Quantitative Analysis of Designer Benzodiazepines in Urine by LC-MS/MS

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Introduction

Benzodiazepines are among the most often prescribed drugs in the US with significant abuse potential. Analogs to the prescription class of benzodiazepines, often obtained on illicit marketplaces, are not U.S. Food and Drug Administration (FDA) approved and are increasingly finding its place among the population over the last two decades. These designer benzodiazepine substances retain the core structure of the approved benzodiazepines. However, some functional groups are altered. This allows them to evade legal restrictions while keeping the desired mind-altering effects. It is important to be able to track these substances as they arise so they can be monitored and detected. Due to cross reactivity of these analogs with benzodiazepine immunoassay kits, developing effective LC-MS/MS techniques is increasingly important for proper identification and quantitation. In this study, we focused on 14 compounds in a urine matrix utilizing a high sensitivity fast (less than 4 minutes) LC analysis. The panel comprises of 13 designer benzodiazepines. Alprazolam was also included because Deschloroetizolam is isomeric to it, and differentiation is necessary. Solid phase extraction (SPE) was used for sample cleanup to achieve a low limit of detection. A high efficiency 2.6 µm, 50 x 3.0 mm Kinetex™ Biphenyl LC column was used for chromatographic separation, coupled with a SCIEX® 4500 triple quad for MS/MS detection.

Materials and Methods

Reagents and Chemicals

Analytical reference standards and internal standards were purchased from Cerilliant® (Round Rock, TX, USA) and Cayman Chemical (Ann Arbor, MI). Certified drug free urine was sourced from UTAK Laboratories, Inc. (Valencia, CA). Beta-Glucuronidase enzyme was purchased from Integrated Micro-Chromatography Systems (Irmo, SC).Certified drug free urine was sourced from UTAK (Valencia, CA). Water was obtained via Sartorius® arium® Comfort II from Sartorius Corporation (Bohemia, NY, USA). All other chemicals were obtained from Sigma-Aldrich® Company (St. Louis, MO, USA).

LC Conditions – Quantitative Analysis for Designer Benzodiazepine Analytes

Column: Kinetex 2.6 µm Biphenyl
Dimensions: 50 x 3.0 mm
Part No.: 00B-4622-YO
Mobile Phase: A: 0.1% Formic Acid in Water
B: 0.1% Formic Acid in Methanol
Gradient:

Time (min)	%B
0	60
0.5	95
2.5	95
2.51	60
4	60

Flow Rate: 0.5 mL/min
Injection Volume: 5 µL
Temperature: 40 °C
LC System: Agilent® 1260 Infinity
Detection: MS/MS
Detector: SCIEX 4500 Triple Quad

