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Superior detection of clonazepam use by a urine benzodiazepine immunoassay

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ABSTRACT

The widely-used anti-convulsant and anxiolytic drug clonazepam (Klonopin®) requires monitoring, as the drug is subject to both abuse and diversion. Urine is the most-used and convenient specimen for monitoring benzodiazepine use in the general population. Urine benzodiazepine immunoassays detect clonazepam use very poorly however, as they are generally insensitive at detecting 7-aminoclonazepam-related species, the clonazepam derivatives that predominate in urine. Simultaneously collected urine-serum pairs were obtained from 106 patients taking clonazepam and judged to be free of other benzodiazepines. The authors tested these specimens using three urine benzodiazepine immunoassays: the Roche Kinetic Interaction of Microparticles in Solution (KIMS), Syva EMIT II+, and Biosite Triage. The KIMS assay was tested using three different cut-offs: 100, 200, and 300 ng/mL. The KIMS assay detects the primary urinary metabolite 7-aminoclonazepam with equal sensitivity as the parent drug, while the others do not. Analysis of 55 urine samples from confirmed clonazepam users free of other benzodiazepines, yielded the following sensitivities for detecting clonazepam use: KIMS (100 ng/mL cutoff), 82%; KIMS (200 ng/mL cutoff), 56%; KIMS (300 ng/mL cutoff), 36%; Syva EMIT II+ (200 ng/mL cutoff), 27%; Biosite Triage (300 ng/mL cutoff), 18%. This order of sensitivity matched the sensitivity of each assay's ability to detect clonazepam's major urinary metabolite, 7-aminoclonazepam. Excluding dilute urines (creatinine < 0.50 mg/mL) KIMS100 was 96% (87 of 91) sensitive at detecting clonazepam use. Urine creatinine concentration was more useful than serum clonazepam concentration in rationalizing a patient's urine benzodiazepine immunoassay results. The KIMS100 sensitivity, 96%, far exceeds that of the other urine benzodiazepine immunoassays studied here, rivaling LC-MS-MS at estimating patient compliance with clonazepam use.

KEYWORDS: clonazepam, urine drug testing, urine creatinine, immunoassay, comparison, prescription compliance

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INTRODUCTION

Clonazepam is a benzodiazepine (BZ) drug with both anxiolytic and anticonvulsant properties. It is marketed in the US by Roche under the trade-name Klonopin® and is used in chronic epilepsy management and short-term relief of the symptoms of anxiety and panic disorders, particularly social phobia and generalized anxiety disorder [1-3]. It is also an abused drug, with significant street-market value and potential for diversion [4]. Monitoring of prescription compliance is essential. As an anticonvulsant, the maximum recommended daily adult dose is 20 mg [5]. Psychotropic-related doses in adults are typically 0.5-4.0 mg/day [6]. Serum therapeutic levels when used as an anticonvulsant are 20-70 ng/mL [7]. Therapeutic ranges for non-anticonvulsant uses are not well-defined. Clonazepam is extensively metabolized by the liver, and has an elimination

half-life of 19-60 hours with less than 0.5% of a daily dose appearing unchanged in the urine [8]. Clonazepam's principle metabolite is 7-aminoclonazepam, which is further metabolized via acylation to yield 7-acetamidoclonazepam [9]. Clonazepam and 7-aminoclonazepam also undergo 3-hydroxylation with subsequent glucuronidation. 7-aminoclonazepam derivatives are the predominant clonazepam metabolites present in urine [9].

