Diagnostic Accuracy

KIMS, CEDIA, and HS-CEDIA Immunoassays Are Inadequately Sensitive for Detection of Benzodiazepines in Urine from Patients Treated for Chronic Pain

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Background: Patients treated for chronic pain may frequently undergo urine drug testing to monitor medication compliance and detect undisclosed prescribed or illicit drug use. Due to the increasing use and abuse of benzodiazepines, this class of medications is often included in drug screening panels. However, immunoassay-based methods lack the requisite sensitivity for detecting benzodiazepine use in this population primarily due to their poor cross-reactivity with several major urinary benzodiazepine metabolites. A High Sensitivity Cloned Enzyme Donor Immunoassay (HS-CEDIA), in which beta-glucuronidase is added to the reagent, has been shown to perform better than traditional assays, but its performance in patients treated for chronic pain is not well characterized.

Objectives: To determine the diagnostic accuracy of HS-CEDIA, as compared to the Cloned Enzyme Donor Immunoassay (CEDIA) and Kinetic Interaction of Microparticles in Solution (KIMS) screening immunoassays and liquid chromatography-tandem mass spectrometry (LC-MS/MS), for monitoring benzodiazepine use in patients treated for chronic pain.

Study Design: A study of the diagnostic accuracy of urine benzodiazepine immunoassays.

Setting: The study was conducted at an academic tertiary care hospital with a clinical laboratory that performs urine drug testing for monitoring medication compliance in pain management.

Methods: A total of 299 urine specimens from patients treated for chronic pain were screened for the presence of benzodiazepines using the HS-CEDIA, CEDIA, and KIMS assays. The sensitivity and specificity of the screening assays were determined using the LC-MS/MS results as the reference method.

Results: Of the 299 urine specimens tested, 141 (47%) confirmed positive for one or more of the benzodiazepines/metabolites by LC-MS/MS. All 3 screens were 100% specific with no false-positive results. The CEDIA and KIMS sensitivities were 55% (78/141) and 47% (66/141), respectively. Despite the relatively higher sensitivity of the HS-CEDIA screening assay (78%; 110/141), primarily due to increased detection of lorazepam, it still missed 22% (31/141) of benzodiazepine-positive urines. The KIMS, CEDIA, and HS-CEDIA assays yielded accuracies of 75%, 79%, and 90%, respectively, in comparison with LC-MS/MS.

Limitations: This study was limited by its single-site location and the modest size of the urine samples utilized.

Conclusions: While the HS-CEDIA provides higher sensitivity than the KIMS and CEDIA assays, it still missed an unacceptably high percentage of benzodiazepine-positive samples from patients treated for chronic pain. LC-MS/MS quantification with enzymatic sample pretreatment offers superior sensitivity and specificity for monitoring benzodiazepines in patients treated for chronic pain.

Key words: High sensitivty immunoassay, benzodiazepine, beta-glucoronidase, pain management, compliance, LC-MS/MS, screening

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Physicians managing patients with chronic pain have the difficult job of ensuring availability of pain-relieving medications to patients with a legitimate medical need while minimizing inappropriate use or diversion of prescription drugs. To curb the escalating rate of controlled and illicit substance abuse, urine drug screens are frequently employed as one tool to monitor compliance and/or detect the use of other prescribed, undisclosed, or illicit substances in patients undergoing chronic pain treatment (1-5). Detection of prescribed drugs/metabolites and the absence of other non-prescribed or illicit drugs in urine is reassuring because it suggests adherence to the patient's treatment plan.

While opioids are the most frequently prescribed drugs in patients with chronic pain, benzodiazepines, a group of psychoactive drugs, are also increasingly prescribed in the pain management setting due to their anxiolytic and muscle relaxant properties (6,7). The rate of benzodiazepine abuse is rising and the number of emergency department visits related to benzodiazepines is reaching a level similar to opioids, illustrating a nationwide problem with benzodiazepine misuse (8-10).

In the pain management population at Brigham and Women's Hospital (BWH), approximately 27% of patients tested screen positive for benzodiazepines. However, immunoassay-based testing lacks the requisite sensitivity for detecting benzodiazepine use in this population primarily due to their poor cross-reactivity with several major urinary benzodiazepine metabolites (6,11,12). In fact, a previously published study concluded that point-of-care immunoassays should not be utilized for monitoring benzodiazepine compliance in pain management due to their poor sensitivity (6). We have observed high rates of false-negative benzodiazepine screening results with both the Kinetic Interaction of Microparticles in Solution (KIMS) and Cloned Enzyme Donor Immunoassay (CEDIA) methods. False-negative benzodiazepine screening results can generate confusion and lead clinicians to presume patients are diverting their medications and/or allow patients taking undisclosed medications to go undetected.

