Detection of the Marijuana Metabolite 11-Nor-Δ^9-Tetrahydrocannabinol-9-Carboxylic Acid in Oral Fluid Specimens and Its Contribution to Positive Results in Screening Assays

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Abstract

The detection of the marijuana metabolite 11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid (THC-COOH) in oral fluid specimens is described, and its contribution to an immunoassay for the detection of cannabinoids is investigated. Oral fluid specimens, screened using an enzyme-linked immunosorbent immunoassay (ELISA), were carried forward to confirmation for both tetrahydrocannabinol (THC) and THC-COOH using gas chromatography–mass spectrometry (GC–MS). One hundred and fifty-three specimens were analyzed, of which 143 screened positive for cannabinoids. Ninety-five (66.4%) of these specimens were positive for both THC and THC-COOH; 14 (9.7%) were positive for THC-COOH only, and 27 (18.8%) were positive for THC only. The GC–MS assay for the detection of THC-COOH in oral fluid was linear to 160 pg/mL with a limit of quantitation of 2 pg/mL. The detection of the marijuana metabolite, THC-COOH, in 76.2% of oral fluid specimens screening positive for cannabinoids is reported. As a potential defense against passive exposure claims, proposed SAMHSA regulations may require the simultaneous collection of a urine sample when oral fluid samples are used. The detection of the metabolite, THC-COOH, is a significant alternative to this approach because its presence in oral fluid minimizes the argument for passive exposure to marijuana in drug testing cases.

Introduction

Tetrahydrocannabinol (THC) is the active ingredient in marijuana and is generally administered orally or by smoking, resulting in euphoria and hallucinations, and it is the main drug detected in oral fluid following marijuana intake (1). The detection of THC in oral fluid following a single marijuana ingestion via smoked and oral routes was reported by Niedbala et al. (2), with the average length of detection being 34 h when gas chromatography–tandem mass spectrometry (GC–MS–MS) was used at a 0.5 ng/mL cutoff concentration. Corresponding urine samples tested for the metabolite, 11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid (THC-COOH), were positive greater than 15 ng/mL for 58 h. Huestis et al. (3) reported an average detection time of THC-COOH in urine following a single high-dose smoked administration of marijuana (3.55% THC cigarette) as 88 h using a GC–MS cutoff concentration of 15 ng/mL. However, an improved detection time for THC-COOH in oral fluid has not been studied, but its presence in saliva indicates the potential for increasing the detection window of marijuana while minimizing concerns due to passive exposure.

The detection of THC-COOH in oral fluid is extremely difficult because of the very low concentrations and problems with the sensitivity of detection. In 2005, Day et al. (4) presented data on the detection of THC-COOH in oral fluid using GC–MS–MS to achieve the sensitivity required for the detection of THC-COOH in oral fluid. They reported a quantitation limit of 10 pg/mL and concentrations up to 240 pg/mL present in the oral fluid specimens (4).

As a potential defense against passive exposure claims, proposed SAMHSA regulations may require the simultaneous collection of a urine sample when oral fluid samples are used (5). The detection of the metabolite, THC-COOH, is a significant alternative to this approach because its presence in oral fluid minimizes the argument for passive exposure to marijuana in drug testing cases.

Further, screening procedures for cannabinoids in oral fluid are often positive, but they do not confirm for the parent drug. Because immunoassay format screens are also sensitive to other cannabinoids, a principal objective of our study was to

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determine whether a major metabolite, THC-COOH, was present in oral fluid and therefore possibly contributing to unconfirmed screen positives. In order to approach the required concentration using a single-quadrupole MS system, we employed a Dean's switch two-dimensional GC–GC instrument. Previously, we reported on the use of this technology for the detection of low amounts of THC-COOH in hair specimens (6), and in this report, we have adapted the assay to the quantitation of THC-COOH in oral fluid (7).

Materials and Methods

Specimens

One hundred and fifty-three oral fluid specimens were received from the Redwood Toxicology Laboratory (Santa Rosa, CA). The specimens had been screened using ELISA technology, and positive screening results were confirmed for the presence of THC using the confirmatory method with GC–MS instrumentation. Upon receipt of the specimens at Immunalysis, they were analyzed for the presence of THC-COOH. Ten specimens that screened negative were chosen randomly and also analyzed for THC-COOH. The screening and confirmatory results from the Redwood Toxicology Laboratory were blinded to the analysts at the Immunalysis until the analyses had been performed.