Monitoring of clonazepam prescription compliance is problematic compared to other BZs. Specific clonazepam detection and quantitation is readily accomplished by high-complexity laboratory-based techniques such as liquid (LC) or gas chromatography (GC), using a variety of detection techniques including photodiode-array (PDA) and mass spectrometry (MS) [9]. Class-specific urine BZ immunoassays generally detect the use of most BZs with very good sensitivity and specificity. An exception is clonazepam, one of three BZs (the others are nitrazepam and flunitrazepam) having a $–NO_2$ moiety instead of a -Cl one at the para (#7) position on the fused benzene ring. While these three parent drugs are recognized reasonably well by immunoassays, their 7-amino derivatives are frequently not (Table 1) [10-15]. The poor detection of clonazepam use by typical urine BZ immunoassays can be rationalized as due to the combination of negligible parent drug present in urine and insensitivity of immunoassays to 7-aminoclonazepam-related species (which are the principle clonazepam derivatives in urine). At Massachusetts General Hospital (MGH) the inability to detect clonazepam use using urine samples (that were tested for multiple other drug classes by immunoassay screens in addition to BZ) was judged by many clinicians to be a major flaw in clinical laboratory toxicology services. The only options available to clinicians to verify clonazepam prescription compliance was to either order a quantitative serum clonazepam level, or a chromatography-based urine screen.

In an effort to eliminate this clonazepam-related flaw in our toxicology services we evaluated the Roche KIMS (Kinetic Interaction of Microparticles in Solution) urine BZ assay [16, 17]. Based on the cross-reactivity tables in the manufacturer's literature

Table 1. Limits of detection (in ng/mL) for select benzodiazepines and metabolites by different immunoassays.

Analyte	Triage	EMIT II+	KIMS100	KIMS200	KIMS300
Clonazepam	650	255	148	307	445
7-Aminoclonazepam	7,500*	3238	144	288	489
Flunitrazepam	150	138	142	283	424
7-Aminoflunitrazepam	2,000*	590	97	212	333
3-Hydroxyflunitrazepam	NA	NA	175	355	584
Lorazepam	200	603	163	341	487
Lorazepam-glucuronide	300	> 10,000	19,615	> 20,000	> 20,000
Oxazepam	3,500	253	122	259	398
Nitrazepam	2,600	142	114	246	359
7-Aminonitrazepam	NA	NA	103	239	340
7-Acetomidonitrazepam	NA	NA	43,026	91,765	175,497
Temazepam	200	138	145	256	409
Temazepam-glucuronide	1,000	> 10,000	> 20,000	> 30,000	> 20,000
Nordiazepam (KIMS Calibrator)	700	109	100	200	300
Lormetazepam (EMIT Calibrator)	250	200	163	307	503
Estazolam (Triage Calibrator)	300	110	108	213	325

The number is the minimum concentration needed to produce a positive benzodiazepine result as listed in the manufacturer's package insert as of 2009 unless noted.
*Biosite Triage documentation dated 8/1/2005. NA = Not available.

the Roche assay appeared to have similar detection sensitivities for both clonazepam and 7-aminoclonazepam. We decided to compare KIMS with two other urine immunoassays in use at our institution for their ability to detect clonazepam use, using serum LC-PDA as the "gold standard" [18]. Here we report the results of our studies.

MATERIALS AND METHODS

Sample inclusion criteria

All samples during two different three-month periods that had a "comprehensive" serum drug screen performed using LC-PDA [18] were included. Of these screens, all samples with a serum clonazepam finding of < 7 ng/mL or evidence of use of other benzodiazepines were then excluded. The limit-of-detection for other significant benzodiazepine parent drugs is: diazepam (21 ng/mL), nordiazepam (13 ng/mL), chlordiazepoxide (23 ng/mL), temazepam (15 ng/mL), oxazepam (11 ng/mL), lorazepam (11 ng/mL), and alprazolam (10 ng/mL). Finally, we excluded all patients that did not have a simultaneously-collected urine specimen tested for benzodiazepines. Paired serum/urine samples from 55 patients were collected in the first three-month period. These 55 urine samples were stored at -20 °C for up to two weeks before analysis by three different immunoassays (see below). In the second three-month period we identified 51 more serum/urine pairs (these urine samples were tested by only one immunoassay, KIMS100) and results were combined with the original 55 urine samples to estimate the KIMS100 sensitivity of detection of clonazepam use reported here.