The High Sensitivity CEDIA (HS-CEDIA), which employs a beta-glucuronidase enzymatic pre-treatment to convert glucuronidated benzodiazepine metabolites to their free form prior to analysis, has been shown to provide improved sensitivity compared to traditional screening assays (13). However, HS-CEDIA sensitivity for detecting benzodiazepine use in patients treated for chronic pain has not been well characterized. This study was designed to evaluate the sensitivity and specificity of HS-CEDIA compared to traditional benzodiazepine screening assays for monitoring medication compliance in pain management using liquid chromatography-tandem mass spectrometry (LC-MS/MS) as the reference method.

METHODS

This study was approved by the Partners Human Research Committee.

CEDIA, HS-CEDIA, and KIMS Screening

A total of 299 consecutive urine specimens sent to the BWH laboratory for benzodiazepine screening for monitoring medication compliance in pain management were included in this study. Samples were frozen (-20°C) in our laboratory until testing ($\leq$ 3 months). Each sample was screened utilizing the KIMS assay (Roche Diagnostics, Indianapolis, IN) on Roche Cobas c501 and CEDIA and HS-CEDIA assays (Thermo Scientific, Fremont, CA) on Beckman AU 480 (Beckman Coulter, Inc., Brea, CA).

The KIMS assay was performed according to manufacturer instructions and calibrated to a positive/negative cutoff of 100 ng/mL. This qualitative assay is based on the measurement of changes in light transmission over time. KIMS reagents contain an anti-drug antibody and a labeled microparticle (i.e. microparticle-drug conjugate). A free drug competes with the drug-microparticle conjugate for antibody binding (14). In the absence of a drug in the patient, the antibody complexes with the labeled microparticle, forms an aggregate, and leads to an increase in absorbance at 505 nm. The presence of a drug inhibits aggregate formation resulting in a decrease in absorbance.

The CEDIA and HS-CEDIA assays were performed qualitatively according to manufacturer instructions and calibrated to a positive/negative cutoff of 200 ng/mL. The CEDIA method uses recombinant DNA technology. The enzyme beta-galactosidase is engineered into 2 inactive fragments, the enzyme donor (ED) and the enzyme acceptor (EA). The drug in the specimen competes with the drug conjugated to the ED fragment for antibody binding. In the absence of a drug, the ED-antibody conjugate remains intact preventing the formation of an active enzyme resulting in no change in absorbance over time. In the presence of a drug, the ED is free to combine with the EA to form the active enzyme, cleave the substrate, and form a product which

absorbs light at 570 nm. The amount of drug present in the patient is proportional to the increase in absorbance. In the HS-CEDIA, the reaction is identical except an optional enzyme is utilized to hydrolyze glucuronide metabolites of benzodiazepines. The technologist performing the testing is instructed to add a manufacturer-defined volume of beta-glucuronidase reagent (Glusulase, Patella Vulgata, Perkin Elmer) to the reconstituted EA solution prior to testing.

Diagnostic Accuracy

TP (true-positive), FN (false-negative), TN (true-negative), and FP (false-positive) results were assigned based on LC-MS/MS results. Diagnostic sensitivities and specificities of KIMS, CEDIA, and HS-CEDIA were calculated as $100 \times TP/(TP + FN)$ and $100 \times TN/(TN + FP)$, respectively. Diagnostic accuracy was calculated as $100 \times (TP + TN)/(TP + FN + TN + FP)$. Samples containing detectable (≥ 50 ng/mL) 7-aminoclonazepam, alpha-hydroxyalprazolam, lorazepam, nordiazepam, oxazepam, and/or temazepam by LC-MS/MS were considered positive. The sensitivity and FN rate, calculated as $100 \times FN/(TP+FN)$ (equal to $1 -$ specificity), were individually calculated for each of the 6 benzodiazepines/metabolites detected by LC-MS/MS for each immunoassay. TP were identified based on the presence of one or more benzodiazepine/metabolites by LC-MS/MS at a concentration ≥ 50 ng/mL.