Extraction efficiency from the Quantisal™ device

The Quantisal collection device consists of a pad that is placed into the mouth for saliva collection. A blue line becomes visible when 1 mL (± 10%) of oral fluid has been collected. The pad is then placed in stabilization buffer (3 mL) and capped, and the specimen sent to the laboratory for analysis. The buffer causes the amount of oral fluid in the testing sample to be diluted 1:4.

Previously, we reported over 80% extraction efficiency of THC from the collection pad into the oral fluid transportation buffer (8). More recently, two independent research groups reported on the extraction efficiency for THC-COOH from the collection pad, which averaged a recovery of over 80% (7,9).

Reagents and consumables

Tri-deuterated THC-COOH [used as the internal standard (100 µg/mL in methanol)], unlabelled drug (1 mg/mL in methanol), tri-deuterated THC (100 µg/mL in methanol), and unlabelled THC (1 mg/mL in methanol) were obtained from Cerilliant (Round Rock, TX). Methanol, toluene, ethyl acetate, hexane, and glacial acetic acid were obtained from Spectrum Chemicals (Gardena, CA). All solvents were high-performance liquid chromatography grade or better, and all chemicals were American Chemical Society grade. The derivatizing agent, *N,O*-bis-(trimethylsilyl)trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (TMCS), was purchased from Pierce Chemical (Rockford, IL). GC columns were obtained from J&W Scientific (Palo Alto, CA). The screening kits, Saliva/Oral fluids Cannabinoids ELISA (catalog # 224), were purchased from Immunalysis (Pomona, CA).

Screening assay

Oral fluid specimens collected using the Quantisal device were screened according to the manufacturer's package insert. Briefly, calibrators, standards, and specimens (75 µL) were added to each well of the micro-plate. Following incubation at room temperature (60 min), enzyme conjugate (75 µL) was added to each well and the plate was incubated in the dark (30 min). The plate was washed six times, and substrate reagent (150 µL) was added. After a final incubation (30 min), the reaction was stopped by the addition of acid (100 µL), and the absorbance of the specimens was read at a dual wavelength of 450 and 650 nm. Specimens showing a lower absorbance than the cut-off calibrator of 4 ng/mL of THC were considered positive and carried forward to confirmation.

Confirmatory assay: THC

Calibration standards at 2, 4, 10, 20, and 40 ng/mL were included in every batch. Because the calibrators, controls, and specimens are all diluted 1:4 in extraction buffer, the actual equivalent concentrations of neat oral fluid were 0.5, 1, 2.5, 5, and 10 ng/mL.

Calibrators, controls, and/or specimens (1 mL) were pipetted into screw-cap test tubes. Internal standard (200 µL of 25 ng/mL solution) and 4 mL THC extraction solvent (hexane/ethyl acetate/acetic acid, 90:10:3, v/v) were added to each tube. The tubes were centrifuged for 5 min. The upper organic layer was then transferred to clean tubes and evaporated to dryness under air. The specimens were reconstituted in BSTFA + 1% TMCS (50 µL), capped, and heated at 70°C for 12 min. The final derivatives were transferred to autosampler vials for analysis. The extracts were analyzed on a Shimadzu QP2010 GC–MS. The injection port was held at 260°C and the detector at 280°C. The oven was programmed from 125°C for 0.5 min to 300°C at a rate of 20°C/min and held for 2.3 min. The ions monitored were *m/z* 374 and 389 for THC-d_3 and *m/z* 371, 386, and 303 for THC with a sampling time of 0.2 s. Specimens were considered positive if they contained more than 1 ng/mL of THC.