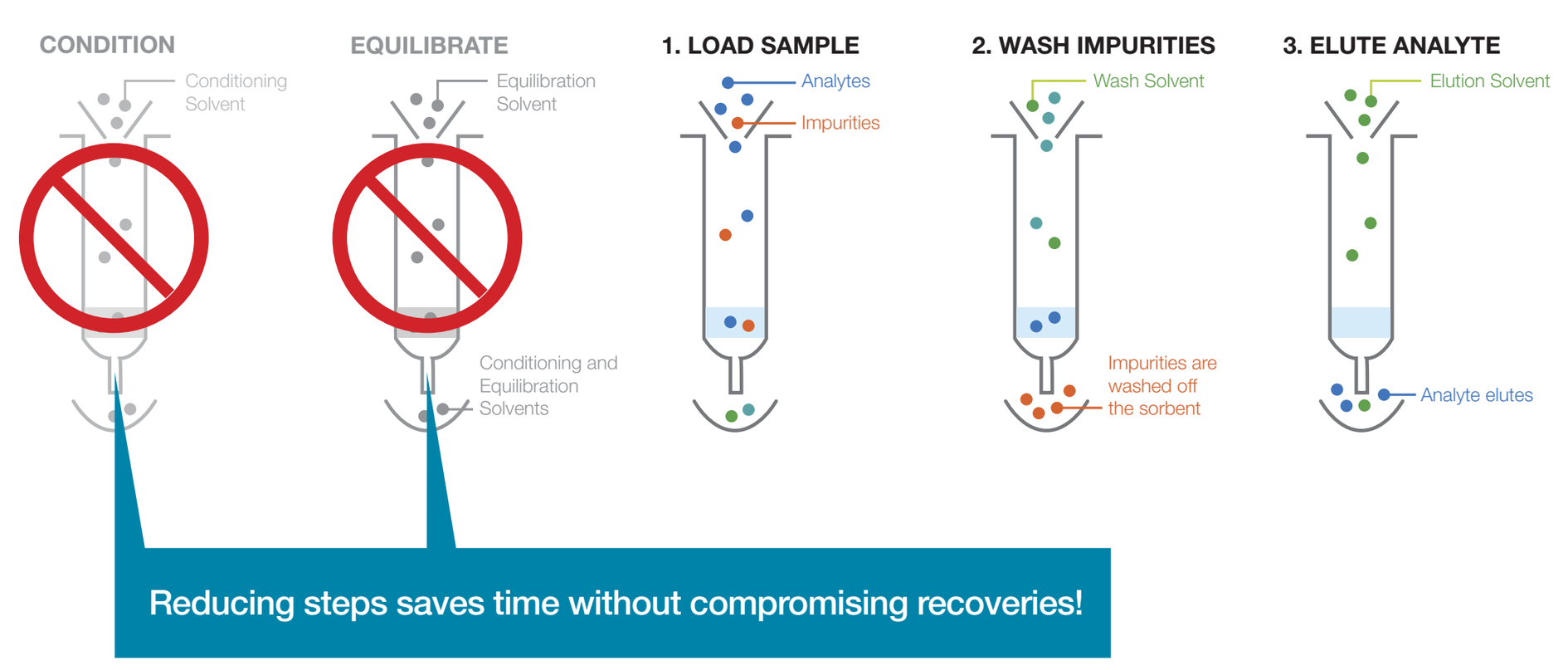
MS/MS Conditions

Ion Source: ESI
Polarity: Positive
Source Temperature: 550 °C
GS1: 40
GS2: 60
CUR: 20
IS: 4500

Feature, Function and Benefit of Strata®-X-Drug B Plus SPE

Enzymatic hydrolysis in a 96-well SPE plate!

- Incubation (hydrolysis) followed by SPE in the same well
- No leaking of aqueous (solvent Shielding Technology™) for 6 hours or more!
- Save time and eliminate transfer steps
- Save money (no extra consumables cost for hydrolysis)
- Simplified SPE (No Conditioning or Equilibration)



Solid Phase Extraction (SPE) Sample Preparation

Sample Pretreatment:	Combine 200 µL of urine sample spiked with 20 mL of internal standard (0.5 ug/mL), 60 µL RT hydrolysis buffer, 20 µL of IMCSzyme® RT enzyme on the Strata-X-Drug B Plus, 30 mg plate (Part No.: 8E-S128-TGB-P). Incubate at room temperature for 15 minutes.
Condition:	Not required.
Equilibrate:	Not required.
Load:	Add 200 µL 0.1 % Formic Acid to the plate, shake/vortex for a minute. Apply vacuum to absorb sample on SPE media.
Wash 1:	1 mL of 0.1 % Formic Acid in Water.
Wash 2:	1 mL of 30 % Methanol in Water.
Dry:	5 minutes at high vacuum (15-20 in Hg).
Elute:	Option 1: 2 washes of 0.5 mL 5 % Ammonium Hydroxide in Methanol. Option 2: 2 washes of 0.5 mL 5 % ammonium hydroxide in (Methanol / Acetonitrile (1:1). Option 3: 2 washes of 0.5 mL Ethyl Acetate / Isopropanol / Ammonium Hydroxide (7:2:1).
Dry down:	Evaporate sample under gentle stream of Nitrogen at 40-45 °C.
Reconstitute:	Resuspend dried samples in 200 µL initial mobile phase.

Results

Figure 1. Separation of 14 Benzodiazepines Using a Kinetex 2.6 µm Biphenyl Column.