Point-of-care (POC) type immunoassay

The Triage TOX BZO benzodiazepine test is a lateral-flow immuno-chromatographic format immunoassay as described by Melanson [19]. The Triage BZ test is a point-of-care (POC), fluorescence-based immunoassay used in conjunction with the Triage Meter (Biosite Triage, Alere, Waltham MA) for the qualitative detection of benzodiazepines in urine specimens. A 300 ng/mL estazolam cut-off calibrator is used to distinguish positive from negative samples.

Instrument-based immunoassays

The Syva EMIT II+ BZ assay is a homogenous enzyme immunoassay reagent kit (Seimens, Newark, DE). Testing was performed on a Hitachi 911 analyzer (Roche Diagnostics, Indianapolis, IN). The 200 ng/mL lormetazepam cut-off calibrator was used to distinguish positive from negative samples.

The Roche Benzodiazepines Plus homogenous immunoassay (Roche Diagnostics, Indianapolis, IN) is based on the KIMS principle. The assay was performed on a cobas 501 analyzer (Roche Diagnostics, Indianapolis, IN) using the 100, 200, and 300 ng/mL nordiazepam calibrators to distinguish positive from negative samples.

Other assay information

The limits-of-detection for all urine immunoassays used in this study for clonazepam, clonazepam metabolites, and related benzodiazepines/metabolites are listed in Table 1. Urine creatinine was measured on a cobas 501 analyzer with rate-Jaffe reagents from Roche Diagnostics. Serum BZ quantitation was performed by LC-PDA [18]. The detection limit for serum clonazepam was 7 ng/mL. This investigation was performed as part of a quality assurance program, and so institutional review board approval was not needed.

RESULTS

Table 2 tabulates the results of the split-sample correlation study performed on the samples collected during the first three-month collection period. Each of the 55 patient's paired serum/urine results are listed, including the serum clonazepam concentration, the creatinine concentration of the simultaneously-collected urine samples, the nominal, unverified, clonazepam dose per day (if available), and the qualitative urine benzodiazepine results by the five different immunoassays. Table 3 is a summary of the performance of each immunoassay, using the serum LC-PDA as the "gold standard". KIMS100 was the most sensitive at detecting clonazepam use, followed (in order) by KIMS200, KIMS300, EMIT II+, and TRIAGE. This sensitivity order mimics the order of reactivity of each assay towards 7-aminoclonazepam (Table 1). As expected, lowering the positive cutoff threshold from 300 to 200 and to 100 ng/mL for the KIMS assay resulted in progressively better detection of clonazepam use. Such lower cutoffs can also result in increased false-positives. The lower KIMS100 cutoff we have used for over five years in clinical service at this institution has not resulted in an increased rate

Table 2. Results of 55 patient serum/urine specimen pairs analyzed by LC-PDA (serum) and three different benzodiazepine immunoassays (urine), with three different cutoffs used for the urine KIMS assay.

Patient #	Clonazepam Dose (mg/day or ingestion)	Serum Clonazepam (ng/mL)	Urine Creatinine (mg/mL)	KIMS 100	KIMS 200	KIMS 300	EMIT II+	Triage
1	1.5	7	1.26	+	-	-	-	-
4	?	8	0.86	-	-	-	-	-
5	1	8	0.63	+	-	-	-	-
9	?	9	0.24	+	+	+	+	+
11	2	10	1.36	+	+	-	-	-
12	2	10	1.08	+	+	-	-	-
13	?	10	0.20	-	-	-	-	-
14	0.75	10	0.66	+	-	-	-	-
15	?	10	0.99	-	-	-	-	-
19	1.5	11	0.37	-	-	-	-	-
20	0.5	11	0.28	-	-	-	-	-
24	?	13	0.48	+	-	-	-	-
25	2	13	0.36	+	-	-	-	-
26	?	13	2.60	+	+	+	-	-
29	?	14	0.86	+	-	-	+	+
32	1	15	4.34	+	+	-	-	-
35	2	16	2.99	+	+	+	-	-
36	1	16	1.31	+	-	-	-	-
37	1	16	3.05	+	+	+	+	-
42	2	17	0.50	+	-	-	-	-
45	1.5	18	0.98	+	+	-	-	-
46	?	18	1.35	+	+	-	-	-
51	?	19	0.84	+	-	-	-	-
52	1.5	19	1.58	+	+	-	-	-
53	20	20	1.09	+	-	-	-	+
54	?	20	1.08	+	+	-	-	-
55	4	21	0.04	-	-	-	-	-
58	1	23	1.72	+	+	+	-	-
60	2	25	1.66	+	+	-	-	-
63	?	26	0.43	+	+	-	+	+
64	1.5	27	1.94	+	+	+	+	+
65	3	27	0.34	+	-	-	-	-

