

# Determination of Benzodiazepines in Oral Fluid using LC–MS–MS

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## Abstract

A rapid, simple, highly sensitive procedure for the simultaneous analysis of bromazepam, alprazolam, clonazepam, lorazepam, oxazepam, diazepam, midazolam, flurazepam, flunitrazepam, nordiazepam, triazolam, temazepam, nitrazepam, and chlordiazepoxide in oral fluid is described, using liquid chromatography coupled to a triple quadrupole mass spectrometer in positive electrospray mode. Benzodiazepines in oral fluid samples (1 mL) were analyzed using the Quantisal™ collection device, quantified using solid-phase extraction, and detected using liquid chromatography with tandem mass spectrometric detection. For confirmation, two transitions were monitored and one ion ratio determined, which was within 20% of the ratio for known calibration standards. The limits of quantitation ranged from 0.5–5ng/mL of neat oral fluid; the intraday precision of the assays ( $n = 5$ ) ranged from 2.8–7.29%; and the interday precision ranged from 1.42–6.8% ( $n = 5$ ). The percentage recovery of the drugs from the collection pads ranged from a low of 81.4% for midazolam to the highest of 90.17% for nitrazepam. The procedure can be applied to authentic oral fluid specimens.

## Introduction

Benzodiazepines are the most commonly prescribed class of drugs in the USA for the treatment of anxiety and insomnia, particularly in the elderly (1). They are also used as muscle-relaxants and anti-convulsants. They are often detected in incidents of driving under the influence of drugs (DUID), and in combination with other medications (2,3). Oral fluid is becoming increasingly used as a specimen in many areas of forensic and clinical interest, including collection at the roadside during traffic stops. Its ease of collection, difficulty of adulteration, and applicability to routine testing have promoted its use as a valid test specimen. However, the detection of benzodiazepines is not without difficulty, particularly in oral fluid, because the saliva–plasma ratio for most of the drug class is low.

One of the main issues with the quantitation of drugs in oral fluid is the difficulty of collection in terms of specimen volume. Many of the currently available devices do not give an indication of how much oral fluid is collected, thereby rendering any quantitative results questionable without further manipulation in the laboratory (4,5). Furthermore, devices incorporating a pad or material for the saliva collection do not always indicate how much of each drug is recovered from the pad before analysis, again calling into question any quantitative result. The drug concentration reported is dependent on the collection procedure used (6).

This work employed the Quantisal™ oral fluid collection device, which collects a known amount of neat oral fluid. The efficiency of recovery of the benzodiazepines from the collection pad into the transportation buffer was determined, in order to increase confidence in the quantitative value.

Several publications have addressed the issue of the analysis of benzodiazepines in oral fluid. Quintela et al. (7) determined 9 benzodiazepines in neat oral fluid using a liquid chromatographic method with mass spectrometric detection (LC–MS). They included lormetazepam and tetrazepam, which were not in our profile; however, clonazepam, chlordiazepoxide, nordiazepam, temazepam, oxazepam, flurazepam, and nitrazepam were not included.

A recent publication from Oiestad et al. reported the screening of oral fluid using tandem LC–MS for several drugs, including benzodiazepines (8). They analyzed fenazepam and some benzodiazepine metabolites, which we did not include (see below), but they did not include the commonly prescribed drugs triazolam, temazepam, midazolam, flurazepam, or chlordiazepoxide. Smink et al. (9) analyzed urine and oral fluid for 33 benzodiazepines using liquid chromatography with tandem mass spectrometric detection (LC–MS–MS). With the exception of diazepam, where a limit of quantitation (LOQ) of 0 ng/mL was reported, the lower limit of quantitation for the other analytes was significantly higher than in our application.

In our research, we did not include the metabolites such as 7-aminoflunitrazepam, 7-aminoclonazepam, 7-aminonitrazepam,  $\alpha$ -hydroxy alprazolam,  $\alpha$ -hydroxytriazolam, or desalkylflurazepam because the parent drug is found more often in higher concentration than metabolites in oral fluid. We did,

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however, include metabolites such as nordiazepam, temazepam, oxazepam, and lorazepam (a metabolite of lormetazepam), because they can be prescribed as individual drugs.

