J Forensic Sci, July 2005, Vol. 50, No. 4
Paper ID JFS2004539
Available online at: www.astm.org

TECHNICAL NOTE

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Comparison of GC-MS and EIA Results for the Analysis of Methadone in Oral Fluid

ABSTRACT: The purpose of these studies was to evaluate the performance characteristics of the Cozart® Microplate Enzyme Immunoassay (EIA) for the determination of methadone in oral fluid from patients in a drug misuse treatment program. Oral fluid specimens were collected using the Cozart® RapiScan Collection system from 198 donors who were receiving treatment for their addiction and were monitored for drug misuse. Oral fluid specimens were also collected from forty volunteer donors who were not drug users. The specimens were analyzed in the laboratory by EIA and then analysed for methadone and its main metabolite EDDP by gas chromatography-mass spectrometry (GC-MS). A total of 103 samples were confirmed positive for methadone. The Cozart® Microplate EIA for d-Methadone in oral fluid using a cutoff of 30 ng/mL in diluted oral fluid had a sensitivity of 91.3% ± 2.8% and a specificity of 100% ± 1.0% vs. GC-MS.

KEYWORDS: forensic science, forensic toxicology, methadone, EIA, microtiter plate assay, GC/MS, oral fluid, sensitivity, specificity

Cozart® Microplate EIA assays are widely used in forensic laboratories worldwide for the analysis of drugs in whole blood (1–5) and hair (6–8). The same properties of high sensitivity, low cutoffs and cross-reactivity with parent drugs that makes these assays useful for whole blood and hair analysis are also advantageous for the analysis of drugs in oral fluid (9–15).

Drug misusers frequently develop high levels of tolerance to illicit drugs such as heroin and as a substitute, methadone is commonly prescribed in high doses and as a consequence blood concentrations are high and levels in oral fluid are elevated. Saliva/plasma ratios for methadone and metabolites range from 0.89 (EDDP) to 7 (methadone) (16–18). This produces a long window of detection for drugs in oral fluid testing in these subjects (19).

Collection of oral fluid is preferred by both donors and caregivers for monitoring drug misusers in treatment and incarceration because the collection of oral fluid is non-invasive, can be repeated often and does not require observation by a person of the same sex. Oral fluid can be correlated with being under the influence of a drug (10) and is useful for the investigation of drugged driving and assessing fitness for duty (9). Due to potential legal consequences it is necessary to validate the microplate based enzyme-linked immunoassay (ELISA's) for oral fluid screening for methadone and to establish the sensitivity and specificity for this application.

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* This paper was presented in part at the AAFS Meeting in Atlanta, GA (2002).
Received 23 Dec. 2004; and in revised form 9 March 2005; accepted 12 March. 2005; published 25 May 2005.

Methods

Specimens

Oral fluid specimens were collected from 198 donors who were receiving methadone treatment and were monitored for their drug misuse. Oral fluid was also collected from forty volunteer donors who were not drug users. All specimens were collected using the Cozart® RapiScan Collection System for oral fluid. Whilst personal information was not collected, the donors were both male and female with an approximate ratio of male:female of 2:1.

One milliliter of oral fluid was collected from each subject on each occasion using the Cozart® RapiScan Oral Fluid Collector. The collector has an indicator in the plastic handle that turns blue when one milliliter of fluid is collected. The oral fluid-soaked collector pad was placed in the Cozart® RapiScan Collector test tube with 2 ml of run buffer giving a final 1:3 dilution of the oral fluid. The tubes of oral fluid-buffer mixture were capped and sent by post to the analytical laboratory within Cozart Bioscience Ltd, where the diluted oral fluid was tested using the Cozart® Microplate EIA for d-Methadone. The remaining fluid was stored frozen at −20°C for several months until all specimens were collected and then thawed for GC-MS analysis.

Cozart® Microplate EIA (d-Methadone)

Cozart® Microplate enzyme immunoassays employ antibody-coated microtiter plates and a drug-derivative that is labeled with horseradish peroxidase. For analysis, 10 μL of sample, calibrator or control was added to each well of the coated microtiter plate followed by 100 μL of working enzyme conjugate. After 30 min incubation, the plate was washed four times with 350 μL wash buffer. Then 100 μL of substrate solution containing 3,3′,5,5′-tetramethyl benzidine as the chromagen was added to each well and incubated

TABLE 1—*Cross reactivity of the Cozart® Microplate EIA (d-Methadone).*

Compound	ng/mL Tested	% Cross Reactivity
LAAM	1,000	1.6%
	500	1.6%
EDDP	100,000	>0.006%
EMDP	100,000	>0.006%

for a further 30 min. Finally 100 μL of stop solution (1M sulphuric acid) was added to each well and the absorbance was read at 450 nm using the manufacturer's instructions. Concentrations were determined from the assay calibration curve run on the same plate as the oral fluid-buffer specimens. Concentrations shown in this paper are corrected for the 1:3 dilution and are expressed as nanogram methadone equivalents per milliliter of neat oral fluid.

