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7-Aminoclonazepam is superior to clonazepam for detection of clonazepam use in oral fluid by LC–MS/MS

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ABSTRACT

Background: Clonazepam (CLON) is not only frequently prescribed in addiction management 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 the detection of CLON use using OF.
Methods: We measured CLON and its primary metabolite 7-aminoclonazepam (7AC) by liquid chromatography–tandem mass spectrometry in OF specimens over a 2 month period. The samples were collected using the Orasure Intercept OF sample collection device.
Results: One hundred samples were presumptive-positive for 7AC and/or CLON. 91 (91.0%) confirmed positive for 7AC (median, range: 4.2, 0.5–316.7 ng/ml) using the ion ratio test, while only 44 of the 100 (44.0%) samples confirmed positive for CLON (median, range: 3.7, 0.5–217.2 ng/ml) using the ion ratio test. In OF the levels of 7AC were approximately 2.4-fold higher than CLON. The use of 7AC as an analyte for the detection of both CLON compliance and undisclosed use is also recommended.
Conclusions: 7AC should be the analyte measured in OF for the detection of CLON use.

1. Introduction

Oral fluid (OF) is gaining popularity as a specimen type for the detection of therapeutic and abused drugs as well as monitoring compliance in the addiction and pain management settings [1–5]. Unlike urine, OF is difficult to adulterate, can be collected in oliguric and anuric patients and has higher concentrations of some parent drugs, particularly amines such as the heroin metabolite 6-acetylmorphine and methamphetamine [3–7]. Drugs also appear earlier in OF as compared to urine which makes OF a better indicator of recent use (within 6 to 8 h of ingestion) [4]. However, OF has some limitations including lower concentrations of a few drug/drug classes and potential for improper sampling technique [3,5,7]. It may also be challenging to obtain a sufficient volume of OF in patients with dry mouth or related conditions [7,8].

Benzodiazepines (BZ) are one class of drugs that can be challenging to detect using OF [7,9–10]. BZ are weakly acidic and are therefore excluded from OF, resulting in low OF concentrations [7]. In addition, BZ are highly protein bound, reducing the free fraction of drug capable of entering the OF [6]. Finally, most BZ possess one or more chlorine atoms, which reduce the signal produced in a unit-resolution mass spectrometer by ≥25%, due to the 75:25 isotopic distribution of Cl^{35} to Cl^{37}. The combination of these properties necessitates that the OF detection limits for BZ be 50 to 100 times lower than urine to achieve similar clinical sensitivity.

Clonazepam (CLON) is a frequently prescribed BZ in the US and has a much higher potency than older BZ like diazepam and chlordiazepoxide [11]. In our outpatient population CLON is detected in serum and oral fluid approximately three times more frequently than any other BZ. CLON is also widely abused [12,13]. Both of these facts emphasize the importance of detecting CLON use. CLON is metabolized by nitro reduction and 3-hydroxylation of the diazepine ring to 7-aminoclonazepam (7AC), an active metabolite, and 3-hydroxyclonazepam, respectively. In blood, concentrations of CLON and 7AC after oral ingestion are both relatively low (approximately 25–125 μg/l) [14]. In urine, 7AC predominates, with <1% of a dose remaining as CLON [14]. Less is known about CLON and 7AC in OF with the exception that BZ concentrations are 50 to 100-fold lower than in urine. Some reports describe BZ testing in OF using liquid chromatography–tandem mass spectrometry (LC–MS/MS), but most of these articles test only for the parent compound CLON [4,9,10,15–17]. A few other reports briefly describe the performance of 7AC and/or CLON in OF as compared to urine, but only as

Abbreviations: CLON, clonazepam; 7AC, 7-aminoclonazepam; OF, oral fluid; BZ, benzodiazepine; LC–MS/MS, liquid chromatography–tandem mass spectrometry; MDMA, 1,3-methylenedioxy-methamphetamine; MS, mass spectrometry; OXAZ, oxazepam; PRN, pro re nata or when necessary.

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minor parts of larger studies focused on the comparison of many drugs in urine versus OF [2,3,17]. To our knowledge, there is no literature comparing the performance of CLON and 7AC in OF for the detection of CLON use.

We perform an increasingly high volume of OF testing for our addiction clinics (approximately 2400 tests per year). Anecdotal within-lab investigations and physician user feedback suggested OF testing for BZ, particularly CLON was producing false-negative results compared to urine testing. In an effort to improve OF testing for the detection of CLON use, we investigated the performance of CLON versus 7AC.

2. Methods

2.1. Patient samples and inclusion criteria

All samples over a 2 month period that had an Oral Fluid Drug Screen Panel ordered by Massachusetts General Hospital physicians as part of their treatment of patients in an addiction-psychiatry/medicine outpatient setting were included in the study. The screen panel included testing for CLON and twenty-five other therapeutic and abused drugs and their metabolites. The Partners Human Research Committee approved this study.

2.2. Sample collection

We used the Orasure Intercept sample collection device (Orasure Technologies) and followed the collection instructions recommended by the manufacturer. Upon receipt in the lab (usually 2–10 h after collection) specimens were frozen at −20 °C and mixed after thawing prior to subsequent testing; which usually started within 24–72 h of specimen collection.

2.3. LC–MS/MS confirmation

All samples were tested for CLON and 7AC by LC–MS/MS using the following summarized protocol.

