SENSITIVE AND RAPID HOMOGENEOUS IMMUNOASSAY FOR THE DETECTION OF HYDROCODONE IN URINE

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INTRODUCTION

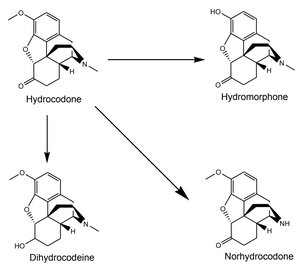
Hydrocodone is a semi-synthetic derivative of codeine and produces opioid-like effects similar to morphine. Hydrocodone (Trade names: Vicodin, Lortab, Hycodan, Vicoprofen) is one of the most frequently prescribed opioids in the U.S. as an antitussive (cough suppressant) and narcotic analgesic agent for the treatment of moderate to severe pain. Its ease of prescription and opioid-like effects has led to widespread drug diversion and abuse. The two commercially available homogeneous immunoassays for the detection of hydrocodone in urine at a cutoff of 300 ng/mL suffer undesirable cross-reactivity to oxycodone and morphine at concentrations below 25,000 ng/mL. ARK Diagnostics has developed the ARK™ Hydrocodone Assay to detect hydrocodone at a cutoff of 300 ng/mL with no cross-reactivity to oxycodone, morphine, and codeine at concentrations below 100,000 ng/mL.

METHOD

The ARK™ Hydrocodone Assay is a liquid stable homogeneous enzyme immunoassay, consisting of two reagents, with a cutoff of 300 ng/mL and semi-quantitative range up to 800 ng/mL. The performance of this assay was evaluated on the Beckman Coulter AU680 Automated Clinical Chemistry Analyzer. Precision, analytical recovery, specificity, Histogram Overlap Analysis of ±25% controls and the cutoff, and method comparison with LC-MS/MS were evaluated.

Hydrocodone Metabolites

Hydrocodone is rapidly metabolized to hydromorphone and its glucuronide. The metabolic pathways of hydrocodone include O-demethylation catalyzed by cytochrome P450 2D6 (CYP2D6) to its active metabolite, hydromorphone; N-demethylation by cytochrome P450 3A4 to form norhydrocodone; and C6-keto reduction to form approximately equal amounts of 6-α-hydrocol(dihydrocodeine) and 6-β-hydrocol. In addition, the active metabolite hydromorphone undergoes phase II glucuronidation predominate metabolite hydromorphone-3-glucuronide.



RESULTS

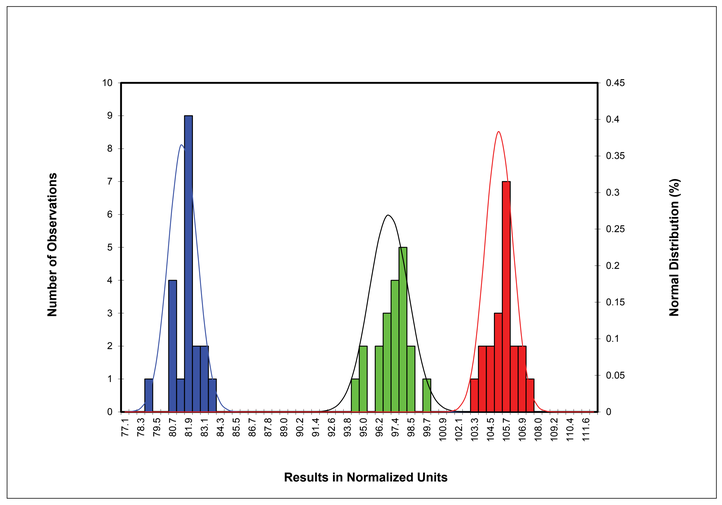
PRECISION

Pooled human urine was spiked with hydrocodone to achieve concentrations at 25% increments apart from the cutoff calibrator (300 ng/mL). Samples were tested in quadruplicate twice per day for 20 days (N=160). Semi-quantitative mode of analysis was evaluated according to the Clinical Laboratory Standards Institute (CLSI) Protocol EP05-A3.