Results and Discussion

Method validation

THC-COOH. The extraction and analytical procedures for THC-COOH in oral fluid have been previously described (7). Briefly, the assay was linear to 160 pg/mL with a correlation coefficient of $r^2 = 0.999$ and a limit of quantitation of 2 pg/mL. The precision was determined by analyzing five oral fluid specimens containing THC-COOH at a concentration of 2, 5, 10, and 20 pg/mL on the same day (intraday precision) and on different days (interday precision; $n = 5$). At 20 pg/mL, the coefficient of variation was 3.42% and 7.47% for intra- ($n = 5$) and interday ($n = 5$) studies, respectively. Interference studies revealed that cocaine, norcocaine, cocaethylene, benzoylecgonine, methamphetamine, amphetamine, 3,4-methylenedioxymethamphetamine, 3,4-methylenedioxyamphetamine, morphine, codeine, 6-acetylmorphine, hydrocodone, hydro-

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morphone, phencyclidine, cannabinol, 11-hydroxy-THC, THC, and cannabidiol did not interfere with the assay when injected at concentrations higher than 10 ng/mL.

THC. The method was validated for accuracy and precision by the analysis of known standards and calculation of percent deviation from the target at three concentrations. The linearity of the method and the limit of quantitation were determined, and no interference was observed at concentrations of 500 ng/mL of methamphetamine, amphetamine, benzoylecgonine, cocaine, codeine, morphine, hydrocodone, hydromorphone, oxycodone, 6-acetylmorphine, phencyclidine, and methadone. The validation data is presented in Table I.

Authentic specimens

The method was applied to oral fluid specimens received from Redwood Toxicology Laboratory. The specimens had previously been screened using the ELISA method described and, if positive, confirmed for THC using GC–MS. The results are given in Table II.

Overall, 153 samples were received. 143 had screened positive for cannabinoids using ELISA techniques at a cut-off of 4 ng/mL.

Negative screens. Ten specimens screened negative and nine were confirmed negative for THC-COOH. One sample was positive for the presence of THC-COOH at a concentration of 16 pg/mL. These specimens were not confirmed for THC.

Positive screens. Of the 143 positive screens, 95 (66.4%) contained both THC and THC-COOH; 14 (9.7%) contained only THC-COOH at or greater than 2 pg/mL; and 27 (18.8%) were positive for THC only, at or greater than 1 ng/mL. The number of true positives was 136 (95.1%); the number of positive screens, which did not confirm for either THC or THC-COOH, was seven (Figure 1). The contribution of THC-COOH to ELISA-positive screening results was determined to be 9.7%.

Though the majority of oral fluid specimens (66.4%) contained both parent and metabolite, the addition of THC-COOH to the confirmatory profile increased the confirmation rate of the screening test from 85.3% to 95.1%. The positive predictive value was determined to be 95.1%, and the negative predictive value was 90%. Overall, the efficiency of the test was 94.77%.

Cross-reactivity

The specificity of the ELISA plate was determined by generating inhibition curves for various cannabinoids. The cross reactivity of cannabinol was 4% and less than 1% for cannabidiol.

However, the assay was much more sensitive to Δ^8-THC (133%), 8-11-dihydroxy-Δ^9-THC (150%), and 800% cross-reactive to THC-COOH at an equivalent THC concentration of 4 ng/mL. In order to cause a positive screening result, the concentration of THC-COOH in the specimen would need to be greater than 500 pg/mL. Therefore, it is unlikely that the positive screen results from specimens not confirming for THC were caused by THC-COOH alone, but they were perhaps caused by a combination of THC-COOH, other marijuana metabolites in the specimen, and cannabinoids in the plant material.

Potential conversion of THC to THC-COOH at room temperature

The presence of THC in oral fluid has been claimed to be "contamination" from recently inhaled marijuana. In order to determine whether the presence of THC-COOH could occur in vitro, perhaps because of amylase activity in real oral fluid, the following study was carried out. Data analysis of THC concentrations detected revealed a mean level of 57 ng/mL and a high concentration of 1870 ng/mL. Subsequently, THC was added to neat drug-free saliva and to the Quantisal buffer at concentrations of 200 ng/mL (approximately 4 × the mean) and 2000 ng/mL (above the highest concentration detected). The specimens were stored at room temperature. Every other day for six days, an aliquot of each was extracted (0.25 mL of neat oral fluid; 1 mL of buffer) and analyzed according to the protocol described. A conversion or impurity level of 0.0001% was observed in both the oral fluid and the buffer containing 2000 ng of THC after two days and 0.0002% after four days. No THC-COOH was noted in the 200 ng/mL specimens. In the authentic specimen containing 1870 ng/mL of THC, the THC-COOH concentration was 285 pg/mL. Therefore, any in vitro conversion or impurity did not contribute significantly to the THC-COOH result.

