

## INTRODUCTION

Urine samples are frequently analyzed by toxicology laboratories to identify the presence of benzodiazepine metabolites. Benzodiazepine compounds are used to sedate patients, help them to sleep, prevent seizures, ease anxiety and relax muscle spasms. Laboratories that run analyses to determine exact benzodiazepine concentrations in urine use a LC-MS/MS technique with a runtime duration of minutes.

We developed a method using Extraction Tips (DPX-Wax) for a fast cleanup followed by a Liquid-Liquid Extraction (LLE) and quantification using LDTD-MS/MS system for the simultaneous determination of 12 benzodiazepine compounds in urine in less than 10 seconds. The method is validated and compared to LC-MS/MS values.

### LDTD™ Ionization Source:

The LDTD uses a Laser Diode to produce and control heat on the sample support (Figure 1) which is a 96 wells plate. The energy is then transferred through the sample holder to the dry sample which vaporizes prior to being carried by a gas in a corona discharge region. High efficiency protonation and strong resistance to ionic suppression characterize this type of ionization, and is the result of the absence of solvent and mobile phase. This allows for very high throughput capabilities of 9 seconds sample-to-sample analysis time, without carry over.

## METHOD

A solution made of 125 µL of urine and 50 µL of buffered internal standard was hydrolyzed with a 25 µL β-glucuronidase enzyme solution (2 hours/60°C). The hydrolyzed samples are cleaned with an Extraction Tip (DPX-WAX). 250 µL of MeOH:Water (1:1) was added to hydrolysis sample and loaded in the extraction tip. After the washing step, benzodiazepines were eluted with 500 µL Acetonitrile. This rapid cleaning is intended to cover a wide range of benzodiazepines. Finally, LLE with 1-chlorobutane was used to improve the cleaning. 4 µL of the extract is transferred onto an EDTA pre-coated LazWell plate and allowed to dry.

### Instrumentation

- LDTD model S-960 & AB Sciex API5500 Qtrap

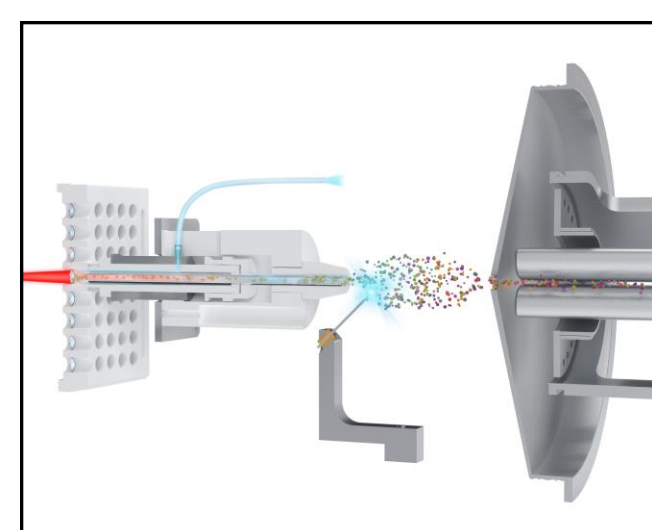
### LDTD Parameters

- Laser power pattern :
  - Increase laser power to 65 % in 3.0 s
  - Decrease laser power to 0 %
- Carrier gas flow : 3 L/min (Air)

