

Detection of Conjugated 11-nor- Δ^9 -Tetra-hydrocannabinol-9-carboxylic Acid in Oral Fluid

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Abstract

The presence of the conjugated marijuana metabolite 11-nor- Δ^9 -tetrahydrocannabinol-9-carboxylic acid (THCA) glucuronide in oral fluid specimens is described for the first time. Oral fluid specimens were collected using a QuantisalTM device and analyzed for the presence of THCA using two-dimensional gas chromatography (GC) with mass spectrometric (MS) detection both before and after hydrolysis. The nature of the conjugation was determined by analyzing specimens from a marijuana user without hydrolysis, with base hydrolysis, with β -glucuronidase treatment, and hydrolysis using sulfatase only. Treatment with sodium hydroxide proved to be the most efficient hydrolytic procedure. Specimens collected over 48 h showed an average conjugation of over 64.5%. The specimens were also analyzed for the active component, tetrahydrocannabinol (THC), which was detected in the oral fluid, in most cases, for up to 24 h. Parent THC was not found to be glucuronide bound. Specimens were then subjected to commercially available immunoassays in order to determine their utility as screening procedures. The metabolite, THCA, was detected in all samples up to and including the specimen 48 h after smoking, using the more sensitive screening assay and two dimensional GC-MS. Moreover, proof that the THCA is conjugated in oral fluid minimizes concerns associated with passive inhalation.

Introduction

Marijuana is the most commonly used illicit drug in the USA and throughout the world. The active constituent, tetrahydrocannabinol (THC), is rapidly oxidized to 11-hydroxy- Δ^9 -tetrahydrocannabinol (11-OH-THC) and then to the inactive metabolite, 11-nor- Δ^9 -tetrahydrocannabinol-9-carboxylic acid (THCA). In some biological matrices, these undergo further

metabolism and conjugation to form glucuronides, which are more polar than their precursors and can be eliminated from the body more rapidly.

Oral fluid is becoming more widespread as the specimen of choice for routine drug analysis in workplace and roadside settings. Its ease of collection and difficulty of adulteration are distinct advantages over the more common urinalysis. The identification of marijuana intake by the detection of THC in oral fluid has been reported (1,2). In the smoking process, the THC is deposited into the oral cavity, and it appears that fluid from this depository is the main source of the THC collected and measured in oral fluid analysis, rather than drug which has circulated through the body. This potential disadvantage to oral fluid analysis was largely removed by the recent identification of the metabolite THCA in saliva (3,4). Its detection minimizes the argument of passive exposure to marijuana being responsible for a positive THC result.

Huestis and Cone (2) reported that THC concentrations generally dropped below 1 ng/mL approximately 12 h after smoking. Similarly, Niedbala et al. (5) reported a slightly longer detection time for THC in oral fluid than in plasma, with an average detection time for consecutive positives of 13 h. However, some subjects appeared to be positive after 72 h having previously fallen below the limit of quantitation (LOQ). They conclude that the fast and slow release of THC in saliva is due to its sequestration in the oral cavity. With a view to further extending the window of detection of marijuana in oral fluid and eliminating both sequestration and passive exposure concerns, this study investigated whether THC and THCA were conjugated in saliva. Oral fluid specimens collected from a marijuana user following smoking were analyzed for THC and free THCA, then hydrolyzed using three separate procedures, and were also subjected to screening by two different immunoassays. Further, specimens routinely received into our laboratory for testing were analyzed using immunoassay and confirmed for the presence of THC and THCA following base hydrolysis.

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Materials and Methods

Reagents and consumables

Enzyme linked immunosorbent assay kits [Saliva/Oral fluids Cannabinoids ELISA kit (Catalog #224) and Ultra-sensitive Cannabinoids ELISA kit (Catalog #230)] were obtained from Immunalysis Corporation (Pomona, CA). Methanol, toluene, ethyl acetate, hexane, and glacial acetic acid were obtained from Spectrum Chemicals (Gardena, CA). All solvents were HPLC grade or better and all chemicals were ACS grade. Trace-N (TN-315) solid-phase extraction (SPE) columns and the positive pressure extraction manifold were purchased from SPEWare (San Pedro, CA). The derivatizing agent, trifluoroacetic anhydride (TFAA), was purchased from Pierce Chemical Company (Rockford, IL), and 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) was purchased from Campbell Science (Rockton, IL). The derivatizing agent, *N,O*-bis (trimethylsilyl)trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (TMCS) was purchased from Pierce. Gas chromatographic columns were obtained from J & W Scientific, an Agilent Company (Palo Alto, CA).

