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Syva EMIT II Plus 6-Acetylmorphine Assay:

Analytical Performance and Comparison Studies

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Important Characteristics of Drug Testing Assays

Immunoassays are the most commonly used methods to screen for drugs of abuse due to their ease of use and relatively low cost. However, the antibodies used in these assays may react not only with the drug of interest, but also with other structurally similar compounds (either another drug or a drug metabolite). This undesirable phenomenon is referred to as cross-reactivity. Another important assay characteristic is specificity—how well the antibodies recognize the target drug. For example, a specificity of 100% indicates that the antibodies recognize only the target drug and do not bind to any structurally similar drugs. False-positive results are less likely to occur with assays that have high specificity for the target drug.

Screening for Heroin

Heroin (3,6-diacetylmorphine) is a naturally occurring opiod analgesic that is found in the seedpod resin of the opium poppy *Papaver somniferum*. Heroin can also be synthesized by the acetylation of morphine.[1]

In the U.S., heroin is a Schedule I drug, which means it has no acceptable medical use. For many years, the procedure for detection of heroin abuse was an initial opiate screen for codeine/morphine by immunoassay testing, with positive specimens subsequently tested via gas chromatography/mass spectrometry for detection of the heroin metabolite 6-acetylmorphine (6-AM).

In 2008, the U.S. Substance Abuse Mental Health Services Administration (SAMHSA) released revised guidelines for federal workplace drug testing that required the inclusion of 6-AM testing as part of an initial screen; these guidelines became effective October 1, 2010.[2] The added requirement for the inclusion of detection of 6-AM was due to the fact that many of the individuals who tested positive with the codeine/morphine screen were not abusing heroin, but rather had a positive result due to use of a codeine/morphine medication for which they had a valid prescription, or positive results could be attributed to ingestion of foods containing poppy seeds. Such unconfirmed results could have a significantly negative impact on an individual.

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Opioid Metabolism

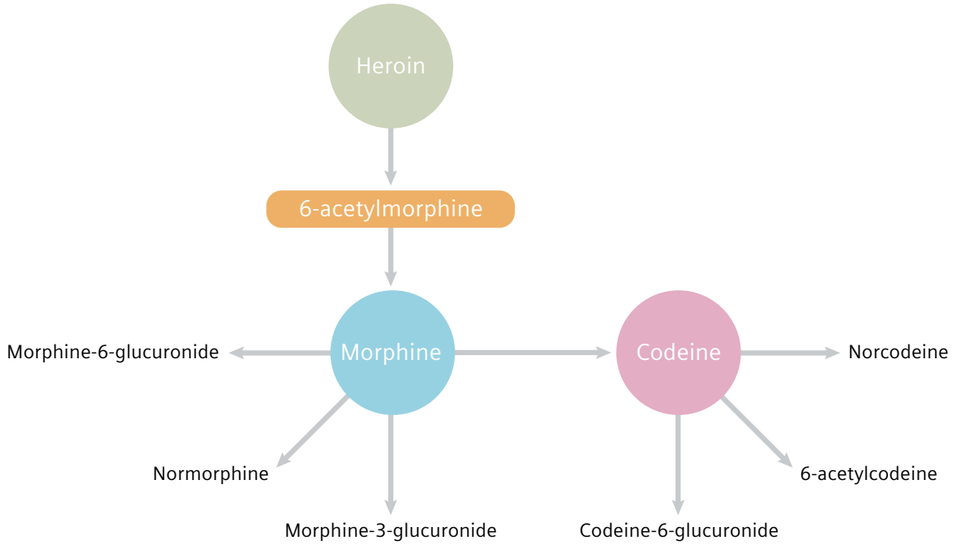
Figure 1 illustrates the metabolism of heroin, morphine, and codeine. As shown, 6-AM is found in the body only in response to heroin intake—it does not arise from intake and subsequent metabolism of either codeine or morphine. To minimize the risk of a false result suggesting heroin abuse, it is important to use a 6-AM assay that has little to no cross-reactivity to morphine, codeine, or any of their metabolites.

