Extraction of Benzodiazepines in Urine with Polymeric SPE Cation Exchange, Agilent Bond Elut Plexa PCX

Application Note

Forensic Toxicology

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Introduction

Benzodiazepines are a large class of drugs and include compounds such as diazepam (Valium), chlordiazepoxide (Librium), oxazepam (Serax), lorazepam (Ativan), alprazolam (Xanax), clonazepam (Clonopin), and others. 1,4-Benzodiazepines, such as diazepam, nordiazepam, and temazepam, are metabolized and excreted as oxazepam and oxazepam glucuronide. The nitrobenzodiazepines, such as clonazepam and flunitrazepam, are metabolized to a 7-amino metabolite in urine. Flurazepam is rapidly desalkylated.

Quantitative analysis of benzodiazepines in urine by LC/MS can be difficult due to the high level of matrix components. Organic salts as well as pigments and proteins cause ion suppression and the loss of signal intensity. Agilent Bond Elut Plexa PCX SPE products are a member of the Plexa family based on a polymeric cation exchanger. Plexa PCX products use a generic and simplified method to remove neutral and acidic interferences from the matrix and concentrate basic analytes, resulting in improved analytical performance and sensitivity in the quantification of basic compounds.

In addition, Bond Elut Plexa PCX SPE products offer faster and highly reproducible flow rates, resulting in excellent tube-to-tube and well-to-well performance. Bond Elut Plexa PCX SPE products exhibit significantly reduced ion suppression because their highly polar, hydroxylated surfaces are entirely amide free. Therefore, the particle exterior minimizes strong binding of proteins and phospholipids. An LC/MS/MS method is presented for the quantitative determination of benzodiazepines and their target metabolites in human urine specimens with Bond Elut Plexa PCX SPE tubes. Hydrolysis may also be necessary by adding 5,000 units of β-glucuronidase to a 1 M acetic acid (pH = 3.8) buffered urine sample. The sample was vortexed and incubated for 2 hours at 60 °C prior to extraction.

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Materials and Methods

Table 1. SPE reagents and solutions.

2% Formic acid	Add 2 mL of concentrated formic acid to 100 mL of DI water
Methanol	Reagent grade or better
50% Methanol	Add 50 mL of methanol to 50 mL of DI water
5% Ammonia in methanol	Add 5 mL of concentrated ammonia to 100 mL of methanol

Table 2. SPE method.

Column:	Agilent Bond Elut Plexa PCX 30 mg 3 mL tube (p/n 12108303)
Sample pretreatment:	1 mL human urine. Dilute 1:2 with 2% formic acid.
Condition:	1. 1 mL CH_3OH 2. 1 mL H_2O
Load:	Apply sample and extract under low or no vacuum
Wash 1:	2 mL 2% formic acid
Wash 2:	2 mL 50% CH_3OH in water
Elution:	1 mL 5% NH_3 in methanol

All samples are evaporated to dryness and reconstituted in 200 µL of 50:50 0.1% aqueous formic acid: CH_3OH.

Table 3. MS conditions.

Compound	Q1	Q3	CE
Clonazepam	316.0	270.0	16.5 V
7-Aminoclonazepam	285.8	121.0	24.5 V
Flurazepam	388.0	315.0	18.0 V
Desalkylflurazepam	288.9	140.0	24.0 V
Midazolam	326.4	290.9	21.5 V
Alprazolam	309.0	204.9	37.0 V
Flunitrazepam	314.0	268.0	21.0 V
7-Aminoflunitrazepam	284.1	135.0	22.0 V
Chlordiazepoxide	300.3	227.0	19.5 V
Diazepam	285.0	222.0	20.5 V
Lorazepam	321.0	274.9	18.0 V
Oxazepam	286.8	241.0	16.5 V
Nordiazepam	271.0	165.0	23.0 V
Temazepam	301.0	255.0	17.0 V

LC conditions

Mobile phase:	A: 0.1% Formic acid	
	B: Methanol	
Gradient:	t = 0-1 minutes	40% A : 60% B
	t = 2.0-4.30 minutes	20% A : 80% B
	t = 4.31-5.30 minutes	40% A : 60% B
Flow rate:	0.2 mL/min	
Column:	Agilent Pursuit XRs$^{Ultra\ 2.8}$ C18, 2.0 × 100 mm (p/n A7511100X020)	
Capillary:	70 V	
Dry gas temperature:	350 °C, 30 psi	
CID:	Argon	
Polarity:	Negative	