2.3.1. Standards and reference materials

Stock standards of CLON, Oxazepam (OXAZ)-d5, 7AC, and 7AC-d4 were from Cerilliant Inc. as 1.0 mg/ml solutions in methanol or acetonitrile. Working standard solutions of CLON (at 0.0 and 10.0 ng/ml) and 7AC (at 0.0 and 20.0) ng/ml were prepared by appropriate dilution with deionized water. Working controls were prepared to contain 0.5, 1.0, 2.0, and 50.0 ng/ml of CLON, and 1.0, 2.0, 4.0, and 100.0 ng/ml of 7AC.

2.3.2. Calibration and quality control

Each batch of patient samples was calibrated using the 0.0, 10.0 and 20.0 ng/ml working standards of CLON and 7AC diluted 1 ≥ 3 with negative calibrator (Orasure). This 1 ≥ 3 dilution compensates for the Orasure swab's projected collection of 400 μl of neat OF from a patient, and subsequent dilution by 800 μl preservative fluid in the Orasure swab's storage container. Therefore, all OF sample concentrations reported in this study are the neat, undiluted-by-preservative oral fluid concentration. Quality control materials were run to validate each calibration.

2.3.3. Sample preparation and chromatography

Two hundred microliters of each diluted standard, diluted quality control material, and patient sample was added to a 96-well plate and mixed with 50 μl of a working internal standard solution containing 80 ng/ml OXAZ-d5 and 7AC-d4. 75 μl of each mixture was injected directly into a TLX2 chromatograph (Thermo Scientific) fitted with a 50 × 0.5 mm Cyclone-P turbulent-flow extraction column (ThermoFisher) and an Ascentis Phenyl (150 × 4.6 mm, 5 μm ave. particle diameter) analytical column (Supelco). The analytes were first deposited on the extraction column using a 0.005% formic acid in water mobile phase. The extracted analytes were then transferred to the analytical column with 200 μl of an eluent containing 80:20 (by volume) 0.005% formic acid in water:acetonitrile. The analytes were then separated using a gradient elution program. Starting conditions were 99% A (0.005% formic acid in water) and 1% B (a solution of 30% isopropanol and 70% 0.04% formic acid in acetonitrile). At 1.37 min the gradient ramped to 78% A and 22% B, ramping again at 2.03, 2.70, 3.37, and 4.03 min to 72:28, 68:32, 62:38, and 40:60 A%:B%, respectively. At 13.00 min the gradient returned to the initial analytical column conditions, 99:1% A:B. The cycle time from injection to injection was 16.0 min. Mass spectrometer (MS) data was collected during the 3.50–12.50 min interval post-injection. Samples above the linear range were diluted 1:1 with deionized water and re-tested.

2.3.4. Mass spectrometric detection

The detector was a Thermo Quantum Ultra (Thermo Scientific) triple quadrupole MS equipped with a heated electrospray interface (HESI-II) operated in the positive ion mode. Settings included a vaporizer temperature of 450 °C, spray voltage of +3000 V, an Argon collision gas pressure of 1.5 mTorr, a Q1 resolution of 0.2 FWHM, *m/z* and a Q3 resolution of 0.7 FWHM, *m/z*. Analyte-specific MS settings are listed in Table 1. CLON and 7AC quantitative results were calculated by ratioing the observed area of the quantifier ion of a patient's sample to that of the internal standard, and then calculating the result from the two-point standard curve.

2.4. Assay performance

Within-day linearity studies were performed per EP-6A [18] in triplicate using 1, 2, 20, and 200 ng/ml aqueous solutions of CLON and 7AC. Within-run and total imprecision studies were performed per EP-5A [19]. Each control material was tested twice in a daily run, for twenty or more consecutive daily runs. Carryover was assessed by running a high sample with 1000 or 200 ng/ml of CLON and/or 7AC prior to a blank sample processed in triplicate.

The percent absolute recovery for CLON and 7AC was assessed at different concentrations ranging from 1–200 ng/ml, in triplicate in a single run [20]. Each solution was processed in two ways, producing a test and a reference sample. The test solution of each pair was diluted exactly as the batch's calibrators and controls i.e., they were first diluted 1 ≥ 3 with negative calibrator. The reference solution of each pair was diluted 1 ≥ 3 with water instead of a negative calibrator. Subsequently both the test and reference pair were processed alike as an unknown patient sample (IS addition, etc.) with one important difference: 75 μl of the test solution was injected as normal into the TLX2 chromatograph, whereas 75 μl of the reference solution of the pair was injected directly onto the TLX2's analytical column, thereby bypassing the Turbo Flow column completely. The percent absolute recovery was subsequently calculated as: ((analyte area from test solution / analyte area from the reference solution) * 100) [20].

For interference testing we prepared aqueous solutions (ng/ml concentration in parenthesis) of the following compounds containing 10.0 ng/ml of the analytes 7AC and CLON, and processed the solutions exactly as the calibrators and controls, evaluating their effect on the analytes and IS: 6-Monacetylmorphine (20), Alprazolam (10), Amphetamine

Table 1
Analyte-specific mass spectrometer settings.

Analyte	Parent ion (*m/z*)	Quantifier ion (collision energy)	Qualifier ion (collision energy)	Scan time (msec)
CLON	316	270 (25)	214 (38)	20
7AC	286	222 (24)	250 (19)	20
OXAZ-d5	292	246 (23)	274 (14)	20
7AC-d4	290	226 (23)	254 (18)	20