β -glucuronidase (Type L-II) from limpets with an activity of 2380700 units/g (Catalog # G8132-500KU) and sulfatase (Type V) from *Patella Vulgata* (keyhole limpets) with an activity of 37 units/mg (Catalog # S8629-1KU) were purchased from Sigma (St. Louis, MO).

Standards

The internal standards, tri-deuterated THC and tri-deuterated THCA (100 μ g/mL in methanol), as well as the unlabeled drugs (1 mg/mL in methanol) were obtained from Cerilliant, (Round Rock, TX). The internal standard concentration for D₃-THCA was prepared at a concentration of 50 pg/mL of oral fluid, and for d₃-THC the concentration was 40 ng/mL.

Drug recovery from the Quantisal™ device

The Quantisal™ collection device consists of a pad, which is placed into the mouth, and a blue adequacy line becomes visible when 1 mL (\pm 10%) of oral fluid has been collected. The recovery of the THC and THCA from the collection pad and buffer has been previously documented. Briefly, collection pads were placed into oral fluid fortified with THC and THCA until 1 mL had been collected. The pads were then placed into the transportation buffer and allowed to remain at room temperature overnight. The following day, the pads were removed using serum separators, and the drug concentration remaining in the oral fluid was determined. The result was compared to the amount detected in the transportation buffer fortified at the same concentration, but with no collection pad in the system, in order to determine drug recovery from the pad. The efficiency of the collection system for the recovery of these cannabinoids was greater than 80% (6,7). Because THCA-glucuronide is not commercially available as a standard compound, it was not possible to determine its recovery from the collection pad.

Gas chromatography–mass spectrometry conditions

All the specimens were analyzed for both THC and THCA. A

calibration curve, oral fluid specimens and drug-free negative controls were included in every batch. The drug-free controls were prepared using synthetic oral fluid and diluted in the Quantisal buffer to simulate extraction matrix effects. For the calibration curve, unlabelled THCA was added to the oral fluid at concentrations of 2, 5, 10, 20, 40, 80, and 160 pg/mL of oral fluid, respectively. For THC, concentrations of 1, 2, 4, and 8 ng/mL were prepared in synthetic oral fluid. The calibrators were then diluted 1 part to three parts with transportation buffer.

Hydrolysis methods

Each specimen was analyzed without hydrolysis and following hydrolysis by one of three different procedures: base hydrolysis, enzyme hydrolysis using β -glucuronidase, and enzyme hydrolysis using sulfatase.

Base hydrolysis. 1N sodium hydroxide (0.2 mL) was added to 1 mL of each specimen (oral fluid + buffer). The samples were heated at 60°C for 15 min and then allowed to cool. Glacial acetic acid (0.5 mL) was added and the samples were extracted according to the later described extraction procedure.

Enzyme hydrolysis using β -glucuronidase. The β -glucuronidase was reconstituted in 0.1M phosphate buffer in order to inhibit any sulfatase activity, which may have been present.

Sodium acetate buffer (2M) (0.1 mL, pH 5) was added to the samples along with β -glucuronidase (50 μ L), and the samples were incubated at 45°C for 2 h. A separate aliquot was incubated at 37°C overnight, but results showed there was no significant difference in the total concentration of THCA measured between the two procedures, therefore subsequent specimens were treated with β -glucuronidase for 2 h at 45°C. After cooling, the samples were extracted according to the later described extraction procedure.