### MS Parameters

- APCI (+) & MRM mode
- DP 100 & Discharge current 3 µA
- Dwell time 5 msec

Compound	Q1	Q3	CE
Nordiazepam	271	140	32
7-Aminoflunitrazepam	284	236	36
Diazepam	285	154	32
7-Aminoclonazepam	286	222	30
Oxazepam	287	241	32
Estazolam	295	205	48
Temazepam	301	255	25
Alprazolam	311	274	40
Lorazepam	321	275	23
α-OH-alprazolam	325	205	54
2-OH-Ethylflurazepam	333	211	46
α-OH-Midazolam	342	203	35
α-OH-Triazolam	359	331	36
Nordiazepam-d <sub>5</sub>	276	140	32
7-Aminoclonazepam-d <sub>5</sub>	290	226	30
Diazepam-d <sub>5</sub>	290	198	36
7-Aminoflunitrazepam-d <sub>7</sub>	291	243	36
Oxazepam-d <sub>5</sub>	292	246	32
Estazolam-d <sub>5</sub>	300	210	48
Temazepam-d <sub>5</sub>	306	260	25
Alprazolam-d <sub>5</sub>	316	279	40
Lorazepam-d <sub>5</sub>	325	198	40
α-OH-Alprazolam-d <sub>5</sub>	330	210	54
α-OH-Midazolam-d <sub>4</sub>	346	203	35
α-OH-Triazolam-d <sub>5</sub>	363	335	36

**Table 1** MS parameters for each compound optimized

**Figure 1** Schematic of the LDTD ionization source

- The recovery percentages (Figure 2) are over 40% for all compounds and most are over 55%.
- None of 6 matrices tested seem to cause interference with the analysis of the 12 benzodiazepines (Table 2).
- No interference is caused by other drugs present in the matrix during the analysis of 12 benzodiazepines (Table 3; Drug Inter). The drugs added are caffeine, ibuprofen, warfarin, nicotine and acetaminophen.
- Freeze & Thaw Stability (Table 3; F/T) demonstrate that after 4 cycles, no significant degradation is noticed.
- The Bench Test Stability (Table 3; BT) revealed that compounds are stable 24 hours at room temperature.
- The extracts (Table 3; Wet stab) are stable 3 days at 4°C.
- Spotted onto Lazwell, the Dry Stability is 3 days (Table 3; Dry stab).
- Long Term Stability at -20°C (Table 3; LT) is established at 52 days.
- The linearity (Table 4) of almost all compounds is over 0.99
- For the Intra-Run and Inter-Run (Table 5 & Table 6) all Medium QCs show an accuracy between 90% to 115% and a precision below 15%. Same results were obtained for low QCs and High QCs (not showed on the poster).

Compound	M1	M2	M3	M4	M5	M6	
Oxazepam Conc.: 367 ng/mL N: 3	Mean 423.5	409.6	389.2	422.0	411.6	418.6	
Nordiazepam Conc.: 387 ng/mL N: 3	Mean 375.7	394.7	352.0	391.2	383.1	357.5	
Temazepam Conc.: 194 ng/mL N: 3	Mean 191.3	194.4	184.8	199.5	194.8	188.8	
Lorazepam Conc.: 348 ng/mL N: 3	Mean 337.2	327.5	388.3	311.1	347.4	360.2	
α-hydroxyalprazolam Conc.: 290 ng/mL N: 3	Mean 287.1	282.0	337.5	259.2	358.0	323.4	
α-hydroxytriazolam Conc.: 73 ng/mL N: 3	Mean 72.5	73.2	70.5	77.4	74.4	70.1	
7-Aminoclonazepam Conc.: 69 ng/mL N: 3	Mean 62.9	72.0	84.1	73.0	N.Av	80.2	
Estazolam Conc.: 109 ng/mL N: 3	Mean 103.1	113.9	114.3	123.8	108.5	102.3	
Diazepam Conc.: 87 ng/mL N: 3	Mean 77.8	73.0	81.0	77.2	62.6	79.5	
2-Hydroxyethylflurazepam Conc.: 436 ng/mL N: 3	Mean 407.4	413.2	449.4	429.2	346.8	411.8	
α-hydroxymidazolam Conc.: 93 ng/mL N: 3	Mean 93.2	91.7	85.2	86.4	90.3	95.8	
Alprazolam Conc.: 372 ng/mL N: 3	Mean 389.1	368.0	363.1	428.8	397.5	398.4	
	%RSD	7.1	5.2	5.0	6.1	3.9	8.7
	%Nom	104.6	99.0	97.6	115.3	106.9	107.1

