disadvantage of studying OF is that people are sometimes unable to produce sufficient amounts of fluid for analysis. The greatest advantage of analysing OF is that samples can be collected in ways that both respect patients dignity and also assure staff that the sample comes from the patient. Staff can directly observe patients when they produce samples, usually by placing a collection tube or device in their mouth. The possibility of contamination or substitution is minimal. The levels in saliva of substances to be detected tend to be rather lower than those of the drugs metabolites in urine, thus analysis of OF is a more sensitive process.

The usefulness of oral fluid testing for Δ^9tetrahydrocannabinol (Δ^9THC) is related to its use as a diagnostic indicator of recent inhalation of marihuana smoke. Δ^9THC was identified as the major component in oral fluid with a detection time ranging from 2 to 10 h [2]. However, the detection of Δ^9THC in oral fluid is linked to the contamination of the oral cavity during smoking [3–5]. Contrary to what Schramm et al. [6] stated, neither carboxytetrahydrocannabinol nor 11-hydroxytetrahydrocannabinol were detected in oral fluid, and only in some cases were cannabinol and cannabidiol detected with Δ^9THC [7]. Huestis and Cone [8], reported a significant correlation between mean values of oral fluid

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doi:10.1016/j.jchromb.2004.08.017

Table 1
Conditions of measurements of Δ^9THC

Compound	Retention time	*m/z*	Voltage (V)
Δ^9THC	3.1	315.4	20
		193.1	50
Δ^9THC-d3	3.1	318.4	20

Δ^9THC concentrations and performance measure of drug effect, although with high individual variability. According to Idowu and Caddy [9], the oral fluid/plasma concentration ratio of Δ^9THC varies over a wide range but, until now, including recent reviews [10–13], there is not enough data to clarify this relationship. Despite this, oral fluid is an interesting biological sample for forensic interpretation purposes. Its main advantages are: it uses a non-invasive specimen, is easy and rapid to take, and requires no special equipment or facilities; also, the supervision of the collection of this sample is more acceptable for the donor than urine or blood collection.

Most laboratories analyze Δ^9THC in blood and oral fluid by time-consuming GC–MS methods [14–18]. Hughes et al. [19] were pioneers in setting up a LC–MS–MS method for the analysis of Δ^9THC in blood which involved comparing several ionization techniques, columns, SPE procedures and mobile phases. The main problem encountered was a low recovery rate (30%). Maralikova and Weinmann [20] reported a sensitive LC–MS–MS method for the simultaneous analysis of three forensic most relevant cannabinoids, Δ^9THC, 11-hydroxy-Δ^9THC and 11-nor-9-carboxy-Δ^9THC, in plasma. Recently, Valiveti and Stinchcomb [21] applied LC–MS to quantify Δ^9THC and metabolites in plasma and obtained high levels of recovery when applying the method to pharmacokinetic studies in rats. However, no data are yet available on the application of LC–MS to detect Δ^9THC in oral fluid.

The method here reported for the determination of Δ^9THC in oral fluid only requires 200 μl of sample, and achieves limits of detection of 2 ng/ml, using a simple liquid–liquid extraction procedure with hexane.