Enzyme hydrolysis using sulfatase. The sulfatase was reconstituted in deionized water. Sodium acetate buffer (2M) (0.2 mL, pH 5) was added to the samples along with sulfatase (50 μ L), and the samples were incubated at 45°C for 3 h. After cooling, the samples were extracted according to the later described extraction procedure. It should be noted that the transportation buffer in the Quantisal device contained some phosphate buffer, so it was possible that the activity of the sulfatase was quenched prior to the incubation.

Extraction procedure

The extraction procedure was the same for both THC and THCA. The internal standards, THCA-d₃ at a concentration of 50 pg/mL, and THC-d₃ at a concentration of 40 ng/mL, were added to each specimen (1 mL, equivalent to 0.25 mL neat oral fluid). For the calibration curve, unlabelled THCA was added to the oral fluid at concentrations of 2, 5, 10, 20, 40, 80, and 160 pg/mL of oral fluid and THC was added at concentrations of 1, 2, 4, 8, and 16 ng/mL. Two separate aliquots of each specimen were required since the final derivative was different for each analyte.

Trace-N (TN-315, SPEWare) extraction columns were conditioned with methanol (0.5 mL), and 0.1M acetic acid (0.1 mL). The samples were loaded onto the respective columns and allowed to dry. The columns were washed with deionized water/

glacial acetic acid (80:20, v/v, 1 mL), then they were washed with deionized water/ methanol (40:60, v/v, 1 mL). The columns were allowed to dry for 5 min. The THCA was eluted with hexane/ acetic acid (98:2, 1 mL) into silanized glass tubes. The entire extraction procedure was carried out using a positive pressure manifold, which allowed the flowrate through the columns to be highly uniform. The eluent was evaporated to dryness under nitrogen at 40°C.

Derivatization THCA. The residue was reconstituted in TFAA (50 µL) and HFIP (30 µL). The mixture was transferred to autosampler vials with glass inserts and capped. The vials were heated at 70°C for 15 min, and left at room temperature for 10 min. Finally, the extracts were evaporated to dryness in a vacuum oven. The samples were finally reconstituted in toluene (25 µL) for injection into the

gas chromatography–mass spectrometry (GC–MS) system.

Derivatization THC. The residue was reconstituted in ethyl acetate (20 µL). The derivatizing agent (BSTFA + TMCS) was added (20 µL) and the vials were heated at 60°C for 15 min. The samples were injected into the GC–MS system.

Confirmatory assay

THCA. The complete validation details of the GC–MS method for the detection of THCA have been previously published (8).

THC. For the detection of parent tetrahydrocannabinol, an Agilent 6890 GC with a 220/240 V oven, coupled to a 5975 mass selective detector with an inert source operating in electron impact mode, was employed. The column was a DB5-MS (15 m · 0.25 mm · 0.25 µm) and the injector was operated in splitless mode at a temperature of 250°C. The oven ran from 125°C for 0.5 min to 250°C at a rate of 40°C/min, where it was held for 1.3 min, then ramped at 70°C/min to 300°C. The selected ions monitored were: **374.2** and 389.1 for the tri-deuterated internal standard (THC-d₃) and **371.2**, 386.1 and 303.2 for unlabelled THC. The quantitation ions are shown in bold type. The retention time for deuterated THC was 4.27 min. Calibration using deuterated internal standardization was carried out using linear regression analysis over a concentration range of 0–16 ng/mL. Peak area ratios of target analytes and the internal standard were calculated for each concentration. The data were fit to a linear least squares regression curve, not forced through the origin, and with equal weighting. For confirmation, three ions were monitored for THC, and two ion ratios were determined, which were required to be within 20% of those of the known calibration standards (calculated at 4 ng/mL) in order to be acceptable.

The selectivity of the method was determined by the analysis of five drug-free oral fluid specimens collected using the Quantisal device. An aliquot of each was taken and subjected to extraction and analysis as described, in order to assess potential interferences associated with endogenous compounds or the transportation buffer.

