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Diagnostic Accuracy Study

Comparison of Clonazepam Compliance by Measurement of Urinary Concentration by Immunoassay and LC-MS/MS in Pain Management Population

Robert West, MS, Amadeo Pesce, PhD, Cameron West, PhD, Bridgit Crews, PhD, Charles Mikel, PhD, Perla Almazan, MT, Murray Rosenthal,DO, and Sergey Latyshev, MS

From: Millennium Laboratories Research Institute, San Diego, CA

Dr. Mike is Chief Scientist at Millennium Laboratories Research Institute, San Diego, CA. Dr. Rosenthall is Chief Medical Officer, Millennium Laboratories Research Institute, San Diego, CA. Ms. Almazan, MT is Associate Director Clinical Operations, Millennium Laboratories Research Institute, San Diego, CA. Dr. West is Co-laboratory Director Millennium Laboratories Research Institute, San Diego, CA. Dr. Pesce is Co-Laboratory Director, Millennium Laboratories Research Institute, San Diego, CA Dr. Crews is the Mass Spectrometry Specialist at Millennium Laboratories Research Institute, San Diego, CA. Latyshev is Mass Spectrometry Specialist at Millennium Laboratories Research Institute, San Diego, CA.

Address correspondence:
Cameron West, Ph.D.
Millennium Laboratories
16981 Via Tazon
San Diego CA 92127
E-mail: cwest@becausepainatters.com

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Background: Physicians determine patient compliance with their medications by use of urine drug testing. It is known that measurement of benzodiazepines is limited by immunoassay specificity and cutoff limits and therefore does not offer physicians an accurate picture of their patients' compliance with these medications. A few studies have used lower cutoffs to demonstrate patient compliance.

Objectives: To define more appropriate cutoffs for compliance monitoring of patients prescribed clonazepam as determined using immunoassay and liquid chromatography-tandem mass spectrometry (LC-MS/MS).

Study Design: A diagnostic accuracy study of the urinary excretion of clonazepam.

Methods: Millennium Laboratories performed measurements on the urinary excretion of pain patients prescribed clonazepam as the indicator test. This benzodiazepine was chosen because it forms one major metabolite, 7-aminoclonazepam which is specific for that drug. Patients whose only benzodiazepine medication was clonazepam were selected as the test population.

The Millennium Laboratories test database was filtered first to select patients on clonazepam, then a second filter was used to eliminate patients with any other listed benzodiazepine medications. Samples were tested using the Microgenics DRI ® benzodiazepine assay with a 200 ng/mL cutoff. The same samples were quantitatively assessed for 7-aminoclonazepam by LC-MS/MS with a cutoff of 40 ng/mL. The results from the immunoassay were scored as positive or negative while the quantitative results from the LC-MS/MS were also scored as positive or negative depending upon their concentration.

Results: Samples from 180 patients met these medication criteria. The positivity rates were 21% (38 samples) by immunoassay. The positivity rate was 70% (126 samples) if the LC-MS/MS cutoff was set at 200 ng/mL. However, the positivity rate was 87% (157 samples) if the LC-MS/MS was set at 40 ng/mL. Concentration distributions revealed a significant fraction (7%) in the 40 – 100 ng/mL range.

Limitations: A limitation of the study was the inability to measure lower than 40 ng/mL. There may be another fraction of the population that was positive below the cutoff value.

Conclusions: The difference in positivity rate between the immunoassay and the LC-MS/MS result showed that the nominal 200 ng/mL cutoff of the immunoassay did not apply to 7-aminoclonazepam. This low immunoassay positivity rate is inconsistent with the manufacturer's published cross reactivity data for clonazepam and 7-aminoclonazepam. These data illustrate the limitations of using a 200 ng/mL cutoff to monitor clonazepam compliance and suggest that a cutoff of 40 ng/mL or less is needed to reliably monitor use of this drug.

Key words: Clonazepam, 7-aminoclonazepam, pain management, LC-MS/MS, immunoassay, patient compliance

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Pain management patients are often prescribed benzodiazepines as well as opiates as part of their regimen of pain management medication (1-6). The drug clonazepam (Klonopin®) is one of the most frequently prescribed Schedule IV medications in the United States and is used for the treatment of anxiety and epilepsy (7). Potential problems associated with improper use or abuse of this drug include physical and psychological dependence, suicidal thoughts or actions, worsening of depression, sleep disorders, and aggression (8,9). As with many benzodiazepines, Klonopin can have drug interactions and can cause a number of unwanted physical symptoms upon withdrawal such as faintness, dizziness, sweating, tremors, etc. (10,11).