Table 2 continued..

67	2	28	1.26	+	+	+	-	-
68	?	28	1.45	+	+	+	+	+
69	0.5	29	1.46	+	+	+	-	-
70	3	29	0.20	-	-	-	-	-
72	3	30	2.08	+	+	+	-	-
73	3	31	0.53	+	-	-	-	-
74	?	32	0.45	-	-	-	-	-
75	6	32	0.18	-	-	-	-	-
77	?	33	0.14	-	-	-	-	-
80	?	35	0.84	+	-	-	-	-
81	?	37	1.23	+	+	+	-	-
83	1	39	3.08	+	+	+	-	+
84	4	39	1.69	+	+	+	+	+
91	?	51	2.16	+	+	+	+	-
93	4	58	1.46	+	+	+	+	-
94	6	61	0.28	+	-	-	-	-
97	2	94	2.16	+	+	+	+	-
98	1	111	1.04	+	+	+	+	+
99	48	122	1.14	+	+	+	+	-
100	30	131	0.41	+	+	-	+	-
102	11	147	0.94	+	+	+	+	-
105	?	174	2.85	+	+	+	+	+
106	?	183	0.24	+	+	-	-	-

- = Negative result.
+ = Positive result.
? = Unknown clonazepam dose.

Table 3. Clinical performance of three different urine benzodiazepine immunoassays, with three different cutoffs for the KIMS assay, in 55 serum/urine pairs. Serum HPLC is the reference or "gold standard".

Characteristic	KIMS 100	KIMS 200	KIMS 300	EMIT II+	Triage
True Positives	45	31	20	15	10
False Negatives	10	24	35	40	45
Agreement with Serum HPLC (%)	82	56	36	27	18

of positive urine BZ results that are challenged by MGH physicians. Our only negative experience using the lower 100 ng/mL BZ cutoff is that we have had two instances in which borderline positive KIMS100 results were not confirmed by a chromatographic assay with slightly lower clonazepam or 7-aminoclonazepam detection limits.

Of the 15 samples from 15 patients prescribed 0.5-1.5 mg clonazepam per day, 13 tested positive by KIMS100. Patients # 19 and 20 tested negative, and those samples had creatinine values of 0.37 and 0.28 mg/mL, respectively. Of the 12 samples from 12 patients prescribed 2-3 mg clonazepam per day, 11 tested positive by KIMS100. Patient 70 was the

lone negative in the 2-3 mg/day group, and had a creatinine value of 0.20 mg/mL. In the larger, overall study group of 106 serum/urine pairs, KIMS100 was positive in 87 urine samples (87 of 106, or 82%). Table 4 compiles the available data from the nineteen urine samples (of all 106 samples) that were KIMS 100 negative and serum clonazepam positive by LC-PDA.

DISCUSSION

The urine sample selection criteria chosen guaranteed that there was clonazepam in the patient's blood at the time the urine sample was collected, and ruled out the presence of other parent BZ down to the serum LC-PDA method's limit of detection. With our inclusion criteria choice we did not have to make assumptions about patient's clonazepam prescription compliance or whether the patient was secretly ingesting other BZs. A potential drawback of our sample selection criteria is that the serum BZ method may not detect all benzodiazepine metabolites (hydroxy-alprazolam, etc.), particularly the glucuronidated ones. If this happens, other non-clonazepam-related BZ species would likely be present in the urine and may falsely add to the urine BZ immunoreactivity we attribute to that of clonazepam-only use.