Calibrators are supplied in oral fluid:buffer mixture at concentrations equivalent to 0, 6, 30 and 300 ng/mL of d/l-methadone in neat oral fluid and cross-reactivities are summarized in Table 1.

Sample Preparation—GC-MS

For GC-MS analysis, calibration standards were prepared by adding 0.5 mL blank oral fluid buffer mixture to a vial. Then appropriate amounts of standard mixtures of methadone and EDDP (100 and 1000 ng/mL) were added to make the following spiked concentrations: 0, 30, 60, 120 and 180 ng/mL oral fluid. An internal standard concentration equivalent to 120 ng/mL was added to standards, controls (60 ng/mL) and samples using a 1000 ng/mL internal standard solution (d_3-EDDP and d_9-methadone). 0.5 mL of pH7.4 phosphate buffer (0.1 M) was added and the samples mixed.

Solid-Phase Extraction (SPE)

The samples were extracted by solid-phase (Bond Elut Certify, 50 mg, 3 mL). The columns were conditioned with 1 mL methanol followed by 1 mL phosphate buffer (pH7.4, 0.1 M). The samples were then loaded onto the columns and washed with 1 mL deionized water. The column pH was adjusted with 0.5 mL of 0.01 M acetic acid. The columns were then dried on full vacuum for 10 min., 50 μL of methanol was added and dried on full vacuum for a further 1 min. The column was washed with 3 mL acetone:chloroform (1:1) and dried briefly to remove residual solvent. Methadone and EDDP were eluted with 1 mL 2% ammoniated ethyl acetate.

The eluates were evaporated to dryness at 40°C under nitrogen and then reconstituted in 100 μL ethyl acetate.

GC-MS Parameters

The temperature program consisted of an initial 2.0 min at 80°C that was then ramped at 30°C/min to 230°C, then to 300°C (10°C/min) and held for 2 min. The following ions were monitored: EDDP: 262, 276, **277**; EDDP-d_3: 265, 279, **280**; methadone: 162, 294, **72** and methadone d_9: 165, 303, **78**. The abundances found for the ions noted in bold were used for quantitation. With a 500 μL oral fluid sample, the limit of detection and the limit of quantitation of the GC-MS procedure was 5 ng/mL, equivalent to 15 ng/mL in neat oral fluid for both methadone and EDDP.

Matrix Controls

Oral fluid samples previously analyzed were retested on subsequent ELISA and GC-MS runs as controls and the results compared to the previous values. From one to ten repeated oral fluid specimens were included in each microtiter plate or GC-MS batch.

Sensitivity and Specificity

Sensitivity, the true positive rate, was calculated from the tally of true positives and false negatives as: Sensitivity = TP/(TP + FN). Specificity was calculated as: Specificity = TN/(TN + FP). The number of true positives, false negatives, false positives and true negatives was determined by comparison of the EIA results to GC-MS as the referee method. Sensitivity and specificity are probabilities; therefore their uncertainty is expressed as a Standard Error. The standard error of a probability (SEp) is equal to $SEp = \sqrt{(pq/n)}$. Where p is the probability, q is one minus the probability and n is the number of specimens analyzed.

Results

Table 2 shows the Cozart® Microplate and/or GC-MS positive results for methadone and EDDP in the oral fluid specimens. Microplate assay concentrations shown in Table 2 are methadone and methadone metabolites expressed as nanogram methadone equivalents in milliliters of neat oral fluid. The concentrations reported for GC-MS analysis in Table 2 are corrected for the 1:3 dilution with buffer in the Cozart® RapiScan Collection System and are for ng/mL in neat oral fluid.

Methadone was confirmed by GC-MS at a 30 ng/mL cutoff in 103 of the oral fluid specimens screened using the Cozart® Microplate EIA for d-Methadone. Methadone concentrations ranged from 0 to 2052 ng/mL in neat oral fluid. Methadone metabolite, EDDP, was present in 10 of the oral fluid specimens at concentrations ranging from 15 to 175 ng/mL. Employing the manufacturers recommended cutoff for oral fluid of 30 ng/mL methadone equivalents, all of the oral fluid specimens screened positive by the Cozart® Microplate EIA for Methadone were confirmed positive by GC-MS. An additional 9 samples initially screened negative were found to contain methadone at concentrations ranging from 23–54 ng/mL. All forty samples from the non-drug using volunteers were screened and confirmed negative.