LC-MS/MS Confirmation

LC-MS/MS testing for benzodiazepines (7-aminoclonazepam, alpha-hydroxyalprazolam, lorazepam, nordiazepam, oxazepam, and temazepam) was performed on all 299 urine specimens. The defined lower limit of reportability for all 6 benzodiazepines was 50 ng/mL. The samples were prepared following LC-MS/MS testing protocol: 20 μL of sample urine was added to 100 μL 4.5 % (v/v) beta-glucuronidase (Glusulase, Patella Vulgata, Perkin Elmer) in 1.1 M, pH 5.0 acetate buffer, 100 μL aqueous internal standard working solution (100 ng/mL deuterated form of all analytes of interest), and 500 μL water. The resulting aliquots were capped and incubated at 65oC for 3.5 hours. Samples were allowed to cool to room temperature and spun in a centrifuge for 5 minutes at 14,000 rpm. The caps of the microcentrifuge tubes were removed and the samples were placed directly into a 24 well plate and analyzed using a Waters ACQUITY-Xevo TQ equipped with an electrospray ionization (ESI) probe in positive ionization mode.

Chromatographic separation was achieved on a Phenomenex Kinetex 1.7μ, C18, 50 mm x 2.10 mm column (Phenomenex, USA) maintained at a temperature of 30oC with a solvent flow rate of 0.45 mL/min and a gradient beginning with 70% mobile phase A (0.1% formic acid in water) and 30% mobile phase B (0.1% formic acid in methanol) and increasing to 95% B over 2 minutes. The composition was held at 95% B for 2.25 minutes and returned to 30% B for 0.25 minutes before the next injection. Two ions were monitored for each analyte in multiple reaction monitoring mode. Each run contained a set of calibrators (0, 50, 100, and 500 ng/mL) that were injected at the beginning and end of the run formulating a best fit calibration curve. A coefficient of determination ≥ 0.98 was used as the minimum threshold for calibration acceptance.

RESULTS

Of the 299 urine specimens tested, 141 (47%) confirmed positive for one or more of the benzodiazepines/benzodiazepine metabolites quantified by LC-MS/MS. 7-aminoclonazepam, alpha-hydroxyalprazolam, lorazepam, nordiazepam, oxazepam, and temazepam were positive in 14%, 7%, 16%, 13%, 17%, and 15% of specimens, respectively, with many specimens containing detectable amounts (≥ 50 ng/mL) of multiple benzodiazepine/benzodiazepine metabolites. Due to the complex metabolic pathways of benzodiazepines (15) (Fig. 1), the presence of specific combinations of temazepam, oxazepam, and/or nordiazepam can suggest ingestion of different parent benzodiazepines. For example, the presence of temazepam and/or oxazepam with nordiazepam suggests prior ingestion of diazepam, while the presence of temazepam and/or oxazepam without nordiazepam could be consistent with diazepam or temazepam ingestion. Oxazepam alone could be due to diazepam, temazepam, oxazepam, or chlordiazepoxide ingestion (Fig. 1A). On the other hand, the metabolism of alprazolam and clonazepam are more straightforward; the presence of alpha-hydroxyalprazolam and 7-aminoclonazepam suggest prior ingestion of alprazolam or clonazepam, respectively (Fig. 1B).

