TECHNICAL NOTE

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The Detection of Cocaine in Hair Specimens Using Micro-Plate Enzyme Immunoassay*

REFERENCE: Moore C, Deitermann D, Lewis D, Feeley B, Niedbala RS. The detection of cocaine in hair specimens using micro-plate enzyme immunoassay. J Forensic Sci 1999;44(3): 609–612.

ABSTRACT: The analysis of hair for drugs of abuse is becoming increasingly popular and is under consideration by the Division of Health and Human Services as a possible alternative or adjunct to urinalysis in workplace programs. The detection of cocaine in human hair using a commercially available micro-plate enzyme immunoassay is described for the first time. Sample size and incubation time were the major variables in the optimization of the method. In order to validate the procedure, the method was applied to 105 consecutive hair samples routinely received into our laboratory. The samples were simultaneously analyzed by the Micro-Plate immunoassay (EIA), as well as our current fluorescence polarization immunoassay (FPIA) procedure and gas chromatography-mass spectrometry (GC/MS). The sensitivity of the EIA and FPIA assays were 75% and 67.8% respectively; specificity 97.4% and 80.5% respectively; and efficiency 91.4 and 77.1% respectively. The Micro-Plate EIA was shown to be a valid alternative to other immunoassay screening methods for the detection of cocaine in hair by demonstrating increased sensitivity, specificity and efficiency over our current technique.

KEYWORDS: forensic science, forensic toxicology, hair analysis, micro-plate enzyme immunoassay, cocaine

The use of hair as an alternative specimen to common drug testing matrices such as urine and blood has been increasing in popularity over the last few years and has many applications (1,2). Hair offers distinct advantages over urine for the detection of drug use since, depending on its length, it can provide information on historical drug ingestion and external exposure. Additionally, hair is easy to collect and store, and has been adopted by various companies for workplace drug testing in place of urinalysis.

When cocaine is ingested, the major compound detected in hair is usually the parent drug itself and not the urinary metabolite benzoylecgonine (3,4). Screening procedures for urine drug testing are directed towards cross-reactivity with benzoylecgonine and not cocaine, so their use in high volume screening for hair has been limited. The determination of cocaine in hair specimens has been reported using a variety of immunological procedures including fluorescence polarization (FPIA), enzyme multiplied immunoassay technique (EMIT), and radioimmunoassay (RIA) (5). These methods were recently reviewed by Tagliaro et al. (6). Preliminary data regarding the use of enzyme linked immunosorbent assays (ELISA) to detect various drugs in hair was presented at an International conference (7). This paper describes the use of a commercially available EIA system for the screening of cocaine in hair. The method can be easily automated to improve analysis times and to help eliminate operator error.

Materials and Methods

Materials

Mixed-mode solid-phase extraction columns (200 mg; 10 mL; Isolute HCX) were obtained from Jones Chromatography, Lakewood, CO. Cocaine, benzoylecgonine, cocaethylene and deuterated standards were obtained from the Radian Corporation, Austin, TX; derivatizing reagents were obtained from Regis Chemical, Morton Grove, IL. STC Technologies Inc., Bethlehem, PA loaned the plate washer (ELP-40 Micro-Plate Strip Washer, Bio-Tek Instruments) and absorbance reader (Dynatech MR 5000). Additionally, they supplied various Cocaine 5-plate Saliva Micro-Plate kits (Lot numbers: 057828, 087815). All chemicals were of ACS grade and solvents were of HPLC grade or better.

Sample Preparation

Drug-free hair specimens from laboratory workers and children of laboratory staff were powdered in a "ball-beater" (BioSpec Products, OK) using stainless steel balls for 30–60 seconds. The powdered hairs were weighed out into aliquots of 50 mg. Cocaine hydrochloride in methanol was added to the hair to attain concentrations of 0.5, 2.0, 10.0, and 20.0 ng/mg. "Positive" hairs were spiked at 1.5 ng/mg and negative hairs had no drug added. Hydrochloric acid (0.1M, 3 mL) was added to each of the hairs and the samples were incubated overnight at 60°C. The following day, the acid was decanted, ethanol (200 μL), deionized water (2 mL) and 12N sodium hydroxide (200 μL) were added and the hair was incubated at 80°C for 30 min. After cooling, glacial acetic acid (1

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* Presented in part at the 50th Annual Meeting, American Academy of Forensic Sciences, San Francisco, CA, February 1998.