Table 3 summarizes the sensitivity and specificity for the Cozart® Microplate EIA for d-Methadone for different immunoassay and GC-MS cutoffs. The true positives, true negatives, false positives and false negatives that are the basis for these calculations are shown in Fig. 1. The sensitivity for each cutoff was plotted vs. one minus the specificity as a Relative Operating Curve (ROC) and is plotted in Fig. 2. From the ROC analysis, the optimum cutoff was 20 ng/mL methadone equivalents in neat oral fluid. The Cozart® Microplate EIA for d-Methadone using a cutoff of 20 ng/mL methadone in neat oral fluid had a sensitivity of 92.2% ± 3.2% and a specificity of 97.8% ± 1.5% vs. GC-MS. Using the recommended cutoff of 30 ng/mL methadone equivalents the sensitivity was 91.3% ± 3.3% and the specificity was 100% ± 0.3%.

Discussion

Bennett et al. (19) found the sensitivity of oral fluid equal to that of urine in detecting methadone (91% and 94%) in a blind study of urine and oral fluid testing of clients from a British addiction treatment service. The specificity for methadone in oral fluid and

TABLE 2—*Cozart® Microplate EIA methadone and GC-MS positive results on oral fluid specimens.*

No.	Cozart® Microplate EIA Methadone and Metabolites (ng/mL)*	GC-MS	
		Methadone (ng/mL)	EDDP (ng/mL)
1	54	128	0
2	425	>180	0
3	458	>180	0
4	187	218	0
5	245	>180	0
6	94	111	0
7	271	>180	0
8	205	177	0
9	55	63	0
10	223	224	0
11	28	40	0
12	147	160	0
13	43	138	0
14	580	>180	0
15	40	67	0
16	503	>180	0
17	383	>180	0
18	132	>180	0
19	507	>180	0
20	591	>180	0
21	101	148	0
22	357	>180	0
23	105	146	0
24	157	>180	0
25	309	>180	0
26	36	68	0
27	159	>180	0
28	93	186	0
29	411	>180	0
30	237	>180	0
31	402	>180	0
32	32	61	0
33	145	>180	0
34	457	>180	0
35	184	209	0
36	66	109	0
37	251	>180	0
38	80	81	0
39	64	127	0
40	0	45	0
41	148	161	0
42	39	55	0
43	240	>180	0
44	116	204	0
45	34	60	0
46	41	77	0
47	21	0	0
48	221	>180	0
49	85	93	0
50	237	>180	0
51	235	218	0
52	578	>180	0
53	231	>180	0
54	235	>180	0
55	38	70	0
56	0	39	0
57	123	149	0
58	0	35	0
59	10	36	0
60	15	23	0
61	10	37	0
62	13	54	0
63	30	60	0
64	52	57	0
65	157	192	0
66	434	2052	0
67	34	53	0
68	313	>180	15
69	482	>180	175
70	258	147	0
71	276	>180	0
72	281	>180	0
73	232	>180	0
74	169	93	0
75	361	>180	0
76	421	>180	75
77	401	>180	19
78	125	71	0
79	329	>180	0
80	395	>180	0
81	319	115	0
82	420	>180	16
83	357	>180	0
84	369	>180	34
85	388	>180	27
86	192	50	0
87	303	164	0
88	417	>180	19
89	339	170	0
90	311	128	0
91	234	93	0
92	289	126	0
93	272	118	0
94	271	154	0
95	386	>180	0
96	0	46	0
97	313	180	0
98	304	136	0
99	382	>180	17
100	10	0	0
101	25	0	0
102	71	67	0
103	63	54	0
104	35	59	0
105	180	47	0
106	0	46	23
107	18	0	0
108	261	65	0

TABLE 2—*Continued.*

* Expressed as ng/mL methadone equivalents in neat oral fluid.

urine was 90% and 95% respectively. They concluded that oral fluid testing is as accurate as urinalysis in detecting the presence or absence of methadone in their client population (19).

The detection of methadone and EDDP in oral fluid with the Cozart® Microplate EIA for d-Methadone is a sensitive measure for monitoring compliance in methadone treatment programs. This was true whether a cutoff of 30 ng/mL or the LOD for the GC-MS was used as the reference standard for the ROC analysis of true positives, true negatives, false positives and false negatives. The cutoff indicated as most efficient by ROC analysis was 20 ng/mL methadone equivalents in diluted oral fluid. The manufacturer recommends a conservative cutoff of 30 ng/mL methadone equivalents for the EIA.

Further investigation into the ratios of d:l methadone present in the oral fluid samples could provide an explanation as to why samples initially screened negative using the d-methadone ELISA were confirmed positive by GC-MS.