While a positive 6-AM result confirms heroin use, a negative result for 6-AM does not rule out heroin abuse, because 6-AM has a short half-life of 36 minutes in the blood system and is detected in the urine for only a few hours after heroin ingestion.[3]

There are also other opioids, including prescription opioids, that could potentially bind to the antibodies used in a 6-AM assay due to their structural similarity to 6-AM. These include compounds such as hydrocodone, hydromorphone, oxycodone, and oxymorphone; all are semisynthetic narcotic analgesics that structurally resemble other opioids.

Hydrocodone is prepared from codeine and used in a number of prescription analgesics, including the popular VICODIN. Hydromorphone used in medications including DILAUDID, and oxycodone is used in a number of medications, including OXYCONTIN, PERCOCET, and PERCODAN. Oxymorphone is a metabolite of oxycodone and is also used in prescription medications. All these compounds have been reported to cross-react in some morphine and opiate immunoassays.[4]

Figure 1. Metabolism of codeine, morphine, and heroin. Note that 6-acetylmorphine arises only from the metabolism of heroin.



The Syva EMIT II Plus 6-Acetylmorphine Assay

The Syva® EMIT® II Plus 6-Acetylmorphine Assay (EMIT 6-AM assay) was introduced in 2011, and is available for use on several automated immunoassay platforms. The assay can be run in either a qualitative or semi-quantitative mode. In the qualitative mode, a sample containing 6-acetylmorphine (6-AM) at a concentration greater than the cutoff of 10 ng/mL would be identified as positive. The assay range for the semi-quantitative mode is 0–20 ng/mL, with an analytical sensitivity of 1.1 ng/mL. The antibodies used in the assay are specific for 6-AM, and greatly reduce the number of false-positive results seen in samples containing other opioids or their metabolites.

Studies Using the EMIT 6-AM Assay

Two studies that examined the performance of the EMIT 6-AM assay have recently been completed.

Study 1: Analytical Performance

The first study was conducted on a Viva-E® Drug Testing System and examined precision, linearity, accuracy, cross-reactivity with structurally related opioids, and potential interference from endogenous substances and structurally unrelated drugs.[5] In the semi-quantitative mode, the authors reported repeatability and within-lab precision of < 4% CV. Testing of 20 replicates of the 10 ng/mL cutoff calibrator and two negative urine pools contrived with 6-AM to concentrations of 7.5 and 12.5 ng/mL resulted in a good separation (< 5% overlap) between the three levels; this indicates that in the "gray area" around the cutoff (6-AM concentrations between 7.5 and 12.5 ng/mL), samples should be correctly classified as positive or negative (see Fig. 2).[5] One hundred five samples were tested with both the EMIT 6-AM assay and gas chromatography/mass spectrometry (GC/MS), and an overall agreement of 99% was obtained. Morphine and morphine metabolites did not show any cross-reactivity at the concentrations tested (> 100,000 ng/mL) in the semi-quantitative mode, and gave negative results in the qualitative mode. Similarly, the structurally related drugs hydrocodone, hydromorphone, oxycodone, oxymorphone, and codeine gave negative results in the qualitative mode and exhibited < 0.01% cross-reactivity in the semi-quantitative mode.

Study 2: Comparison of EMIT 6-AM and CEDIA 6-AM Assays

The second study was conducted at the South Bend Medical Foundation, South Bend, Indiana, U.S., a regional, independent, commercial reference laboratory, and compared the performance of the EMIT 6-AM assay to that of the Thermo Scientific CEDIA Heroin Metabolite (6-Acetylmorphine) Drugs of Abuse Assay (CEDIA 6-AM assay).

Study Protocol

The EMIT 6-AM assay and the CEDIA 6-AM assay were run concurrently on a Beckman Coulter AU2700 Analyzer. Testing for all studies was performed in the qualitative mode.