Results and Discussion

The procedure describes a method for extracting and determining 14 different benzodiazepines in human urine. The limit of detection (LOD) of the combined solid phase extraction and LC/MS/MS analysis was 1.0 ng/mL. Recoveries were calculated from a first order regression with RSD values based on a sampling of n = 6. Excellent absolute recoveries were achieved demonstrating good retention and elution, as well as minimal ion suppression. Response for all the compounds evaluated was linear up to three orders of magnitude from 1.0 ng/mL to 1.0 µg/mL with correlation coefficients all above 0.995. To demonstrate reproducibility, samples were analyzed at two concentrations (n = 6). Table 4 shows that the extractions produced very reproducibly high recoveries.

Table 4. Analyte relative recoveries.

Analyte	% Rec (1 ng/mL)	% RSD	% Rec (100 ng/mL)	% RSD
Clonazepam	116	13	103	7
7-Aminoclonazepam	102	10	99	2
Flurazepam	117	14	106	8
Desalkylflurazepam	115	13	99	6
Midazolam	108	13	110	4
Nordiazepam	113	15	107	7
Alprazolam	113	17	110	8
Flunitrazepam	107	16	101	3
7-Aminoflunitrazepam	112	18	95	9
Chordiazepoxide	119	15	92	10
Diazepam	111	12	99	8
Temazepam	118	4	97	8
Lorazepam	102	14	94	10
Oxazepam	113	10	97	5

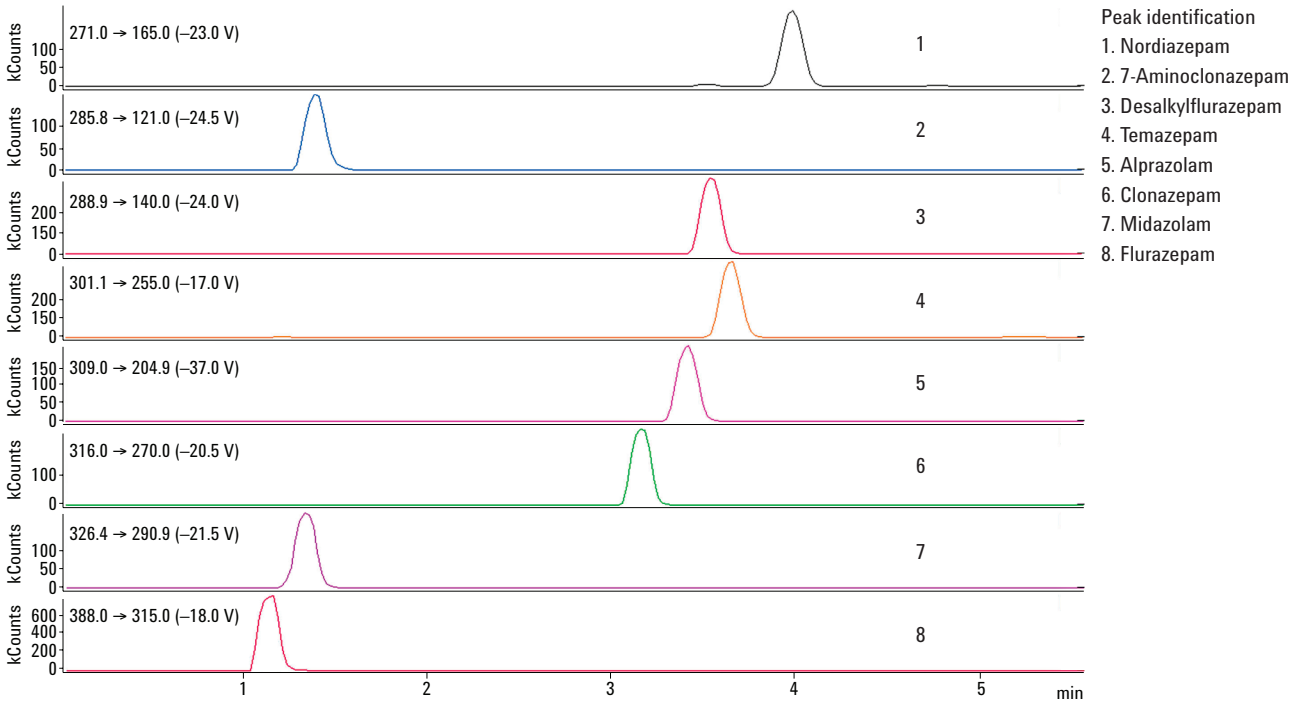

Figure 1a. Chromatograms of a 100 ng/mL urine extract (peaks 1-8).