Additionally, to other aliquots of the drug-free fluid, common drugs of abuse were added at concentrations of 500 ng/mL. Codeine, morphine, 6-acetylmorphine, amphetamine, methamphetamine, methylenedioxyamphetamine (MDMA), methylenedioxyamphetamine (MDA), methylenedioxyethylamphetamine (MDEA), pseudoephedrine, phenter-

mine, phencyclidine, cocaine, benzoylecgonine, hydrocodone, propoxyphene, meperidine, tramadol, and methadone were all added to drug-free oral fluid, extracted, and analyzed as described.

The LOQ of the THC analytical method was defined as the lowest point at which the signal-to-noise ratio (peak height) was at least 10, and the chromatography in terms of peak shape and resolution, retention time (within 2% of calibration standard), and qualifier ion ratio (\pm 20%) compared to the 4 ng/mL calibration standard were acceptable. Because all specimens were to be quantitated, the limit of detection (LOD) was not determined.

The precision of the assay at four levels was evaluated. Specimens fortified at concentrations of 1, 2, 4, and 8 ng/mL were prepared and six aliquots of each concentration were analyzed according to the described procedure each day (interday precision) for 4 consecutive days (intraday precision).

Application to authentic samples

Specimens were collected on multiple occasions from a volunteer who was a habitual marijuana smoker, according to the Internal Review Board protocol (Immunalysis 2006–04). The subject was requested to collect a sample immediately before smoking, then at regular intervals after smoking for as long as possible. Quantisal oral fluid collection devices were used and the specimens were analyzed using the described procedures.

Immunoassay screening

The observation that THCA was significantly conjugated in oral fluid indicated the possibility that specimens actually containing THCA were screening negatively with commercially available screening assays since the target analyte was parent THC. In order to investigate the potential use of ELISA to detect the metabolite in oral fluid for a longer period of time, the specimens, including calibrators and controls were analyzed using two separate ELISA kits, which displayed different sensitivities and cross-reactivity towards cannabinoids.

Screening kit A. The first screening kit was intended for the analysis of THC in saliva or oral fluids using a cutoff concentration of 4 ng/mL. A calibration curve was analyzed with each batch at concentrations of 2, 4, 8, 25, 50, and 100 ng/mL. An aliquot of the calibrator, control, or specimen (50 µL) was added to each well of the microplate, and the plate was allowed to remain in the dark at room temperature for 1 h. The enzyme conjugate (pH 8.5, 100 µL) was added to each well, and again the plate was incubated for 30 min at room temperature. The plate was washed six times with deionized water, then 3,3',5,5' tetra-methylbenzidine with hydrogen peroxide in buffer (TMB) was added as the chromogenic substrate (100 µL). The plate was allowed to remain at room temperature in the dark for 30 min, then 1 N hydrochloric acid was added (100 µL) to stop the reaction. The absorbance of each well of the plate was read at a dual wavelength of 450 and 650 nm.

Screening Kit B. The second screening kit was intended for use with hair specimens, therefore the sensitivity of the system had been improved to allow a cutoff concentration of 1 pg/mg (equivalent to 4 pg per well at the cutoff concentration). How-

ever, because the concentration of THCA in oral fluid was so low, the ELISA kit was employed to determine whether it could detect marijuana use. A sample size of 200 μL of the oral fluid + buffer (equivalent to 50 μL of neat oral fluid) was added to the microplate, and the plate was allowed to remain in the dark at room temperature for 1 h. The plate was washed three times, then the enzyme conjugate (pH 8.5, 200 μL) was added to each well, and again the plate was incubated for 1 h at room temperature. The plate was washed six times with deionized water, then 3,3',5,5' tetra-methylbenzidine with hydrogen peroxide in TMB was added as the chromogenic substrate (200 μL). The plate was allowed to remain at room temperature in the dark for 30 min then 1 N hydrochloric acid was added (100 μL) to stop the reaction. The absorbance of each well of the plate was read at a dual wavelength of 450 and 650 nm. A calibration curve with concentrations of 2, 5, 10, 20, 40, and 100 pg/mL was analyzed with each batch (equivalent to 8, 20, 40, 160, and 400 pg/mL of neat oral fluid).

