TECHNICAL NOTE

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Blind Trials of an Onsite Saliva Drug Test for Marijuana and Opiates*

REFERENCE: Jehanli A, Brannan S, Moore L, Spiehler VR. Blind trials of an onsite saliva drug test for marijuana and opiates. J Forensic Sci 2001;46(5):1214–1220.

ABSTRACT: The objective of these clinical trials was to calculate the performance, limit of detection, specificity and sensitivity of a novel, semi-quantitative immunoassay for drugs of abuse in saliva and to determine operator bias when measured blind by four different operators. The test is based on lateral flow gold particle technology coupled with digital photography to provide a semi-quantitative end point. The performance of the test was compared with that of enzyme immunoassays and GC/MS methods. Volunteers consumed marijuana or codeine and their saliva was collected 0.25 to 24 h later with the Cozart RapiScan collection device. The sensitivity and specificity of the opiate test were both 100% ± 10.4% for codeine for 9 h after dosing. The cutoff of the marijuana test at 10 ng/mL THCA was too high to detect marijuana use for more than a few hours after smoking. There was no operator bias because the results were presented in written form either as "positive" or "negative for each of the five drug classes on the screen of the hand-held reader.

KEYWORDS: forensic science, forensic toxicology, saliva, immunoassay, sensitivity and specificity, operator bias

Chemical testing of biological fluids is the most common objective means of diagnosis of use of drugs of abuse. The presence of a drug analyte in a biological specimen can be used as evidence of recent exposure. Saliva has many advantages over both blood and urine (1–4). Saliva collection is noninvasive. Saliva testing detects primarily the parent drug. Presence of the parent drug in blood and therefore in saliva may correlate with impairment. The feasibility of detecting drugs in saliva samples obtained from impaired drivers was first investigated by Peel et al. (1). They found that the presence of drugs in saliva correlated well with officer judgments of driving while intoxicated.

We describe here a recently developed rapid saliva test that is based on the principle of competitive lateral flow immunoassay. The outcome of the test was expressed electronically through the use of digital photography.

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Methods

Saliva was collected using the Cozart RapiScan collection pad and tube and tested using the Cozart RapiScan Saliva Drugs Test (Cozart Bioscience Ltd., Abingdon, UK). When placed in the mouth, the collection pad absorbed exactly 1 mL of saliva as indicated by a blue indicator in the handle. The saliva-soaked pad was then placed in the tube containing elution fluid and separated from the plastic handle. The 1 mL of saliva on the cellulose pad was diluted with 2 mL of run buffer fluid to a final volume of 3 mL. Four drops of the saliva/run fluid mixture was placed in the immunoassay cartridge using a transfer pipette (Fig. 1). Once fluid started to flow by capillary action, the cartridge was inserted into the hand-held Cozart RapiScan instrument for incubation. The saliva/run fluid rehydrated gold-labeled anti-drug antibodies contained within the cartridge. This mixture traveled by capillary action across an array of immobilized drug sites. Absence of or reduction in color development at an immobilized drug position indicated drug presence. The quality control position contained anti-IgG to ascertain that complete lateral transfer of specimen had been achieved. Incubation is timed for 4 to 12 min depending on the number of test bands on the cartridge.

The test results are read electronically, processed by a computer chip and displayed as a written message. After 12 min for the 5 panel test (4 min for the single tests), if the quality control was satisfied, the screen on the Cozart RapiScan reader, displayed the results as "Positive" or "Negative" for each of the five drug classes. However, the instrument can be adapted to express the outcome as percent of drug line intensity. Presence of a given drug in the saliva results in a decrease in the corresponding standard drug line intensity. Detection limits were set based on testing a large number of negative saliva samples. These do not necessarily correspond to total absence of the drug line. The back-lit screen for reading results, timing, quality control, and error messages is similar to those used in mobile phones, onsite glucose analyzers, and hand-held computers. In addition to the message, if all results are negative, a green light appears above the power switch. If any of the results are positive a red light appears.

The collector pad has a dead volume of approximately 1 mL and the Cozart RapiScan cartridge requires between 0.12 and 0.15 mL for completion. The same volume is required for single, dual, or multiple drug panels. The excess volume of saliva/run fluid mixture was designed to allow confirmation to be performed. For the purposes of this study, it allowed multiple testing from the same sample tube.

Crossreactivity

Crossreactivity for the Cozart RapiScan was determined by dropping Cozart RapiScan run buffer spiked with drugs at the concentrations indicated in the tables into the sample well and determining the response of the Cozart RapiScan test. In all the five tests performed simultaneously, the colloidal-gold labeled drug derivatives have been tested in the same manner at 10 000 ng/mL and found not to cause a positive for the other Cozart RapiScan drug tests.