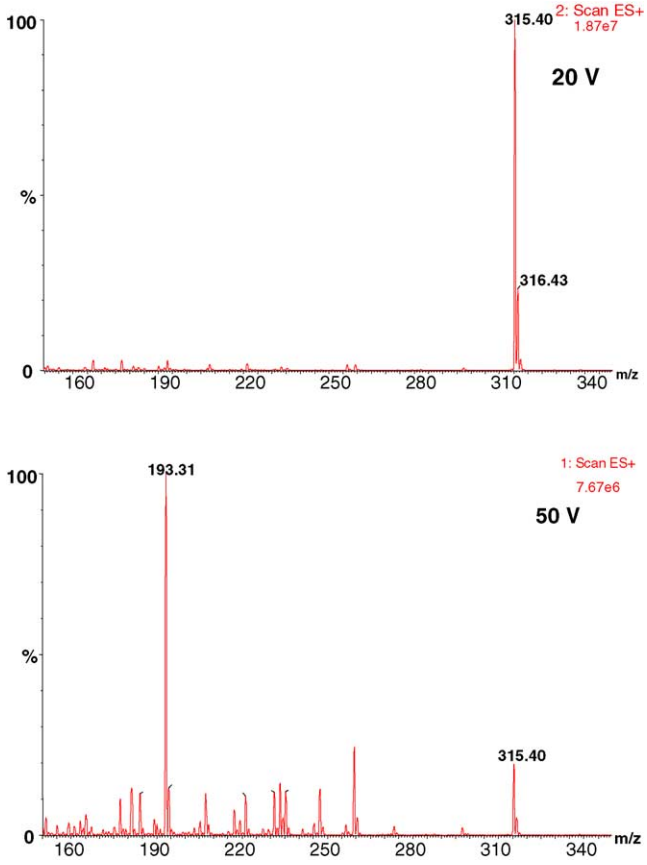

Fig. 1. Full scan mass spectrum of Δ^9THC at different cone voltages.

2. Experimental

2.1. Chemical and reagents

Tetrahydrocannabinol (Δ^9THC) 1 mg/ml in metanol and Δ^9THC deuterated (d3) (Δ^9THC-d3) 100 μg/ml in methanol as pure standards were supplied by Radian International (Austin TX, USA). LC–MS Chromasolv® grade Acetonitrile (99.98% pure) was from Riedel de Häen Sigma–Aldrich Chemie (Schnelldorf, Germany). Hexane, formic acid (98–100%), potassium dihydrogen phosphate and di-sodium hydrogen phosphate were from Merck (Barcelona. Spain).

Table 2
Repeatability and reproducibility results

Compound	Concentration (ng/ml)	Recovery (%)	Repeatability (n = 6)			Reproducibility (n = 6)		
			Concentration mean (ng/ml)	C.V. (%)	Bias (%)	Concentration mean (ng/ml)	C.V. (%)	Bias (%)
Δ^9THC	2		2.08	6.3	3.92	1.89	14.7	−5.5
	5		4.54	6.8	−9.3	4.87	4.3	−2.6
	10	88.3	10.20	10.0	2.0	11.06	8.0	10.6
	25		24.83	5.6	−0.7	24.68	6.5	−1.3
	50		50.45	9.0	0.9	51.66	6.0	3.3
	125	84.9	120.26	5.4	−3.8	115.70	6.3	−7.4
	250		247.61	5.3	−1.0	257.13	2.6	2.8

Calibration curve: slope 0.0123 ± 0.009, intercept 0.073 ± 0.004, coefficient of determination (R^2) 0.995 ± 0.003.

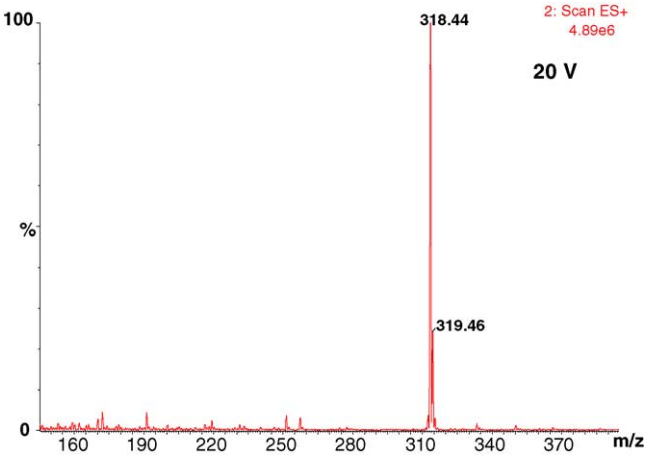

Fig. 2. Full scan mass spectrum of Δ^9THC-d3 at 20 V.

2.2. Preparation of standards and sample extraction

Calibration standards of Δ^9THC were prepared in drug-free oral fluid by spiking with concentrated standards in order to obtain a concentration range between 2 and 250 ng/ml. 25 μl of internal standard Δ^9THC-d3 (1 μg/ml) was added for all concentrations.