Specificity of the kits

Screening kit A was targeted at the parent drug, $\Delta^9\text{-THC}$ (100%), and showed a cross-reactivity of 133% towards $\Delta^8\text{-THC}$, 800% towards THCA, and 150% cross-reactivity towards 8-11-dihydroxy- $\Delta^9\text{-THC}$. Cannabinol and cannabidiol were less than 5% cross-reactive with the assay.

Screening kit B was targeted towards the metabolite, $\Delta^9\text{-THCA}$ (100%), and showed a cross-reactivity of 11% towards $\Delta^8\text{-THCA}$, 16.6% towards 11-hydroxy- $\Delta^9\text{-THC}$, and less than 5% towards $\Delta^9\text{-THC}$, cannabinol and cannabidiol.

Results and Discussion

GC-MS method validation for THC in oral fluid

The method for the detection of THCA in oral fluid has been previously described. The procedure for THC was validated

using drug-free oral fluid specimens diluted in 0.1% bovine serum albumin (BSA) buffer, fortified with various concentrations of the analytes as described. Specimens from real users with concentrations higher than the range analyzed were diluted into the linear range of the assay. No endogenous interference was noted from drug-free extracts or for exogenous interference from any of the commonly encountered drugs, which were analyzed at high concentration. For THC, linearity was obtained for over the range 0–16 ng/mL , and the LOQ was 0.5 ng/mL . The inter-day precision of the assay was 1.46% at 4 ng/mL ($n = 4$) and the intra-day precision was 1.39% ($n = 6$).

Authentic specimens

THCA has been reported to be significantly glucuronide bound in urine, plasma, and meconium (9–11). It predominantly forms an ester bond between the carboxy-acid and glucuronic acid, which is effectively cleaved using alkaline or enzyme hydrolysis. For THC, however, alkaline conditions have been reported to be inadequate for the cleavage of the ether bond formed between the hydroxyl group and the glucuronic acid. Therefore, hydrolysis using β -glucuronidase has been recommended for urine, plasma, and meconium analysis of cannabinoids (12,13). The concentration of THC in the oral fluid did not increase following treatment with β -glucuronidase or base hydrolysis.

Frequent marijuana smoker

The methods were applied to oral fluid specimens collected from a frequent user of marijuana. The subject, a 46-year-old male (210 lbs), willingly consented to sample collection. He has been a marijuana smoker for over 20 years and smokes at least every other day. Samples were collected almost immediately after the subject smoked, then at various intervals up to 48 h after smoking. The experiment was repeated three times following daily smoking (Sessions 1,2, and 3). Though additional smoking by the user cannot be ruled out, the subject had no reason to give inaccurate information.

Table 1. Hydrolysis of Oral Fluid Samples Collected from a Frequent Marijuana User (Session 1)

Hours after smoking	THC (ng/mL)	THCA (pg/mL)				% Total Binding	Ratio: THCA bound /THCA
		Free	Hydrolytic treatment		Base		
			Sulfatase	Glucuronidase			
Before smoking	38	33	36	67	91	63.7	1.76
0.5	67	46	50	84	128	64.0	1.78
1	33	51	52	92	138	63.0	1.71
2	18	65	71	134	174	62.6	1.68
8	8.3	64	73	163	229	72.0	2.58
12	2.3	53	55	84	138	61.5	1.6
16	2.1	35	40	73	103	66.0	1.94
24	0	38	40	71	108	64.8	1.84
36	0	46	49	82	129	64.3	1.8
48	0	77	91	227	314	75.4	3.0
					Mean	65.7	1.98
					SD	4.45	0.47
					CV (%)	6.76	23.91

The initial sample set (Session 1) was analyzed for parent THC, free THCA, THCA following hydrolysis with β -glucuronidase, sulfatase, and sodium hydroxide. The results are given in Table I. The average glucuronide conjugation of THCA in oral fluid was 49.8%, the average sulfatase conjugation was 8.1%, and the mean total conjugation was 65.7% [coefficient of variation (CV) 6.7%]. Analysis of the data set indicated that the hydrolytic procedure using sulfatase could be eliminated from the experimental pattern, because the nature of the conjugation due to sulfate was minimal (Figure 1). For Sessions 2 and 3, the samples were analyzed before hydrolysis, after glucuronidase treatment, and after base hydrolysis. The glucuronide and total conjugation for the subject was relatively constant throughout the data sets (Tables II and III). Interestingly, in Session 2, the parent THC could not be measured in the oral fluid beyond 2 h, yet the THCA was present throughout the 16-h collection period.

