Method Validation of 12 Benzodiazepines by LDTD-MS/MS Analysis in 6 seconds per sample with correlative data from LC-MS/MS for 200 real patient urine samples

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INTRODUCTION

Overview:

Benzodiazepine metabolites in urine are drugs frequently analyzed in Pain management and Toxicology laboratories. High-throughput is a definite requirement due to the quantity of samples involved in routine analysis. Minimal analysis time is achieved by using a sample introduction shotgun approach with no chromatographic separation. The 12 benzodiazepines include molecules with similar chemical structures and molecular weight that require specific MS/MS transitions to allow selectivity and differentiation between one another. Assessment of the method passed through all requirements from FDA Guidance (September 2013, revision 1) for industry in bioanalysis. Direct cross validation with LC-MS/MS, the standard gold method, on real samples confirms the validity of using LDTD-MS/MS for quantitation of benzodiazepines in urine samples.

LDTD Ionization Source:

The LDTD uses a Laser Diode to produce and control heat on the sample support which is a 96 well plate**.** The energy is then transferred through the sample holder to the dry sample which vaporizes prior to being carried by a gas in an APCI region. High efficiency protonation with strong resistance to ionic suppression characterize the ionization due to the absence of solvent and mobile phase. This allows very high throughput capabilities of 6 seconds sample-to-sample analysis time, without carry over.

METHOD

Sample preparation:

Sample hydrolysis

125 µl of urine
50 µl of Buffer solution Sodium acetate 1M (pH 4.5) containing IS
25 µl of β-glucoronidase
(Incubate for 2 hours at 60 °C)
Add 250 µl MeOH-Water (1:1 volume)

Solid phase extraction (SPE)

SPE WAX tips Load hydrolyzed samples by aspirating and dispensing 2 times
Wash with 500 µl Water
Wash with 500 µl Water-MeOH (75-25%)
Elute with 500 µl of Acetonitrile

Liquid-liquid extraction

250 µl of elution solution
250 µl of Sodium carbonate 0.5M (pH 9)
500 µl of Chlorobutane
Transfer 4 µl in Lazwell plate and dry prior analysis

Lazwell plate coating

96-wells plates for analysis are pre-coated with 8 µl of an EDTA solution (100 µg/ml in MeOH/H_2O/NH_4OH (75/20/5%)) and dried before sample deposition.

Instruments setting:

Mass spectrometer AB Sciex 5500 QTrap operated in APCI positive mode.

MRM transitions used with DP=100, 3 µA discharge current and 5 msec dwell time

LDTD model S-960 operated with a gas flow rate of 3 L/min and a laser pattern ramp from 0 to 65% in 3 seconds, maintaining this power level for 2 seconds before dropping it back to 0.

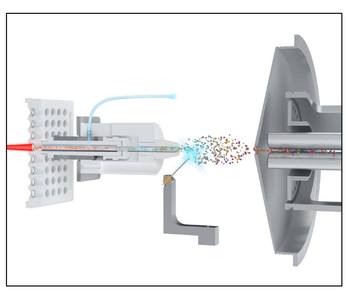

Figure 1 Schematic of the LDTD ionization source.

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
Alpha-Hydroxyalprazolam	325	205	54
2-Hydroxyethylflurazepam	333	211	46
Alpha-Hydroxymidazolam	342	203	35
Alpha-Hydroxytriazolam	359	331	36
D5-Nordiazepam	276	140	32
D4-7-Aminoclonazepam	290	226	30
D5-Diazepam	290	198	36
D7-7-Aminoflunitrazepam	291	243	36
D5-Oxazepam	292	246	32
D5-Estazolam	300	210	48
D5-Temazepam	306	260	25
D5-Alprazolam	316	279	40
D4-Lorazepam	325	198	40
D5-Alpha-OH-alprazolam	330	210	54
D4-Alpha-OH-midazolam	346	203	35
D4-Alpha-Hydroxytriazolam	363	335	36

Table 1 MS/MS transitions.

RESULTS

Intra-Run reproducibility and accuracy

Reproducibility and accuracy intra-run result at QC medium level are reported in **Table 2**.

Compound	Conc.	N	Mean	%RSD	%Nom
	(ng/ml)		(ng/ml)	(%)	(%)
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
Alph-OH-alprazolam	110	4	112,6	13,0	102,1
Alpha-OH-triazolam	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-OH-ethylflurazepam	90	4	91,3	9,2	101,2
Alpha-OH-midazolam	103	4	108,3	4,6	105,1
Alprazolam	103	4	106,0	9,4	102,5

Table 2 Intra-run reproducibility and accuracy.

