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Extraction of Benzodiazepines From Human Urine Using ISOLUTE® SLE+ in Column Format Prior to GC-MS analysis

This application note describes the extraction of a range of benzodiazepines from human urine using ISOLUTE® SLE+ supported liquid extraction columns prior to derivatization and GC-MS analysis.

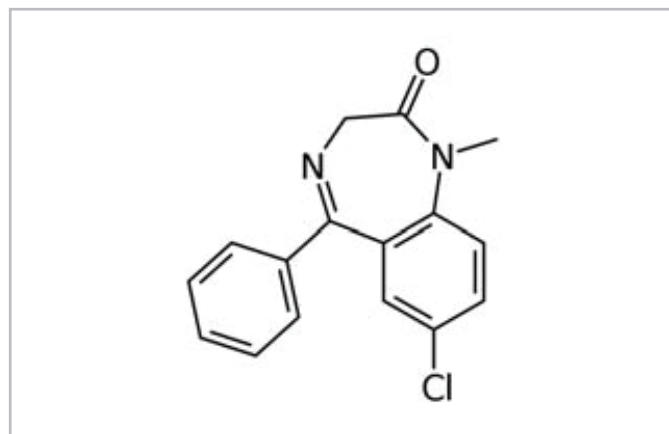


Figure 1. Structure of Diazepam.

Introduction

Benzodiazepines are psychoactive drugs used to treat various conditions including anxiety, insomnia, seizures and alcohol withdrawal. Long-term use can cause physical dependence and withdrawal upon cessation of use, due to this their application as medication has to be monitored and measured. This application note details an optimized extraction protocol for the use of ISOLUTE SLE+ supported liquid 1 mL sample volume columns to extract a range of benzodiazepines from human urine. This simplified and efficient extraction method has analyte recoveries from 90% with LOQs as low as 40 ng/mL. The application has also been performed with halogenated and non-halogenated extraction solvents in the interests of providing for green chemistry.

ISOLUTE® SLE+ Supported Liquid Extraction plates and columns offer an efficient alternative to traditional liquid-liquid extraction (LLE) for bioanalytical sample preparation, providing high analyte recoveries, no emulsion formation, and significantly reduced sample preparation time.

Analytes

Diazepam, nordiazepam, midazolam, flunitrazepam, 7-aminoflunitrazepam, bromazepam, oxazepam, nitrazepam, flurazepam, temazepam, 7-aminoclonazepam, lorazepam, hydroxyethylflurazepam, estazolam, alprazolam, triazolam, α -hydroxyalprazolam, α -hydroxytriazolam.

ISOLUTE® SLE+ Procedure

- Format:** ISOLUTE SLE+ 1 mL Sample Volume columns, part number 820-0140-C
- Sample pre-treatment:** Dilute human urine (500 μ L) with sodium acetate (100 mM, pH 5) (500 μ L). Perform enzymatic hydrolysis with β -glucuronidase (H3) ex. H.pomatia (Sigma-Aldrich G8885) 50 μ L/mL urine (approx. 4500 U/mL urine) at 60 $^{\circ}$ C $\pm$ 2 $^{\circ}$ C for 2 hours. Adjustment to pH 7 using 25 % ammonium hydroxide (conc.), 10 μ L/mL urine.
- Sample loading:** Load pre treated urine (1 mL in total) onto the ISOLUTE SLE+ column followed by a pulse of vacuum to initiate flow (VacMaster-10 Sample Processing Manifold 121-1016), allow the sample to absorb for 5 minutes.
- Analyte elution:** Apply dichloromethane (2.5 mL), wait five minutes to allow the solvent to adsorb, apply a short pulse of vacuum if solvent not fully adsorbed. Apply a second aliquot of dichloromethane (2.5 mL), allow to adsorb for a further 5 minutes and then apply a final pulse of vacuum.
- Post Extraction:** Evaporate the eluate to dryness with 80 L/min air at 35 $^{\circ}$ C and reconstitute in 50 μ L of ethyl acetate. Sample then derivatized 1:1 with with TBDMS-TFA, N-tert-butyldimethylsilyl-N-methyltrifluoroacetamide with 1% tert-butyldimethylchlorosilane (Sigma-Aldrich 375934) at 70 $^{\circ}$ C $\pm$ 2 $^{\circ}$ C for 20 minutes.

GC Conditions

Instrument:	7890A GC / 5975A MSD (Agilent Technologies Inc., Santa Clara CA, USA).
Column:	Zebron ZB-5Msi, 30 m x 0.25 mm, 0.25 µm df. Deactivated fused silica, 5 m x 0.25 mm pre-column.)
Carrier gas:	Helium 1.2mL min ⁻¹ (constant flow)
Inlet:	Pulsed Splitless, 9.8 psi at 250 °C Pressure pulse 20 psi to 0.75 min Purge flow 50 mL/min at 1.5 min
Injection:	2 µL
Oven:	50 °C for 1.5 min, 25 °C/min to 150 °C then 10 °C/min to 320 °C, hold 6 min
Transfer Line:	280 °C

MS Conditions

Source:	230 °C
Quadrupole:	150 °C
Solvent Delay:	6 min
MSD mode:	SIM
SIM Groups:	1 – 6.1 min to 18.4 min / 2 – 18.4 min to 19.1 min 3 – 19.1 min to 20.2 min / 4 – 20.2 min to 22.6 min 5 – 22.6 min to 23.9 min / 6 – 23.9 min to 25.3 min 7 – 25.3 min to 28 min