## Methods and Materials

### Oral fluid collection devices

Quantisal devices for the collection of oral fluid specimens were obtained from Immunalysis Corporation (Pomona, CA). The devices contain a collection pad with a volume adequacy indicator, which turns blue when 1 mL of oral fluid ( $\pm 10\%$ ) has been collected. The pad is then placed into a transport buffer (3 mL), allowing a total specimen volume available for the analysis of 4 mL (3 mL buffer + 1 mL oral fluid). This is specifically advantageous in cases where the specimen is positive for more than one drug and the volume of specimen available for analysis may be an issue. The oral fluid concentration is diluted 1:3 when using Quantisal collection devices, and detected drug concentrations were adjusted accordingly.

### Standards and reagents

Deuterated internal standards: D5-diazepam, D5-temazepam, D5-alprazolam, D5-oxazepam, and D4-clonazepam, as well as unlabelled drug standards: bromazepam; clonazepam; nitrazepam; triazolam; alprazolam; flunitrazepam; flurazepam; lorazepam; midazolam; chlordiazepoxide; diazepam; oxazepam; nordiazepam; temazepam were purchased from Cerilliant (Round Rock, TX). Mixed mode solid-phase extraction columns (CSDAU020) were purchased from United Chemical Technologies (Bristol, PA). All solvents were of HPLC grade or better; all reagents were ACS grade and purchased from Spectrum Chemical (Gardena, CA).

### Calibrators and controls

Calibration standards and controls were prepared from synthetic oral fluid, which comprised 25mM phosphate buffered saline (pH 7.0), 30mM sodium bicarbonate, 0.1% albumin, amylase, and 0.1% Proclin 300 as a preservative. The calibrators and controls prepared at concentrations of 0.5, 1, 5, 10, 20, and 40 ng/mL were then diluted by four with Quantisal transportation buffer. Throughout the development of the assay, multiple Quantisal collection devices were selected from different lots. The drug concentration used to fortify the synthetic oral fluid was adjusted according to the dilution factor for all calibration standards and controls. In this way, the final result obtained from the instrument did not need to be recalculated for dilution factors. For each analysis, a six-point calibration curve (0.5, 1, 5, 10, 20, and 40 ng/mL of neat oral fluid; actually equivalent to 0.125, 0.25, 1.25, 2.5, 5, and 10 ng being measured) was run with each batch; the internal standard concentration was 40 ng/mL. The corresponding deuterated internal standard was used for the quantitation of clonazepam, oxazepam, diazepam, and temazepam; for all other benzodiazepines, d5-alprazolam was used as the internal standard.

### Extraction procedure

In order to improve sensitivity and minimize matrix or ion suppression effects, the specimens were extracted prior to analysis. Sodium phosphate buffer (0.1M, pH 6.0, 1 mL) was added to the Quantisal buffer (1mL) and the samples were mixed. Extraction tubes were placed onto the vacuum manifold and conditioned: with methanol (3 mL), deionized water (3 mL), and 0.1M phosphate buffer (pH 6.0, 2 mL). The column bed was not allowed to dry. Each sample was poured through the column and allowed to dry, then rinsed with deionized water (3 mL), 0.1M phosphate buffer pH 6.0: acetonitrile (80:20; 2 mL) and allowed to dry (10 min; 30 psi). Hexane was allowed to flow through the column (1 mL), and the columns were again dried. Finally the drugs were eluted in ethyl acetate + 2% ammonium hydroxide (2 mL). The eluates were evaporated to dryness under nitrogen (20 psi /37 C) and reconstituted in ethyl acetate (50 mL) for analysis.