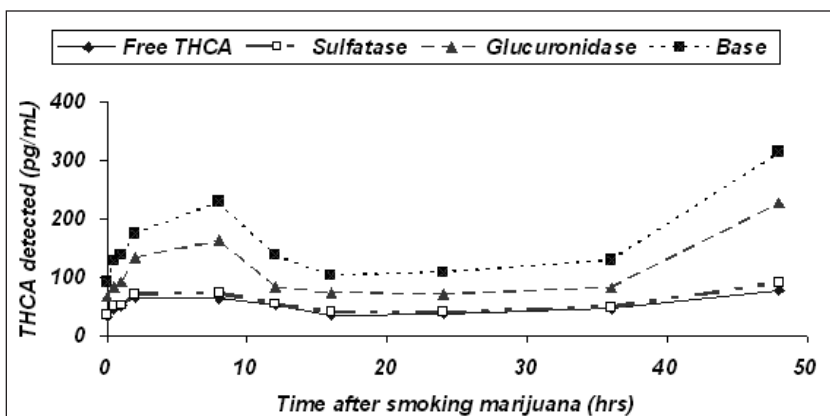


Figure 1. Concentration of THCA detected in oral fluid samples following hydrolytic treatment.

Table II. Glucuronic and Base Hydrolysis of Oral Fluid Samples Collected from a Frequent Marijuana User on Two Separate Occasions (Time 0 to 16 h After Smoking)

Hours after smoking	THC (ng/mL)	THCA (pg/mL)				Ratio: THCA bound/THCA
		Free	Glucuronidase	Base	% Total Binding	
Before smoking	10	50	119	137	63.5	1.74
Directly after smoking	87	71	133	201	64.6	1.83
0.5	15	79	115	223	64.5	1.82
1	8	34	77	95	64.2	1.79
2	4	63	150	178	64.6	1.83
3	0	33	53	75	56	1.27
4	0	30	73	63	52.3	1.1
5	0	27	57	71	61.9	1.62
6	0	44	120	66	33.3	0.5
16	0	64	173	297	78.4	3.64
			Mean	60.3	1.72	
			SD	11.6	0.80	
			CV (%)	19.3	46.83	

Various groups have measured both THCA and its glucuronide simultaneously using liquid chromatography with tandem mass spectrometry. In urine, the ratio of THCA glucuronide to free THCA was reported as 1.3–4.5 (14). In plasma the ratio at peak plasma concentrations was reported as 2.0 shortly after marijuana ingestion (15). In our study for oral fluid, the average THCA total conjugation to free THCA ratio was 1.74 (CV 12.9%, $n = 3$), which was within the reported range for other body fluids.

Glucuronide conjugation. For the three separate collections where the subject admitted smoking marijuana the previous day, the THCA appeared to be glucuronide-bound in oral fluid. The concentration of the acid increased markedly following all base and glucuronidase hydrolysis procedures. The average glucuronide conjugation was $48.6\% \pm 9.3\%$ and the mean total conjugation (base) was $64.8\% \pm 6.4\%$.

Sulfatase conjugation. The sulfatase appeared to have a minimal effect on the THCA concentration detected, indicating THCA was not sulfate-bound in oral fluid to any great degree because the correlation between the non-hydrolyzed concentrations and the levels following sulfatase hydrolysis was 0.991. The sulfatase released approximately 8% of conjugated THCA, however, as mentioned previously, potential inhibition by the phosphate buffer present in the transportation buffer may have impacted sulfatase activity.

Infrequent smoker

The subject was asked to abstain from smoking for 5 days before more specimens were collected, and the results are shown in Table IV. THC was not detected in the specimen collected directly before smoking; however, THCA was detected at a concentration of 40 pg/mL prior to smoking. In contrast, when the subject smoked each day, THC was detected in the specimens prior to smoking, and the residual THCA detected in oral fluid averaged 120 pg/mL.

