In Oral Fluid 7-Aminoclonazepam is Superior to Clonazepam for Detection of Clonazepam Use

Stacy E.F. Melanson[1,3], David Griggs[2], Tahira Khaliq[2], and **James G. Flood[2,3]**

[1]Clinical Chemistry Laboratory, Brigham and Women's Hospital, Boston MA USA
[2]Chemistry/Core Laboratory, Massachusetts General Hospital, Boston MA USA
[3]Department of Pathology, Harvard Medical School, Boston MA USA

Clonazepam (CLON) is not only frequently prescribed in addiction management due to its anxiolytic properties but is also commonly abused. Therefore many addiction clinics perform oral fluid (OF) testing, which unlike urine is not subject to adulteration, to monitor CLON compliance. However, CLON and other benzodiazepines can be challenging to detect in OF due to their weakly acidic nature and their presence in low concentrations. We determined the optimal technical and clinical approach for detection of clonazepam in OF. We measured CLON and its primary metabolite 7-aminoclonazepam (7AC) by liquid chromatography-tandem mass spectrometry (LC-MS/MS) in OF samples from outpatient addiction medicine clinics over a 4 month period. The samples were collected using the Orasure Intercept OF sample collection device.

OF sample preparation for LC-Tandem MS is described in Table 1. The Internal Standards used for 7AC and CLON were 7AC-d4 and Oxazepam-d5, respectively. The chromatographic instrumentation used is depicted in Figure 1 and further described in Table 2. A representative chromatogram of a calibrator containing both analytes and ISs is shown in Figure 2. Absolute recovery from 2-200 ng/ml ranged from 65 to 69 % for 7AC and 52-72 % for CLON. Both 7AC

and CLON were linear from 1.0 to at least 200 ng/ml. Daily calibration of batches was accomplished with a blank and a single calibrator containing 10 and 20 ng/ml of CLON abd 7AC, respectively. QC materials at twice and half of each analyte's LOD were also run to validate each calibration. 7AC total imprecision (CV) was 12.4 % at 1.81 ng/ml and 5.7 % at 3.94 ng/ml 7AC. CLON total imprecision (CV) was 31.0 % at 0.83 ng/ml and 19.2 % at 1.76 ng/ml.

102 samples were positive for 7AC and/or CLON. 93 (91.2%) were confirmed using the ion ratio (IRC) to contain 7AC (median, range: 4.15, 0.5-316.7 ng/ml), while only 49 of the 102 (48.0%) samples were confirmed using the ion ratio to contain CLON (median, range: 3.44, 0.1-217.2 ng/ml). Table 3 shows the superior IRC performance of 7AC over CLON as analyte concentration in calibrators decreases. Table 4 shows the % of patient samples passing the IRC test stratified by analyte concentration. IRC confirmation performance deteriorates for CLON and 7AC as the analyte concentration go below about 3 and 1 ng/ml, respectively. No samples were confirmed positive for CLON and negative for 7AC. In oral fluid the levels of 7AC were on average 2.4 fold higher than CLON. 7AC exhibited significantly more carryover than CLON, as shown in Table 5. We conclude that 7AC should be the analyte measured in OF for the detection of recent CLON use because of 7ACs superior precision, LOD, and IRC characteristics.

Table 1. OF Sample Prep for LC-MS-MS

- 200 ul OF sample in Orasure Preservative
- Add 50 ul IS solution, Mix
- Inject 75 ul using PAL Autosampler into TLX2-QQQ MS (Quantum Ultra)

Table 2. OF Chromatography for LC-MS

- Analytes/ISs isolated on TurboFlow P column (0.5x50 mm)
- Transfer analytes/ISs w Loop Contents/Focus Mode to Phenyl analytical column
- Separate analytes using a binary gradient on a Supelcosil Phenyl column at 26 °C (5um particle size, 4.6x150 mm) for elution to the H-ESI source/Tandem MS
- Sample-to-sample injection cycle time is 16 min., reducible to nearly 8 min. by duplexing the TLX2. Yes, that's SLOW!
- Shorter column allows 16 m=>9 m cycle time reduction (pending)

Table 3. IRC Run-to-Run Imprecision

Analyte	Conc. (ng/ml)	Transition (m/z) Q1=>Q3 (Qual, Quant)	Mean (%)	CV (%)	IRC Failures (out of 26)
CLON	1.0	316=>214, 270	27.67	38.4	14
CLON	10.0	316=>214, 270	34.76	10.0	1
7AC	1.0	286=>250, 222	104.90	12.0	2
7AC	10.0	286=>250, 222	101.76	7.4	0

Table 4. IRC Confirmation Summary

Conc. (ng/ml)	CLON		7AC	
	#	% IRC PASS	#	% IRC PASS
< 1.0	42	19.0	20	70.0
1.0-1.9	21	33.3	22	86.4
2.0-2.9	12	66.7	6	100.0
3.0-3.9	5	80.0	6	100.0
4.0-4.9	4	100.0	4	100.0
5.0-9.9	7	100.0	18	100.0
> 9.9	11	100.0	26	100.0
TOTAL	**102**	**48.0**	**102**	**91.2**

Table 5. 7AC/CLON Carryover

Analyte (ng/ml)	Absolute Carryover (ng/ml)	% Carryover	Comments
7AC (20.0)	0.13 0.16	0.65 0.80	8/8/14 7/18/14
7AC (200.0)	0.90 1.50	0.45 0.75	8/8/14 7/18/14
CLON (1000.0)	1.00	0.10	N = 3; 2012

Figure 1. The TLX2 Chromatograph

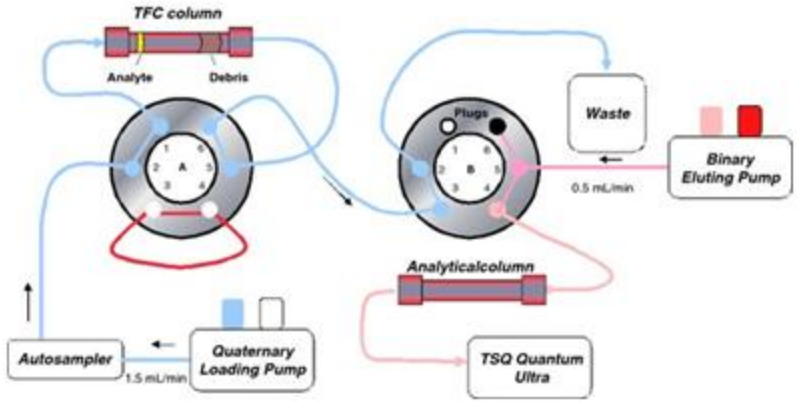

Figure 2. C'grams of Calibrator w ISs