Automated Extraction of Benzodiazepines in Urine Using Dispersive Pipette Extraction (DPX) Tips with a Tecan® Robotic System

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Abstract

A dispersive pipette extraction (DPX) Hummingbird tip was designed to be used with a Tecan® Liquid Handling system. The Tecan® system used was equipped with an eight pipetting liquid handler which can process 8 samples simultaneously. Following urine sample hydrolysis, the complete automated extractions took 3 minutes to perform. The extractions were performed hands free with 8 samples extracted at one time! The extractions were found to be reproducible (with C.V.'s less than 8%) and provided high recoveries (greater than 70%) for 7 benzodiazepines. The automated DPX method provided accurate results for over 180 case samples tested (accuracies were greater than 90% for most cases).

Introduction

Analysis of benzodiazepines in urine is commonly performed using enzymatic hydrolysis followed by solid-phase extraction (SPE). SPE methods are often performed manually or though various types of automated SPE platforms . The traditional type of SPE uses large volumes of organic solvents, ammonium hydroxide and chemist time. To reduce the organic waste cost and time consuming bottle neck step, a 3 minute DPX method was developed. The DPX technology uses the same robotic platform that is currently used in many labs and may be utilized for other high throughput applications. Using common liquid handling systems to pipette samples and perform dispersive pipette extraction, the bottle neck seen in traditional SPE methods is eliminated.

In this study, Hummingbird (HB) dispersive pipette extraction (DPX) tips were developed to be used with a Tecan® robot. The HB-DPX tips contain a "disperser" that promotes mixing of the sorbent particles with the sample solution. The "dispersive" property of the HB-DPX tips is unique and permits robotic platforms to reproducibly extract compounds from urine with high efficiency. An application of HB-DPX using the Tecan® robotic system is shown for the analysis of common benzodiazepines (benzos) including diazepam, nordiazepam, oxazepam, temazepam, α-hydroxy-alprazolam, 7-aminoclonazepam, and lorazepam.

Key Words

dispersive pipette extraction, benzodiazepines, LC/MS/MS, DPX, automation

Experimental

Materials

All drug standards were purchased from Cerilliant Corporation (Round Rock, TX, USA). These standards included nordiazepam, lorazepam, temazepam, oxazepam, 7-aminochlonazepam, alpha-hydroxy-alprazolam, diazepam and deuterated analogues of each target compound.

The HB-DPX tips (Hb-T WAX 20 mg) were provided by DPX Labs (Columbia, SC). The DPX tips were made using a new Patent pending technology to ensure "dispersive" extractions occur reproducibly during the automated extractions.

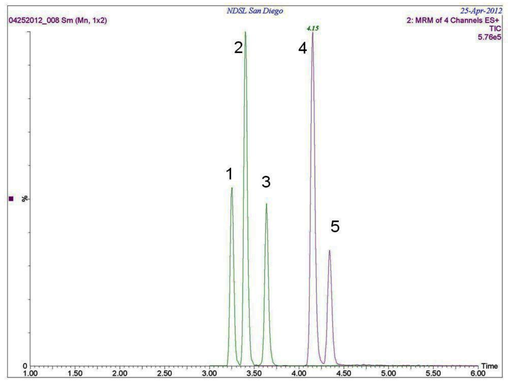


Figure 1. Total ion chromatogram of the 5 common benzodiazepines using LC conditions for optimal separation. The benzodiazepines are: 1-alpha-hydroxyalprazolam, 2-oxazepam, 3-temazepam, 4-lorazepam and 5-nordiazepam.

Instrumentation

Automated DPX extractions were performed on a Tecan® EVO-100 liquid handling platform with 8-channels.

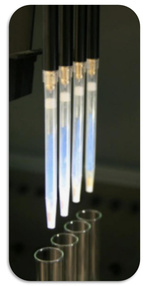
Two LC/MS/MS systems were used in this study. One LC/MS/MS system was a Waters TQD MS-MS with Acquity uPLC pumps and autosampler. The other system was an AB SCIEX 3200 Qtrap™ coupled to an Agilent 1100 LC system equipped with a Zorbax C18 column.

Methods

Urine samples (500 uL) were first enzymatically hydrolyzed using B-glucuronidase and buffer (pH 5). The HB-DPX tips were first conditioned with 30% methanol in water. After dispensing the solution to waste, the sample solution was aspirated into the Hb-DPX tips, mixed by aspirating air bubbles, and then dispensed into the corresponding sample vial. The sorbent was subsequently washed with 500 uL of DI water by aspirating, mixing and dispensing to waste. The benzodiazepines were eluted by aspirating 500 uL of methanol, mixing by aspirating with air bubbles, and then dispensing into clean vials. The automated HB-DPX method was able to process 8 samples simultaneously in approximately 3 min.