Patients are tested to ensure compliance with this group of drugs. Failure to observe the presence of the prescribed benzodiazepine can lead to patient dismissal with dire consequences for their pain control. Therefore the analyst is challenged to provide accurate information regarding compliance with benzodiazepine medications.

Fraser (12) has pointed out that there has been an evolution in the prescription use of benzodiazepines in that "the cutoff values for benzodiazepines were established many years ago when most benzodiazepines were prescribed in doses of 5 to 20 mg/d". As a consequence, lower concentrations of this class of drugs should be found on urine drug screening. Therefore, the current 200 ng/mL cutoff is most likely too high to establish patient compliance for benzodiazepines. Few studies have been conducted to establish the appropriate cutoff level for benzodiazepines (13-16).

Clonazepam is structurally in the benzodiazepine class and its metabolism results in the products 7-aminoclonazepam and 7-acetamidoclonazepam. Less than 0.5 percent of the drug is eliminated as the parent or unchanged drug (17). The cross-reactivity of the 7-aminoclonazepam varies considerably depending upon the immunoassay vendor. Many vendors do not list the cross-reactivity of the 7-aminoclonazepam; rather, they only describe the parent drug (17). Several of those show that 7-aminoclonazepam has a cross-reactivity of less than 2 percent in their "benzodiazepine immunoassay." With this in mind, we chose to measure the excreted concentrations of 7-aminoclonazepam from patients prescribed clonazepam. To define the cutoff, we compared our immunoassay results with those from Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) performed as previously described (18).

Methods

Participants

Participants were patients being treated for pain who were given urine drug testing to monitor compliance as part of their standard treatment protocol. No patients were harmed in this study. This human research was approved by the Aspire Independent Review Board 9320 Fuerte Dr. Suite 105, La Mesa, CA, 91941.

Test Methods

This cohort was tested using the Microgenics DRI® benzodiazepine assay with a 200 ng/mL cutoff on Olympus AU640 and AU5400 analyzers (19). The manufacturer lists the concentration at which the assay will be positive for 7-aminoclonazepam and clonazepam at 200 and 250 ng/mL respectively. No mention is made of the glucuronidated forms. Quantitative analysis of 7-aminoclonazepam was performed on the same samples by LC-MS/MS with a cutoff of 40 ng/mL using the Agilent model 6410 triple quad in MRM mode. Samples were prepared for MS analysis using a simple "dilute and shoot" technique that incorporates glucuronidase hydrolysis. Creatinine was measured for each sample to insure that there was no adulteration.

The LC-MS/MS procedures were performed on Agilent 6410 instruments (Agilent Corporation, 5301 Stevens Creek Blvd, Santa Clara CA 95051, USA). The method was that described by Moore, Coulter and Crompton as modified by Millennium Laboratories (18,20,21).

An Agilent 1200 series binary pump SL LC system, well plate sampler, thermostatted column compartment paired with an Agilent triple Quadrupole mass spectrometer and Agilent Mass Hunter software were used for analysis of 7-aminoclonazepam (18). Chromatographic separation was performed using an acetonitrile, formic acid, water gradient running at 0.4 mL/min and a 2.1 x 50 mm, 2.7µm Ascentis Express C18 column (Supelco, Bellefonte, PA). Mobile phase A = 0.1% formic acid in water, B = 0.1% formic acid in acetonitrile, and column temperature was set to 50° C. Samples were prepared for injection by incubating 25 µL of urine with 50 units of glucuronidase b-Glucuronidase Type L-II from Patella vulgata (keyhole limpet) Sigma Product number G 8132 (Sigma-Aldrich Corp 3050 Spruce Street Saint Louis, MO 63103) in 50µL 0.4M pH 4.5 acetate buffer for 3 hours at 65° C. The samples were then diluted with 100 µL of acetonitrile and filtered using Millipore MultiScreen Solvinert filter plates. Five µL of this material was injected into the LC-MS/MS.