The HS-CEDIA assay demonstrated the highest sensitivity, 78% (110/141), of the 3 immunoassays in this study (Table 1), compared to 55% (78/141) and 47% (66/141), for CEDIA and KIMS, respectively (Table 1). For all 3 assays, the highest sensitivities in comparison with LC-MS/MS were observed for samples containing alpha-hydroxyalprazolam and the lowest sensitivities for samples containing lorazepam and 7-aminoclonazepam (Fig. 2). The HS-CEDIA, CEDIA, and KIMS assays
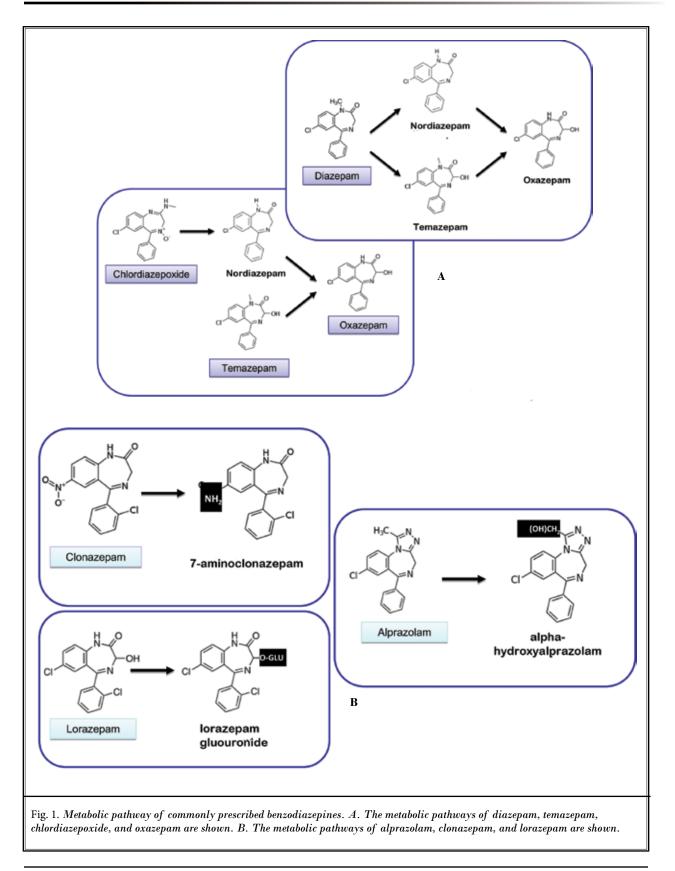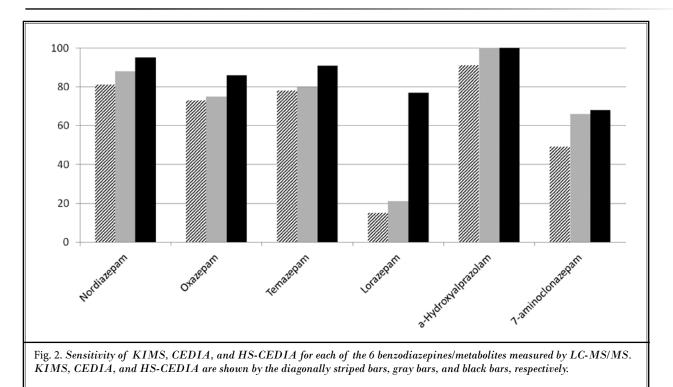
Fig. 1. *Metabolic pathway of commonly prescribed benzodiazepines. A. The metabolic pathways of diazepam, temazepam, chlordiazepoxide, and oxazepam are shown. B. The metabolic pathways of alprazolam, clonazepam, and lorazepam are shown.*

Table 1. *Sensitivity, specificity, and accuracy of KIMS, CEDIA, and HS-CEDIA urine benzodiazepine immunoassays in comparison with LC-MS/MS.*

Assay	Cut-off (ng/mL)	Sensitivity, %	Specificity, %	Accuracy, %	TP	FP	TN	FN
KIMS	100	47 (66/141)	100 (158/158)	75 (224/299)	66	0	158	75
CEDIA	200	55 (78/141)	100 (158/158)	79 (236/299)	78	0	158	63
HS-CEDIA	200	78 (110/141)	100 (158/158)	90 (268/299)	110	0	158	31

TP = true-positive, FP = false-positive, TN = true-negative, and FN = false-negative.
Sensitivity (%) = 100% × TP/(TP + FN), Specificity (%) = 100% × TN/(TN + FP) TP (true positive), Accuracy (%) = 100% × (TP + TN)/(TP + FN + TN + FP).

Fig. 2. *Sensitivity of KIMS, CEDIA, and HS-CEDIA for each of the 6 benzodiazepines/metabolites measured by LC-MS/MS. KIMS, CEDIA, and HS-CEDIA are shown by the diagonally striped bars, gray bars, and black bars, respectively.*

were all 100% specific with no FP screening results identified. Overall, the accuracy of the HS-CEDIA, CEDIA, and KIMS compared to LC-MS/MS was 75%, 79%, and 90%, respectively (Table 1).

The FN rate using HS-CEDIA was 22% (31/141), a substantial improvement over CEDIA (45%, 63/141) and KIMS (53%, 75/141), which each missed approximately half of benzodiazepine-positive samples. Twenty-two percent (22%, 31/141) of LC-MS/MS benzodiazepine-positive samples were detected by the HS-CEDIA assay only. The majority (84% or 25/31) of the samples detected only by HS-CEDIA contained lorazepam only (concentrations ranging from 160 to 9,489 ng/mL). Fig.