**Table 2** Matrix interference

Compound	Conc. (ng/mL)	N	Mean (ng/mL)	%RSD (%)	%Nom (%)
Oxazepam	114	4	112.4	9.3	98.9
Nordiazepam	103	4	111.0	2.5	107.8
Temazepam	97	4	93.9	2.5	96.9
Lorazepam	100	4	112.8	11.7	112.8
α-hydroxyalprazolam	110	4	112.6	13.0	102.1
α-hydroxytriazolam	103	4	106.8	3.4	103.8
7-Aminoclonazepam	56	4	64.1	5.2	114.0
Estazolam	99	4	102.2	9.9	102.8
Diazepam	91	4	86.7	5.0	95.7
2-Hydroxyethylflurazepam	90	4	91.3	9.2	101.2
α-hydroxymidazolam	103	4	108.3	4.6	105.1
Alprazolam	103	4	106.0	9.4	102.5

**Table 5** Intra-run test

## RESULTS

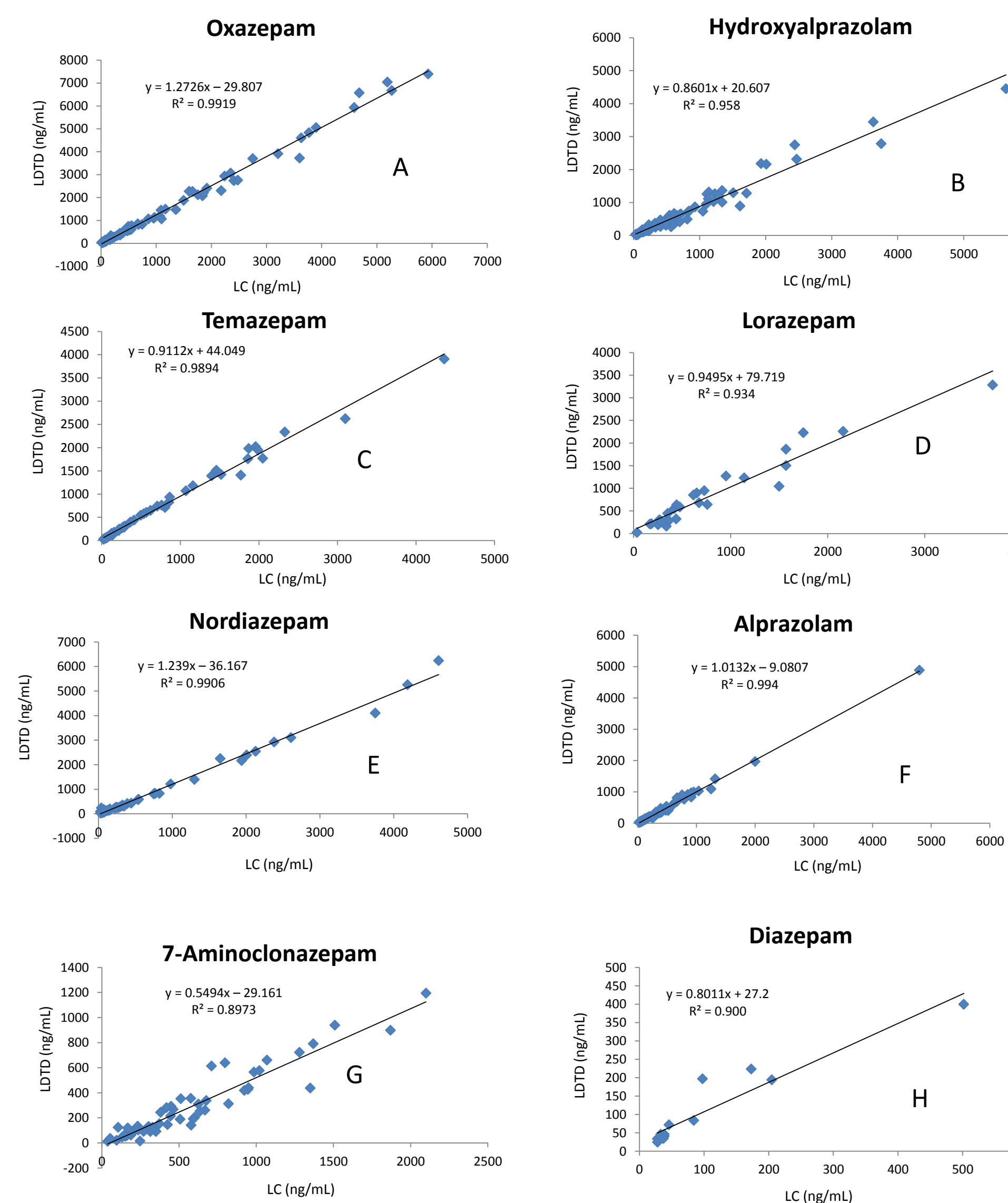
Compound	Drug Int.	F/T	BT	Wet stab	Dry stab	LT	
Oxazepam Conc.: 367 ng/mL N: 3	Mean 370.0	354.3	385.2	382.0	387.4	376.7	
Nordiazepam Conc.: 387 ng/mL N: 3	Mean 356.9	347.4	353.8	366.5	368.7	333.0	
Temazepam Conc.: 194 ng/mL N: 3	Mean 184.4	168.0	177.2	181.6	184.0	176.6	
Lorazepam Conc.: 348 ng/mL N: 3	Mean 386.2	365.8	383.3	394.7	350.1	352.6	
α-hydroxyalprazolam Conc.: 290 ng/mL N: 3	Mean 253.0	287.1	285.1	317.7	275.3	296.9	
α-hydroxytriazolam Conc.: 73 ng/mL N: 3	Mean 72.0	66.7	69.7	70.6	70.0	66.8	
7-Aminoclonazepam Conc.: 69 ng/mL N: 3	Mean 65.7	64.1	72.1	59.4	55.2	66.5	
Estazolam Conc.: 109 ng/mL N: 3	Mean 108.0	97.7	103.7	94.2	110.6	99.5	
Diazepam Conc.: 87 ng/mL N: 3	Mean 74.8	70.9	78.4	76.2	73.9	73.4	
2-Hydroxyethylflurazepam Conc.: 299 ng/mL N: 3	Mean 294.0	282.4	299.0	314.0	333.5	327.3	
α-hydroxymidazolam Conc.: 93 ng/mL N: 3	Mean 87.8	84.5	89.7	88.3	84.2	86.6	
Alprazolam Conc.: 372 ng/mL N: 3	Mean 342.6	312.7	345.4	360.4	345.6	315.0	
	%RSD	0.4	8.0	1.6	7.6	11.5	4.8
	%Nom	92.1	84.1	92.9	96.9	92.9	84.7