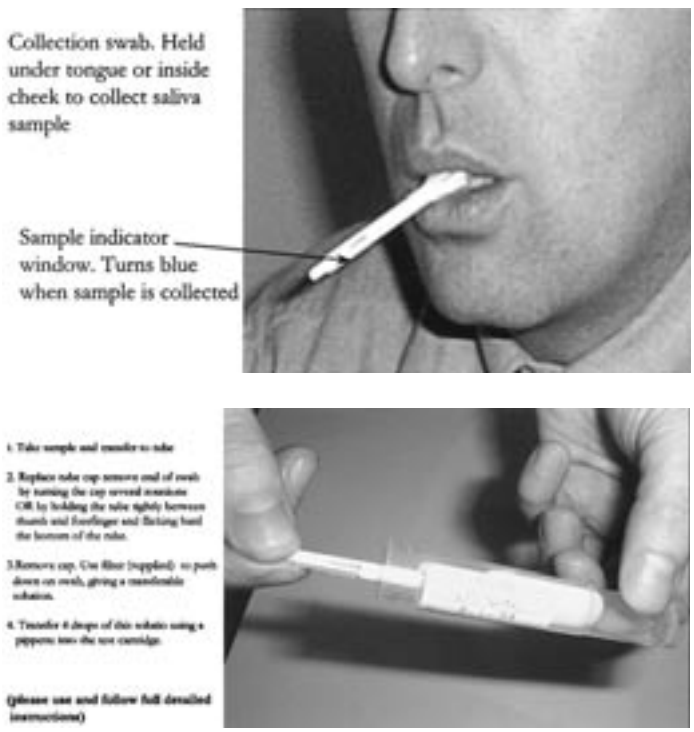

FIG. 1*a* and 1*aa—The Cozart RapiScan Saliva Collection. Sample indicator in window in handle of collection pad turns blue when 1 mL of sample is collected. Transfer to tube.*

EIA Microtiter Plate Assays

The Cozart Microplate EIA assays were used to assay the mixed saliva and run buffer fluid which remained in the collection tubes. These are antibody-coated microtiter plates employing a drug derivative that is labeled with horseradish peroxidase. In the assay 25 μL sample, calibrator, or control is added to each well of the coated microtiter plate followed by 100 μL of working enzyme conjugate. After a 30 min incubation the plate is washed four times with 350 μL wash buffer. Then 100 μL of substrate solution containing a 3,3′5,5′-tetramethyl benzidine is added to each well and incubated for a further 30 min. Finally, 100 μL of stop solution (1 M sulfuric acid) is added to each well and the absorbance is read at 450 nm within 30 min (5). Concentrations were determined from the assay calibration curve run on the same plate as the saliva-buffer specimens. Concentrations shown in Table 3 are of total opiates in the run buffer-saliva mixture.

Cannabis Study

The ability of the Cozart Saliva Test to detect cannabinoids was studied using cannabis resin. Fifty mg of resin was suspended in 1

FIG. 1*b—The Cozart RapiScan Drug testing cartridge. Buffer fluid from the collection tube is transferred into the cartridge by pipetting "4 drops" of fluid into the collection well.*