Received 3 April 1998; and in revised form 2 Oct. 1998; accepted 5 Oct. 1998.

mL) was added dropwise. Following centrifugation (3000 rpm; 5 min) the supernatant was combined with the acid and the hair was discarded. Deionized water (9 mL) was added to ensure polarity of the extract.

Solid-Phase Extraction

Mixed-mode solid-phase extraction columns (200 mg; 10 mL, Isolute HCX) were conditioned with methanol (3 mL), deionized water (3 mL) and 1.93M acetic acid (1 mL). The sample was added to the column through a filter, and drawn through the sorbent bed slowly. The bed was washed with deionized water (3 mL), 0.1N hydrochloric acid (1 mL) and acetonitrile-deionized water (20:80v,v; 1 mL). Collection tubes were placed in the rack and any acid-neutral drugs present were eluted with hexane-ethyl acetate (50:50 v,v; 3 mL). This was to simulate our full screening procedure which includes the isolation of acid and neutral drugs. However, for this experiment, further work was only carried out on the basic fraction. The collection tubes were removed and the columns were washed with methanol (3 mL). Collection tubes were placed back into the rack and the basic drugs were eluted in methylene chloride-isopropanol-ammonium hydroxide (78:20:2 v,v; 3 mL). The eluent was evaporated to dryness in a TurboVap (Zymark) using air at a temperature of 50°C and the residues were reconstituted in Stabilzyme buffer (pH 7.0; 120 μL).

Screening Procedure-EIA

A modified Micro-Plate enzyme immunoassay kit, originally designed for use with oral fluid (saliva) samples was chosen (STC Technologies Inc.). The initial kit chosen was designed for testing parent cocaine in sweat, since cocaine is the predominant compound detected in sweat following cocaine use, and therefore it was postulated that this kit would be most useful for hair specimens. However, the oral fluid kit gave better initial results, so this was used in method development.

The reconstituted sample, calibrator or control (100, 50, 25, or 10 μL) was added to each well of the micro-plate. All specimens were tested in duplicate. The working dilution of stock enzyme conjugate (100 μL) was added and the plate was incubated at room temperature in the dark for 15, 30, 45, or 60 min. The plate was emptied and washed with deionized water (6 × 300 μL) and substrate reagent was added (100 μL). The plate was again incubated in the dark, at room temperature, for 30 min. This second incubation time was not further optimized. After incubation, stopping reagent was added (100 μL) and the absorbance at 450 nm was read immediately after removing the plate from the incubation, and again 15 min later. Calibrators supplied with the assay kit were used with each plate, serving as a means of quality control for the plate. Extracted hair calibrators at 0.5, 2.0, 10.0, and 20.0 ng/mg were run with each plate along with spiked positive hairs (1.5 ng/mg) and negative hair.

Screening Procedure-FPIA

For analysis using our current procedure, the specimen was reconstituted in Adx buffer (250 μL) and analyzed by fluorescence polarization immunoassay (FPIA) using our validated methodology. The screening cut-off currently used on this system is 0.5 ng/mg of hair.

Gas Chromatography-Mass Spectrometry

Immediately after powdering the hair, deuterated internal standards were added (10 μg/mL; 10 μL) and the extraction was carried out as described previously with the elimination of the acidic drug elution step. Following evaporation of the eluates to dryness, the extracts were reconstituted in butyronitrile (40 μL), transferred to autosampler vials and capped. N-methyI-N-(tert-butyldimethylsilyl) trifluoroacetamide, MTBSTFA (30 μL) was added and the extract was heated at 80°C for 20 min. Analysis of the extracts was carried out using gas chromatography-mass spectrometry (GC/MS) in electron impact, selected ion monitoring mode using a Hewlett Packard 5890 gas chromatograph coupled to a 5971A mass selective detector. The column was a DB-5 MS (25 m length × 0.2 mm i.d. × 0.33 μm film thickness). The injector temperature was set at 270°C, the detector at 310°C and the oven program was as follows:

Initial temperature 100°C for 1 min; ramped at 30°C/min to 230°C, ramped at 3°C to 249°C, ramped at 30°C/min to 310°C; held for 6.8 min. The injection mode was splitless and the ions monitored were: D3-cocaine 306, 185; Cocaine 303, 182, 198; D3-cocaethylene 320, 199; Cocaethylene 317, 196, 212; D3-benzoylecgonine 285, 406 and benzoylecgonine 282, 403, 346. The retention times for cocaine, cocaethylene, and benzoylecgonine were 12.22, 12.78, and 14.65 min respectively. The ion ratios were set to fall within ±30% of the calibration standards and the limit of quantitation was 0.01 ng/mg.

