QUANTITATIVE ANALYSIS OF Δ9-TETRAHYDROCANNABINOL IN PRESERVED ORAL FLUID BY LIQUID CHROMATOGRAPHY-TANDEM MASS-SPECTROMETRY

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OBJECTIVES

To develop and validate a simple and sensitive method for the analysis of THC in oral fluid collected using the Intercept® device.

Figure 1. Cannabis sativa.

INTRODUCTION

- Cannabis is the collective term for the psychoactive substances of the Cannabis sativa plant (Figure 1) and one of the most frequently used illicit drugs in the western world.
- Delta-9-tetrahydrocannabinol (THC), the main psychoactive constituent of cannabis, is deposited in the oral cavity during cannabis smoking.
- Over the last few years there has been an increasing interest in the use of oral fluid to document drug use. The advantage of this specimen over the more traditional matrices e.g. urine and blood, is that collection is almost non-invasive, relatively easy to perform, and may be achieved under close supervision to prevent adulteration or substitution of the sample.
- LC/MS/MS is a technique that lends itself well to the high-throughput determination of multiple analytes in oral fluid samples due to its high specificity, sensitivity and short analysis times[1,2].
- The Intercept® is a FDA approved oral fluid collection device that is used on a large scale in the U.S. for workplace testing[3]. It is also the device of choice to collect the samples in a current joint roadside study between the European Union and the U.S. to detect driving under the influence of drugs[4].
- The Intercept® collection system utilises a variety of ingredients to ensure stability and to maintain the integrity of the sample. However, these ingredients can also cause interferences e.g. ion suppression during LC/MS/MS analysis in the absence of a suitable clean-up method[5].
- The purpose of this study was to develop and validate a rapid and sensitive LC/MS/MS method that would be suitable for the analysis of THC in oral fluid samples collected with the Intercept®.

METHODS AND INSTRUMENTATION

Samples

Calibrators and quality control (QC) samples

Oral fluid samples used for the preparation of blanks, calibrators and QC samples were obtained from healthy volunteers and collected with the Intercept® collection device (OraSure Technologies, Bethlehem, PA) according to the manufacturer's instructions. Briefly, after gently wiping the collector pad between gum and cheek for approximately 2 minutes the device is placed in the supplied vial and sealed. Following centrifugation, the recovered fluid was spiked with THC to yield a series of calibrators ranging from 0.1 to 100 ng/mL. QC samples were also prepared by spiking control oral fluid with THC.

Authentic samples

Oral fluid samples were collected by the police at roadblocks, the purpose of which, was to intercept drivers who were driving under the influence of drugs. The samples were collected at the roadside using the same procedure as described for the blank samples.

An additional series of authentic samples were obtained from volunteers with a history of cannabis use. Once a week, and over 2 consecutive weeks, subjects received either a placebo cigarette (where the THC had been extracted) or a marijuana cigarette which contained 300 µg cannabis per kg). Samples were collected 0.5 hour prior to drug administration and at various times following drug administration (0.25, 0.5, 1, 1.25, 1.5 hour).

The study protocol was approved by the ethics committee of the University Hospital of Maastricht in the Netherlands.

Internal standard solution

An internal standard (IS) working solution of THC-d3 at a concentration of 10 ng/mL was prepared in methanol.

Sample preparation

Extraction was performed using either 100 or 500 µL of the collected specimen. When using 500 µL, 50 µL of the IS working solution and 4 mL of hexane were added; when only 100 µL of oral fluid was used, an additional 400 µL of deionised water was added. After mechanical shaking (30 min) and centrifugation (10 min at 3000 g), the organic phase was collected and then evaporated to dryness at 40 °C under nitrogen. The extract was reconstituted in 100 µL of mobile phase.