The LC conditions

The LC column was a Waters Acquity CSH C18, 2.1 x 100 mm, 1.7 microns. Solvent A was 10 mM ammonium formate in 0.1% formic acid, and solvent B was 15/85 isopropanol/ acetonitrile. The flow was set to 0.4 mL/min starting at 75% A and ramping to 50% A at 6.0 min with a linear ramp. The sample volume injected was 20 uL for the Waters system, and 5 uL for the AB Sciex system.



igure 2. HB-DPX tips on a Tecan® robotic platform (4 hannel). The tips are shown just above the sample solutions. he total extraction time takes less than 3 minutes to perform ith sample extraction, wash and elution steps.

MS/MS parameters:

α-OH-alprazolam:	325.1 to 297.1	325.1 to 205
D5-α-OH-alprazolam	330.1 to 302.1	330.1 to 210
Lorazepam	321 to 275	321 to 229
Oxazepam	287.1 to 269	287.1 to 241
Oxazepam D5	292.1 to 274	292.1 to 246
Nordiazepam	271.1 to 165	271.1 to 140
D5-Nordiazepam	276.1 to 165	276.1 to 140
Temazepam	301.1 to 283.1	301.1 to 255.1
Temazepam D5	306.1 to 288.1	306.1 to 260.1
Diazepam	285.0 to 193.2	
7-aminoclonazepam	286.1 to 121.0	

Results & Discussion

Figure 1 shows the chromatogram achieved using the LC parameters delineated in the experimental section. The Navy Lab wanted to ensure the benzos were optimally separated with unique retention times. Figure 2 shows a picture of the HB-DPX tips on the Tecan® robotic platform. The HB-DPX tips required dispersers in order to ensure reproducible mixing and high recoveries.

Spiked urine samples were extracted using the automated method developed with the Tecan® robot and HB-DPX tips. Recoveries of all 7 benzos were greater than 70% with %RSDs (CVs) of less than 8% (Tables 1 and 2).

In addition to the spiked samples, urine case samples were also analyzed. Approximately 30 random case samples that were previously analyzed and confirmed were processed with this new method and are shown in Table 3. No statistical difference was found when compared to the results of the previously validated method. The % differences were less than 17% for all case samples tested with most being less than 10% (Table 3).

Conclusions

Analysis of benzodiazepines was successfully analyzed using HB-DPX tips with Tecan® robotics. CV's for 7 common benzos were less than 8% from 100 ng/mL to 5,000 ng/mL. Direct comparison of results using this automated method with a validated method calculated accuracies with less than 15% difference for app. 30 random samples tested.

Table 1. Coefficient of variation and accuracy for analysis of standards.

Calibrator		1	2	3	4	Mean	SD	Cv
100	Oxazepam	103	102	101	99	101	1.7	1.7%
	Nordiazepam	99	102	101	97	100	2.2	2.2%
	Temazepam	103	100	103	99	101	2.1	2.0%
	Lorazepam	101	97	100	98	99	1.8	1.8%
	α-hydroxy Alprazolam	100	99	101	102	101	1.3	1.3%
	7-amino Clonazepam	102	102	102	101	102	0.5	0.5%
	Diazepam	101	101	102	101	101	0.5	0.5%
200	Oxazepam	205	204	197	200	202	3.7	1.8%
	Nordiazepam	215	207	203	205	208	5.3	2.5%
	Temazepam	210	215	201	206	208	5.9	2.9%
	Lorazepam	187	211	195	201	199	10.1	5.1%
	α-hydroxy Alprazolam	207	210	207	198	206	5.2	2.5%
	7-amino Clonazepam	210	211	206	206	208	2.6	1.3%
	Diazepam	208	209	205	199	205	4.5	2.2%
400	Oxazepam	396	407	394	398	399	5.7	1.4%
	Nordiazepam	397	416	398	403	404	8.7	2.2%
	Temazepam	397	397	394	405	398	4.7	1.2%
	Lorazepam	411	405	407	373	399	17.5	4.4%
	α-hydroxy Alprazolam	393	388	393	407	395	8.2	2.1%
	7-amino Clonazepam	410	413	398	401	406	7.1	1.8%
	Diazepam	404	397	399	393	398	4.6	1.1%
800	Oxazepam	796	787	806	803	798	8.4	1.1%
	Nordiazepam	788	773	798	794	788	11.0	1.4%
	Temazepam	789	786	801	790	792	6.6	0.8%
	Lorazepam	795	786	797	827	801	17.8	2.2%
	α-hydroxy Alprazolam	800	802	798	792	798	4.3	0.5%
	7-amino Clonazepam	777	773	793	792	784	10.2	1.3%
	Diazepam	786	789	794	802	793	7.0	0.9%

Table 2. Coefficient of variation and accuracy for analysis of standards at upper limits of linearity.