Linearity and Precision

The test solutions for the linearity, within-run precision, and accuracy studies were contrived to the desired concentrations using mixtures of the Syva calibrators. Eight test solutions (0, 5.0, 7.5, 10.0, 12.5, 15.0, 17.5, and 20.0 ng/mL) were prepared, and each was tested 20 times. All testing for both assays was conducted concurrently in one run, using the qualitative mode. The average absorbance change rate obtained from the 20 replicates was used to calculate the linearity. Between-run precision was assessed using two levels of a commercial 6-AM control (DETECTABUSE Liquid Control Urine obtained from Biochemical Diagnostics, Inc., Edgewood, NY). Each test solution was tested 20 times in one run.

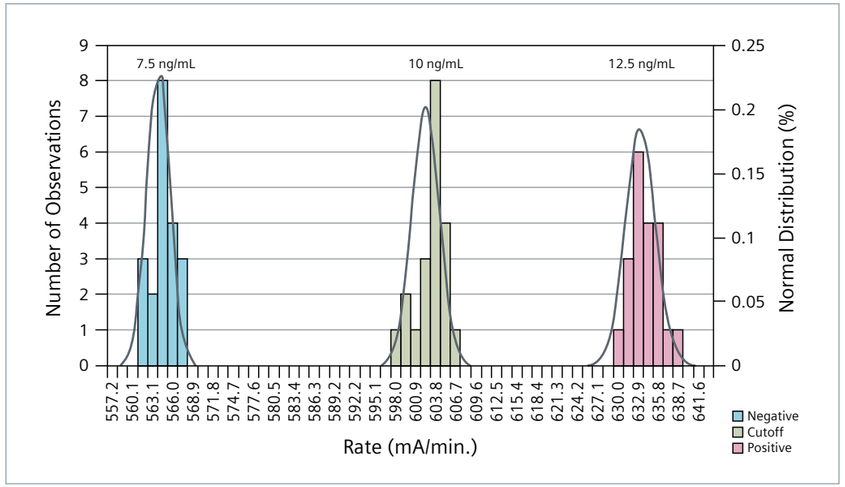
Specificity Study

Test solutions for the specificity study were prepared in SURINE (Cerilliant Corporation, Round Rock, TX), a synthetic negative urine, using pure drug samples (Grace Davidson Discovery Sciences). Next, 10 mL HPLC-grade methanol were added to 10 mg of the pure drug to produce a 1 mg/mL methanolic solution of the drug. The appropriate volume was added to an aliquot of Surine to produce a test solution of the desired drug concentration.

Method Comparison Study

The Syva EMIT II Plus Opiate Assay was used in addition to the two 6-AM assays to test 203 patient samples at the 300 and the 2,000 ng/mL cutoffs. Discrepancies in the results for the two 6-AM assays underwent GC/MS analysis to determine the cause.

Figure 2. Results obtained from testing 20 replicates of controls containing 7.5 and 12.5 ng/mL 6-AM and the 10 ng/mL cutoff calibrator. (Reprinted with permission from Ref. 4.)



Results and Discussion

The linearity results are shown in Figure 3. The two assays gave comparable coefficient of correlation results (R2 = 0.98) for both the EMIT 6-AM and CEDIA 6-AM assays. As shown in Table 1, the precision results obtained for the two assays were also comparable.

The specificity data is shown in Table 2. In the absence of any cross-reactivity to the added compounds, all samples should have read negative, as no 6-AM was present. Note, however, that positive results were obtained with the CEDIA 6-AM assay at a morphine concentration of 15,000 ng/mL and a hydromorphone concentration of 20,000 ng/mL.

Figure 3. Linearity study.

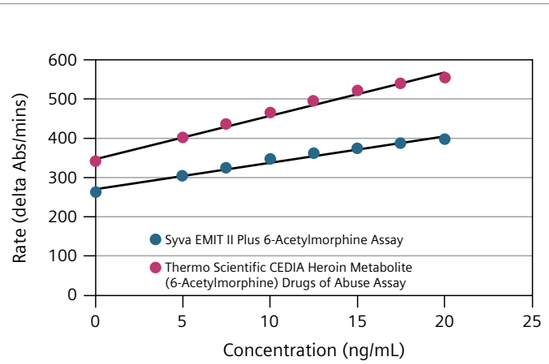


Table 1. Within-run precision results.