The procedure was carried out with 200 μl of oral sample mixed with 1 ml of phosphate buffer pH 6 Soërensen buffer (1/15 M), prepared by dissolving 9.07 g of potassium dihydrogen phosphate into 1 l of deionised water, and 11.6 g of disodium hydrogen phosphate anhydrous into 1 l of deionised water (the Na_2HPO_4 solution was used to adjust the KH_2PO_4 solution to pH 6) and 5 ml of hexane in a 25 ml borosilicate glass tube. After mechanical shaking (30 min) and centrifugating (10 min at 2792.5 × *g*), the organic phase was transferred to a borosilicate tube and evaporated under N_2 at 45 °C. The dry extract was reconstituted in 40 μl of mobile phase. The samples were transferred into autosampler vials, and 15 μl was injected onto the LC–MS.

2.3. Chromatographic conditions

The final optimized LC separation was performed using a Waters Alliance 2795 separation module system. Chromatography was carried out at ambient temperature, using a XTerra® MS C18 column (2.1 mm × 100 mm, 3.5 μm), eluted isocratically using 0.1% formic acid/acetonitrile 15:85, (v/v), delivered at a flow rate of 0.25 ml/min.

Various tests were carried out with different formic acid and acetonitrile ratios. For our purposes the ratio 0.1% formic acid/acetonitrile 15:85 (v/v), was ideal for rapid analysis following previous elution of coextracted endogenous substances, and the high content in acetonitrile improved the ionization of the analyte. The best separation results were obtained using a XTerra® MS column as contrasted with others (Symmetry™ and SymmetryShield™). Data acquisition was controlled using MassLynx NT 3.5 software.

MS procedure was performed using a Micromass ZMD 2000 detector fitted with a Z-spray ion interface. Ionization was achieved using electrospray in the positive ionization mode (ESI+). The following conditions were found to be optimal for the analysis of Δ^9THC: capillary voltage, 3.5 kV, source block temperature, 115 °C and desolvation gas (nitrogen) heated to 300 °C and delivered at 500 l/h. Table 1 summarizes the conditions for the measurement of Δ^9THC and its deuterated analogue.

The specificity (the ability of analytical method to differentiate and quantify the analyte in the presence of other