LC conditions

LC system:	Waters® Alliance® System
Column:	Waters XTerra® MS C_{18} column (2.1 x 150 mm, 3.5 µm) at 40 °C
Mobile phases:	(A): 1 mM ammonium formate (B): methanol Isocratic elution 10:90 (A:B)
Flow rate:	0.2 mL/min
Injection volume:	20 µL

Mass spectrometry conditions

Mass spectrometer:	Waters Micromass® Quattro Premier™ tandem mass spectrometer	
Ionisation mode:	ES +	
Capillary voltage:	2 kV	
Source temperature:	120 °C	
Desolvation gas:	Nitrogen at 700 L/Hr, 280 °C	
MS/MS:	THC	m/z 315.2>193.1 (quantification ion) m/z 315.2>259.3 (qualifier ion)
	THC-d3	m/z 318.2>196.1
	Cannabinol	m/z 311.2>223.1
	Cannabidiol	m/z 315.2>193.1
Collision gas:	Argon at 3.5 x 10^{-3} mbar	

RESULTS AND DISCUSSION

Figure 2 shows the MRM chromatograms obtained following the analysis of a sample enriched with THC and the internal standard *i.e.* THC-d3.

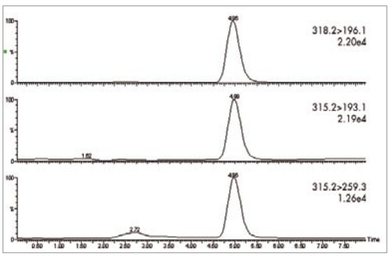

Figure 2. MRM chromatograms obtained with a single injection of a 100 µL extracted oral fluid sample enriched with 5 ng/mL THC and 5 ng/mL THC-d3. The figure shows the response for THC-d3 (top trace) and for the two transitions of THC (quantifier and qualifier middle and bottom trace respectively). Peak intensity is shown in the top right-hand corner of each chromatogram.

The usefulness of the liquid/liquid extraction step was assessed by a comparison of the effect of the matrix both before and after sample clean-up. Matrix effects were monitored throughout the whole of the chromatographic run by performing post-column infusion experiments[6]. The effect on THC response obtained following the injection a sample prior to extraction and the same sample after extraction of 100 µL and 500 µL of oral fluid are given in Figure 3. The results clearly demonstrate the usefulness of the liquid-liquid extraction step prior to LC/MS/MS analysis.

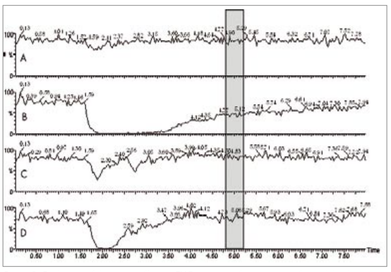

Figure 3. Evaluation of the matrix effect on THC response of an injection of a mobile phase control (A), a blank sample prior extraction (B), the reconstituted extract after extraction of 100 µL (C) and the reconstituted extract after 500 µL of oral fluid (D). The shaded area indicates the elution position of THC. Peak intensity for THC is shown in the bottom right-hand corner.

To assess method linearity, limit of quantitation (LOQ), precision, accuracy and analytical recovery a series of oral fluid calibrators were prepared and a 100 or 500 µL aliquot extracted with hexane prior to analysis using LC/MS/MS. Quantification was achieved by integration of the area under the specific MRM chromatogram. For THC, the response was calculated in reference to the integrated area of THC-d3.

Linear responses (r = >0.999, *1/x* weighting) were obtained up to 100 ng/mL when 100 µL of sample was extracted and up to 10 ng/mL when 500 µL sample was extracted. Linearity and sensitivity data are summarised in Table 1. The limit of quantification was defined as the concentration of the lowest calibrator which was calculated to be within ± 20% of the nominal value and with a % CV less than 20%. This criteria was met by the lowest calibrator i.e. 0.5 and 0.1 ng/mL when either 100 or 500 µL respectively of the collected sample was extracted.

Linearity data					Sensitivity data
volume oral fluid	slope°	intercept°	CV of slope (% over 5 consecutive days)	r^2 (range of 5 consecutive days)	LOQ (ng/mL)
100 µL	1.0635	0.0209	2.9	0.9993-0.9999	0.5
500 µL	5.3976	-0.0009	4.1	0.9992-0.9999	0.1

Table 1. Linearity and sensitivity data for THC in oral fluid.
Samples were prepared by the liquid-liquid extraction method as described in the text.
° Reported values are the mean of five determinations over 5 consecutive days.