All spectra were collected using positive electrospray ionization. The optimized instrumental parameters were as follows: gas temperature, 350° C; drying gas, 12 L/min; nebulizer gas (nitrogen), 35 psi (~ 24,100 Pa); capillary voltage, 3000 V; fragmentor voltage, 60V. Multiple reaction monitoring (MRM) mode was used for quantitation.

In MRM mode 2 transitions were used to identify and quantitate a single compound. A quantitative transition was used to calculate concentration based on the quantifier ion and a qualitative transition was used to ensure accurate identification of the target compound based on the ratio of the qualifier ion to the quantifier ion. The following quantitative transitions were used: 7-aminoclonazepam: m/z 286 → 222 with fragmentation voltage set to 120 volts, 7-aminoclonazepam-D4: m/z 290 → 226 with fragmentation voltage set at 120 volts. The following qualitative transitions were used: 7-aminoclonazepam: m/z 286 → 121 with collision energy set to 25 volts. Dwell times were set to 50 msec.

A 4-point calibration curve was created by using the following concentrations of 7-aminoclonazepam: 40 ng/mL, 100 ng/mL, 3200 ng/mL, and 6400 ng/mL. The calibration curve was established as a linear fit of the four points and forcing the curve to pass through the origin. The accepted accuracy for calibrators was ± 20% of the target value and the coefficient of determination (R2) of the linear fit was required to be greater than or equal to 0.95 as verification of linearity and goodness-of-fit. The imprecision (CV) as determined by the quality control specimens set at 80 and 900 ng/mL were 13% and 11%.

HPLC grade H2O, acetonitrile, methanol, and formic acid were obtained from VWR (Westchester, PA). 7-aminoclonazepam and 7-aminoclonazepam D4 were obtained from Cerriliant Corp (Round Rock, Texas). The deuterated internal standard was diluted to 1,000 ng per mL by adding it to synthetic urine (Microgenics corp., Fremont CA).

The LC-MS/MS limit of quantitation for 7-aminoclonazepam was set from the calibration curve at 40 ng/mL. The linear range of values for 7-aminoclonazepam was from 40 to 100,000 ng/mL.

The assay was tested for interferences against a library of 120 representative drugs and metabolites encountered in abuse, therapeutic treatment, and commonly used over the counter drugs (Table 1). Certified samples of these drugs were obtained from Cerrilliant Corp. and diluted in synthetic urine to the concentrations listed in Table 2. The potentially interfering drugs were treated, chromatographed, and analyzed using the parent and daughter ion settings for 7-aminoclonazepam. The potentially interfering drugs were first tested to determine if they could be detected in the assay.

The listed drug levels produced no false positives or chromatographic interferences in the form of peak broadening, shoulders, or q-value distortion for any of our current LC-MS/MS confirmatory assays at the listed concentrations. This confirmed the specificity of the analytical procedure and the mass ion settings and Q-values used to validate the presence of the 7-aminoclonazepam.

False negative results were assessed using solutions spiked with the confirmatory analyte at the lower limit of quantitation (LLOQ) and the questionable interferant at 50,000 ng/mL (Table 2). The reported quantitative values were correctly measured at the LLOQ within 20%.

Statistical Methods

A retrospective analysis of the Millennium Laboratories test database was conducted by members of Millennium Laboratories Research Institute over a 2-month period to compare the ability of immunoassay and LC/MS/MS to detect clonazepam in a pain patient population. This allowed an evaluation of immunoassay responses representative of clonazepam and its metabolites that were not influenced by other benzodiazepines.

The test data were filtered to include patients with prescriptions for clonazepam and exclude samples containing oxazepam, nordiazepam, temazepam, alpha-hydroxyalprazolam, and lorazepam.

One-hundred and eighty sequential urine specimens from 180 pain patients that listed clonazepam as a prescribed drug were selected for analysis of 7-aminoclonazepam. All patients were in treatment for chronic pain. No other exclusion criteria were used.

Results

Figure 1 illustrates specimen selection flow chart.