Hydrocodone (ng/mL)	Cutoff (%)	Mean	Precision Results	Repeatability (Within-Run Precision)		Between Day Precision		Total Precision (Within-Laboratory Precision)	
				SD	CV (%)	SD	CV (%)	SD	CV (%)
0.00	-100	0.2	160 Negative	1.2	N/A	0.4	N/A	1.2	N/A
75.0	-75	78.2	160 Negative	2.3	3.0	1.8	2.3	3.0	3.9
150.0	-50	141.6	160 Negative	6.3	4.4	4.0	2.8	7.5	5.3
225.0	-25	229.1	160 Negative	7.8	3.4	5.5	2.4	10.1	4.4
300.0	Cutoff	313.5	24 Negative; 136 Positive	11.1	3.5	7.9	2.5	14.7	4.7
375.0	+25	388.0	160 Negative	14.3	3.7	11.2	2.9	18.8	4.8
450.0	+50	459.5	160 Negative	20.9	4.5	16.5	3.6	27.9	6.1
525.0	+75	539.2	160 Negative	31.7	5.9	24.2	4.5	43.6	8.1
600.0	+100	619.7	160 Negative	50.4	8.1	38.3	6.2	61.4	9.9

HISTOGRAM OVERLAP ANALYSIS (QUALITATIVE ANALYSIS)

Frequency of distribution of Hydrocodone values for each sample is shown by histogram analysis. Twenty replicates each of Negative Control (225.0 ng/mL), Cutoff Calibrator (300.0 ng/mL), and Positive Control (375.0 ng/mL) were assayed together in a single run. The distributions of measurements did not overlap.



ANALYTICAL RECOVERY

Spike recovery was evaluated using in-house prepared samples. Ten (10) samples were tested in semi-quantitative mode using the AU680 analyzer. One calibration curve was generated and 5 replicates of each sample were assayed. Mean, SD, %Nominal and %CV were calculated for each level. Percent nominal ranged from 86.2 to 102.9%.

Samples (ng/mL)	Mean (ng/mL)	SD	CV (%)	Nominal (%)	N
80.0	79.5	1.2	1.5	99.3	5
160.0	151.1	1.5	1.0	94.5	5
240.0	246.8	1.7	0.7	102.9	5
320.0	321.7	5.5	1.7	100.5	5
400.0	385.8	17.5	4.5	96.5	5
480.0	472.3	19.6	4.2	98.4	5
560.0	537.4	35.4	6.6	96.0	5
640.0	606.4	40.7	6.7	94.7	5
720.0	620.6	63.6	10.3	86.2	5
800.0	737.5	91.9	12.5	92.2	5

LIMIT OF QUANTITATION

The following characteristics were determined according to CLSI EP17-A2. Pooled human urine was supplemented with known amounts of hydrocodone and assayed 40 times. The LLOQ of the ARK Hydrocodone Assay is defined as the lowest concentration for which acceptable precision (≤20% CV) and recovery (±20%) is observed. At 55.0 ng/mL, the precision was 4.2% CV and the recovery was 118.6%.

Nominal Concentration (ng/mL)	N	Mean (ng/mL)	Recovery (%)	RMS SD	CV (%)
55.0	40	65.2	118.6	2.8	4.2

SPECIFICITY – METABOLITES

The following metabolites of hydrocodone were prepared in drug-free negative human urine. Their corresponding concentration approximately equivalent to the 300 ng/mL hydrocodone cutoff was investigated using a dose-response curve. Dihydrocodeine tested negative at 100,000 ng/mL.

Compound	Concentration Approximately Equivalent to the Cutoff (ng/mL)	Test Level (µg/mL)
Hydrocodone	292	102.7
Hydromorphone	299	100.3
Hydromorphone-3β-Glucuronide	45,439	0.7
Norhydrocodone	2,277	13.2
Dihydrocodeine	>100,000	<0.3

SPECIFICITY – OPIATES/STRUCTURALLY SIMILAR COMPOUNDS

The following opiates and structurally related compounds were tested in drug-free negative human urine.

Compound	Concentration Tested (ng/mL)	Result (POS/NEG)	Percent Cross-reactivity (%)
6-Acetyl morphine	100,000	NEG	<0.3
Buprenorphine	100,000	NEG	<0.3
Buprenorphine-3β-D-glucuronide	50,000	NEG	<0.6
Codeine	100,000	NEG	<0.3
Codeine-6β-D-glucuronide	100,000	NEG	<0.3
Dextromethorphan	250,000	NEG	<0.1
EDDP	100,000	NEG	<0.3
EMDP	100,000	NEG	<0.3
Ethyl morphine	100,000	NEG	<0.3
Fentanyl	100,000	NEG	<0.3
Heroin	100,000	NEG	<0.3
Levorphanol	100,000	NEG	<0.3
Meperidine	100,000	NEG	<0.3
Methadone	100,000	NEG	<0.3
Morphine	100,000	NEG	<0.3
Morphine-3β-D-glucuronide	100,000	NEG	<0.3
Morphine-6β-D-glucuronide	100,000	NEG	<0.3
Nalbuphine	100,000	NEG	<0.3
Naloxegol	100,000	NEG	<0.3
Naloxone	100,000	NEG	<0.3
Naltrexone	100,000	NEG	<0.3
Norbuprenorphine	100,000	NEG	<0.3
Norcodeine	100,000	NEG	<0.3
Normorphine	100,000	NEG	<0.3
Noroxycodone	100,000	NEG	<0.3
Nortilidine	100,000	NEG	<0.3
Oxycodone	100,000	NEG	<0.3
Oxymorphone	100,000	NEG	<0.3
Oxymorphone-3β-D-glucuronide	50,000	NEG	<0.6
Pentazocine	100,000	NEG	<0.3
Tapentadol	100,000	NEG	<0.3
Thebaine	100,000	NEG	<0.3
Tilidine	100,000	NEG	<0.3
Tramadol	100,000	NEG	<0.3