Intra-assay and interassay variation (as % CV) were all found to be highly satisfactory at <6% (Table 2). Analytical recovery was estimated by comparing the responses of a 5 ng/mL calibrator (using 100 µL of oral fluid) when the non-deuterated compounds were added *before* the extraction step (n= 3) with those obtained when the non-deuterated analytes were added *after* sample preparation (n= 3). The recovery was found to be satisfactory at 85.6 ±0.5%

volume oral fluid	concentration of QC (ng/mL)	Intra-assay precision: mean concentration found (ng/mL)	%CV	accuracy (%)	Interassay precision: mean concentration found (ng/mL)	%CV	accuracy (%)
100µL	2.5	2.5	3.6	-1.0	2.4	2.9	-2.5
	25.0	24.8	0.4	-0.7	24.0	5.4	-4.1
500µL	0.5	0.5	2.5	-2.4	0.5	4.1	-0.5
	5.0	4.9	0.4	-2.0	4.7	3.8	-6.8

Table 2. Precision and accuracy data for THC for the extraction of 100 µL and 500 µL of spiked oral fluid samples.
° Intra-assay precision was evaluated by the preparation and analysis of four replicates of a low and a high in a single assay for both volumes of oral fluid used. Interassay precision was evaluated by the preparation and analysis of each QC over 8 consecutive days.

The stability of THC in oral fluid collected by the Intercept® device was assessed by spiking oral fluid with THC at 3 different concentrations (1, 10 and 100 ng/mL) and then monitoring the stability at 4 °C and at room temperature over a period of 48 hours. No statistical significant differences could be observed for the three different concentrations in both conditions.

The stability of the samples post extraction was assessed by repeated injections of extracted samples over a period of 15 hours. No instability was noted over the course of this experiment.

Cannabidiol and cannabinol are two components that are also naturally-occuring in the *Cannabis sativa* plant. Since the *m/z* for the precursor mass of cannabinol is different to that of THC, it does not interfere in its quantitation. On the other hand, the protonated molecular species of cannabidiol *i.e. m/z* 315.2 is the same as that of THC. Furthermore it shows the same product ions after collision induced dissociation. Thus chromatographic separation is essential to distiguish between these 2 isobaric compounds. Analysis of standards showed cannabidiol to be chromatographically resolved from THC (Figure 4).

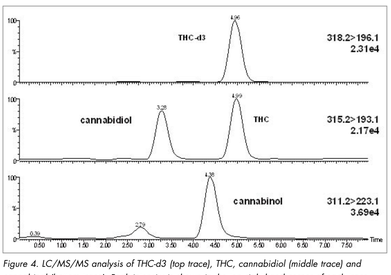

Figure 4. LC/MS/MS analysis of THC-d3 (top trace), THC, cannabidiol (middle trace) and cannabinol (bottom trace). Peak intensity is shown in the top right-hand corner of each trace.

The utility of the LC/MS/MS method was demonstrated by the analysis of 102 authentic samples collected from volunteers who smoked a placebo or marijuana cigarette. Figure 5 shows the values for THC in oral fluid collected after smoking the marijuana cigarette; mean values are plotted as a function of time. All specimens collected prior to smoking were negative, with the exception of 3 samples where concentrations were very low (maximum 2.2 ng/mL). Peak concentrations occurred 0.5 hour after smoking. Thereafter concentrations decreased steadily. There was considerable inter-individual variation in the observed concentrations; this has also been reported by other authors[7] and may also be as a result of the lack of exact volume measurement in the device.

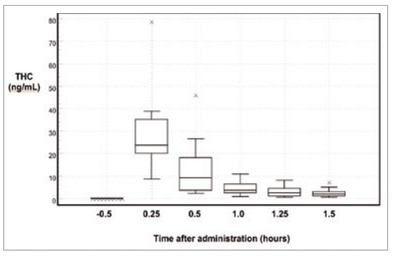

Figure 5. Box- and whisker plots of THC levels in oral fluid samples following smoking of a single marijuana cigarette. Oral fluid samples were taken prior to administration i.e. at –0.5 h, 0.25 h, 0.5 h, 1 h, 1.25 h and 1.5 h after smoking. Concentrations plotted on the Yaxis are expressed as ng/mL. The central box represents the values from the lower to upper quartile (25 to 75 percentile). The middle line represents the median. The horizontal line extends from the minimum to the maximum value, excluding "outside" (not present) and "far out" values (cross marker) which are displayed as separate points.