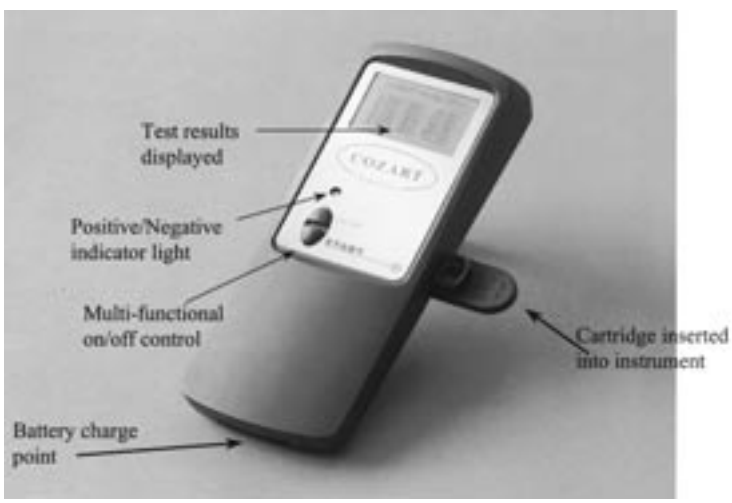

FIG. 1*c—The Cozart RapiScan Instrument with cartridge inserted.*

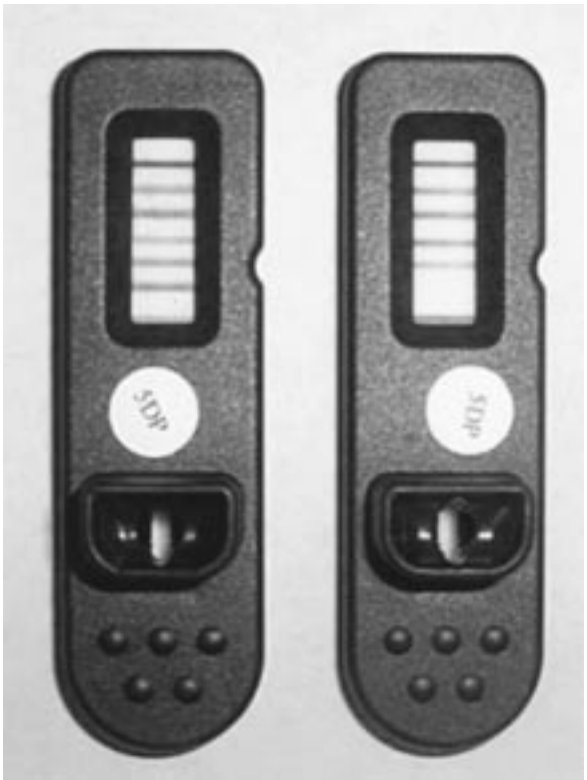

FIG. 1*d—the Cozart RapiScan Drug testing cartridge after incubation and test development. The cartridge on the left is negative for all drugs, the cartridge on the right is positive for one of the five drug classes.*

mL of run buffer and allowed to mix for 24 h. Dilutions of this mixture were tested using the Cozart RapiScan cannabinoids cartridge. The detection limit for delta-9-THC was determined by spiking several saliva samples with the drug. The detection limit was calculated as the buffer-only value plus two standard deviations.

Three volunteers smoked marijuana cigarettes and saliva samples were obtained using the Cozart RapiScan collector. Three saliva samples were obtained from the smoker prior to smoking and at time intervals of 10 to 30 min for up to 10 h to after smoking.

Codeine Study

Controlled dosing studies of codeine were carried out to assess the sensitivity and specificity of the Cozart RapiScan Saliva Drug Test for opiates. Five volunteers (four males and one female) received 16 mg codeine, 2 × 8 mg codeine and 500 mg paracetamol (Codamol tablets, Sterwin Medicines, Guildford, Surry, UK). Saliva specimens were obtained and measured at 0, 15 min, 30 min, 60 min, 4 h, 6 h, 8 h, 24 h after dosing. One mL of saliva was obtained at each time point using the Cozart RapiScan saliva collection pad and indicator. The saliva was tested with the Cozart RapiScan Saliva Opiates test (cutoff value 10 ng/mL) which reported the results as positive or negative. The saliva was also tested by GC/MS and by the Cozart microplate immunoassay for opiates (5).

Blind Trials—Saliva samples were collected from 43 volunteers. These volunteers covered a range of 15 to 65 years of age with approximately equal numbers of males and females (22 males, 21 females). Details on over-the-counter and prescription medicines were recorded. These included anti-hypertensives, antibiotics, non-steroidal anti-inflammatory drugs and antihistamines.

Each sample was tested independently by four operators using the 5 panel cartridge (amphetamines, benzodiazepines, cannabis, cocaine, and opiates) to give a total number of 860 tests (43 × 5 × 4). The samples were also subjected to EIA analysis using Cozart microplates modified for use by incorporating calibrators prepared in Cozart RapiScan run fluid. The same cut-off concentrations, 10 ng/mL drug equivalents for morphine, amphetamine, THCA, benzoylecgonine, and 100 ng/mL for temazepam, were used for both systems.

Sensitivity and Specificity

Sensitivity was calculated as the TP/(TP + FN) and specificity as TN/(TN + FP) where TP is the number of true positive results in persons administered the drug, TN is the number of true negative results in people not taking the drug, FN is the number of false negative results in persons given the drug and FP is the number of false positive results in persons not taking the drug. The standard error of the sensitivity and specificity was calculated as SE = square root of pq/n where p is the sensitivity or specificity expressed as a decimal number, $q = 1\text{-}p$ and n is the sample size.

Results

Cannabis Study

Dose-response relations for the different drugs were investigated using both buffer and saliva spiked with the drugs. The test was assessed using cannabis as an example. Figure 2*a* shows a typical dose-response plot for delta-9-THC and delta-9-carboxylic-THC with spiked buffer; Figure 2*b* shows the dose response curve for spiked saliva. Figure 2*b* shows that the test can detect the equivalent of 10 μg of cannabis resin. The cutoff concentration was set at 50 ng/mL delta-9-THC and 10 ng/mL delta-9-THC carboxylic acid respectively. Figure 2*c* shows the Cozart RapiScan response with spiked and unspiked buffer-saliva mixture (2:1) which was equivalent to 150 ng/mL delta-9-THC in neat saliva. Table 1 shows the cross-reactivity of the Cozart RapiScan Saliva Test for the different cannabinoids.