The selection criteria also allowed us to evaluate if there was any effect of serum clonazepam concentration on urine benzodiazepine immunoreactivity. KIMS100 and KIMS200 detected clonazepam use in all seven urine samples from patients with a supra-therapeutic serum clonazepam concentration (those > 70 ng/mL). KIMS300 detected five, EMITII+ six, and TRIAGE two. Otherwise, there appeared to be little correlation between serum clonazepam concentration and urine KIMS100 BZ positivity. Within each serum/urine pair, urine concentration, as estimated by the urine creatinine, seemed to have a much more pronounced effect

Table 4. Characteristics of KIMS100 negatives.

Patient #, Sex	Clonazepam Dose (mg/day or ingestion)	Serum Clonazepam (ng/mL)	Urine Creatinine (mg/mL)	% Immunoreactivity of 100 ng/mL Nordiazepam KIMS100 Calibrator
2, F	1	7	0.27	60
3, F	1	7	0.52	75
4, M	?	8	0.86	95
7, M	1.5	8	0.21	41
10, M	?	9	0.46	72
13, F	?	10	0.20	64
15, F	?	10	0.99	42
19, F	1.5	11	0.37	62
20, F	0.5	11	0.28	65
23, M	?	12	0.57	86
27, M	2	13	0.44	77
31, M	2	14	0.41	94
47, F	1.5	18	0.23	92
55, M	4	21	0.04	70
70, F	3	29	0.20	90
74, M	?	32	0.45	29
75, M	6	32	0.18	60
77, M	?	33	0.14	64
79, F	12.5	33	0.24	64

? = Unknown clonazepam dose.

than serum clonazepam concentration regarding whether a urine sample would be KIMS100 BZ positive. As an example, in the 20-39 ng/mL serum clonazepam range (Table 2) KIMS100 was always positive, except when the urine creatinine was less than 0.46 mg/mL (patients # 55, 70, 74, 75, 77), despite the similar serum clonazepam concentrations. Table 5 compiles a single patient's serum clonazepam, urine creatinine, and KIMS100 reactivity (as a % immunoreactivity of the 100 ng/mL nordiazepam KIMS100 calibrator) over a six-month period on the patient's constant 2.5 mg/day dose of clonazepam (these results are not part of the 106 serum/urine pairs reported in this study). Notably, the patient's serum clonazepam is in the 21-37 ng/mL range, yet the KIMS100 immunoreactivity varies nearly seven-fold, likely due to the vastly different hydration states of the patient between urine collections (as estimated by the urine creatinine).

Evaluating a patient's compliance with their clonazepam prescription is problematic. Automated urine BZ immunoassays detect clonazepam use poorly [10-15]. Specific clonazepam assays for serum, urine, and oral fluid exist, but typically require chromatography coupled with sensitive detection techniques such as tandem mass spectrometry or photo-diode array spectrophotometry. Such assays typically are found only at reference laboratories or large medical centers. Published estimates of clonazepam compliance rates are rare. West *et al.* in a study of 180 samples from patients prescribed clonazepam found that a urine immunoassay with a positive cutoff threshold for BZ of 200 ng/mL had only a 21% positivity/compliance estimate rate [13]. Using LC-MS-MS and a 40 ng/mL limit of detection for 7-aminoclonazepam on the same samples, the positivity or estimated compliance rate soared to 87%, similar to the KIMS100 positivity/ compliance rate of 82% reported here, despite the different cutoffs (144 ng/mL for KIMS100 versus 40 ng/mL for LC-MS-MS).