Of the oral fluid specimens from this larger study, 109 specimens were positive for various opiates other than methadone and 116 were positive for cocaine and cocaine metabolites. These results have been published elsewhere (15,20).

TABLE 3—*True positives, true negatives, sensitivity and specificity of the Cozart® Microplate EIA (d-Methadone) for oral fluid vs. GC-MS.*

	GC-MS 30 ng/mL		GC-MS LOD	
	Methadone 30 ng/mL Cutoff	Methadone 20 ng/mL Cutoff	Methadone 30 ng/mL Cutoff	Methadone 20 ng/mL Cutoff
True Positives	94	95	94	95
False Negatives	9	8	10	9
False Positives	0	2	0	2
True Negatives	95	93	94	92
Sensitivity	91.3% ± 2.8%	92.2% ± 2.6%	90.4% ± 2.9%	91.4% ± 2.9%
Specificity	100% ± 1.0%	97.9% ± 1.5%	100% ± 1.0%	97.9% ± 1.5%

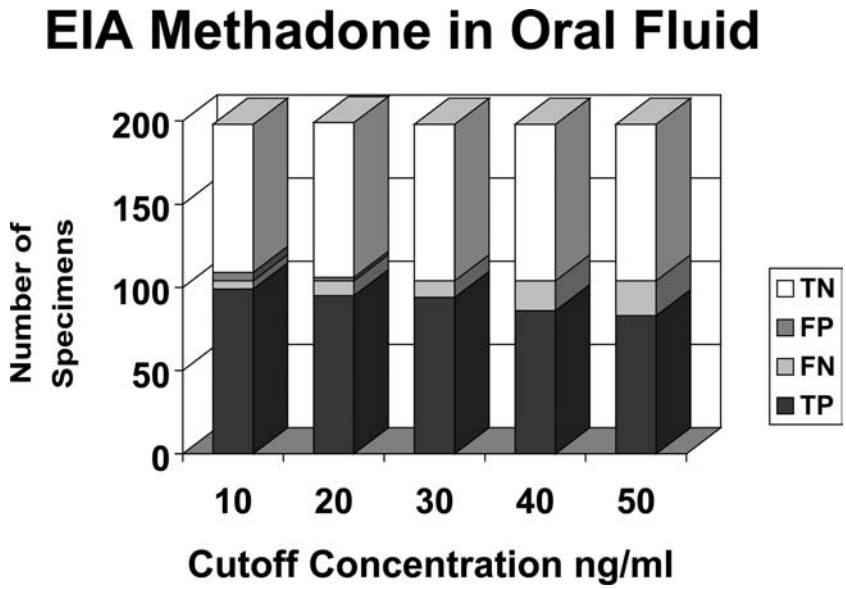

FIG. 1—*True positives, false negatives, false positives and true negatives vs. cutoffs for the Cozart® Microplate d-Methadone oral fluid assay.*

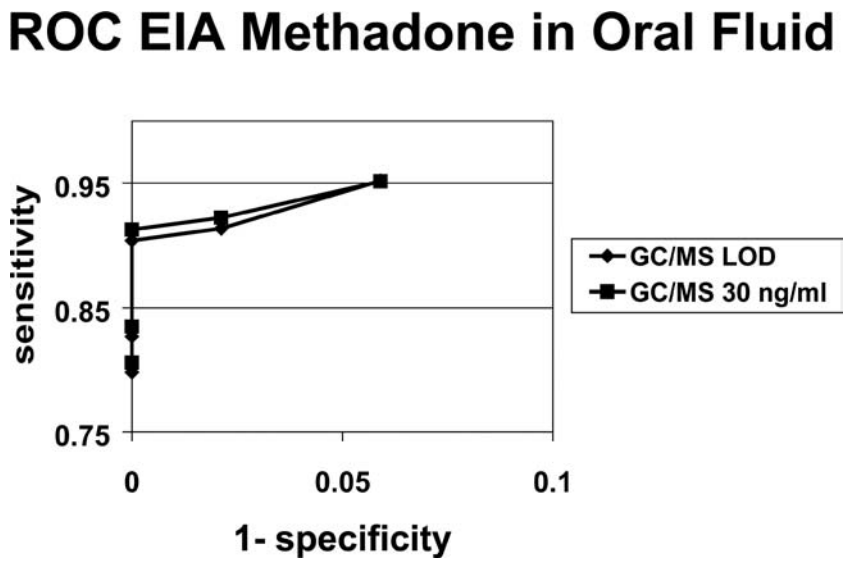

FIG. 2—*Receiver operating curve: sensitivity vs. 1-specificity for the Cozart® Microplate EIA (d-Methadone) in oral fluid.*

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