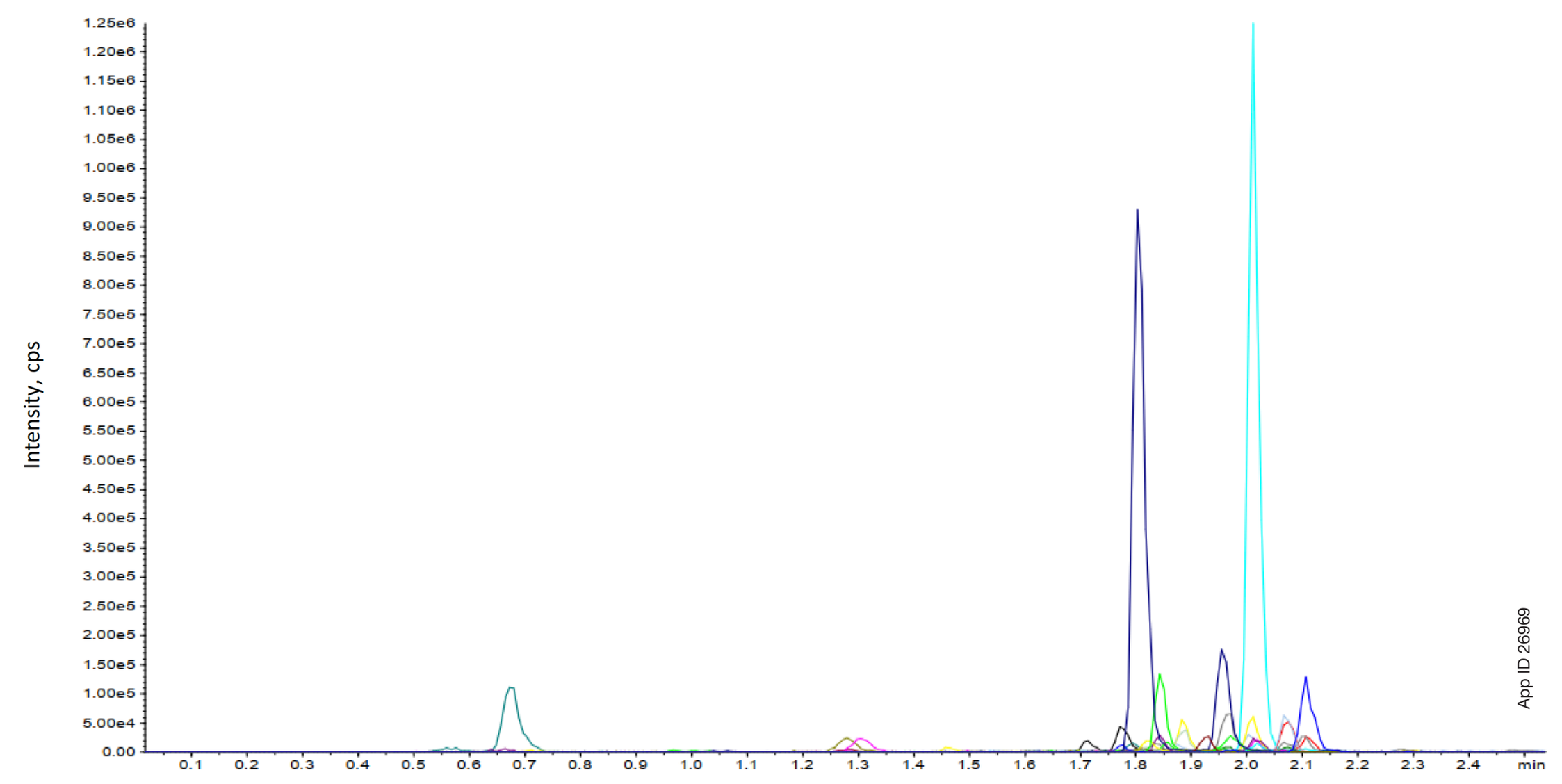


Figure 2. Separation of Isomeric Alprazolam and Deschloroetizolam Using a Kinetex 2.6 µm C18 Column.