	Upper limit of Linearity	Precision						
		1	2	3	4	Mean	SD	Cv
Oxazepam	5000	4880	4800	4700	5420	4950	321.9	6.5%
Nordiazepam	5000	5170	5000	4840	4920	4983	141.0	2.8%
Temazepam	4000	3840	3950	3810	3850	3863	60.8	1.6%
Lorazepam	5000	4470	5076	5300	4890	4934	351.8	7.1%
α-hydroxy Alprazolam	5000	4520	5060	4889	5002	4868	242.5	5.0%
7-amino Clonazepam	4000	3866	4026	4025	3990	3977	75.7	1.9%
Diazepam	4000	4030	3987	3985	3999	4000	20.8	0.5%

The main advantage of this robotic procedure is that the samples are processed from start to finish without manual handling of the samples. The system is also capable of complete barcode tracking. Other advantages include speed, with up to eight samples being ready for analysis in under 3 minutes. That means it is possible to extract 96 samples in app. 36 minutes. Also, low volumes of organic solvent is utilized, with approximately 0.6 mL of methanol for the extraction of each sample.

Disclaimer

* The views expressed in this presentation are those of the author and do not necessarily reflect the official policy or position of the Department of the Navy, Department of Defense, or the U. S. Government.

Acknowledgement

We are grateful to Gerard Lawther from Molecular BioProducts for the design of the wide bore pipette tips and frits used to make this product.

Table 3. Comparison of DPX-Tecan® method with validated method for app. 30 case samples.

Oxazepam	DPX Hb-T Tips	Validated method	%Diff
Urine sample 1	960	1028	-6.6%
Urine sample 2	586	570	2.8%
Urine sample 3	171	167	2.2%
Urine sample 4	886	866	2.3%
Urine sample 5	5525	4835	14.3%
Urine sample 6	5635	5285	6.6%
Urine sample 7	2965	2635	12.5%
Urine sample 8	3505	3275	7.0%
Urine sample 9	1715	1695	1.2%
Urine sample 10	201	210	-4.3%
Urine sample 11	232.8	230	1.2%
Urine sample 12	1465	1395	5.0%
Urine sample 13	3105	3000	3.5%
Urine sample 14	114	110	3.6%
Urine sample 15	364	368	-1.1%
Urine sample 16	348	369	-5.7%
Urine sample 17	749	762	-1.7%
Urine sample 18	706	664	6.3%
Noradiazepam	**Tecan Tips**	**Standard extraction**	
Urine Sample 1	199	183	8.7%
Urine Sample 2	120.3	113	6.5%
Urine Sample 3	126.3	134	-5.7%
Urine Sample 4	934	890	4.9%
Urine Sample 5	103	102	1.0%
Urine Sample 6	3216	3656	-12.0%
Urine Sample 7	675	670	0.7%
Urine Sample 8	573	594	-3.5%
Urine Sample 9	100	107	-6.5%
Urine Sample 10	115	112	2.7%
Urine Sample 11	493	463	6.5%
Urine Sample 12	110	116	-5.2%
Urine Sample 13	103.9	108	-3.8%
Urine Sample 14	300	265	13.2%
Temazepam	**Tecan Tips**	**Standard extraction**	
Urine Sample 1	255	253	0.8%
Urine Sample 2	267	242	10.3%
Urine Sample 3	188	178	5.6%
Urine Sample 4	3913	3903	0.3%
Urine Sample 5	12833	12533	2.4%
Urine Sample 6	5163	5023	2.8%
Urine Sample 7	1733	1593	8.8%
Urine Sample 8	1203	1220	-1.4%
Urine Sample 9	403	408	-1.2%
Urine Sample 10	108	107	0.9%
Urine Sample 11	785	763	2.9%
Urine Sample 12	6073	5653	7.4%
Urine Sample 13	217	209	3.8%
Urine Sample 14	2173	2523	-13.9%
Urine Sample 15	9593	9763	-1.7%
Urine Sample 16	160	151	6.0%
7-amino Clonazepam	**Tecan Tips**	**Standard extraction**	
Urine Sample 1	1295	1205	7.5%
Urine Sample 2	355	337	5.3%
Urine Sample 3	467	455	2.6%
Urine Sample 4	1265	1295	-2.3%
Urine Sample 5	105.3	104	1.3%
Urine Sample 6	1085	1105	-1.8%
Urine Sample 7	532	476	11.8%
Urine Sample 8	194	209	-7.2%
Urine Sample 9	503	502	0.2%
α-hydroxy Alprazolam	**Tecan Tips**	**Standard extraction**	
Urine Sample 1	6386	6086	4.9%
Urine Sample 2	305	305	0.0%
Urine Sample 3	124	120	3.3%
Urine Sample 4	277	283	-2.1%
Urine Sample 5	299	318	-6.0%
Urine Sample 6	1756	1736	1.2%
Urine Sample 7	108	106	1.9%
Lorazepam	**Tecan Tips**	**Standard extraction**	
Urine Sample 1	4522	4712	-4.0%
Urine Sample 2	1493	1490	0.2%
Diazepam	**Tecan Tips**	**Standard extraction**	
Urine Sample 1	144.9	143	1.3%
Urine Sample 2	156.8	144	8.9%
Urine Sample 3	164.1	160	2.6%
Urine Sample 4	147.4	147	0.3%
Urine Sample 5	143.5	150	-4.3%