The mean glucuronide conjugation was 43.68% (CV 18.5%), and the average total conjugation was 68.8% (CV 13.2%). These numbers were remarkably consistent with the data collected following frequent smoking: 48.6% glucuronide conjugation and 64.8% total conjugation, indicating the half-life of THCA in oral fluid appears to be extensive. Though our data supports the hypothesis that THCA can be detected in oral fluid for at least 48 h, it also suggests that the detection time may be even longer in a frequent user because the subject reportedly abstained from smoking for 5 days and his initial oral fluid sample contained 40 pg/mL of THCA.

In all sessions, the concentration of THCA increased within the first few hours, then ap-

peared to reach a plateau before increasing again. As stated previously, while additional smoking cannot be ruled out, the subject had no reason to provide incorrect information.

ELISA results

The data indicated that conjugated THCA was present in the specimens, therefore the possibility of its contribution to screening activity was considered. The specimens from the fourth data set (collected after the subject had abstained from smoking for 5 days) were subjected to two commercially available immunoassays, which were targeted at different sensitivity levels, one for oral fluid specimens and one for hair samples. The results are shown in Table V. Using the oral fluid screening kit (A), the samples showed inhibition below the cutoff as far as the specimen collected at 24 h, indicating a positive screening result. The sample collected at 36 h showed inhibition between the 2 and 4 ng/mL controls, but not enough to cause a screen positive result. Because the sample did not show the presence of any THC using GC-MS, it must be de-

duced that the antibody in this particular screening kit showed significant cross-reactivity with the THCA. For the detection of parent THC in oral fluid, screening kit A showed adequate sensitivity, coupled with routine single quadrupole GC-MS in electron impact mode.

Using the hair screening kit (B), which was specifically targeted at low concentrations of THCA, all the samples taken from the marijuana user were well above the screening cutoff concentration of 5 pg/mL (equivalent to 20 pg/mL of neat oral fluid) even after 48 h. For the detection of the metabolite THCA in oral fluid, screening kit B was appropriate coupled with two-dimensional GC-MS in negative ion chemical ionization mode for confirmation of the metabolite.

Routine specimens

In the second stage of the experiment, 21 oral fluid specimens received into the laboratory from unknown donors, which screened positively using ELISA screening kit A, were analyzed for THC, free THCA and THCA following base hydrolysis. The results are shown in Table VI. In addition, 24 oral fluid samples, which screened negatively using ELISA screening kit A, were re-screened using ELISA kit B and also analyzed for THCA following base hydrolysis.

For the initially screened positive samples, the total conjugation of THCA in oral fluid was $60.8\% \pm 13.1$ (CV 21.6%), which was very similar to the 64.8% observed in the oral fluid of the marijuana smoker. The variation in conjugation was expected to be higher because the specimens were from 21 separate individuals.

Using screening kit B, with a cutoff concentration of 5 pg/mL (equivalent to 20 pg/mL of neat oral fluid), three of the 24 samples that had previously screened negatively using kit A showed significant inhibition below the cutoff and were found to contain THCA following base hydrolysis at concentrations of 3.7, 5.1, and 28 pg/mL. Two other specimens screening above the cutoff concentration (indicating a negative screening result) contained THCA at levels of 2.1 and 4.4 pg/mL. Using the more sensitive ELISA kit, three of the 24 reported negative samples (12.5%) would have been identified as marijuana positive.