High concentrations of purchased standards of THC and deuterated THC-d_3 (2000 ng) were diluted in water and also allowed to remain at room temperature for six days, with no buffer or oral fluid. They were then extracted, derivatized, and analyzed according to the THCA procedure. No conversion to THC or impurity was noted from the deuterated standards, but 7 pg/mL of 11-nor-9-carboxy-Δ^9-tetrahydrocannabinol was detected following addition of 2000 ng of unlabelled THC to Quantisal buffer, a conversion of 0.00035%.

Table I. Validation Data for Analysis of THC in Oral Fluid Using GC–MS

Parameter Accuracy								
Concentration of THC added (n = 3) (ng/mL)			Measured THC concentration (ng/mL)			Mean (ng/mL)	Standard deviation	CV* (%)
5	5	5	5	5	5.1	5.03	0.058	1.15
10	10	10	11.1	10.8	11.2	11.03	0.208	1.89
25	25	25	25.8	25.9	25.3	25.7	0.321	1.25

Precision	1.4% (Mean CV at 3 concentrations)
Linearity	1–200 ng/mL; r^2 = 0.999
LOD†	1 ng/mL
LOQ‡	1 ng/mL
Carryover limit	800 ng/mL THC

* CV = coefficient of variation.
† LOD = limit of detection.
‡ LOQ = limit of quantitiation.

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Table II. THC-COOH Contribution to Positive ELISA Screen Results

Sample #	Amount Remaining for THC-COOH Confirmation	THC-COOH (pg/mL)	THC (ng/mL)	Sample #	Amount Remaining for THC-COOH Confirmation	THC-COOH (pg/mL)	THC (ng/mL)
1	0.25 mL	2	5	56	0.25 mL	16	39
2	0.25 mL	2	1	57	0.25 mL	16	372
3	0.25 mL	2	6	58	0.25 mL	17	53
4	0.5 mL	3	Negative	59	0.25 mL	18	12
5	0.25 mL	3	3	60	0.25 mL	18	Negative
6	0.5 mL	3	4	61	0.25 mL	18	4
7	0.25 mL	3	12	62	0.25 mL	18	15
8	0.25 mL	3	18	63	0.5 mL	18	20
9	0.25 mL	3	35	64	0.25 mL	19	Negative
10	0.25 mL	3	7	65	0.25 mL	19	100
11	0.25 mL	3	3	66	0.25mL	19	65
12	0.5 mL	4	2	67	0.25mL	21	56
13	0.25 mL	4	10	68	0.25mL	21	19
14	0.5 mL	4	Negative	69	0.5 mL	22	168
15	0.25 mL	4	7	70	0.25 mL	23	Negative
16	0.5 mL	4	8	71	0.25 mL	24	56
17	0.5 mL	4	3	72	0.25 mL	25	169
18	0.25 mL	4	14	73	0.25 mL	26	346
19	0.25 mL	4	17	74	0.5 mL	28	92
20	0.25 mL	4	96	75	0.25 mL	29	6
21	0.25 mL	4	6	76	0.5 mL	30	16
22	0.25 mL	4	7	77	0.25 mL	31	198
23	0.25 mL	4	4	78	0.5 mL	34	8
24	0.5 mL	4	24	79	0.25 mL	35	Negative
25	0.5 mL	4	3	80	0.25 mL	38	91
26	0.25 mL	5	4	81	0.5 mL	38	95
27	0.25 mL	5	6	82	0.5 mL	38	Negative
28	0.5 mL	5	6	83	0.25 mL	41	171
29	0.25 mL	6	1	84	0.25 mL	43	6
30	0.25 mL	6	4	85	0.25 mL	44	Negative
31	0.25 mL	7	62	86	0.25 mL	44	49
32	0.25 mL	7	60	87	0.25 mL	44	110
33	0.25 mL	7	Negative	88	0.25 mL	46	7
34	0.25 mL	8	119	89	0.25 mL	47	20
35	0.25 mL	8	338	90	0.25 mL	49	3
36	0.25 mL	9	26	91	0.25 mL	54	25
37	0.5 mL	10	41	92	0.25 mL	59	29
38	0.5 mL	10	19	93	0.25 mL	59	Negative
39	0.5 mL	10	Negative	94	0.25 mL	63	204
40	0.5 mL	11	Negative	95	0.25 mL	63	33
41	0.25 mL	11	5	96	0.25 mL	64	92
42	0.25 mL	11	5	97	0.25 mL	64	Negative
43	0.25 mL	11	24	98	0.25 mL	79	86
44	0.25 mL	11	55	99	0.25 mL	81	43
45	0.25 mL	11	15	100	0.25 mL	89	162
46	0.25 mL	12	25	101	0.25 mL	90	307
47	0.25 mL	12	3	102	0.25 mL	116	Negative
48	0.25 mL	13	5	103	0.25 mL	160	7
49	0.25 mL	14	9	104	0.25 mL	167	44
50	0.25 mL	14	2	105	0.25 mL	190	55
51	0.25 mL	15	39	106	0.25 mL	198	11
52	0.25 mL	15	5	107	0.25 mL	243	124
53	0.25 mL	15	11	108	0.25 mL	285	1870
54	0.5 mL	15	20	109	0.25 mL	352	173
55	0.25 mL	16	21	110	0.25 mL	Negative	4

