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How to Prevent the Next Wave in Overdose Crisis? - Detect Designer Benzodiazepines Using CEDIA™ and DRI™ Assays

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Abstract

Purpose: This study is to demonstrate the potential of detecting designer benzodiazepines in urines by using DRI™ Benzodiazepine and CEDIA™ Benzodiazepine assays

Methods: Designer benzodiazepines were spiked into drug free urine and cross-reactivity was evaluated using DRI Benzodiazepine and CEDIA Benzodiazepine assays.

Results: 14 out of 17 (82%) tested designer benzodiazepines showed high cross-reactivity (> 100%) in DRI Benzodiazepine assay. 12 out of 17 (71%) tested designer benzodiazepines showed high cross-reactivity (> 100%) in CEDIA Benzodiazepine assay with 200 ng/mL cutoff and 300 ng/mL cutoff.

Introduction

Benzodiazepines are a class of drugs used for relieving symptoms of anxiety, insomnia, agitation, muscle spasms, and alcohol withdrawal. Benzodiazepines are CNS depressants enhancing the effect of GABA neurotransmitter at GABA receptors and they are well-known for drug abuse potential and drug dependence. Designer benzodiazepines are new psychoactive substances emerging in the past two decades. Designer benzodiazepines are often taken by individuals who use hallucinogenic and stimulant drugs. The drug abuse can lead to respiratory depression, coma, or death. There exists a need to develop a new homogeneous enzyme immunoassay.[1]

Existing commercial immunoassay antibodies may cross-react with designer benzodiazepines which have chemical structure closely related to classic therapeutic benzodiazepines. Herein, we evaluated designer benzodiazepines cross-reactivity using DRI Benzodiazepine and CEDIA Benzodiazepine assays and demonstrated the potential of detecting designer benzodiazepines in urines.

Materials and methods

Sample Preparation

Designer benzodiazepines tested in this study are 3-Hydroxyphenazepam, Adinazolam, Bromazolam, Clonazolam, Cloniprazepam, Deschloroetizolam, Diclazepam, Etizolam, Flubromazepam, Flubromazolam, Flunitrazolam, Meclonazepam, Nifoxipam, Nimetazepam, N-Desmethylflunitrazepam,, Nitrazolam, and Pyrazolam. Compounds were spiked into drug free urine.

Test Method(s)

CEDIA Technology is based on the bacterial enzyme β-Galactosidase which has been genetically engineered into two inactive fragments, Enzyme Acceptor (EA) and Enzyme Donor (ED). These fragments spontaneously re-associate to form an active enzyme. In the absence of analyte from the sample, the specific antibody binds the ED-drug conjugate causing a decrease in enzyme activity. The free drug in the sample will compete for the limited number of antibody binding sites, making the ED-drug conjugate available for complementation to form an active enzyme. This phenomenon creates a direct relationship between the drug concentration in urine and enzyme activity. The enzyme activity is then determined spectrophotometrically at 570 nm. The performance of the assay was evaluated on the Beckman Coulter AU680 analyzer. The CEDIA assay used 200 ng/mL and 300 ng/mL cutoff calibrators and controls.

DRI assay is based on competition between drug labeled with glucose-6-phosphate dehydrogenase (G6PDH) and free drug in the urine sample for a fixed amount of antibody binding sites. The enzyme activity is determined spectrophotometrically at 340 nm by measuring its ability to convert NAD to NADH. The performance of the assay was evaluated on the Beckman Coulter AU680 analyzer. The DRI assay used 200 ng/mL cutoff calibrators and controls.

The cross-reactivity of spiked samples was tested by Qualitative mode and Semi-quantitative mode on the Beckman Coulter AU680. The lowest tested concentration that produces a positive result was reported.

Results

The structure of designer benzothiazines tested in this study are shown in the Figure 1. The tested cross-reactivity results are shown in Table 1, 2, and 3.

Figure 1. Structure of Calibrator Drug and Tested Designer Benzodiazepines.