### Analytical procedure

An Agilent Technologies 6410 LC coupled to a triple quadrupole mass spectrometer (MS-MS) operating in positive electrospray mode (ESI) was used for the analysis. The LC pump was an Agilent 1200 Series and the column was a Zorbax Eclipse XDB C18 (4.6  $\times$  50 mm  $\times$  1.8  $\mu$ m). The column temperature was held at 35°C; the initial flowrate was 0.2 mL/min,

**Table I. Benzodiazepine Multiple Reaction Monitoring (MRM) Acquisition Parameters**

Compound	Start time (min)	Precursor ion	Fragment ion	Fragment voltage	CE (V)*
<b>Segment 1</b>					
Bromazepam	0	316	288 (209)	160	20 (30)*
<b>Segment 2</b>					
D4-Clonazepam	4.1	320	274	120	25
Clonazepam	4.1	316	270 (214)	120	25 (35)
Lorazepam	4.1	321	275 (229)	140	25 (35)
Nitrazepam	4.1	282	236 (180)	160	25 (35)
D5-alprazolam	4.1	314	286	160	25
Alprazolam	4.1	309	281 (274)	160	25 (30)
Chlordiazepoxide	4.1	300	283 (227)	120	15 (30)
D5-Oxazepam	4.1	292	246	120	20
Oxazepam	4.1	287	241 (269)	120	20 (20)
Triazolam	4.1	343	308 (239)	120	35 (35)
<b>Segment 3</b>					
Flunitrazepam	5.4	314	268 (239)	160	30 (35)
Midazolam	5.4	326	291 (249)	200	30 (40)
D5-Temazepam	5.4	306	260	120	25
Temazepam	5.4	301	255 (177)	120	35 (40)
Nordiazepam	5.4	271	140 (165)	160	30 (30)
<b>Segment 4</b>					
D5-Diazepam	7.2	290	262	160	25
Diazepam	7.2	285	257 (222)	160	25 (25)
Flurazepam	7.2	388	315 (288)	160	25 (25)

\* Values in parentheses indicate qualifying transition

and the injection volume was 5  $\mu$ L. Solvent A consisted of 20mM ammonium formate; solvent B was acetonitrile. The mobile phase composition remained at 50:50 (v,v) although the flowrate changed. The flowrate was held at 0.2 mL/min for 6.5 min, then increased to 1 mL/min. At 8 minutes, the flowrate was decreased back to 0.2 mL. The total run time was 10 minutes, with a 4.5 min post-run allowing mobile phase equilibrium.

The mass spectrometer operated at a capillary voltage of 4500V; an initial nebulizer pressure of 15 psi, which was later raised to 50 psi for improved signal; nitrogen gas flow of 6 L/min and a gas temperature of 300°C. The mass spectrometer was operated in multiple reaction monitoring (MRM) mode. The precursor and product ions, along with optimized fragment voltages and collision energy, are shown in Table I.

**Table II. Slope, Linearity, and Limit of Quantitation for the Assay**

Analyte	Equation	Correlation ( $r^2$ )	Limit of Quantitation (ng/mL)
Alprazolam	$Y = 0.0298x + 0.0114$	0.9995	0.5
Bromazepam	$Y = 0.0096x - 0.0129$	0.9909	1
Chlordiazepoxide	$Y = 0.0146x - 0.0032$	0.9998	1
Clonazepam	$Y = 0.0278x - 0.0108$	0.9991	1
Diazepam	$Y = 0.0305x - 0.0004$	0.9996	1
Flunitrazepam	$Y = 0.007x - 0.0002$	0.9999	0.5
Flurazepam	$Y = 0.2984x - 0.0024$	0.9993	0.5
Lorazepam	$Y = 0.0189x - 0.008$	0.9986	5
Midazolam	$Y = 0.0156x - 0.0143$	0.9960	0.5
Nitrazepam	$Y = 0.0551x + 0.018$	0.9987	1
Nordiazepam	$Y = 0.011x - 0.0013$	0.9999	0.5
Oxazepam	$Y = 0.0228x - 0.0065$	0.9996	5
Temazepam	$Y = 0.0149x - 0.0034$	0.9998	0.5
Triazolam	$Y = 0.0225x + 0.0073$	0.9995	0.5

**Table III. Interday and Intraday Precision of the Assay**

Drug	Inter-Day Precision (%) ( $n = 5$ )	Intra-Day Precision (%) ( $n = 5$ )
Alprazolam	2.03	2.8
Bromazepam	6.8	6.13
Chlordiazepoxide	2.26	6.71
Clonazepam	3.14	4.25
Diazepam	6.04	7.29
Flunitrazepam	5.11	4.64
Flurazepam	5.01	6.95
Lorazepam	3.68	3.64
Midazolam	5.94	3.29
Nitrazepam	1.42	4.41
Nordiazepam	3.27	3.71
Oxazepam	3.07	4.2
Temazepam	3	3.85
Triazolam	2.55	4.96