The measured saliva/plasma ratio for THC after smoking marijuana is 10 and is a function of the time since smoking (2). Cannabinoids in saliva are probably due largely to residual cannabinoids left in the mouth during ingestion or smoking of marijuana or marijuana products. Figure 3*a* shows the time-dose profile for the Cozart RapiScan Cannabis Saliva Test following smoking a "joint" and Fig. 3*b* shows the time course of the reported effect after smoking the joint. In the three volunteers, the Cozart RapiScan test was able to detect the presence of the drug in the saliva for at least 1 h and in some cases up to 2 h post smoking. This is not long enough to coincide with the period of driving impairment which may last from 6 to 8 h or longer after smoking. At the conclusion of these studies the cutoff of the Cozart RapiScan Cannabinoids test was lowered for further field trials. The Cozart RapiScan test also showed good correlation with reported "effect" following cannabis smoking. This is in agreement with Menkes et al. (6) who found that saliva levels of cannabis correlated with rapid heart rate and psychological feelings of "high."

Codeine Study

Controlled dosing studies of codeine were carried out to assess the sensitivity and specificity of the Cozart RapiScan Saliva Drug

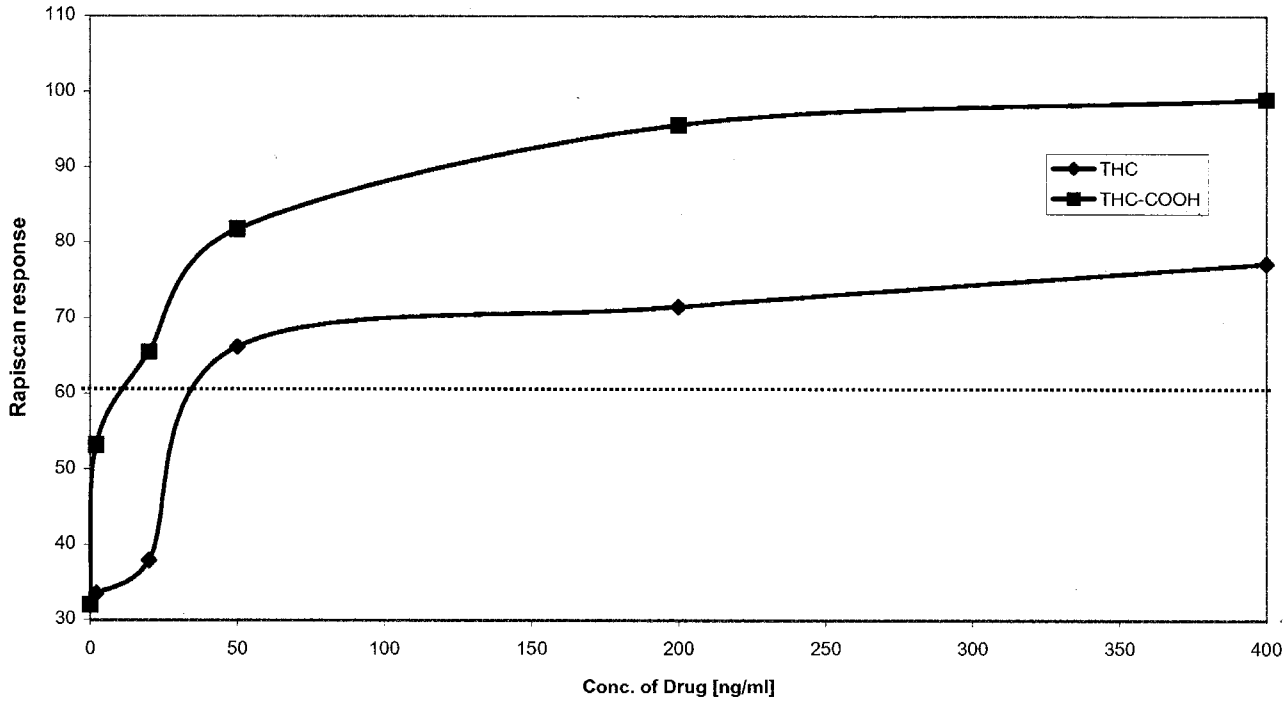

FIG. 2*a—Dose-response plot for the Cozart RapiScan Cannabinoid test. Buffer was spiked with the drug and loaded into the cartridge. Drug concentration in ng/mL is plotted versus RapiScan response.*