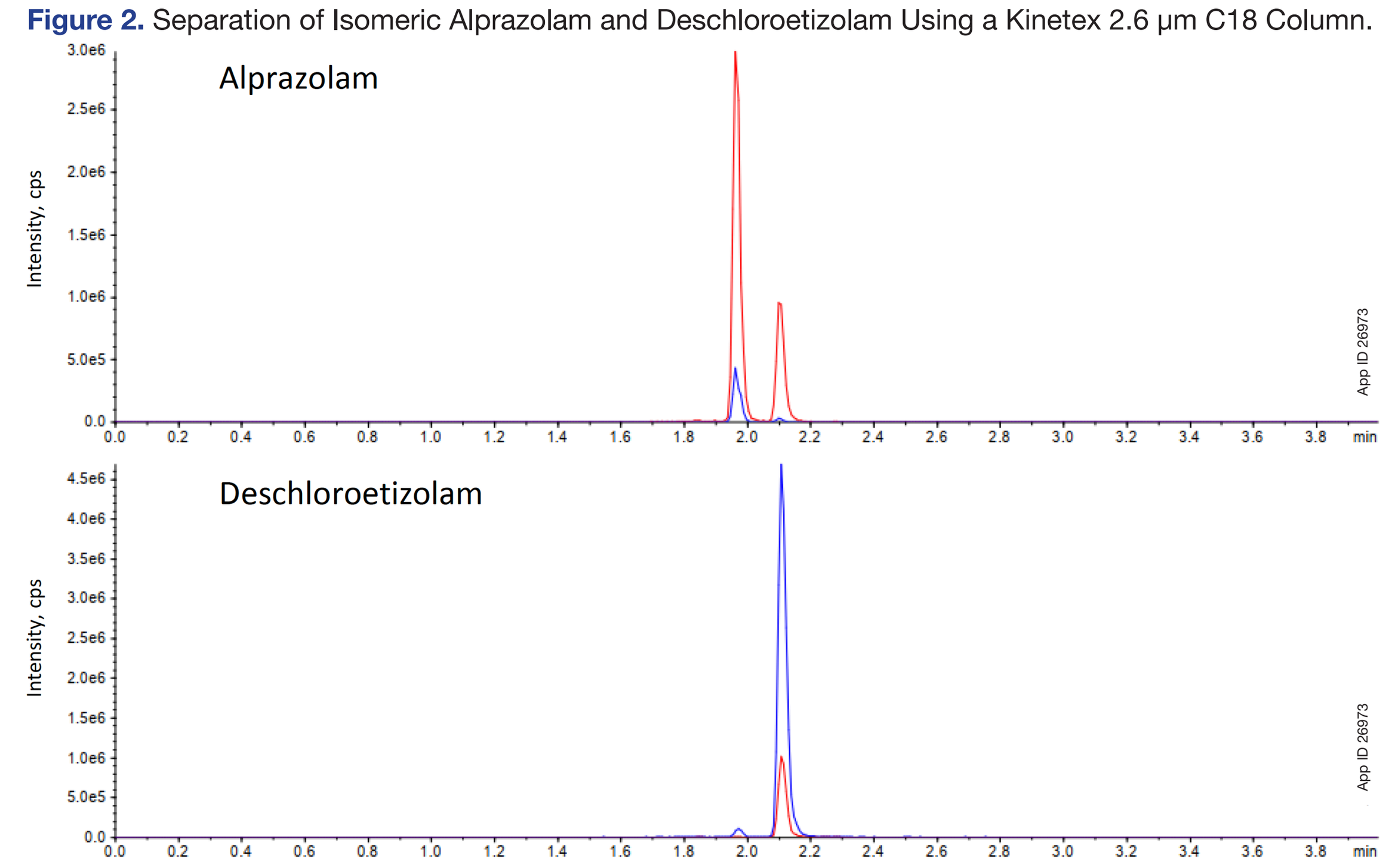


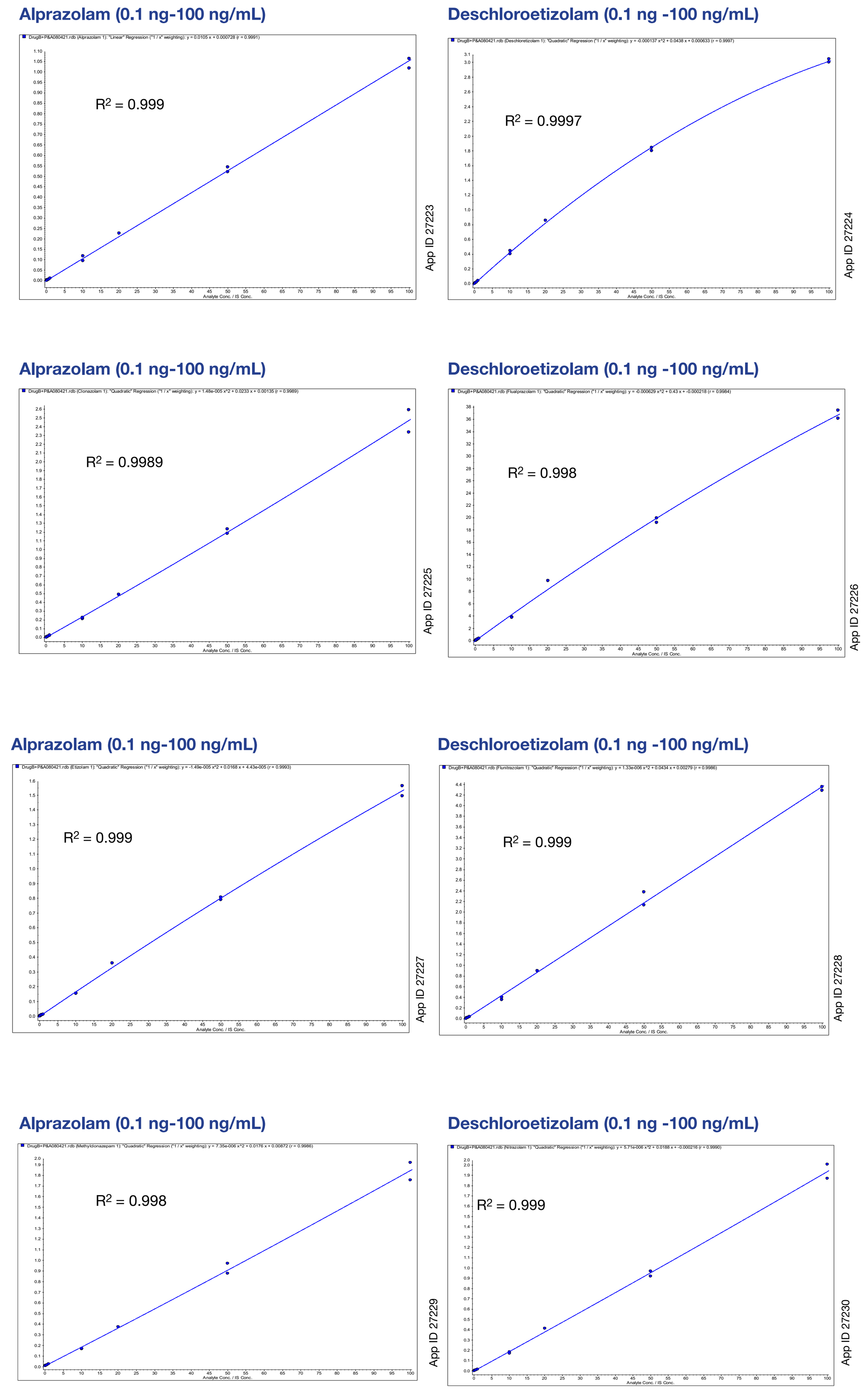
Table 1. SPE Method Optimization for % Recovery and CV Under Three Different SPE Conditions Utilizing a Strata-X-Drug B Plus 96-well Plate.

Spiked conc.	Analyte	% Rec	% CV (N=4)	% Rec	% CV (N=4)	% Rec	% CV (N=4)
(ng/mL)		Option 1		Option 2		Option 3	
		5 % Ammonium Hydroxide in Methanol		5% Ammonium Hydroxide in (Methanol / Acetonitrile (1:1)		Ethyl Acetate / Isopropanol / Ammonium Hydroxide (7:2:1)	
10	Adinazolam	85	11.7	93	1.1	84	6.8
10	Bromazolam	94	17.2	83	6.4	82	8.2
10	Clonazolam	76	7.2	81	11.9	82	9.2
10	Deschloroetizolam	85	11.7	93	1	84	6.8
10	Diclazepam	88	10.9	88	4.5	76	1.3
10	Etizolam	92	14.7	97	2.1	83	1.4
10	Flualprazolam	90	18.6	89	11.2	81	4.4
10	Flubromazepam	99	17.5	80	16.9	85	8.6
10	Flubromazolam	96	26.1	84	7.1	82	1.4
10	Flunitrazolam	73	14.3	77	8.5	93	9.5
10	Methylclonazepam	102	27.9	87	11.3	83	9.2
10	Nitrazolam	75	18.6	86	1.7	78	6.1
10	Phenazepam	90	21.3	86	7.7	77	5.7
10	Alprazolam	95	16.7	91	13.9	99	7.8
		73-102	7.2-27.9	77-97	1.1-16.9	76-99	1.4-9.5

Figure 3. Image Captured for the Resuspended Dried Samples Obtained for Extraction of the Three Different Options in SPE Optimization.

Results

Figure 4. Linearity Curves for Analytes in Urine Samples extracted using a Strata-X-Drug B Plus 96-well Plate Over a 1000-fold Dynamic Concentration Range.