## Method validation

### Linearity, sensitivity, and precision

Calibration of the assay was calculated using linear regression analysis, forced through the origin, over a concentration range of 0.5–40 ng/mL for the benzodiazepines. Five deuterated internal standards were used in order to minimize the number of transitions being monitored per segment, and thereby improve the sensitivity of the assay. The limit of quantitation (LOQ) for each drug was established by entering a signal-to-noise ratio (SNR) of 10 into the method analysis for the quantitative transition. If the response of the primary transition ion did not exceed an SNR of 10, it was not considered to be quantitative. The precision of the assay, both inter- and intraday, was determined at the concentration of 10 ng/mL of neat oral fluid: equivalent to 2.5 ng/mL drug diluted in buffer ( $n = 5$ ).

### Drug recovery from the collection pad

Extraction efficiency of the collection system for benzodiazepines was determined. Oral fluid was fortified with all the drugs at the concentration of 10 ng/mL ( $n = 6$ ). A collection pad was placed into the fluid until the volume adequacy indicator turned blue, showing that 1 mL (+/-10%) of oral fluid had been absorbed. The pads were placed into the Quantisal buffer (3 mL), capped, and allowed to remain at room temperature overnight, to simulate transportation to the laboratory. The following day, the pads were removed and an aliquot (1 mL) of the specimens was analyzed according to the described procedures.

## Results and Discussion

The analytical method was validated according to standard protocols, whereby the limit of quantitation, linearity range,

**Table IV. Percentage Recovery of Benzodiazepines from Oral Fluid Collection System Following Overnight Incubation at Room Temperature (fortified at 10 ng/mL;  $n = 6$ )**

Drug	Mean recovery (%)	CV (%)
Alprazolam	86.76	8.85
Bromazepam	88.42	14.01
Chlordiazepoxide	89.41	6.33
Clonazepam	88.1	2.97
Diazepam	82.82	4.42
Flunitrazepam	85.1	4.46
Flurazepam	81.57	2.85
Lorazepam	83.44	2.52
Midazolam	81.48	5.32
Nitrazepam	90.17	3.64
Nordiazepam	83.28	3.8
Oxazepam	84.65	2.82
Temazepam	84.19	2.96
Triazolam	85.45	8.71

correlation, and intra- and interday precision were determined. The validation results are presented in Tables II and III.

Table II details the equation of the slope of the calibration curves, which were not forced through the origin, the non-weighted correlation coefficient, and the limit of quantitation associated with each drug.

The precision of the assays was excellent, with both within-day and between-day variations being below 7.3% for all drugs (Table III). The concentration detectable by a combination of solid-phase extraction and extremely sensitive tandem mass spectrometric detection is necessary for the determination of these drugs in oral fluid.

The variables associated with the collection of oral fluid are not often considered. Some commercially available collection devices are unable to determine how much oral fluid has been collected for analysis, thereby calling into question any quantitative result. For any device incorporating a collection pad, the recovery of drugs from that pad is an important component of assay sensitivity, and must be included in any experimental

protocol. We have reported the efficient recovery of THC and its metabolite THCA from the Quantisal device (10,11), as well as the pain medications meperidine, tramadol, oxycodone, and propoxyphene (12,13); others have published the efficacy of the Quantisal device for various drugs, including oxazepam (14).

Placing the pad in the Quantisal buffer overnight, and analyzing the samples the following day, allowed the recovery of benzodiazepines from the collection pad to be determined. In this way, losses occurring during overnight transportation to a laboratory were assessed. Six replicate analyses were analyzed to assess reproducibility of the extraction efficiency. The extraction recovery of all 14 benzodiazepines from the pad was over 81% (Table IV).

The described procedure allowed the rapid determination of 14 benzodiazepines in oral fluid at an extremely low concentration, as is required for these drugs. The concentration of benzodiazepines present in oral fluid is very low due to the saliva-plasma ratio and the small dosage forms available. Additionally, the benzodiazepine class is wide, and an assay to incorporate all the possible members of the group is difficult.