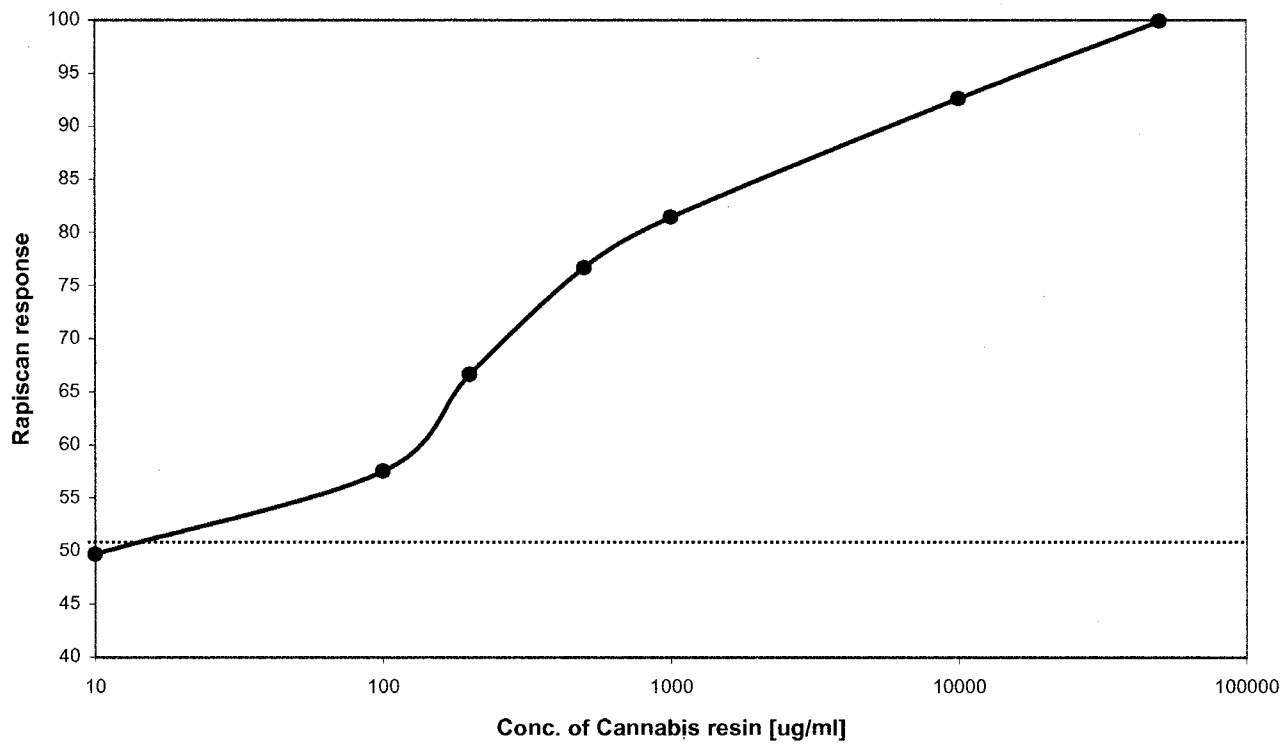

FIG. 2*b—Dose-response plot for Cannabis resin using RapiScan THC single cartridges. Broken line indicates buffer only value (mean + 2SD).*

Test for Opiates. Codeine was used as a model for opiate pharmacokinetics in saliva because, while codeine has pharmacokinetic parameters similar to heroin, 6-monoacetyl morphine and morphine, its pharmacodynamics are relative safe and benign allowing codeine to be used in clinical studies with healthy volunteers (7). The major metabolite found in saliva after heroin use is 6-monoacetyl morphine (6-MAM) (8,9). After codeine administration, codeine is found in saliva with a saliva to plasma ratio of 3.3 and after morphine administration, morphine may be found in a saliva to plasma ratio of 0.2 (7). Table 2 shows the cross-reactivity