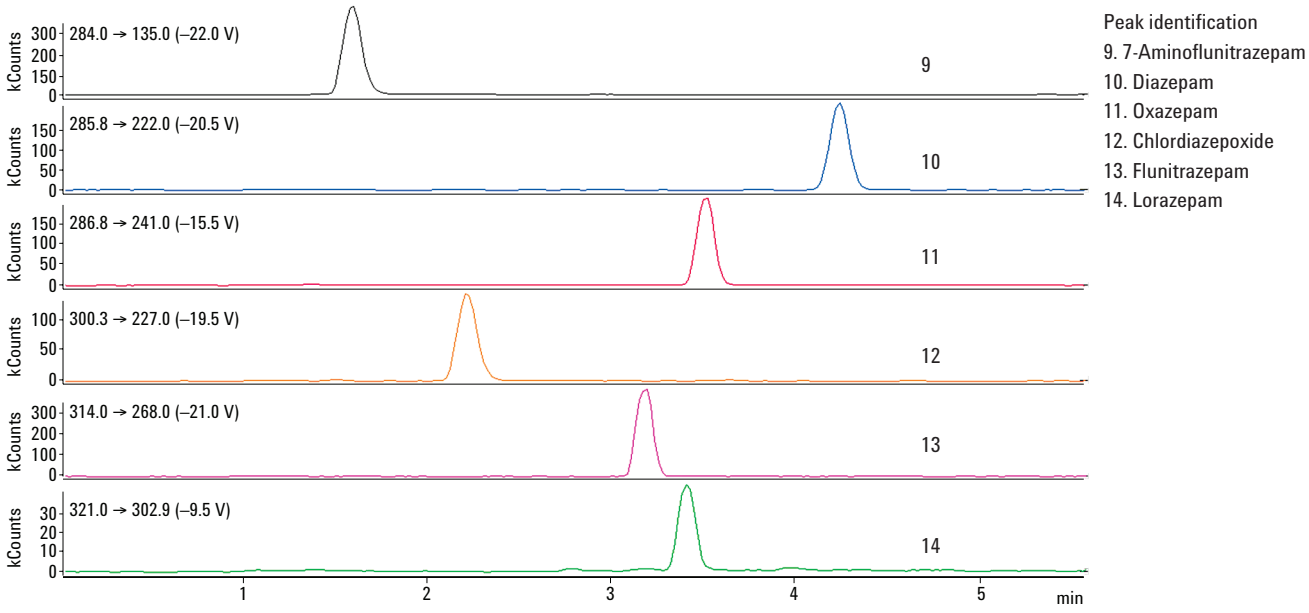

Figure 1b. Chromatograms of a 100 ng/mL urine extract (peaks 9-14).

Conclusions

Agilent Bond Elut Plexa PCX is a useful tool for high throughput SPE applications, which require analysis at low analyte levels, need excellent reproducibility, and must be quickly implemented with minimal method development. Bond Elut Plexa PCX products meet these requirements.

With Bond Elut Plexa PCX, a generic drug extraction protocol can be applied to polar analytes with basic amino functional groups. Under acidic conditions, the charged analyte binds to the cation exchange groups of the sorbent. Polar interferences and proteins are washed away with an acidic, aqueous solution. A wash with 50% aqueous methanol is possible without a significant loss of analytes. The wash elutes neutral compounds retained in the hydrophobic cores of the sorbent. Finally, ammoniated methanol was used to disrupt the cation exchange interaction, resulting in the elution of the benzodiazepines.

Flow rate is fast because Bond Elut Plexa PCX particles have much narrower particle size distribution with no fines to cause blockages, thus resulting in excellent tube-to-tube reproducibility. Bond Elut Plexa PCX tubes are, therefore, a useful tool for high throughput SPE applications, which require analysis at low analyte levels, excellent reproducibility and quick implementation, with minimal method development.

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