In their publication, Quintela et al. (7) detected a concentration of 0.57 ng/mL of midazolam one hour after administration to a patient undergoing midazolam treatment; Smink et al. (9) reported only five positives for benzodiazepines in their comparison with urine specimens. In their study, 5 oral fluid samples were found to be positive: two for oxazepam (concentrations of 18 and 1659 ng/mL) and three for alprazolam (concentrations of 5, 6, and 9 ng/mL). All these concentrations were above the limits described in our method. There is only one description of 7-aminoflunitrazepam being present in slightly higher concentrations in oral fluid than its parent drug, flunitrazepam, which is reportedly unstable; the data was based on a single ingestion of flunitrazepam (15), and the concentration of parent drug detected (0.6–4 ng/mL) would have been identified using the described assay.

From a forensic point of view, the chromatography afforded by the small particle analytical column allowed separation of the peaks in each of the four group segments. Segments allow specific transitions to be monitored over a given time period in order to improve the sensitivity of the assay. The primary transitions associated with the assay are shown in Figure 1 at a concentration of 5 ng/mL, which was the highest limit of quantitation for two of the drugs, lorazepam and oxazepam. The software is able to monitor a secondary transition from the precursor ion and automatically calculate the ratio to the primary ion. If the ratio is not within 20% of a calibration standard, determined at 10 ng/mL, the identification is rejected. This

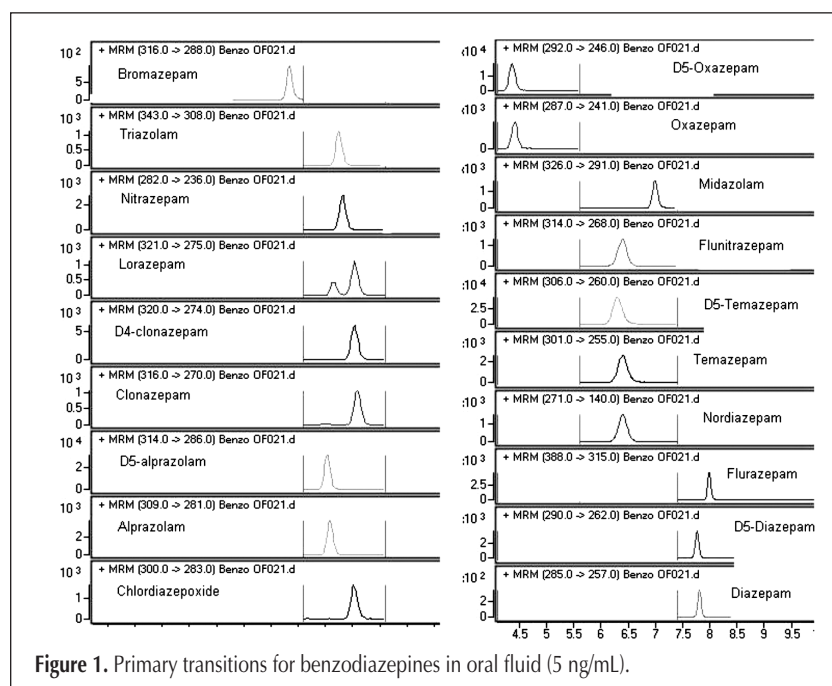


Figure 1. Primary transitions for benzodiazepines in oral fluid (5 ng/mL).