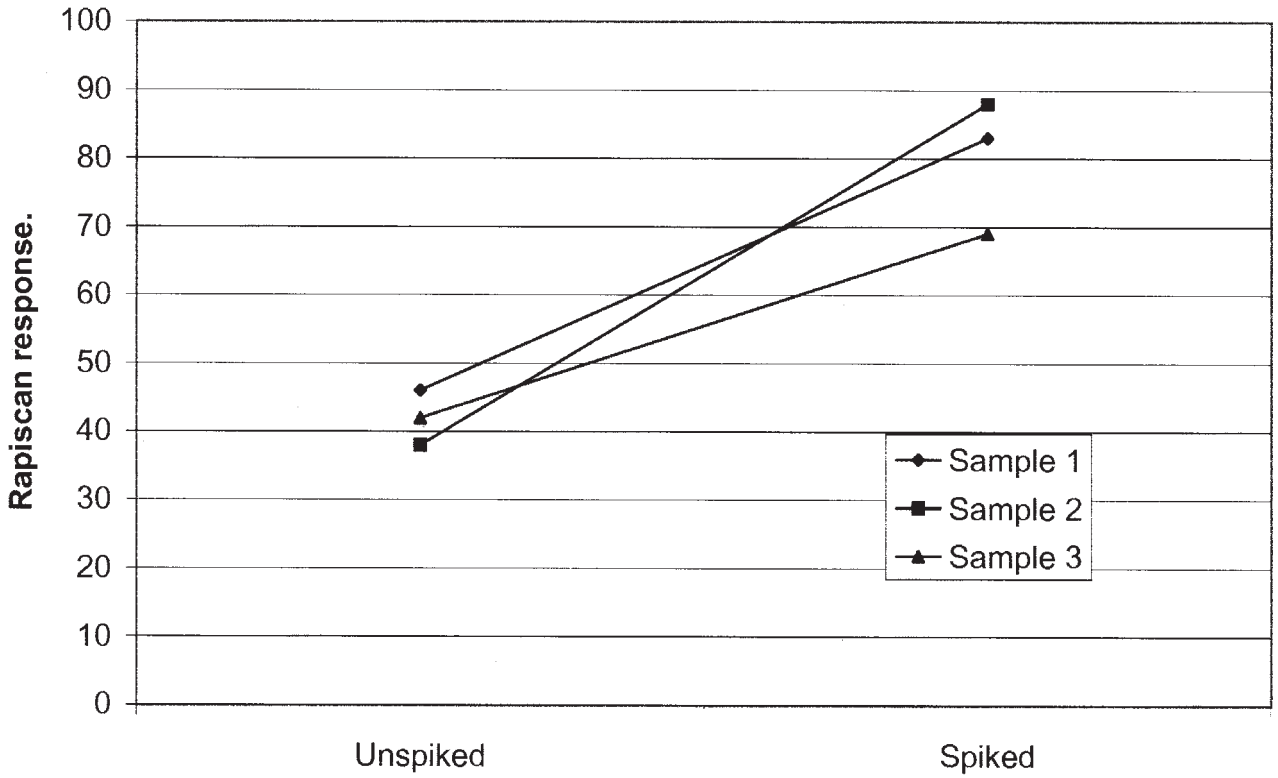

FIG. 2*c—Delta-9-THC was spiked into buffer-saliva mixture (2 + 1) which is equivalent to 150 ng/mL in neat saliva.*

TABLE 1—*Crossreactivity of the Cozart RapiScan Saliva Test for cannabinoids.*

Cannabinoids	ng/mL	Cozart RapiScan Response
Δ9-THC	50	THC Positive +ve
THC-COOH	10	THC Positive +ve
11-OH-THC Δ9-THC	50	THC Positive +ve

NOTE: These derivatives have been tested at 10 000 ng/mL and found not to cause a positive for the other Cozart RapiScan drug tests.

TABLE 2—*Crossreactivty of the Cozart RapiScan Saliva Test for opiates.*

Opiates	ng/mL	RapiScan Response
Morphine	10	OPI Positive +ve
6 Monoacetylmorphine (6MAM)	10	OPI Positive +ve
Heroin (Diacetylmorphine)	10	OPI Positive +ve
Dihydrocodeine	10	OPI Positive +ve
Codeine	10	OPI Positive +ve
Morphine-3-glucuronide	25	OPI Positive +ve
Pholcodeine	30	OPI Positive +ve
Nalorphine	500	OPI Positive +ve

NOTE: These derivatives have been tested at 10 000 ng/mL and found not to cause a positive for the other Cozart RapiScan drug tests.

of the Cozart RapiScan Saliva Opiates test for various opiates and opioids.

All 44 saliva specimens obtained in the first 9 h after codeine administration were positive for opiates by both the Cozart RapiScan and the Cozart microplate enzyme immunoassay (Table 3). Three of the five volunteer's saliva was still positive after 24 h. A Cozart RapiScan Opiates Positive response was present in all subjects at the first 15 min specimen and persisted as long as the saliva codeine concentrations were greater than 5 ng/mL by GC/MS. No "Opiates Positive" responses were obtained from volunteers not taking codeine.

From the 43 drug free volunteers, 41 samples were negative for all five drug classes. One positive result was obtained from a volunteer who was taking Nurofen® Plus (ibuprofen with codeine) on a regular basis, for a total of 45 positive opiate results after codeine containing medications. One result was unexpected, a Cozart RapiScan positive for Amphetamines. The total opiate negative samples from drug-free volunteers was 42. The sensitivity and standard error of the Cozart RapiScan Saliva test for Opiates was calculated to be $45/45 \pm \sqrt{(100)(99)/91} = 100\% \pm 10.4\%$. The specificity of the Cozart RapiScan Test for Opiates was $48/48 \pm \sqrt{(100)(99)/91} = 100\% \pm 10.4\%$. Therefore, both the sensitivity and specificity for the Cozart RapiScan Saliva test for opiates are greater than 90%.

Blind Trials

All results were in complete agreement. There were no differences between operators in the results of the blind specimens. There were no differences between the RapiScan results and the microtiter plate assays (Tables 3). Forty one samples were negative for all five drug classes. One positive opiate result was found as expected as the volunteer was taking Nurofen® Plus (ibuprofen with codeine) on a regular basis. One result was unexpected, a RapiScan positive for Amphetamines. On questioning, the volunteer admitted taking 30 mg Ionamin® (phentermine as resin complex manufactured by Torbet) 12 h prior to giving a saliva sample. This was being taken as an appetite suppressant. Further samples