Discussion

A 2.6 µm, 50 x 3.0 mm Kinetex Biphenyl column was utilized for the chromatographic separation of the benzodiazepine panel, resolving 14 analytes in 4 minutes (**Figure 1**) that includes 1.5 minutes of re-equilibration time and demonstrates baseline separation of the isomeric Alprazolam and Deschloroetizolam pair (**Figure 2**).

The optimized SPE method developed by Strata-X-Drug B Plus results in clean and clear urine extract (**Figure 3**) with absolute recovery of the analytes ranging from 76-114 % with a % CV value < 10 % (**Table 1**). The tested dynamic range of the assay over 1000 – fold (0.1 ng/mL to 100 ng/mL) concentration shows linear regression value (R2) ranging ≥ 0.997 for all analytes while a quadratic fit with 1/x weighting factor was applied (**Table 2**, **Figure 4**). Data for 3 levels of QC showed accuracy and precision ranging 96-114 % and < 10 % respectively (**Table 3**).

The simplified Strata-X-Drug B Plus method (no conditioning or equilibration) that combines in-well hydrolysis and SPE extraction capability in a single 96-well plate, provides faster workflow compared to conventional SPE format and is cost effective. It is amenable for automation and high throughput.

Conclusion

We corroborated a sensitive, accurate, and precise quantitation method for designer benzodiazepines, streamlining the workflow that accommodates for the hydrolysis of urine and SPE extraction in the same well of the Strata-X-Drug B Plus plate. The integration of the Strata-X Drug B Plus plate with in-well hydrolysis and fast LC method using the Kinetex Biphenyl column creates an efficient workflow for expanding a class of novel psychoactive substances.

Results

Table 2. MRM Transitions and Linearity Data from Calibration Curves for 14 Benzodiazepines Extracted from Urine Using the Strata-X-Drug B Plus 96-well Plate.

Analyte Name	Retention Time (min)	Q1 (m/z)	Q3 (m/z)	Linear Regression (R2) (0.1 ng/mL -100ng/mL)
Adinazolam	1.1	352.1	58	0.999
Bromazolam	1.9	352.9	324.8	0.999
Clonazolam	1.8	354	308	0.999
Deschloroetizolam	2.1	309.1	225.1	0.9997
Diclazepam	2	319.0	154	1.0004
Etizolam	2	343.0	314.1	0.9993
Flualprazolam	1.8	327.01	299	0.998
Flubromazepam	1.8	333.03	226	0.998
Flubromazolam	1.9	371	292.0	0.998
Flunitrazolam	1.7	338	292	0.998
Methylclonazepam	1.9	329.8	283.9	0.999
Nitrazolam	1.8	320	246.2	0.999
Phenazepam	1.8	351.0	185.8	0.997
Alprazolam	1.9	309.1	205.2	0.9991
Diclazepam-D4	2.0	323.0	231.0	-
Etizolam-D3	2.1	346.0	317.0	-
Phenazepam-D4	1.8	355.0	183.0	-
Clonazolam-D4	1.82	358.0	312.0	-

Table 3. Precision and Accuracy Data for 14 Benzodiazepines Extracted from Urine Using the Strata-X-Drug B Plus 96-well plate.

	QC-1 (2.0 ng/mL)		QC-2 (25 ng/mL)		QC-3 (75 ng/mL)	
Analyte Name	% Accuracy	% CV (N = 4)	% Accuracy	% CV (N = 4)	% Accuracy	% CV (N = 4)
Adinazolam	104	9.7	103	5.8	104	2.2
Bromazolam	102	9.7	102	5.5	107	3.8
Clonazolam	102	8.9	107	4.5	103	5.4
Deschloroetizolam	105	0.1	104	2	98	3.2
Diclazepam	109	0.1	107	1.2	109	5.1
Etizolam	105	0.1	105	1.7	99	1.6
Flualprazolam	104	1.8	103	4.1	102	3.9
Flubromazepam	97	8.1	103	1.3	101	4.7
Flubromazolam	105	0.2	104	1.1	99	2.3
Flunitrazolam	98	0.3	105	1.2	102	9.2
Methylclonazepam	105	0.3	101	1.8	101.2	2.5
Nitrazolam	102	0.2	100	2.6	99	7.4
Phenazepam	114	7	104	5.9	103	6.8
Alprazolam	104	9.7	103	5.8	104	2.2