We found the assay to be linear over the calibration range used for this study. All of the 50 or more assays used to gather the data presented in this work were from calibration curves with R2 values greater than 0.95. Virtually all the curves were found to have R2 values of 0.99. Matrix effects were found to be within the acceptable range with ion suppression less than 50% based on the ion response of the internal stan-

Table 1. *Drug levels tested for interference.*

Drug	ng/mL	Drug	ng/mL
6-monoacetylmorphine	1,280	ketamine	500
11-nor-9-carboxy THC	512	lamotrigene	5,000
6-acetyl morphine	5	lidocaine	1,000
7-amino clonazepam	125	lorazepam	5,120
7-amino flunitrazepam	125	loxapine	250
acetaminophen	25,000	maprotiline	1,250
alpha-OH-alprazolam	2,560	MCPP	250
Alprazolam	250	MDA	100
amantadine	250	MDMA	12,800
amitiptyline	500	meclizine	600
amoxapine	600	meperidine	500
amphetamine	12,800	meprobamate	6,400
Antipyrine	1000	mesoridazine	500
atomoxetine	500	methadone	6,400
benzocaine	500	methamphetamine	12,800
benzoylecgonine	3,200	methylphenidate	50
bropheniramine	50	metoclopramide	500
bupivacaine	500	midazolam	125
buprenorphine	1,280	midazolam	100
Bupropion	500	mirtazepine	250
Bupropion metabolite	1,000	Morphine	6,400
Butalbital	5,000	Morphine	50
carbamazepine	5,000	Morphine-3-glucuronide	250
carisoprodol	6,400	norbuprenorphine	1,280
chlordiazepoxide	125	nordiazepam	5,120
chlorpheniramine	500	norfentanyl	1,024
chlorpomazine	500	norfluoxetine	500
Citalopram	250	normeperidine	500
clomipramine	125	norpropoxyphene	12,800
clonazepam	500	nortriptyline	500
Clonidine	250	norverapamil	500

Drug	ng/mL	Drug	ng/mL
Clozapine	500	o-desmethylvenlafaxine	500
cocaethylene	100	olanzapine	600
Cocaine	100	oxezepam	5,120
Codeine	6,400	oxycodone	6,400
cyclobenzaprine	100	oxymorphone	6,400
desalkyflurazepam	500	paroxetine	500
desipramine	500	pentazocine	500
Desmethyl doxepin	500	phencyclidine	640
desmethylcitalopram	250	phenobarbital	5,000
desmethylclomipramine	500	phentermine	500
desmethyldoxepin	600	phenytoin	5000
desmethylsertraline	250	promethazine	500
dextromethorphan	500	propoxyphene	12,800
Diazepam	500	pseudoephedrine	1,250
Diltiazem	500	quetiapine	250
diphenhydramine	500	sertailine	500
Doxepin	500	strychnine	500
doxylamine	500	temazepam	6,400
Duloxetine	500	thioridazine	500
ecgonine methyl esther	500	topiramate	5,000
EDDP	12,800	tramadol	3,200
Fentanyl	256	trazodone	1,000
flunitrazepam	125	triazolam	125
Fluoxetine	500	trimethobenzamide	500
Flurazepam	125	trimethoprim	500
hydrocodone	6,400	varapamil	500
hydromorphone	6,400	venlafaxine	500
Ibuprofen	25,000	zolpidem	250
imipramine	500	zopiclone	125