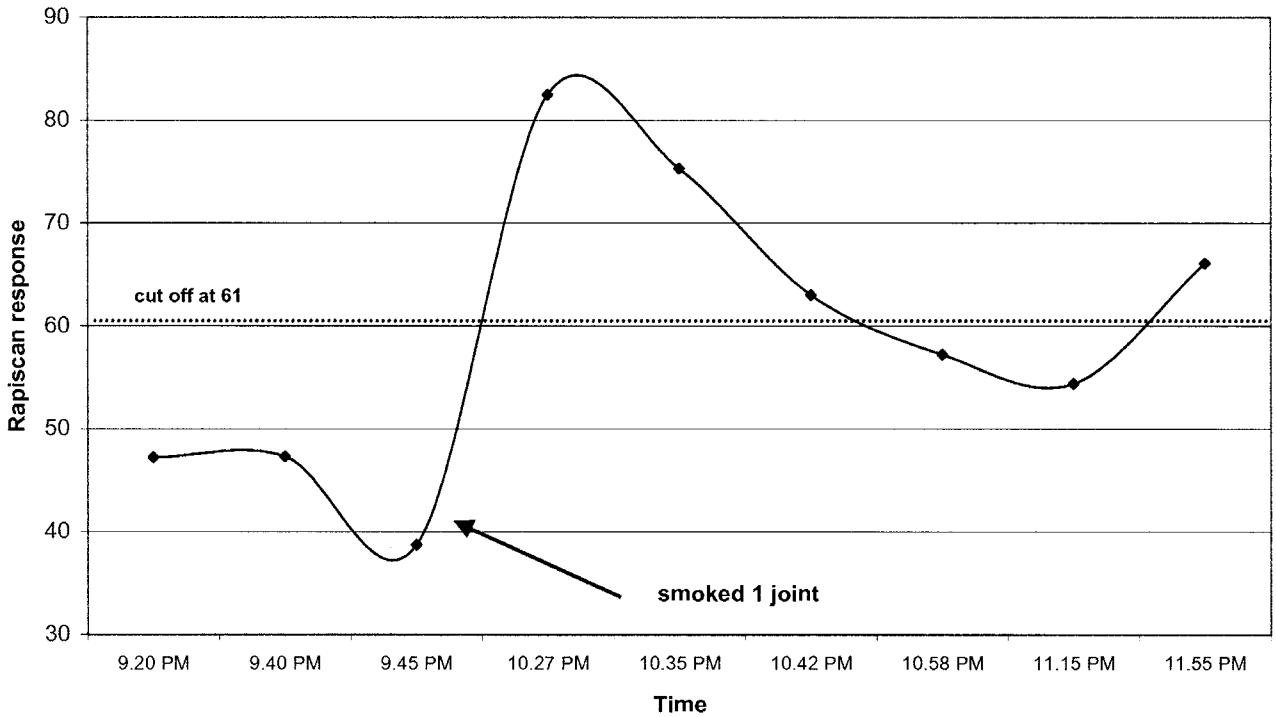

FIG. 3*a*—*Cozart RapiScan THC Saliva test response following smoking of cannabis. Subject 1 began smoking at 9:50 pm and ended at 10:15 pm when the first sample was collected.*