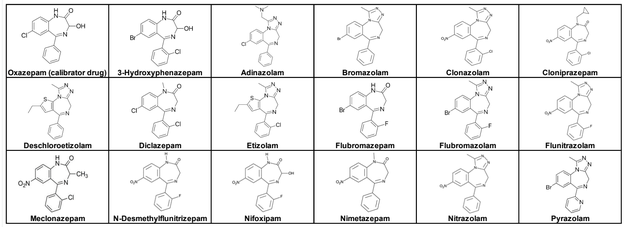

Table 1. Cross-reactivity of Designer Benzodiazepine Tested by DRI Benzodiazepine Assay (200 ng/mL cutoff)

Compounds	Tested Conc. (ng/mL)	Qualitative		Semi-quantitative		% Cross-Reactivity
		Δ Rate to C/O (mA/min)	Pos/Neg	Observed Conc. (ng/mL)	Pos/Neg	
3-Hydroxyphenazepam	400	21	Pos	261	Pos	50%
Adinazolam	120	11	Pos	224	Pos	167%
Bromazolam	120	14	Pos	244	Pos	167%
Clonazolam	145	11	Pos	226.5	Pos	138%
Cloniprazepam	150	19	Pos	251.5	Pos	133%
Deschloroetizolam	115	18	Pos	255.5	Pos	174%
Diclazepam	115	12	Pos	233	Pos	174%
Etizolam	150	16	Pos	241	Pos	133%
Flubromazepam	130	18	Pos	246	Pos	154%
Flubromazolam	115	24	Pos	264.5	Pos	174%
Flunitrazolam	60	10	Pos	229	Pos	333%
Meclonazepam	2,500	18	Pos	255.5	Pos	8%
N-Desmethylflunitrizepam	125	16	Pos	243.5	Pos	160%
Nifoxipam	1,600	12	Pos	236	Pos	13%
Nimetazepam	100	37	Pos	310	Pos	200%
Nitrazolam	100	27	Pos	268	Pos	200%
Pyrazolam	135	15	Pos	242	Pos	148%

Table 2. Cross-reactivity of Designer Benzodiazepine Tested by CEDIA Benzodiazepine Assay (200 ng/mL cutoff)

Compounds	Tested Conc. (ng/mL)	Qualitative		Semi-quantitative		% Cross-Reactivity
		Δ Rate to C/O (mA/min)	Pos/Neg	Observed Conc. (ng/mL)	Pos/Neg	
3-Hydroxyphenazepam	250	14	Pos	290	Pos	80%
Adinazolam	100	24	Pos	270	Pos	200%
Bromazolam	80	11	Pos	256	Pos	250%
Clonazolam	350	14	Pos	276	Pos	57%
Cloniprazepam	160	16	Pos	278.5	Pos	125%
Deschloroetizolam	150	13	Pos	282	Pos	133%
Diclazepam	110	24	Pos	306.5	Pos	182%
Etizolam	125	11	Pos	215	Pos	160%
Flubromazepam	115	11	Pos	220	Pos	174%
Flubromazolam	100	28	Pos	364	Pos	200%
Flunitrazolam	100	17	Pos	218.5	Pos	200%
Meclonazepam	1,000	26	Pos	305	Pos	20%
N-Desmethylflunitrizepam	200	17	Pos	277	Pos	100%
Nifoxipam	1,400	19	Pos	276	Pos	14%
Nimetazepam	150	16	Pos	258	Pos	133%
Nitrazolam	250	11	Pos	211.5	Pos	80%
Pyrazolam	135	12	Pos	232	Pos	148%

Table 3. Cross-reactivity of Designer Benzodiazepines Tested by CEDIA Benzodiazepine Assay (300 ng/mL cutoff)