sample	THC (ng/mL)	sample	THC (ng/mL)
1	5.7	25	60.2
2	7.0	26	3.9
3	4.6	27	52.2
4	18.5	28	25.4
5	2.5	29	193.5
6	95.8	30	111.2
7	0.3	31	7.3
8	84.7	32	14.6
9	0.3	33	1.9
10	0.5	34	4.7
11	4.5	35	100.0
12	3.9	36	23.0
13	31.9	37	57.1
14	50.8	38	88.6
15	34.6	39	3.9
16	56.0	40	375.8
17	81.1	41	3.7
18	11.9	42	4.4
19	107.4	43	4.2
20	92.1	44	4.2
21	10.0	45	4.2
22	17.6	46	4.1
23	94.8	47	4.0
24	37.2	48	4.4

Table 3. Results obtained applying the method to 48 oral fluid samples collected by the police at the roadside.

Forty-eight samples were also collected from drivers intercepted at Belgian roadblocks. Table 3 summarises the quantitative results for all positive samples and Figure 6 shows a MRM chromatogram for one such marijuana user; the presence of cannabidiol was also noted (at 3.28 min) in this specimen.

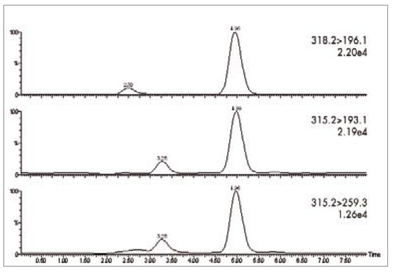

Figure 6. Typical MRM chromatograms obtained following the analysis of an authentic oral fluid specimen obtained from a driver in a roadside setting. The calculated concentrations was 5.7 ng/mL. The figure shows the response for THC-d3 (top trace) and for the two transitions of THC (quantifier and qualifier middle and bottom trace respectively). Peak intensity is shown in the top right-hand corner of each trace.

CONCLUSIONS

- To the very best of our knowledge, the method presented here is the first demonstration of the use of LC/MS/MS for the analysis of THC in oral fluid samples collected with the Intercept® device.
- The method is simple and comprises simple liquid/liquid extraction followed by LC/MS/MS.
- The method demonstrates high recovery, excellent precision and accuracy when using either 100 or 500 µL sample.
- The LOQ is sufficiently low to meet the requirements of SAMHSA (2 ng/mL) for oral fluid testing.
- Pharmacokinetic studies may require lower LOQ's; these requirements can be met by using larger volumes of oral fluid.
- The method was successfully applied to the analysis of samples collected in a controlled cannabis smoking study and to samples collected at the roadside by Belgian police.

REFERENCES

1. K.A. Mortier, K.E. Maudens, W.E. Lambert, K.M. Clauwaert, J.F. Van Boxlaer, D.L. Deforce, C.H. Van Peteghem and A.P. De Leenheer, J. Chromatogr. B 779 (2002) 321–330.
2. R. Dams, C.M. Murphy, R.E. Choo, W.E. Lambert, A.P. De Leenheer and M.A. Huestis, Anal. Chem. 75 (2003) 798–804.
3. E.J. Cone, L. Presley, M. Lehrer, W. Seiter, M. Smith, K.W. Kardos, D. Fritch, S. Salamone, S. Niedbala, J. Anal. Toxicol. 26 (2002) 541.
4. EU Project ROSITA Roadside Testing Assessment. http://www.rosita.org.
5. M. Wood, M. Laloup, M. Ramirez Fernandez, K.M. Jenkins, M.S. Young, J.G. Ramaekers, G. De Boeck, N. Samyn, Forensic Sci. Int, *in press*.
6. R. Bonfiglio, R.C. King, T.V. Olah, K. Merkle, Rapid Commun. Mass Spectrom. 13 (1999) 1175.
7. R.S Niedbala, K.W. Kardos, D.F. Fritch, S. Kardos, T. Fries, J. Waga, J. Robb, E.J. Cone, J. Anal. Toxicol. 25 (2001) 289.

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