The presence of minute amounts of THCA in THC standards may constitute contamination and not in vitro conversion.

Potential interferences

Because the quantitation limit for THC-COOH was low (2 pg/mL), the possible interference from other cannabinoids was studied. Cannabinol, cannabidiol, and THC itself did not interfere at a concentration of 10 ng/mL. The pyrolytic precursors to tetrahydrocannabinol in the marijuana plant are predominantly 2-carboxy-THC, where the carboxy group is present at the 2-carbon position (THCA-A), and 4-carboxy-THC, where the carboxy group resides at the 4-carbon position (THCA-B). However, THCA-A predominates over THCA-B, and it is present in marijuana plants, accounting for up to 5% of the THC level (10). In hemp flowers and in some hashish samples, the concentration is as high as 18%, and 34.9% of the material has been reported to be 2-carboxy-THC (11). In order to assess any potential interference, a concentration of 10 ng/mL of 2-carboxy-THC, derivatized with the same derivative as THC-COOH, was injected into the system and found not to interfere with the assay.

Benefits of the study

With the improvement in instrumentation, the ability to detect trace levels of THC-COOH using a single quadrupole GC–MS instrument has allowed routine analysis of once difficult assays. To date, claims of passive exposure to marijuana were predominantly refuted using data showing the THC concentrations in passive smokers to be significantly lower than those in active smokers (12). However, this data was generated using a device collecting an unknown amount of oral fluid (13,14). Kauert et al. (14) report that the percentage of THC recovered from the Orasure Intercept device ranged from as low as 37.8% to only 55.6% ($n = 5$), and unless gravimetric analysis and a modified elution procedure employing methanol is used, the concentration of THC cannot be accurately determined (14). This report calls into question previously published findings, often used to refute exposure claims (12,15) because no indication is given that methanol elution and/or gravimetric analysis was considered.

The major benefit of our study is the ability to determine THC-COOH in oral fluid at trace concentrations, eliminating the need for either simultaneous urine collection (as proposed in the SAMHSA regulations) or reliance

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Table II. (continued) THC-COOH Contribution to Positive ELISA Screen Results

Sample #	Amount Remaining for THC-COOH Confirmation	THC-COOH (pg/mL)	THC (ng/mL)	Sample #	Amount Remaining for THC-COOH Confirmation	THC-COOH (pg/mL)	THC (ng/mL)
111	0.5 mL	Negative	10	124	0.25 mL	Negative	8
112	0.25 mL	Negative	3	125	0.25 mL	Negative	3
113	0.25 mL	Negative	8	126	0.5 mL	Negative	3
114	0.25 mL	Negative	2	127	0.25 mL	Negative	5
115	0.25 mL	Negative	36	128	0.25 mL	Negative	10
116	0.25 mL	Negative	2	129	0.5 mL	Negative	12
117	0.25 mL	Negative	2	130	0.25 mL	Negative	5
118	0.25 mL	Negative	2	131	0.25 mL	Negative	5
119	0.25 mL	Negative	5	132	0.25 mL	Negative	3
120	0.25 mL	Negative	8	133	0.25 mL	Negative	19
121	0.25 mL	Negative	14	134	0.25 mL	Negative	4
122	0.25 mL	Negative	4	135	0.5 mL	Negative	3
123	0.25 mL	Negative	5	136	0.5 mL	Negative	3