Inter-Run reproducibility and accuracy

Reproducibility and accuracy inter-run result at QC medium level are reported in **Table 3**.

Compound	Conc.	N	Mean	%RSD	%Nom
	(ng/ml)		(ng/ml)	(%)	(%)
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
Alph-OH-alprazolam	110	12	106,6	13,6	96,7
Alpha-OH-triazolam	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-OH-ethylflurazepam	90	12	94,3	7,9	104,5
Alpha-OH-midazolam	103	12	102,1	6,8	99,1
Alprazolam	103	12	102,4	10,7	99,1

Table 3 Inter-run reproducibility and accuracy.

Cross validation LDTD vs LC

Statistics on cross validation with data run on LC-MS/MS method show that both methods agree, with concordance correlation coefficient of 0.99 (95% confidence interval 0.982 – 0.991) and Person p ≥ 0.99. The passing-Bablok regression revealed no significant deviation from linearity (Cusum test, P=0.11). **Figure 2** illustrates the comparative results for the most detected compounds in the urine samples. Comparative study also demonstrates the ability to confirm negative level. 10 of the 12 molecules agree at 100% for negative at the LLOQ level and the 3 remaining ones agree at 98.1%

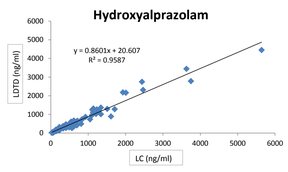

Figure 2a LC-MS/MS and LDTD-MS/MS correlation of OH-Alprazolam

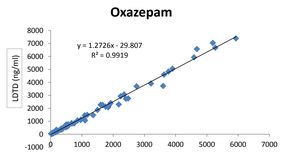

Figure 2b LC-MS/MS and LDTD-MS/MS correlation of Oxazepam

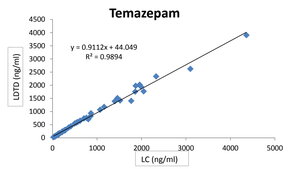

Figure 2c LC-MS/MS and LDTD-MS/MS correlation of Temazepam

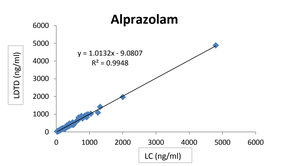

Figure 2d LC-MS/MS and LDTD-MS/MS correlation of Alprazolam

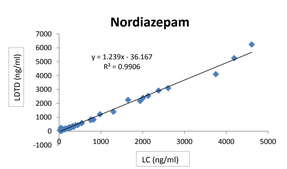

Figure 2e LC-MS/MS and LDTD-MS/MS correlation of Nordiazepam

Matrix selectivity and Matrix effect evaluation

The % of interferences at lower standard for six different matrices was evaluated as measurement of selectivity. Interference less than 20% are obtained for each matrix. Similarly, matrix effects were evaluated by spiking QC in six different negative urines. Results are reported in **Table 5**.