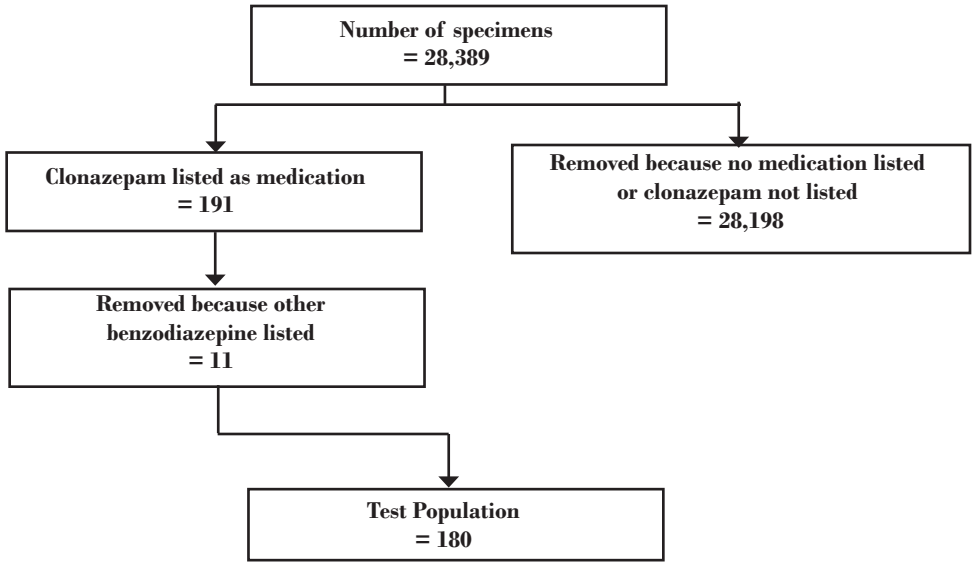

Fig. 1. *Specimen selection flow chart.*

Table 2. *Drugs that did not cause interference.*

Drug	No False Positives Concentration (ng/mL)	No False Negatives Concentration (ng/mL)
Acetaminophen	100,000	50,000
Paraxanthine	100,000	50,000
Naproxen	100,000	50,000
Ibuprofen	100,000	50,000
6-Acetylcodeine	100,000	50,000
Norcodeine	100,000	50,000
dihydrocodeine	100,000	50,000
Normorphine	100,000	50,000
Noroxycodone	100,000	50,000
Phenylpropanolamine	100,000	50,000
MDEA	100,000	50,000
MDA	100,000	50,000
Ephedrine	100,000	50,000
psuedoephedrine	100,000	50,000
phentermine	100,000	50,000

dard of the patient samples versus the calibration and control internal standard ion responses. As described in the methods section above, there were no interferences from common prescription and over the counter drugs.

We determined the 7-aminoclonazepam concentrations in those specimens where the only listed benzodiazepine was clonazepam. Using the LC-MS/MS analytical data as the gold standard, the distribution of the urinary concentrations are presented in Fig. 2. Please note the bin size was changed after the 500 ng/mL mark. It is clear that there is a wide distribution of values from 41 ng/mL to 6,000 ng/mL. The mean, median, and standard deviation were 892 ng/mL, 538 ng/ml, and 1,005 ng/mL respectively.

Of the 180 samples only 21% (38 samples) were positive by immunoassay. By contrast, 70% (126 samples) were positive for the metabolite by LC-MS/MS when the same nominal 200 ng/mL cutoff was used.

The difference between the 21% positivity rate observed by immunoassay and the 70% positivity rate observed by LC-MS/MS indicates that the immunoassay is not finding 7-aminoclonazepam at this nominal 200 ng/mL cutoff. However, the low immunoassay positivity rate relative to that of Mass Spectrometric testing is inconsistent with the manufacturer's published cross reactivity data for clonazepam and 7-aminoclonazepam (14). However, the positivity rate increases to 87% when the LC-MS/MS cutoff is lowered to 40 ng/mL. The difference between the 70% and 87% positivity rates is due to the application of 2 different cutoffs. Further, the concentration distributions plotted in Figure 2 reveal a significant fraction (7%) of the urines had 7-aminoclonazepam in the 40 – 100 ng/mL range.

Discussion

Compliance is a major concern of physicians treating patients for chronic pain (2,4,5). They rely on laboratories to provide accurate data to help them detect diversion as well as non-compliance. This study was limited to documenting compliance with patients prescribed clonazepam; the study did not consider examining patients taking the drug without prescription. Immunoassays are used as the initial screen before confirmation. In this study, we observed that our immunoassay only detected clonazepam use in 21% of the patients who had reported taking clonazepam as part of their treatment regime, so a better method is needed. In contrast, in this study the more accurate and sensitive LC-MS/MS procedure indicated that 87% were compliant, and therefore this is the preferred procedure.

Even with the lower LC-MS/MS cutoff (40 ng/mL) compared to the nominal 200 ng/mL, we observed that 23 of the 180 patients, or 13% were possibly non-