The KIMS100 sensitivity at detecting clonazepam use is considerably better than the other immunoassays tested here, but we doubt physicians will feel comfortable making important clinical decisions using a diagnostic tool that may be correct only 82% of the time. In an effort to improve the KIMS100 performance, we reviewed the data from the nineteen false-negatives, which are compiled in Table 4. Fifteen of the nineteen had creatinine in the range of 0.04-0.46 mg/mL. If we require that a negative KIMS100 result must have a urine creatinine > 0.49 mg/mL to be considered an adequate specimen, the combination of KIMS100 and urine creatinine testing has a sensitivity of 96% (87/91). The remaining four false-negatives (patients 3, 4, 15, and 23) had creatinine values in the range of 0.52-0.99 mg/mL. All four had very low serum clonazepam concentrations (range, 8 - 12 ng/mL).

While the overall order of sensitivity of clonazepam detection matched the order of assay sensitivity towards 7-aminoclonazepam, five individual samples did not (29, 53, 63, 83, and 100). All these samples stand out as they have a negative result to the left of a positive one in Table 2. This should not happen, as the columns are ordered in order of decreasing 7-aminoclonazepam sensitivity from left-to-right. This indicates that 7-aminoclonazepam is not the sole significant contributor to the BZ immunoreactivity produced by these five samples. Other 7-aminoclonazepam derivatives likely contribute to the total immunoreactivity, but their cross-reactivities are not reported in the manufacturer's package insert. Extrapolating from nitrazepam (the only 7-NO_2 BZ in Table 1 with acetamido-metabolite data), 7-acetamidoclonazepam is likely not a significant contributor to the KIMS total BZ immunoreactivity. 3-hydroxyclonazepam and 3-hydroxy-7-aminoclonazepam are likely contributors to the KIMS immunoreactivity, based on the Table 1 data for the 3-hydroxylated BZ parent drugs (lorazepam, oxazepam, temazepam) whose immunoreactivities

Table 5. Results from a single patient on a constant 2.5 mg/day clonazepam dose over a six month period.

Day #	1	29	43	57	87	191
Serum Clonazepam (ng/mL)	31	36	21	37	28	24
Urine reactivity (%) of KIMS100 Calibrator	95	64	162	31	113	189
Urine Creatinine (mg/mL)	0.27	0.19	1.43	0.15	0.58	5.51

are similar to the non-hydroxylated KIMS calibrator, nordiazepam. The glucuronides of the 3-hydroxyclonazepam and aminoclonazepam metabolites likely do not contribute significantly to the KIMS total BZ immunoreactivity, based on the poor reactivity of KIMS with the glucuronide metabolites of lorazepam and temazepam (Table 1).

It is possible that other, non-clonazepam-related, BZ parent drugs and/or metabolites may also be contributors to the EMIT II+ and/or Triage anomalous results in samples 29, 53, 63, 83, and 100, despite the efforts at ruling out use of other BZs, as described in the 'Sample inclusion criteria' in the Materials and Methods section. If LC-PDA failed to detect a minute amount of the parent drug lorazepam (Ativan®) in serum, it would be likely that some amount of lorazepam-glucuronide would be present in the urine. KIMS is very insensitive to lorazepam-glucuronide, whereas Triage is very sensitive to it, with EMIT II+ somewhat less so. Hence while KIMS would likely not generate significantly more immunoreactivity from lorazepam-glucuronide being present in the urine, Triage (and to a lesser extent EMIT II+) would. All the five anomalous results except for # 100 can be explained by invoking low level, undetected lorazepam use by the serum HPLC "gold standard" assay.

CONCLUSION

We conclude that the KIMS Benzodiazepines plus urine immunoassay, using a 100 ng/mL cutoff, can be used to monitor clonazepam use/compliance. It was far superior to the other immunoassays tested here at detecting clonazepam use, rivaling the superior compliance estimate made recently using LC-tandem MS [13]. Coupled with the judicious use of urine creatinine measurements, use of KIMS100 appears to be capable of detecting clonazepam use with > 90% sensitivity, with the caveat that other BZs are not present in the urine.

CONFLICT OF INTEREST STATEMENT

None.

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