Compound		M1	M2	M3	M4	M5	M6
Oxazepam	Mean	423,5	409,6	389,2	422,0	411,6	418,6
Conc.: 367 ng/ml	%RSD	6,3	2,7	7,6	4,3	8,6	1,3
N: 3	%Nom	115,4	111,6	106,1	115,0	112,2	114,1
Nordiazepam	Mean	375,7	394,7	352,0	391,2	383,1	357,5
Conc.: 387 ng/ml	%RSD	1,7	6,2	3,4	4,4	0,7	4,8
N: 3	%Nom	97,1	102,1	91,0	101,2	99,0	92,4
Temazepam	Mean	191,3	194,4	184,8	199,5	194,8	188,8
Conc.: 194 ng/ml	%RSD	1,9	1,3	0,3	1,9	0,7	3,0
N: 3	%Nom	98,9	100,5	95,5	103,1	100,7	97,6
Lorazepam	Mean	337,2	327,5	388,3	311,1	347,4	360,2
Conc.: 348 ng/ml	%RSD	4,9	5,7	9,3	23,0	23,9	14,7
N: 3	%Nom	96,9	94,1	111,6	89,4	99,8	103,5
Alpha-OH-alprazolam	Mean	287,1	282,0	337,5	259,2	358,0	323,4
Conc.: 290 ng/ml	%RSD	7,2	15,2	2,7	25,4	11,9	14,5
N: 3	%Nom	98,8	97,1	116,2	89,3	123,2	111,3
Alpha-OH-triazolam	Mean	72,5	73,2	70,1	77,4	74,5	70,1
Conc.: 73 ng/ml	%RSD	5,2	7,0	3,6	5,9	3,3	4,0
N: 3	%Nom	99,1	100,1	95,8	105,9	101,8	95,9
7-Aminoclonazepam	Mean	62,9	72,0	84,1	73,0	N.Av	80,2
Conc.: 69 ng/ml	%RSD	9,4	17,5	7,1	4,7	N.Av	20,2
N: 3	%Nom	91,4	104,5	122,1	106,1	N.Av	116,5
Estazolam	Mean	103,1	113,9	114,3	123,8	108,5	102,3
Conc.: 109 ng/ml	%RSD	4,7	7,6	6,9	7,7	6,1	4,4
N: 3	%Nom	94,5	104,4	104,8	113,5	99,4	93,7
Diazepam	Mean	77,8	73,0	81,0	77,2	62,6	79,5
Conc.: 87 ng/ml	%RSD	2,4	6,1	6,7	3,1	1,7	1,1
N: 3	%Nom	89,8	84,2	93,5	89,1	72,3	91,8
2-OH-ethylflurazepam	Mean	407,4	413,2	449,4	429,2	346,8	411,8
Conc.: 436 ng/ml	%RSD	3,7	6,4	10,7	14,0	12,2	12,9
N: 3	%Nom	93,4	94,8	103,1	98,4	79,5	94,5
Alpha-OH-midazolam	Mean	93,2	91,7	85,2	86,4	90,3	95,8
Conc.: 93 ng/ml	%RSD	3,6	6,3	5,9	4,2	1,8	3,7
N: 3	%Nom	100,2	98,7	91,7	93,0	97,2	103,1
Alprazolam	Mean	389,1	368,0	363,1	428,8	397,5	398,4
Conc.: 372 ng/ml	%RSD	7,1	5,2	5,0	6,1	3,9	8,7
N: 3	%Nom	104,6	99,0	97,6	115,3	106,9	107,1

Table 5 Matrix effect evaluation.

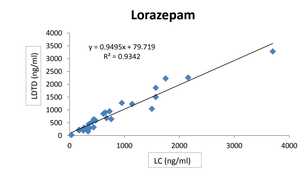

Figure 2f LC-MS/MS and LDTD-MS/MS correlation of Lorazepam

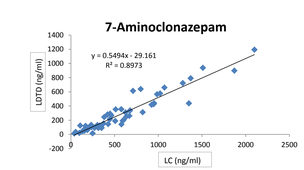

Figure 2g LC-MS/MS and LDTD-MS/MS correlation of 7-Aminoclonazepam

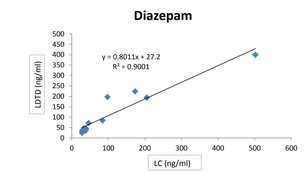

Figure 2h LC-MS/MS and LDTD-MS/MS correlation of Diazepam

Common drug interference and stability

Stability test include freeze and thaw cycle (FT) 4 cycle, bench top (BT) stability solution 24h at room temperature, extraction solution (Wet stability) 72h at 4°C, dry sample (Dry stability) in Lazwell plate 72h room temperature and long term (LT) for 52 days at -20°C. Common drug interferences (Drug Int.) are evaluated by adding Caffeine, Ibuprofen, Warfarin, Nicotine and Acetaminophen to QC. Those results are reported in **Table 4**.