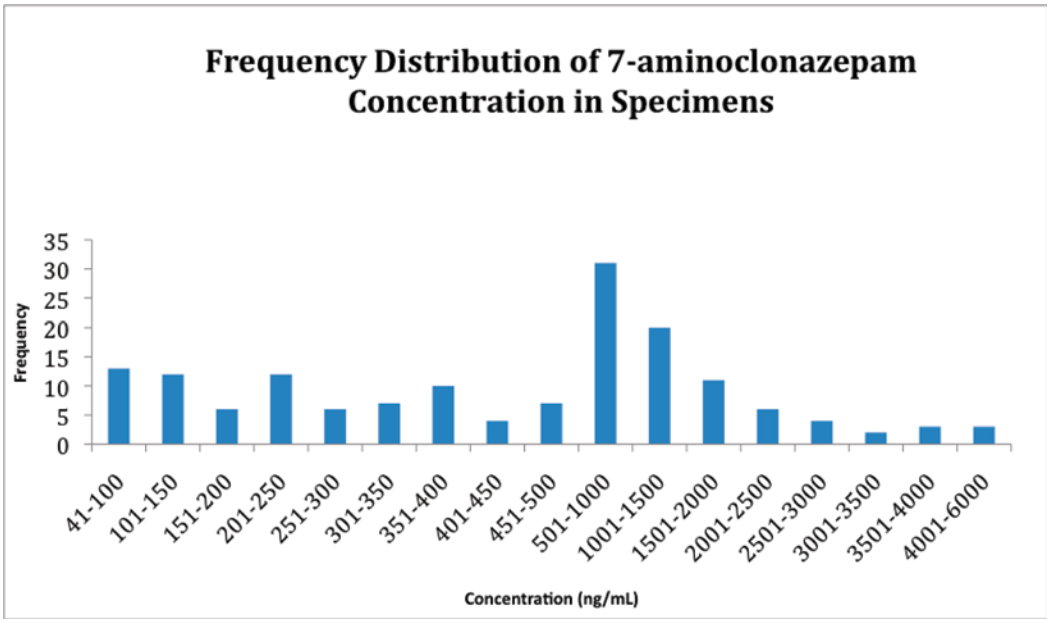

Fig. 2. Frequency distribution of 7-aminoclonazepam concentration in selected specimens. The Y-axis is the number of specimens within the specified concentration range. The X-axis is the bin size for the test specimens. From 0 – 500 ng/mL the bin size is 50 ng/mL. From 500-4000 ng/mL the bin size is 500 ng/mL.

compliant. The question remains as to whether the 40 ng/mL cutoff is most appropriate for this patient population. The median value of the drug was 538 ng/mL and 40 ng/mL represents approximately 1/13th of the median. The half-life of clonazepam is 19 – 60 hrs (12). Therefore, in the usual case, 40 ng/mL represents an approximate time of 4 half-lives or 80-240 hrs after ingestion of the drug. By measuring the presence of the metabolite of clonazepam at 40 ng/mL, this would allow the treating physician to be certain that the patient had taken the drug within the last several days prior to testing. This could be valuable in a number of instances: in cases where the patient is on a very low dose, the lower cutoff helps validate their compliance. Another scenario might be where a normally compliant patient reports having taken the medication on the day of the test, but the results at low cutoff show that they did in fact take the drug but possibly several days earlier. This information might alert the physician to consider that the patient may be experiencing memory loss.

The poor reactivity of some benzodiazepines with immunoassays has been well documented (15,16,22-25). Hydrolysis with beta-glucuronidase has been recommended to improve the sensitivity (14). However, most high-volume laboratories do not use a pre-treatment step, rather, immunoassay analysis on the undiluted urine is performed (26). In addition, it is well known that lowering the detection cutoff results in finding increased numbers of patients positive for test medications as well as illicit drugs (27,28).

Bearing in mind that using a cutoff of 40ng/mL this study only found 13% of the cohort to be "non-compliant," it can be estimated that by using an even lower cutoff even fewer patients would be classified as non-compliant. More studies are needed to decide on the best cutoff.

The limitations of this study include inability to measure lower levels; however, with the available technology this study meets the criteria of diagnostic accuracy studies and also meets the reporting guidelines (29-33).

Conclusions

A number of studies have been published describing patient medication compliance using the traditional methods of immunoassay followed by Mass Spectrometry confirmation. This study raises the question of whether those analyses were flawed because of the

potential for false negative results by both the immunoassay's lack of reactivity with some benzodiazepines as well as the high cutoffs used for these studies. We show that testing by LC-MS/MS at low cutoffs provides an accurate indication of patient compliance when used to detect the presence of 7-aminoclonazepam. Many more patients were compliant. By extension, it can be reasoned that the same techniques and instrumentation may be effective for establishing compliance with prescriptions for other medications used for the treatment of chronic pain.

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