2 demonstrates the striking improvement in lorazepam detection after addition of beta-glucuronidase enzyme to EA reagent (HS-CEDIA). Of the remaining 6 samples detected by HS-CEDIA only, 4 contained some combination of temazepam (53 to 1413 ng/mL), oxazepam (52 to 1542 ng/mL), and/or nordiazepam (52 ng/mL), one contained 7-aminoclonazepam (274 ng/mL), and one contained lorazepam (250 ng/mL) and temazepam (50 ng/mL).

Despite the relatively higher HS-CEDIA sensitivity compared to KIMS and CEDIA, the HS-CEDIA screening assay still missed 31/141 (22%) of benzodiazepine-positive urine specimens by LC-MS/MS (Table 2). While

Table 2. *Concentration ranges and means ($\overline{X}$) of benzodiazepines/metabolites found in the urine samples that tested falsely negative by KIMS, CEDIA, and HS-CEDIA.*

		KIMS	CEDIA	HS-CEDIA
7-Aminoclonazepam	FN	21	14	13
	Range	57 – 1,555	57 – 274	52 – 269
	$\overline{X}$	262	119	107
Alpha-hydroxyalprazolam	FN	2	0	0
	Range	59 – 100	-	-
	$\overline{X}$	80	-	-
Lorazepam	FN	40	37	11
	Range	52 – 9,489	52 – 11,918	52 – 269
	$\overline{X}$	1273	1559	124
Nordiazepam	FN	4	2	1
	Range	51 – 1 175	52 – 1.175	1,175
	$\overline{X}$	389	614	-
Oxazepam	FN	21	21	21
	Range	52 – 3,037	52 – 3,37	52 – 3,037
	$\overline{X}$	440	534	441
Temazepam	FN	10	9	4
	Range	50 – 2,264	50 – 29,623	50 – 2,264
	$\overline{X}$	475	3757	709

- = Not applicable, FN = false negative
Ranges are in ng/mL

HS-CEDIA assay detected all 22 samples with alprazolam (13 with alprazolam only; 9 with alprazolam in combination with other benzodiazepines), one or more samples containing one or more of each of the 5 other benzodiazepines were falsely negative (Table 2). Lorazepam and 7-aminoclonazepam had the highest instances of false negatives for all 3 immunoassays (Table 2).

Discussion

Immunoassay-based benzodiazepine screens are frequently used for medication compliance monitoring of patients treated for chronic pain because they are rapid, inexpensive, automatable, and easily performed on point-of-care devices. Many laboratories, including

our own, have used qualitative benzodiazepine immunoassay screening methods followed by quantitative testing by a more sensitive and specific technique such as LC-MS/MS for specimens with positive and/or unexpected negative results (16).

Although immunoassays have traditionally been the most commonly used tests to screen urine for benzodiazepines, they have several known limitations, including poor cross-reactivity with several major urinary benzodiazepine metabolites, and poor sensitivity for detection of remote benzodiazepine use, when urine drug concentrations are relatively low. Two recent studies illustrated a high false negative rate using point-of-care based benzodiazepine immunoassays as

compared to LC-MS/MS in chronic pain patients (6,10). One study concluded that immunoassays should not be utilized for monitoring benzodiazepine compliance in pain management (6). The other study concluded that all negative urine benzodiazepine immunoassay screen results should be sent out for LC-MS/MS confirmation due to the high rate of false negative results in their study (10). In medication compliance monitoring, false negatives can have a considerable negative impact on patient care by leading to false assumptions of non-compliance and/or diversion, and/or by allowing undisclosed benzodiazepine use to go undetected.

In this study, our primary goal was to determine whether use of beta-glucuronidase in the CEDIA assay (HS-CEDIA) would yield an immunoassay with substantially higher sensitivity than traditional benzodiazepine immunoassays, providing a screening method with sensitivity comparable to our LC-MS/MS methodology. As expected, the HS-CEDIA assay, which incorporates beta-glucuronidase into the reagent to convert the poorly cross-reacting glucuronide metabolites to free drug, markedly increased sensitivity for lorazepam which is excreted in the urine primarily as a glucuronide conjugate (13). The observed improvement in HS-CEDIA sensitivity for detection of samples containing lorazepam (Fig. 2) is consistent with the higher HS-CEDIA manufacturer stated cross-reactivity for lorazepam (Table 3). Lorazepam concentrations of 19,615 ng/mL, 10,000 ng/mL, and 400 ng/mL are required to trigger a positive

results for KIMS, CEDIA, and HS-CEDIA, respectively. However, 23% of specimens with lorazepam were still missed by HS-CEDIA.