Limitations of the Study

Instrumental sensitivity requirements. As previously reported, parent THC is present in ng/mL quantities in oral fluid, therefore, it is much easier to measure than THCA, which requires the improved sensitivity offered by a two-dimensional GC system. However, the technique of GC · GC with MS detection is becoming increasingly applied to drug and

Table III. Glucuronic and Base Hydrolysis of Oral Fluid Samples Collected from a Frequent Marijuana User on Two Separate Occasions (Time 0 to 8 h After Smoking)

Hours after smoking	THC (ng/mL)	THCA (pg/mL)				Ratio: THCA bound/THCA
		Free	Glucuronidase	Base	% Total Binding	
Before smoking	37	47	93	133	64.6	1.83
0.5	93	39	67	82	52.5	1.1
1	25	26	45	50	48	0.92
2	9	40	90	117	65.8	1.93
8	5	85	159	245	65.3	1.88
				Mean	59.2	1.53
				SD	8.3	0.48
				CV (%)	14.1	31.1

Table IV. Specimens Taken Following 5 Days of Marijuana Abstinence

Hours after smoking	THC (ng/mL)	THCA (pg/mL)				Ratio: THCA bound/THCA
		Free	Glucuronidase	Base	% Total Binding	
Before smoking	0	13	21	40	67.5	2.08
Directly after smoking	> 2000	91	126	197	53.8	1.16
0.5	> 2000	24	41	60	60	1.5
1	90	17	31	47	63.8	1.76
2	54	22	45	78	71.7	2.55
12	10	30	66	127	76.3	3.23
24	1.7	23	38	79	70.8	2.43
36	0	12	22	80	85	5.67
48	0	5	10	17	70.5	2.40
				Mean	68.8	2.53
				SD	9.1	1.33
				CV (%)	13.2	52.3

forensic analysis (16–18), and therefore more widely available and less expensive than tandem MS.

Self-reported marijuana use. The data reported here is predominantly from one individual who gave voluntary specimens for our research. Future studies are necessary involving specimens taken from known users in controlled dosages and timing situations in order to validate the observations presented here. Moreover, a controlled study would provide further

insight into the pharmacokinetic behavior, absorption and half-life of THCA-glucuronide in oral fluid.

Conclusions

A commercially available immunoassay can be used for the detection of the marijuana metabolite THCA in oral fluid, for at least 48 h after smoking by incorporating a pre-incubation step to achieve the necessary sensitivity. THCA is conjugated, predominantly to the glucuronide in oral fluid. Base hydrolysis of the specimens was the most efficient hydrolytic mechanism, releasing an average of 63.4% of the THCA for detection. The ratio of conjugated THCA to free THCA ranged from 1.53 to 2.53 with a mean of 1.93 (CV 22.6%), based on four sets of data from the same subject. Enzyme hydrolysis using sulfatase provided a small increase in detection ability (mean = 8.1%), and β -glucuronidase released 48.2% more THCA to considerably increase the concentration detectable. It is recommended that the confirmation of THCA in oral fluid be preceded by base hydrolysis in order to achieve improved detection levels. The inclusion of THCA in the confirmation profile minimizes the argument for passive contamination of the oral cavity, and enhances long-term detection of marijuana use.

	Screening Kit A B/Bo	Confirmation THC (ng/mL)	Screening Kit B B/Bo	Confirmation THCA (pg/mL)
Cutoff B/Bo	68.9		85.4	
Before smoking	86.8	Negative	78.9	40
Directly after smoking	0.36	> 1000	4.6	197
0.5 h	0.26	> 1000	3.4	60
1 h	6.7	90	30.7	47
2 h	9.1	54	34.6	78
12 h	29.3	10	39.3	127
24 h	60.5	1.7	58.7	79
36 h	70.8	Negative	69.3	80
48 h	87.5	Negative	71.2	17

Specimen	Parent THC (ng/mL)	Metabolite, THCA (pg/mL)		
		Free	Base	% Total Binding
1	77	5	22	77.27
2	8	38	159	76.1
3	26	5.5	15	66.67
4	50	106	297	64.31
5	44	113	338	66.57
6	1200	28	60	53.33
7	240	41	107	61.68
8	16	10	39	74.36
9	56	22	37	40.54
10	46	66	136	51.47
11	578	59	133	55.64
12	31	5	11	54.55
13	4	5	25	80
14	4	9	16	43.75
15	2	8	46	82.61
16	1504	54	112	51.79
17	24	9	18	50
18	93	16	25	36
19	5	12	40	70
20	26	8	23	65.22
21	4	10	23	56.52
			Mean	60.87
			SD	13.15
			CV (%)	21.6

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