Compounds	Tested Conc. (ng/mL)	Qualitative		Semi-quantitative		% Cross Reactivity
		Δ Rate to C/O (mA/min)	Pos/Neg	Observed Conc. (ng/mL)	Pos/Neg	
3-Hydroxyphenazepam	350	13	Pos	414.5	Pos	86%
Adinazolam	125	12	Pos	383	Pos	240%
Bromazolam	110	11	Pos	418.5	Pos	273%
Clonazolam	550	11	Pos	425	Pos	55%
Cloniprazepam	175	19	Pos	375	Pos	171%
Deschloroetizolam	250	21	Pos	521	Pos	120%
Diclazepam	125	18	Pos	487.5	Pos	240%
Etizolam	225	30	Pos	585.5	Pos	133%
Flubromazepam	150	24	Pos	519	Pos	200%
Flubromazolam	100	13	Pos	400.5	Pos	300%
Flunitrazolam	150	14	Pos	399	Pos	200%
Meclonazepam	1,500	13	Pos	444.5	Pos	20%
N-Desmethylflunitrizepam	250	14	Pos	441.5	Pos	120%
Nifoxipam	1,800	11	Pos	355.5	Pos	17%
Nimetazepam	250	18	Pos	502.5	Pos	120%
Nitrazolam	450	13	Pos	406	Pos	67%
Pyrazolam	250	10	Pos	384	Pos	120%

Comparison with NPS Benzodiazepine Trend

Both DRI and CEDIA benzodiazepine assays demonstrated good cross-reactivity against most new design benzodiazepines reported in new psychoactive substances (NPS) benzodiazepine trend (Table 4).[2]

Table 4. Comparison of Tested Designer Benzodiazepines and NPS Benzodiazepine Trend Report Identified Designer Benzodiazepines

Tested Designer Benzodiazepines	DRI 200ng/mL cutoff	CEDIA 200ng/mL cutoff	CEDIA 300ng/mL cutoff	NPS Benzodiazepine Trend Report Identified Designer Benzodiazepines											
	% cross-reactivity	% cross-reactivity	% cross-reactivity	2020 Q1	2020 Q2	2020 Q3	2020 Q4	2021 Q1	2021 Q2	2021 Q3	2021 Q4	2022 Q1	2022 Q2	2022 Q3	2022 Q4
3-Hydroxyphenazepam	50%	80%	86%												
Adinazolam	167%	200%	240%		3	17	10	5	3	4					
Bromazolam	167%	250%	273%		2	1	1	3	17	33	39	60	37	23	39
Clonazolam	138%	57%	55%	9	9	1	57	71	79	121	60	33	28	6	6
Cloniprazepam	133%	125%	171%												
Deschloroetizolam	174%	133%	120%			1	9		10	10	9	15	11	10	
Diclazepam	174%	182%	240%	2			1	1	2	1		1			
Etizolam	133%	160%	133%	95	107	180	326	199	248	341	224	267	117	61	16
Flubromazepam	133%	174%	200%		1		4		4	17	29	85	37	17	9
Flubromazolam	174%	200%	300%	18	8	43	71	22	41	71	36	40	16	6	3
Flunitrazolam	333%	200%	200%												
Meclonazepam	8%	20%	20%					3			1	3		3	
N-Desmethylflunitrazepam	160%	100%	120%												
Nifoxipam	13%	14%	17%												
Nimetazepam	200%	133%	120%												
Nitrazolam	200%	80%	67%												
Pyrazolam	148%	148%	120%		3	7		4	1	1		2	1	2	

Conclusions

DRI and CEDIA Benzodiazepine assays showed good cross-reactivity to designer benzodiazepines (> 100%)

- DRI Assay- 14/17 compounds cross-reacted
- CEDIA Assay 200 ng/mL cutoff- 12/17 compounds cross-reacted
- CEDIA Assay 300 ng/mL cutoff- 12/17 compounds cross-reacted

Structural relationship based off cross-reactivity on DRI assay:

- Removal of OH group on seven membered ring then addition of triazolo/methyl groups increases cross-reactivity
- Addition of halogen/NO_2 substituents increases cross-reactivity

Structural relationship based off cross-reactivity on CEDIA assay:

- Not obvious based off 2D structures

The results from NPS benzodiazepines trend report also correlated with the data demonstrating that the DRI and CEDIA Benzodiazepine assays can detect designer benzodiazepines.

References

1. Zawilska JB and Wojcieszak J. “An expanding world of new psychoactive substances–designer benzodiazepines.” *Neurotoxicology* 2019; 73: 8–16.
2. The Center for Forensic Science Research & Education (cfsre) Trends Reports. “NPS Benzodiazepines in the United States.” 2020, 2021, and 2022.

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Note: This was preliminary data that has not been reviewed by any regulatory authorities/cleared by FDA.

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