SPECIFICITY –STRUCTURALLY UNRELATED COMPOUNDS

No interference was observed by the addition of concentrations up to 100,000 ng/mL for 85 structurally unrelated compounds. Acetaminophen, Acetylsalicylic acid, Carbamazepine, Cimetidine, Ibuprofen and Ranitidine were added up to 500,000 ng/mL.

ENDOGENOUS INTERFERENCE

Pooled human urine was supplemented with hydrocodone to contain 225.0 ng/mL or 375.0 ng/mL (equivalent to the quality control concentrations; ±25% of the cutoff concentration) and then endogenous substances were added at the concentrations listed below. No interference was observed except boric acid. Boric acid resulted negative at 1% w/v when tested in the presence of 225 ng/mL and 375.0 ng/mL of hydrocodone.

Substance	Concentration Tested (mg/dL)	Substance	Concentration Tested (mg/dL)
Acetaminophen	10	Galactose	5
Acetone	500	Glucose	1000
Acetyl Salicylic Acid	10	Hemoglobin	150
Ascorbic acid	150	Human Albumin	200
Bilirubin conjugated	2	Human γ- Globulin	500
Bilirubin unconjugated	2	Ibuprofen	10
Boric Acid	1 % w/v	NaCl	1000
Caffeine	10	Oxalic Acid	50
Creatinine	400	Riboflavin	3
Ethanol	10	Urea	1000

Specific Gravity

Urine samples with specific gravity values from 1.000 to 1.030 and with pH range from 3 to 11 in the presence of 225.0 and 375.0 ng/mL hydrocodone were tested. No interference was observed.

METHOD COMPARISON

One-hundred and one (101) positive samples and one-hundred and twenty-five (125) negative samples were analyzed qualitatively and semi-quantitatively by ARK Hydrocodone Assay and by LC-MS/MS. The ARK Hydrocodone assay used a 300 ng/mL hydrocodone as the cutoff concentration and LC/MS/MS had 100 ng/mL cutoff. Results showed an overall agreement of 96.9%, 100.0% clinical specificity, and 92.9% clinical sensitivity.

ARK Hydrocodone Assay 300 ng/mL Hydrocodone Cutoff		LC-MS/MS 100 ng/mL Cutoff Hydrocodone or Hydromorphone	
		(+)	(-)
	(+)	91	0
	(-)	7*	128

* Seven (7) samples were considered discordant. LC-MS/MS had values ≥100 ng/mL for hydrocodone or hydromorphone and ARK semi-quantitative had values <300 ng/mL.

CONCLUSIONS

The ARK™ Hydrocodone assay has high sensitivity with a cutoff of 300 ng/mL in urine screening. The assay detects hydrocodone and its metabolite, hydromorphone, without any significant crossreactivity to other opiate compounds. Method correlation with LC-MS/MS using authentic urine samples showed an excellent agreement of 96.9%. The assay demonstrates superior specificity and simplicity compared to commercially available hydrocodone immunoassays and GC-MS or LCMS/MS methods that require multiple pre-analytical steps or hydrolysis.

PROPOSED INTENDED USE

The ARK™ Hydrocodone Assay is intended for the qualitative detection and/or semi-quantitative estimation of hydrocodone in human urine at a cutoff concentration of 300 ng/mL. The assay is intended for use in laboratories with automated clinical chemistry analyzers.

The semi-quantitative mode is for the purpose of (1) enabling laboratories to determine an appropriate dilution of the specimen for confirmation by a confirmatory method, such as Gas Chromatography/Mass Spectrometry (GC/MS) or Liquid Chromatography/tandem Mass Spectrometry (LC-MS/MS), or (2) permitting laboratories to establish quality control procedures.

The ARK™ Hydrocodone Assay provides only a preliminary analytical test result. A more specific alternative chemical method must be used in order to obtain a confirmed positive analytical result. Gas Chromatography/Mass Spectrometry (GC/MS) or Liquid Chromatography/tandem Mass Spectrometry (LC-MS/MS) is the preferred confirmatory method. Clinical consideration and professional judgment should be exercised with any drug test result, particularly when the preliminary test result is positive.

REGULATORY STATUS

This product is currently pending FDA 510(k) review and is not yet cleared for sale in the US