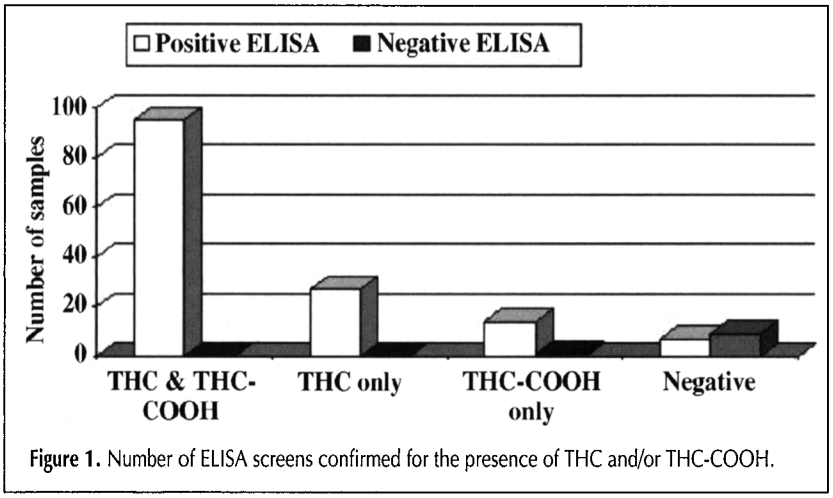

Figure 1. Number of ELISA screens confirmed for the presence of THC and/or THC-COOH.

on questionable data in order to refute passive exposure claims. Further, the detection of THC-COOH in many of the oral fluid specimens demonstrates that its presence may allow a longer history of drug detection than THC alone.

Limitations of the study

Sample volume remaining. Of the 27 specimens with only THC present, four (14.8%) had concentrations higher than 10 ng/mL of parent THC. Five had only 0.5 mL of specimen volume remaining, the others had 0.25 mL of sample to test for THC-COOH. This issue is certainly a factor in the determination of THC-COOH in the specimens because more sample volume may have improved the positivity rate for THC-COOH.

Dynamic range. Parent THC is present in nanogram-per-milliliter quantities in oral fluid. Therefore, it is much easier to measure than THC-COOH, the highest measured concentration of which was 352 pg/mL. The study could be improved by the simultaneous confirmation of the drugs, but the difference in dynamic range is a problem when a joint assay is run. Two separate injections of the same extract is a potential approach to improving the methodology. However, a Dean's switch approach with two "heart-cut" windows is currently being evaluated for use in further research studies.

Conclusions

The detection of the marijuana metabolite, THC-COOH, in oral fluid is described. The procedure makes use of small improvements in methodology to allow the identification of very low amounts of THC-COOH in oral fluid using two-dimensional chromatography and single quadropole MS. The inclusion of THC-COOH in the confirmation profile for cannabinoids in oral fluid increases the confirmation rate by at least 9.7% and minimizes the argument for passive contamination of the oral cavity. Further work will include the possibility of simultaneous confirmation of THC and THC-COOH because the amount of sample volume remaining for confirmation of THC-COOH is often less than 0.5 mL. However, the significantly different dynamic range for the two drugs is an issue in their simultaneous analysis. A controlled administration study for marijuana intake, where the 9-carboxy-THC metabolite and parent drug (THC) are measured in oral fluid, is necessary. We report for the first time, the analysis of THC-COOH in oral fluid and its contribution to ELISA assays commonly used for oral fluid screening. The minimal conversion of high concentrations of THC to its metabolite in oral fluid and/or in buffer eliminates any claim of passive contamination for marijuana-positive drug tests if THC-COOH is detected in the specimen and avoids the need for a simultaneous collection of a urine sample.

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