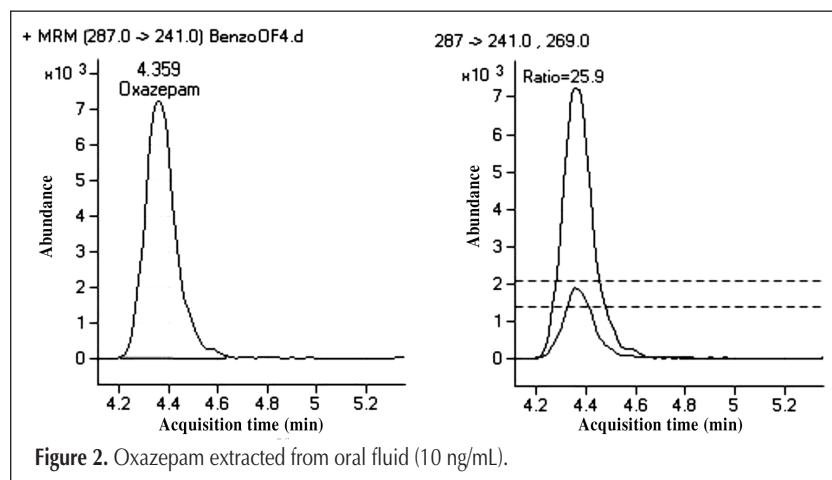
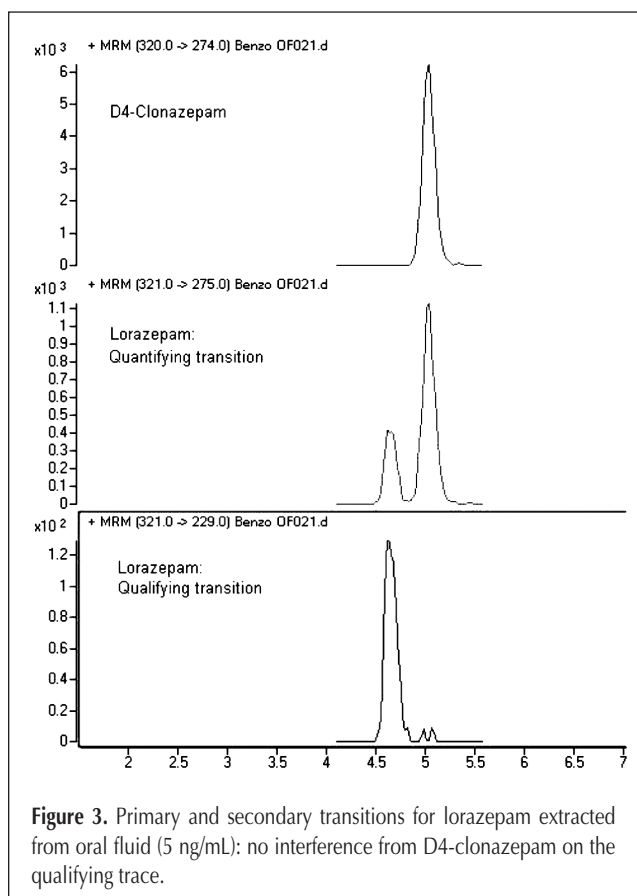


Figure 2. Oxazepam extracted from oral fluid (10 ng/mL).



**Figure 3.** Primary and secondary transitions for lorazepam extracted from oral fluid (5 ng/mL): no interference from D4-clonazepam on the qualifying trace.

is important in forensic analysis where court challenges to laboratory data are frequent. Monitoring a second transition gives additional confidence in the result; applying a ratio to that second transition compared to the primary product ion is a further enhancement to the identification of drugs in oral fluid. The software plots the ratio in the chromatographic window, so the operator is able to visually assess positivity (Figure 2).

The utility of this is can be specifically noted in the MRM for lorazepam. In order to improve the sensitivity of the assay, the resolution for the first quadrupole is set to "Wide", allowing a significant range around the selected ion to be monitored. Unit resolution in the third quadrupole generally eliminates all but the selected transition, however in this case, the transition from  $m/z$  321 to  $m/z$  275 allowed two ions to be detected. The lorazepam is separated from the D4-clonazepam on the basis of retention time, but also on the presence of the secondary qualifying transition:  $m/z$  321 to  $m/z$  229 (Figure 3). There is no corresponding qualifying transition from  $m/z$  321 to  $m/z$  229 in the MRM trace of D4-clonazepam.

## Summary

The procedure described is suitable for the detection of benzodiazepines in oral fluid using a triple quadrupole LC-MS-MS system. The sensitivity of the assay is a significant improvement over that found in other publications. This is the first

method that includes qualifying ions for the identification of benzodiazepines at low concentration in oral fluid, and is currently in routine use in our laboratory.

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