**Table 3** Stability test and drug interference

Compound	Conc. (ng/mL)	N	Mean (ng/mL)	%RSD (%)	%Nom (%)
Oxazepam	114	12	112.0	7.8	98.5
Nordiazepam	103	12	101.3	9.6	98.4
Temazepam	97	12	94.2	3.9	97.3
Lorazepam	100	12	91.4	14.5	91.4
α-hydroxyalprazolam	110	12	106.6	13.6	96.7
α-hydroxytriazolam	103	12	99.8	8.9	97.0
7-Aminoclonazepam	56	12	57.0	12.4	101.4
Estazolam	99	12	98.3	6.7	98.9
Diazepam	91	12	89.3	5.0	98.6
2-Hydroxyethylflurazepam	90	12	94.3	7.9	104.5
α-hydroxymidazolam	103	12	102.1	6.8	99.1
Alprazolam	103	12	102.4	10.7	99.1

**Table 6** Inter-run test

The cross validation between LDTD-MS/MS & LC-MS/MS was done with 200 patient urine samples (Figures 3 A-H). A good concordance correlation coefficient was obtained for each benzodiazepine.


**Figures 3 A-H** Cross validation LC-MS/MS & LDTD-MS/MS

## CONCLUSIONS

- Assessment of the method passed all requirements per FDA Guidance (September 2013, revision 1) for industry in bioanalysis.
- Direct cross-validation with LC-MS/MS on real samples, the standard gold method, confirms the validity of using LDTD-MS/MS for the quantitation of benzodiazepines in urine samples.