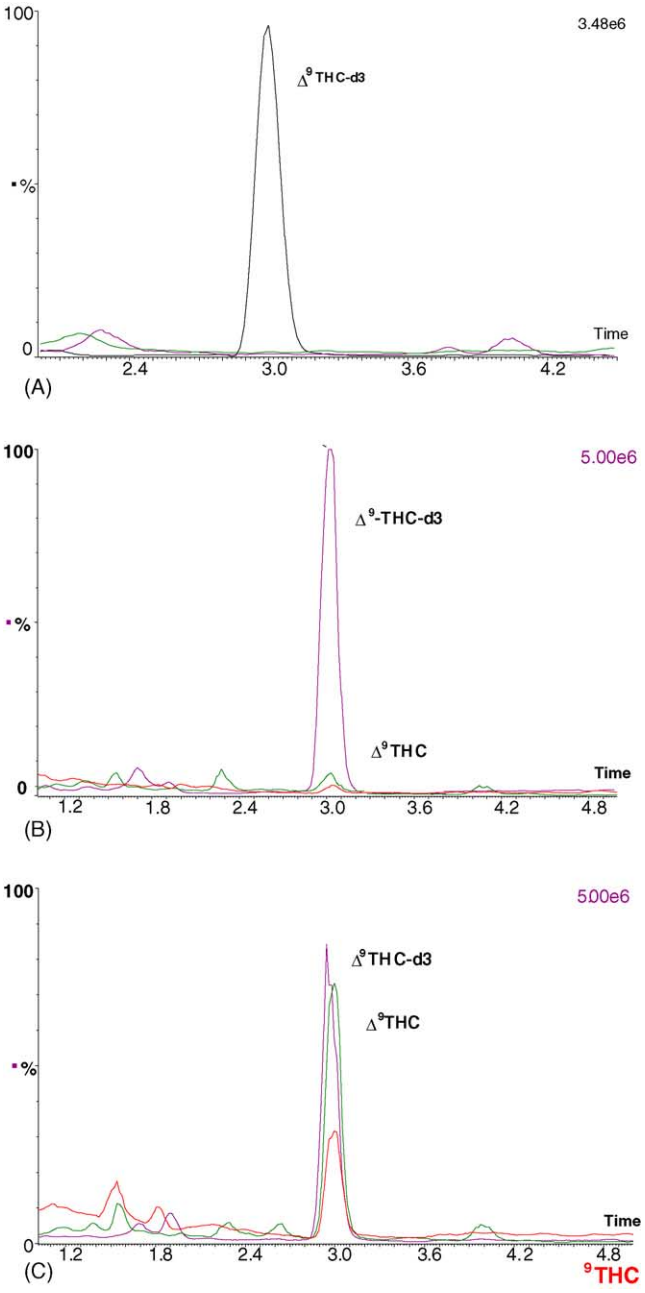

Fig. 3. (A) Blank Oral fluid with internal standard Δ^9THC-d3; (B) Oral fluid drug-free spiked with 5 ng/ml of Δ^9THC; (C) Oral fluid drug-free spiked with 125 ng/ml of Δ^9THC.

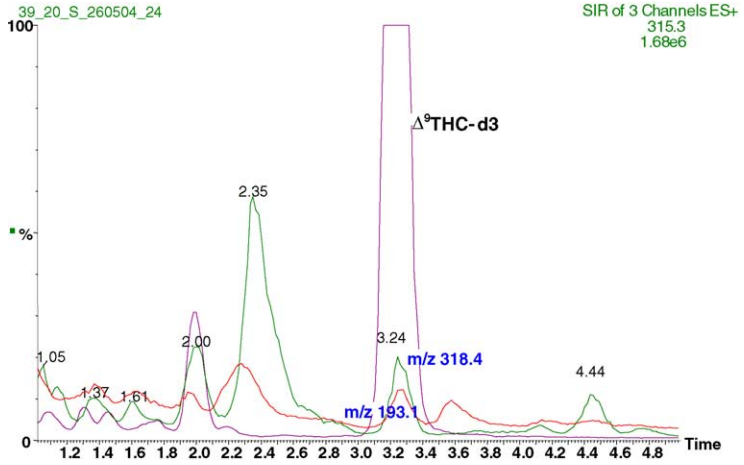

Fig. 4. Chromatogram corresponding to real sample (case 9: 6.79 ng/ml).

components) was achieved by applying the extraction procedure to six different sources (oral fluid collected from the saliva of six non-drug consuming subjects), according Shah et al. [22]. The blank oral fluid extract with the internal standard processed with this procedure showed a typical ion chromatogram (Fig. 3A). Dams et al. [23] achieved selectivity by a unique combination of retention time, precursor and fragment ion (SRM mode in triple quadrupole). In our case, selectivity was obtained by a combination of retention time and SIR mode. Two *m*/*z* ratios for Δ^9THC were monitored in SIR mode, one corresponding to pseudomolecular ion (315.4), and the other to the appropiate fragment of the analyte (193.1). This latter fragment was obtained through collision in the cone with the residual solvent and gas molecules, by a procedure described by Marquet [24] as "collision induced dissociation" (CID), and can be used as confirmation ion for quantitation purposes.

3. Results and discussion

3.1. Validation of the analytical method

The repeatability (within-day precision) and reproducibility (between-day precision), determined by analyzing six quality control samples at seven concentration levels (in the same day and on six separate days, respectively), are shown in Table 2. Results indicated that the accuracy of the assay was >90% and CV did not exceed 15%. According to Causon [25], all parameters obtained fall within the optimal precision and accuracy criteria.

For recovery studies, six replicates of two intermediate concentrations (10 and 125 ng/ml) with internal standard (Δ^9THC-d3) were extracted by applying the previously described extraction procedure. Six blank saliva samples with internal standard (Δ^9THC-d3), were then extracted in the same way and the final dried extracts were spiked with the appropiate amounts of Δ^9THC in mobile phase. Absolute recoveries for Δ^9THC in oral fluid were carried out by comparing the analyte peak areas of the fortified samples with those of the blank samples spiked, after extraction, with the same amount of the analyte.