	EMIT 6-AM Assay			CEDIA 6-AM Assay		
	Mean (Δ Abs)	SD	CV	Mean (Δ Abs)	SD	CV
EMIT Neg Calibrator	259.9	2.2	0.8	341.1	2.5	0.7
BCD* 7.5 ng/mL Control	324.6	3.2	1.0	433.8	3.3	0.8
CEDIA 10 ng/mL Calibrator	339.9	2.5	0.7	465.2	3.1	0.7
BCD 12.5 ng/mL Control	360.6	2.3	0.6	496.0	4.2	0.8

*Biochemical Diagnostics, Inc.

Table 2. Specificity results.

Potential Cross-reactant	Test Concentration (ng/mL)	EMIT 6-AM Result	CEDIA 6-AM Result
Morphine	10,000	Negative	Negative
Morphine	15,000	Negative	**Positive**
Morphine	20,000	Negative	**Positive**
Codeine	10,000	Negative	Negative
Codeine	15,000	Negative	Negative
Codeine	20,000	Negative	Negative
Dihydrocodeine	10,000	Negative	Negative
Dihydrocodeine	15,000	Negative	Negative
Dihydrocodeine	20,000	Negative	Negative
Hydrocodone	10,000	Negative	Negative
Hydrocodone	15,000	Negative	Negative
Hydrocodone	20,000	Negative	Negative
Hydromorphone	10,000	Negative	Negative
Hydromorphone	15,000	Negative	Negative
Hydromorphone	20,000	Negative	**Positive**
Oxycodone	10,000	Negative	Negative
Oxycodone	15,000	Negative	Negative
Oxycodone	20,000	Negative	Negative
Oxymorphone	10,000	Negative	Negative
Oxymorphone	15,000	Negative	Negative
Oxymorphone	20,000	Negative	Negative

The instructions for use (IFU) for each of the assays contain information on the structurally related compounds that the manufacturers tested for cross-reactivity. Table 3 lists the information from both IFUs regarding the concentrations of the compounds that were tested (versus the 10 ng/mL cutoff) and gave negative results. At concentrations higher than than the concentrations listed in the CEDIA 6-AM IFU, positive results may be expected. This is what is observed in this study.

Interference from structurally related compounds is of concern only if the concentrations that result in interference are likely to be encountered in routine sample testing. Table 4 lists information from the literature on the urine levels of various opioids that have been found in different populations. This table shows fairly high levels of these compounds will be encountered; the frequency would depend on several factors, including the populations being studied. It is also important to keep in mind that more than one compound may be present in a sample; in this case, positive results may be obtained at lower concentrations for the individual drug/metabolite.

Table 3. Cross-reactivity data from the IFUs for the two assays; negative results vs. the 10 ng/mL cutoff for 6-acetylmorphine were obtained at the concentrations listed.
(Bold indicates instances when the concentrations tested for the two assays varied significantly.)

Compound Tested	EMT 6-AM[6] Concentration (ng/mL)	CEDIA 6-AM[7] Concentration (ng/mL)
Codeine	500,000	500,000
Dextromethorphan	100,000	100,000
Dihydrocodeine	500,000	500,000
Heroin HCl	80	80
Hydrocodone	300,000	300,000
Hydromorphone	**100,000**	**10,000**
Imipramine	200,000	200,000
Levorphanol	100,000	10,000
Meperidine	800,000	800,000
Morphine	**100,000**	**9,000**
Morphine-3-glucuronide	600,000	600,000
Morphine-6-glucuronide	600,000	600,000
Nalorphine	**100,000**	**7,000**
Naloxone	300,000	300,000
Naltrexone	300,000	300,000
Norcodeine	600,000	600,000
Normorphine	**100,000**	**30,000**
Oxycodone	400,000	400,000
Oxymorphone	80,000	80,000