The less remarkable improvement in HS-CEDIA versus KIMS and CEDIA sensitivities for samples containing oxazepam/oxazepam glucuronide (Fig. 2) was likely due to the fact that many oxazepam-positive samples were also positive for nordiazepam which have relatively high cross-reactivities in the traditional CEDIA and KIMS assays (Table 3). A relatively high percentage of samples containing the major clonazepam metabolite, 7-aminoclonazepam, were missed by all 3 assays. This finding was unexpected given the manufacturer stated cross-reactivity (e.g. 144 ng/mL or 69% in the KIMS assay). It was also unexpected that a sample containing a temazepam concentration of 29,623 ng/mL and oxazepam concentration of 1,515 ng/mL was missed by the CEDIA assay (Table 2). However, in our laboratories, we occasionally obtain aberrant results that are not consistent with manufacturers' claims or prior samples with similar benzodiazepine/metabolite concentrations. These findings illustrate that immunoassays should not be reported semi-quantitatively as results can be inaccurate and unpredictable and that a more specific and sensitive technique such as LC-MS/MS is preferable for monitoring benzodiazepines in the pain management population.

Overall, the HS-CEDIA missed 22% of benzodiazepine-positive samples. Based on our findings of suboptimal immunoassay sensitivities, for our pain management population coupled with the known advantages

Table 3. *Manufacturers' reported cross-reactivity for major benzodiazepines/metabolites in urine.*

Benzodiazepines	Primary Metabolites in Urine	KIMS (Concentration required to trigger a positive result [ng/mL])	CEDIA (Concentration required to trigger a positive result [ng/mL])	HS-CEDIA (Concentration required to trigger a positive result [ng/mL])
Alprazolam	alpha-hydroxyalprazolam	118	163	115
Chlordiazepoxide	demoxepam	92	1,900	1,000
	oxazepam glucuronide	-	10,000	800
Clonazepam	7-aminoclonazepam	144	-	200
Diazepam	nordiazepam	100	150	120
	oxazepam glucuronide	-	10,000	800
	temazepam glucuronide	>20,000	10,000	750
Lorazepam	lorazepam glucuronide	19615	10,000	400
Oxazepam	oxazepam glucuronide	-	10,000	800
Temazepam	oxazepam glucuronide	-	10,000	800
	temazepam glucuronide	>20,000	10,000	750

- = not provided by manufacturer

of LC-MS/MS, including the ability to specifically quantify multiple individual benzodiazepines/metabolites simultaneously, our laboratories now bypass immunoassay screens and use LC-MS/MS as the front-line test for detecting benzodiazepine use in patients treated for chronic pain. For laboratories that continue to rely on benzodiazepine immunoassays screens, a reflex testing algorithm that confirms unexpected negatives (i.e., negative screening results for patients prescribed benzodiazepines) by a more sensitive method could be used to minimize false-negative results. However, for this option, patient medications must be available to the laboratory at the time of testing. Furthermore, laboratories using benzodiazepine screens should educate their clinicians on the sensitivity limitations of benzodiazepine screening.

Many laboratories, including the BWH laboratory, do not require a medication list to be submitted with the urine specimen. Without data on the patients' medications at the time of testing, we could not distinguish which of the benzodiazepine-positive samples originated from patients who were prescribed benzodiazepines versus patients with undisclosed benzodiazepine use. Thus, the accuracy of the KIMS, CEDIA, and HS-CEDIA immunoassays for monitoring compliance in patients prescribed benzodiazepines versus detection of patients with undisclosed benzodiazepine use could not be differentiated. Also of note, our LC-MS/MS method was not designed to detect the short-acting benzodiazepines which are used less frequently in the pain management setting, flurazepam, midazolam, triazolam, and flunitrazepam, and the sensitivity of the immunoassays for these benzodiazepines could not be determined.

Conclusion

While the HS-CEDIA assay provides improved detection sensitivity over the KIMS and CEDIA assays, it still missed 22% of positive benzodiazepine samples and therefore is unsuitable for urine benzodiazepine screening of pain management patients if further confirmation of negative results is not provided. LC-MS/MS quantification with enzymatic sample pretreatment offers superior sensitivity and specificity for monitoring benzodiazepines in patients treated for chronic pain.

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