The limit of quantitation (LOQ), defined as the lowest concentration of analyte that could be measured reproducibly and accurately (CV <20% and bias ± 20%), was 2 ng/ml. It should be noted that the observed LOQ for this technique was achieved by using 200 μl of oral fluid, whereas normally 500–1000 μl of specimen is used for GC–MS.

Linearity was obtained with an average correlation coefficient (R_2, weighting factor 1/*x*) >0.99 over a range from the LOQ value up 250 ng/ml

The ESI mass spectra of Δ^9THC and its deuterated analogue are shown in Figs. 1 and 2. In order to obtain a best yield for the molecular ions (315.4 for Δ^9THC and 318.4 for Δ^9THC-d3, respectively), 20 V was the optimal cone voltage, while 50 V was chosen for the qualifying ion (193.1).

Drug-free oral fluid spiked at 5 and 125 ng/ml with the analyte and the internal standard, and extracted according the proposal method showed typical ion chromatograms seen in Fig. 3B and C.

Table 3
Results obtained applying the method to 14 oral fluid samples

Sample	Elapsed time (h)	Δ^9THC (ng/ml)
1	<1	>250
2	5	78.78
3	1–2	>250
4	1	>250
5	3	240.64
6	Unknown	22.58
7	>10	27.80
8	8	34.84
9	>10	6.79
10	>10	22.82
11	>10	2.42
12	>10	22.17
13	4–5	117.04
14	1–2	>250

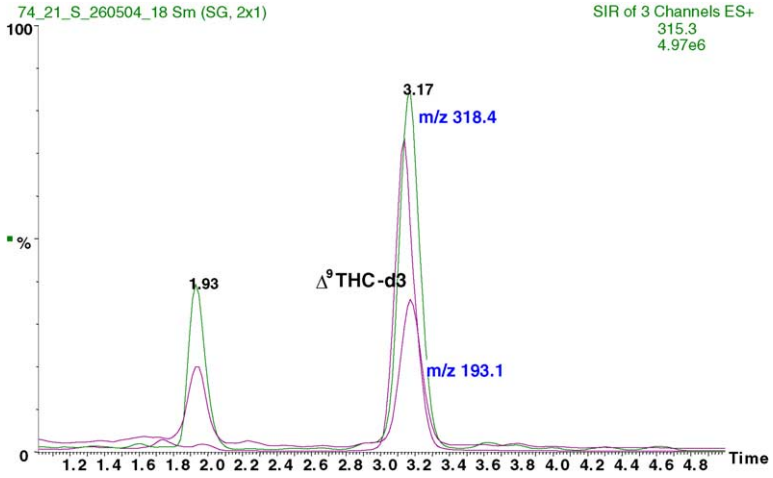

Fig. 5. Chromatogram corresponding to real sample (case 5: 240.64 ng/ml).

Matrix effects were evaluated by postcolumn infusion of Δ^9THC into the MS detector and autosampler injection of extracted blank onto the analytical column, according experimental setup developed by King et al. [26]. The experiment was performed by triplicate to ensure its validity. We detected no suppresive effect.

Oral fluid samples from drivers who had admitted taking cannabis when they tested positive to roadside controls were provided without stimulation by spitting into a polypropilene tube to obtain 1–2 ml. The samples were frozen at −20 °C with dry ice until analysis in the laboratory. Stability of the samples were no tested.

Some drivers were asked to give the lapsed time from the last cannabis intake. Table 3 shows the results obtained by applying the proposed method, and Figs. 4 and 5 show typical chromatograms of cases 9 and 5.

4. Conclusion

The use of oral fluid as a non-invasive specimen alternative to blood as an indicator of recent drug use is particularly indicated in countries were the legislation restricts blood collection. Further studies to evaluate the usefulness of such specimens should involve pharmacological research to determine the expected concentrations, because at the moment the results are still controversial.

In summary, we have developed a simple and rapid method for THC quantitation in oral fluid by using LC–MS. The procedure is sensitive and specific and involves a very simple liquid–liquid extraction method. It has been validated and applied to fourteen real samples.

Acknowledgements

This work was supported by a grant from Dirección Xeral de Investigación Científica de la Xunta de Galicia (XUGA 2001) to research groups of excellence, and by EU under ROSITA project.

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