Following optimization of the Micro-Plate method, one hundred and five (105) consecutively received hair samples were analyzed by EIA, FPIA, and GCMS.

Results and Discussion

The sample size and initial incubation time were the major variables in the development of this assay. Firstly, specimen volumes of 100, 50, 25, and 10 μL were aliquoted into the micro-plate and tested under the standard protocol supplied by STC (package insert). Secondly, using the optimized sample volume of 25 μL, which had proved to give the best separation of absorbance and concentration values, the incubation time for the sample plus conjugate was varied from 15 min to 1 h at intervals of 15 min. The optimum incubation time was determined using plots of absorbance vs. drug concentration. The plot showing the widest difference in absorbance values between the drug-free hair and the fortified low calibrator was selected. One hour was selected as the optimum incubation period.

At a cut-off value of 1 ng/mg, the separation achieved in absorbance values between negative and positive specimens was sufficient to determine positivity. An absorbance value of 1.5 was normally equivalent to the 1 ng/mg concentrations of extracted hair, and values above and below this were assessed for negativity and positivity. However, the results for each plate were assessed from the absorbance of the extracted hair calibrators and controls.

At 0.5 ng/mg, the separation was able to distinguish positive from negative hairs, but added confidence in the procedure was attained when 1 ng/mg was used as the cut-off concentration. Absorbance values greater than the cut-off were considered negative; absorbance values lower than the cut-off value were considered positive. The plates were read twice, 15 min apart and the mean values were accepted. The results of the 105 hair samples studied are shown in Table 1.

Sensitivity, Specificity, and Efficiency

The sensitivity, specificity, and efficiency of the two screening procedures using GC/MS as the indicator of true positivity and true

TABLE 1—*EIA, FPIA, and GC/MS results for 105 hair specimens.*

Sample #	FPIA	EIA	GC/MS Cocaine (ng/mg)	BZE (ng/mg)	EC (ng/mg)
1	+	–	–	–	–
2	+	–	–	–	–
3	+	–	–	–	–
4	+	+	>100	23	–
5	–	–	0.1	–	–
6	+	–	0.3	0.3	–
7	+	+	2.9	0.7	–
8	+	–	18.5	3.3	–
9	+	–	–	0.2	–
10	+	+	0.4	0.5	–
20	–	+	0.04	–	–
23	–	+	1.3	0.2	–
38	+	–	–	–	–
39	+	–	–	–	–
40	+	–	–	–	–
41	+	–	–	–	–
42	+	–	–	–	–
43	+	–	–	–	–
44	+	–	–	–	–
45	+	–	–	–	–
46	+	+	1.8	0.3	–
47	+	+	1.4	0.2	–
48	+	–	1.1	0.2	–
49	+	+	4.5	0.9	–
50	+	–	2.9	0.4	–
51	+	+	0.6	–	–
52	–	–	0.6	–	–
55	–	+	0.2	0.04	–
59	–	+	–	–	–
74	+	+	13.5	13.8	–
75	+	+	9.8	3.8	21.1
76	+	+	3.0	0.4	2.3
77	+	+	0.4	0.1	–
78	+	+	0.5	0.07	–
79	+	+	30.6	6.7	39.8
80	+	+	1.6	0.6	0.2
81	+	–	–	–	–
83	+	–	–	–	–
84	+	–	–	–	–
85	+	–	–	–	–
86	–	+	0.04	–	–
87	–	+	0.2	–	–
92	–	+	–	–	–
94	–	+	0.05	–	–
103	–	+	0.03	–	–

BZE = benzoylecgonine. EC = cocaethylene.

NOTE: Where sample numbers are not shown, all EIA, FPIA, and GC/MS were negative.