Out of the 203 patient samples, 167 (82%) were positive for opiates using the 300 ng/mL cutoff. Only four of these samples, however, were positive in the two 6-AM assays. Using the 2,000 ng/mL opiates cutoff, 154 samples (76%) were positive. Of the 36 opiate-negative samples, 31 were positive by both 6-AM assays. These results are not surprising. A higher number of opiate-positive results at the 300 ng/mL cutoff compared to the 2,000 ng/mL cutoff is expected; for example, if a sample contained 500 ng/mL of opiate, it would be positive at the 300 ng/mL cutoff and negative at the 2,000 ng/mL cutoff. The positive opiates samples may or may not be positive for 6-AM, as a 6-AM concentration > 435 ng/mL is necessary for a positive opiate result unless other opiate drugs are also present. The 6-AM test is much more sensitive than the opiate test (cutoff of 10 ng/mL), and it is therefore not surprising that opiate negative samples were 6-AM-positive.

Table 4. Levels of opioid concentrations in urine (measured by GC/MS or LC/MS).

Analyte	Reference	Range Observed (ng/mL)
Morphine	3*	3,065 – 267,461
	8†	52 – 1,122,000
	9‡	131 – 297,000
Codeine	3	0 – 8,156
	8	59 – 160,600
	9	0 – 50,100
Dihydrocodeine	8	50 – 6,204
Hydrocodone	8	50 – 71,830
Hydromorphone	8	50 – 51,110
	9	< 10 – 1,440
Oxycodone	8	50 – 548,900
Oxymorphone	8	50 – 172,641

*Samples from patients in a heroin/cocaine abuse treatment program; 55 samples from 27 individuals

†Pain patients; 20,089 samples from 13,126 individuals

‡73 samples from a drug testing laboratory

Table 5 shows the agreement of the two 6-AM assays on the 203 patient samples. Overall, the agreement was quite good (99%); two samples, however, produced a positive result with the CEDIA 6-AM assay, but a negative result with the EMIT 6-AM assay. Four samples—the two that were discordant as well as one randomly chosen sample positive by both assays and one sample negative by both assays—were analyzed by GC/MS; the results of this analysis are shown in Table 6. As clearly shown, the opiate positive results in these four samples were due to the presence of compounds other than 6-AM. One 6-AM-containing sample had a high enough concentration to produce a positive 6-AM result in both the 6-AM specific assays, but this concentration would have been too low to yield a positive opiate result if the other opiates had not been present. Note that the other two CEDIA 6-AM-positive/EMIT 6-AM-negative negative samples contain no 6-AM; the positive CEDIA 6-AM results are due to cross-reactivity with the other opiates present.

Table 5. Agreement of the two 6-AM assays on 203 patient samples.

		CEDIA 6-AM Results	
		Positive	Negative
EMIT 6-AM Results	Positive	31	0
	Negative	2	170

Table 6. GC/MS results.

Opiates Result (300 ng/mL Cutoff)	CEDIA 6-AM Results	EMIT 6-AM Results	GC/MS Results (ng/mL)					
			6-AM	Morphine	Oxycodone	Codeine	Oxymorphone	Hydromorphone
Positive	Positive	Positive	>200	7,000	0	348	0	0
Positive	Positive	Negative	0	>140,000	0	0	0	0
Positive	Positive	Negative	0	236,990	0	459	0	0
Positive	Negative	Negative	0	0	7,973	0	2,087	334

Conclusions

- 6-Acetylmorphine will be found in a sample only as a result of heroin abuse. It is not produced by morphine or codeine, or ingestion of prescription opioid medications in the absence of cross-reactive compounds, a positive 6-AM result confirms heroin ingestion.
- The linearity and precision of the EMIT II Plus 6-Acetylmorphine Assay and the Thermo Scientific CEDIA Heroin Metabolite (6-Acetylmorphine) Drugs of Abuse Assay are comparable.
- Results obtained with the 6-AM assays on patient samples were similar, but the CEDIA 6-AM resulted in false-positive results due to cross-reactivity with other opiates present in the samples.

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