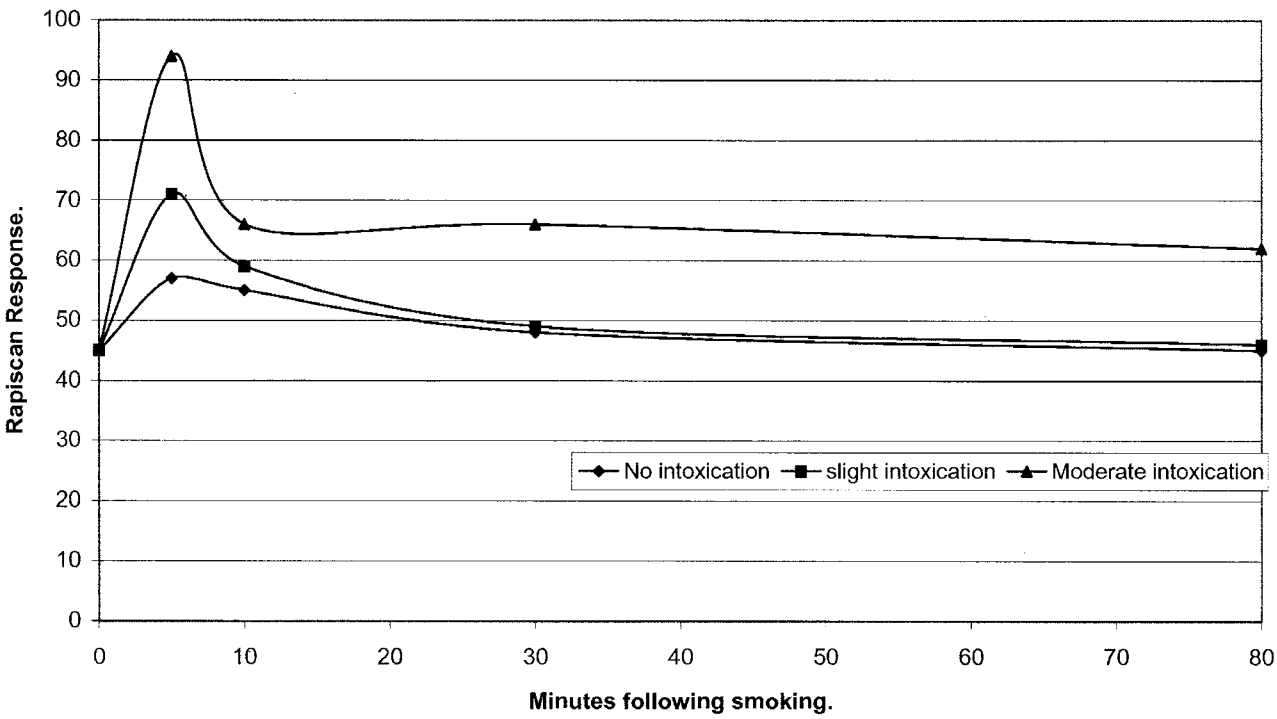

FIG. 3*b*—*Reported "Effect" of smoking cannabis and Cozart RapiScan response. Curve indicated with (diamond) no intoxication, (square) slight intoxication and (triangle) moderate intoxication.*

TABLE 3—*Results with Cozart RapiScan Saliva Test (CRS) and Cozart Microtiterplate EIA after codeine administration.*

Time	Cozart RapiScan Response/Codeine Concentration ng/mL* (EIA)									
	Subject 1		Subject 2		Subject 3		Subject 4		Subject 5	
(hours)	CRS	EIA	CRS	EIA	CRS	EIA	CRS	EIA	CRS	EIA
0	negative	<10	negative	<10	negative	<10	negative	<10	negative	<10
0.25	positive	429	positive	148	positive	227	positive	473	positive	916
0.5	positive	319	positive	593	positive	129	positive	140	positive	1000
1	positive	330	positive	469	positive	641	positive	122	positive	2777
2	positive	568	...	...	...	...	...	...	...	...
3	positive	159	positive	242	positive	142	positive	155	positive	343
4	positive	257	positive	120	...	...	...	...	...	...
4.5	positive	479	...	...	...	...	...	...	...	...
5	positive	97	positive	151	positive	62	positive	184	positive	60
6	...	...	positive	48	...	...	...	...	...	...
7	positive	41	positive	44	positive	16	positive	115	positive	132
8	...	...	positive	41	positive	29	positive	69	positive	71
9	positive	33	positive	55	positive	29	positive	77	positive	26
12	positive	34	positive	13	...	...	positive	45	negative	<10
18	positive	15	...	...	negative	<10	positive	38	positive	15
24	positive	16	negative	<10	negative	<10	positive	15	positive	61

NOTE: CRS Cozart RapiScan Response.
EIA Cozart Microplate Enzyme Immunoassay.
*ng/mL opiate in mixed saliva-run buffer 1:3 dilution.

TABLE 4—*Crossreactivity of the Cozart RapiScan Saliva Test for amphetamines.*

Amphetamines	ng/mL	Cozart RapiScan Response
D Amphetamine	10	AMP Positive +ve
MDA (ADAM)	10	AMP Positive +ve
D Methamphetamine	1 000	AMP Positive +ve
MDMA (Ecstasy)	1 000	AMP Positive +ve
Imipramine	1 000	AMP Positive +ve
MBDB	10 000	AMP Positive +ve
MDEA (EVE)	10 000	AMP Positive +ve
Ephedrine	10 000	AMP Negative −ve
Fenfluramine	10 000	AMP Negative −ve

NOTE: These derivatives have been tested at 10 000 ng/mL and found not to cause a positive for the other Cozart RapiScan drug tests.

were taken a week later at 18 h and 24 h post dose and the Cozart RapiScan response was positive each time for Amphetamines. This is consistent with the known cross-reactivity of the Cozart RapiScan Saliva Test for Amphetamines (Table 4).

Conclusions

The Cozart RapiScan onsite saliva drug tests are as sensitive, specific, and reliable as the immunoassay tests used in the laboratory. The collection and transfer procedures require minimal operator intervention. There is no operator bias because results are presented in written form as either "Positive" or "Negative" for each of the five drug classes on the screen of the hand-held reader. Also they can be printed out with an optional battery-powered printer if a permanent record is required. The Cozart RapiScan Saliva Tests for drugs of abuse is an immunoassay. All positive immunoassay results should be confirmed by a second analytical method based on a different chemical or physical property of the analyte (10). In the case of a positive test result, the remainder of the saliva-buffer mixture can be capped and sent to a laboratory for confirmation testing. In the case of multiple positive results, a second sample, either of saliva or other biological fluid, should be obtained and sent with the remainder of the original saliva-buffer mixture to the confirmation laboratory. To further characterize the performance of the Cozart RapiScan Saliva Drug test, field trials of saliva from actual users of drugs of abuse are currently underway.

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