TABLE 2—*Sensitivity, specificity, and efficiency of screening procedures (*n *= 105).*

Definitions	FPIA	Micro-Plate EIA
True Positives (TP): specimens which screened positively and confirmed using GC/MS	19	21
True Negatives (TN): specimens which screened negatively and did not confirm using GC/MS	62	75
False Positives (FP): specimens which screened positively but did not confirm using GC/MS	15	2
False Negatives (FN): specimens which did not screen positively but were positive using GC/MS	9	7
Sensitivity (%)	67.8	75.0
Specificity (%)	80.5	97.4
Efficiency (%)	77.1	91.4

The sensitivity, specificity, and efficiency were calculated using the following formulae:

Sensitivity = TP/(TP + FN) × 100.

Specificity = TN/(TN + FP) × 100.

Efficiency = (TP + TN)/(TP + TN + FP + FN) × 100.

TABLE 3—*Cross-reactivity of the immunoassays to cocaine and some of its metabolites.*

Compound	% Cross-Reactivity: FPIA	Micro-Plate EIA (Oral Fluid Assay)
Benzoylecgonine	100	100
Cocaethylene	Unknown	143
Cocaine	1.0	102
Ecgonine	0.3	20
Ecgonine Methyl Ester	<0.1	18

negativity were calculated and the results presented in Table 2. Overall, the micro-plate enzyme immunoassay was more sensitive, specific and efficient than the corresponding fluorescence polarization procedure currently in use. This may be due to the differences in cross-reactivity towards parent cocaine and cocaethylene of the two screening systems (Table 3). FPIA is designed for urinalysis so is targeted primarily at benzoylecgonine (cocaine's major urinary metabolite). Concentrations of benzoylecgonine in hair tend to be lower than that of parent cocaine and therefore any immunoassay which specifically targets cocaine should be more sensitive for hair analysis. The micro-plate enzyme immunoassay has a much higher cross-reactivity towards cocaine than the FPIA and this certainly helps to explain the increased specificity and sensitivity of the EIA.

Two specimens were confirmed by GC/MS but did not screen positively by either technique. The concentrations of parent cocaine found were 0.1 ng/mg (sample #5) and 0.6 ng/mg (sample #52). Additionally, micro-plate enzyme immunoassay techniques can also be automated relatively easily. Currently several systems exist for the automated analysis of multiple plates simultaneously, including incubation, washing, reading and printing.

Summary

The STC Micro-Plate EIA was shown to be a valid alternative to other immunoassay screening techniques for the detection of cocaine in hair, since it demonstrates sufficient cross-reactivity with parent cocaine to identify specimens containing cocaine at a level of 1 ng/mg. It shows increased sensitivity, specificity and efficiency over our current technique.

References

1. Pichini S, Altieri I, Pellegrini M, Pacifici R, Zucarro P. The analysis of nicotine in infant's hair for measuring exposure to environmental tobacco smoke. Forensic Sci Int 1997;84:253–8.
2. Lewis DE, Moore CM, Morfissey P, Leikin J. Determination of drug exposure using hair: Application to child protective cases. Forensic Sci Int 1997;84:123–8.
3. Cone EJ, Yousfnejad D, Darwin WD, Maguire T. Testing human hair for drugs of abuse. II. Identification of unique cocaine metabolites in hair of drug abusers and evaluation of decontamination procedures. J Anal Toxicol 1991;15:250–5.

4. Henderson GL, Harkey MR, Zhou C, Jones RT. Cocaine and metabolite concentrations in the hair of South American coca chewers. J Anal Toxicol 1992;16:199–201.
5. Cassani M, Spiehler V. Analytical requirements, perspectives and limits of immunological methods for drugs in hair. Forensic Sci Int 1993; 63:175–84.
6. Tagliaro F, Smith FP, DeBattisti Z, Manetto G, Marigo M. Hair analysis, a novel tool in forensic and biomedical sciences: new chromatographic and electrophoretic/electrokinetic analytical strategies. J Chromatogr 1997;689:261–71.
7. Segura J. Proceedings of the 1995 International Conference and Workshop on Hair Testing, Nov. 1995, Abu Dhabi, editor. DeZeeuw RA, A1 Hosani I, Al Munthiri S, Maqbool A. 351–69.

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