Compound		Drug Int.	F/T	BT	Wet stab	Dry stab	LT
Oxazepam	Mean	370,0	354,3	385,2	382,0	387,4	376,7
Conc.: 367 ng/ml	%RSD	2,8	5,6	1,1	2,1	0,8	1,8
N: 3	%Nom	100,8	96,6	105,0	104,1	105,6	102,7
Nordiazepam	Mean	356,9	347,4	353,8	366,5	368,7	333,0
Conc.: 387 ng/ml	%RSD	4,0	4,0	2,4	2,5	3,8	2,5
N: 3	%Nom	92,3	89,8	91,5	94,8	95,3	86,1
Temazepam	Mean	184,4	168,0	177,2	181,6	184,0	176,6
Conc.: 194 ng/ml	%RSD	3,0	0,7	1,6	2,3	2,3	2,9
N: 3	%Nom	95,3	86,8	91,6	93,9	95,1	91,3
Lorazepam	Mean	386,2	365,8	383,3	394,7	350,1	352,6
Conc.: 348 ng/ml	%RSD	2,3	10,1	13,0	9,2	4,2	9,4
N: 3	%Nom	111,0	105,1	110,2	113,4	100,6	101,3
Alpha-OH-alprazolam	Mean	253,0	287,1	285,1	317,7	275,3	296,9
Conc.: 290 ng/ml	%RSD	5,1	12,3	5,4	14,3	2,4	8,7
N: 3	%Nom	87,3	98,9	98,2	109,4	94,8	102,2
Alpha-OH-triazolam	Mean	72,0	66,7	69,7	70,6	70,0	66,8
Conc.: 73 ng/ml	%RSD	5,2	2,9	3,5	1,9	5,6	2,6
N: 3	%Nom	98,4	91,2	95,2	96,5	95,6	91,3
7-Aminoclonazepam	Mean	65,7	64,1	72,1	59,4	55,2	66,5
Conc.: 69 ng/ml	%RSD	8,5	15,9	14,4	12,3	2,6	11,1
N: 3	%Nom	95,5	93,2	104,7	86,3	80,2	96,5
Estazolam	Mean	108,0	97,7	103,7	94,2	110,6	99,5
Conc.: 109 ng/ml	%RSD	5,9	3,8	3,2	2,1	8,2	3,7
N: 3	%Nom	99,0	89,5	95,0	86,3	101,4	91,2
Diazepam	Mean	74,8	70,9	78,4	76,2	73,9	73,4
Conc.: 87 ng/ml	%RSD	5,2	5,2	4,5	2,6	1,1	6,1
N: 3	%Nom	86,4	81,8	90,5	87,9	85,3	84,7
2-OH-ethylflurazepam	Mean	294,0	282,4	299,0	314,0	333,5	327,3
Conc.: 436 ng/ml	%RSD	2,0	5,8	7,1	2,8	0,6	4,2
N: 3	%Nom	98,3	94,5	100,0	105,0	111,5	109,5
Alpha-OH-midazolam	Mean	87,8	84,5	89,7	88,3	84,2	86,6
Conc.: 93 ng/ml	%RSD	2,7	2,2	2,1	2,5	5,0	5,2
N: 3	%Nom	94,5	90,9	96,5	95,0	90,6	93,2
Alprazolam	Mean	342,6	312,7	345,4	360,4	345,6	315,0
Conc.: 372 ng/ml	%RSD	0,4	8,0	1,6	7,6	11,5	4,8
N: 3	%Nom	92,1	84,1	92,9	96,9	92,9	84,7

Table 4 Potential drug interferences and stability results

Peak desorption

Typical desorption peaks are presented in **Figure 3**.

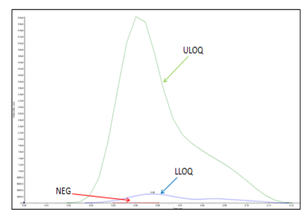

Figure 3 Typical desorption peak

Linearity

Inter-run linearity results are reported in **Table 6**.
Typical standard curve are presented in **Figure 4**.

Compound	Run 1	Run 2	Run 3
	r	r	r
Oxazepam	0,9996	0,9973	0,9951
Nordiazepam	0,9996	0,9978	0,9978
Temazepam	0,9996	0,9955	0,9982
Lorazepam	0,9911	0,9925	0,9913
Alph-OH-alprazolam	0,9909	0,9924	0,9866
Alpha-OH-triazolam	0,9996	0,9977	0,9987
7-Aminoclonazepam	0,9985	0,9964	0,9960
Estazolam	0,9990	0,9961	0,9976
Diazepam	0,9999	0,9995	0,9983
2-OH-ethylflurazepam	0,9997	0,9969	0,9952
Alpha-OH-midazolam	0,9953	0,9948	0,9931
Alprazolam	0,9996	0,9926	0,9959

Table 6 Inter-run linearity result

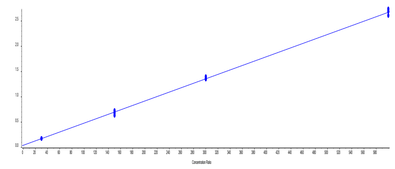

Figure 4 Typical standard curve

Recovery

Recovery quantitation is achieved by spiking the final solution of negative urine at a calculated concentration as benchmark for 100% recovery. Result are presented in **Figure 5**.

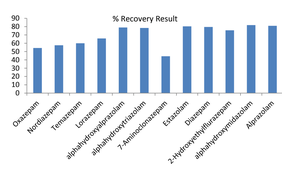

Figure 5 Recovery result

CONCLUSIONS

- **Method Validation of 12 Benzodiazepines analyzed simultaneously by LDTD-MS/MS in 6 seconds per sample was achieved according to the FDA guidelines for industry.**
- **Statistical analysis of the correlative data from LC-MS/MS for 200 real patient urine samples demonstrates the ability of this validated analysis method to generate effective quantitation data at ultra-high-throughput speed.**