Table 1. SIM Parameters

SIM Group	Analyte	Quan Ion	1st Qual Ion	2nd Qual Ion	Dwell / ms
1	diazepam	256	283	221.1	25
2	nordiazepam-TBDMS	327.1	329.1	328.1	25
3	midazolam	310.1	312	325.1	25
3	flunitrazepam	312.1	286.1	266.1	25
3	7-aminoflunitrazepam	283.1	255.1	264.1	25
3	bromazepam-TBDMS	374	372	346	25
4	oxazepam-TBDMS	457.2	513.3	459.2	10
4	nitrazepam-TBDMS	338.1	339.1	292.1	10
4	flurazepam	86.1	87.1	99.1	10
4	temazepam-TBDMS	357.1	283	359.1	10
4	7-aminoclonazepam-TBDMS	342.1	399.2	344.1	10
4	lorazepam-TBDMS	491.1	513.3	493.1	10
4	OH-Et-flurazepam	389.1	391.1	390.1	10
4	estazolam	259.1	294.1	239	10
4	alprazolam	279.1	308.1	204.1	10
5	triazolam	313	238	342	25
6	a-OH-alprazolam-TBDMS	381.1	383.1	384.1	25
7	a-OH-triazolam-TBDMS	415	417.1	419.1	25

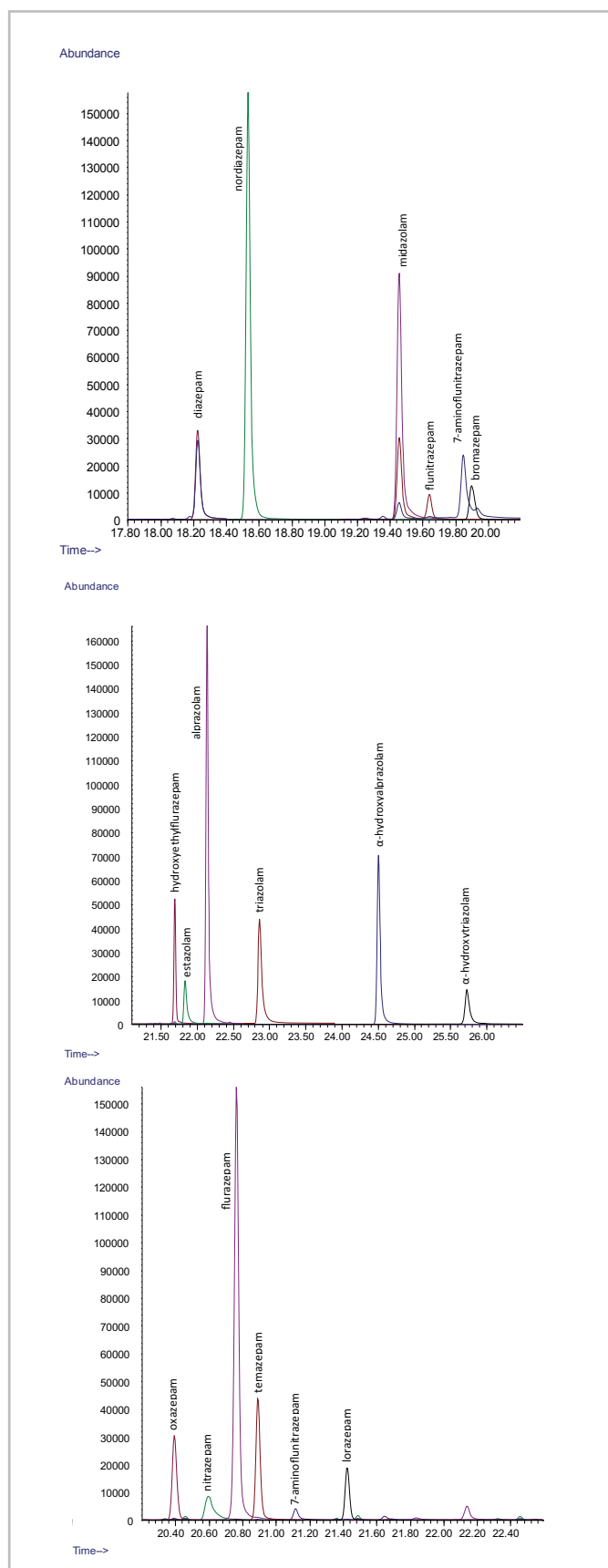


Figure 2. Overlaid Target Ion Chromatograms Sim groups 1-7

Table 1. Peak to peak signal to noise at 40 ng/mL of urine

Analyte	Quan Ion
diazepam	256
nordiazepam-TBDMS	327.1
midazolam	310.1
flunitrazepam	312.1
7-aminoflunitrazepam	283.1
bromazepam-TBDMS	374
oxazepam-TBDMS	457.2
nitrazepam-TBDMS	338.1
flurazepam	86.1
temazepam-TBDMS	357.1
7-aminoclonazepam-TBDMS	342.1
lorazepam-TBDMS	491.1
OH-Et-flurazepam	389.1
estazolam	259.1
alprazolam	279.1
triazolam	313
a-OH-alprazolam-TBDMS	381.1
a-OH-triazolam-TBDMS	415

Figure 2 shows a collection of the overlaid target ion chromatograms for all benzodiazepine analytes spiked at 500 pg/μL. Ethyl acetate was determined to be an acceptable non-halogenated alternative to dichloromethane as an extraction solvent, this is demonstrated alongside the dichloromethane recoveries shown in figure 3. However, recoveries are lower due to decreased signal:noise in proportion to extract cleanliness.

An LOQ of 40 ng/mL was reached (500 μL matrix) for all analytes. Peak-to-peak signal/noise 14:1 or greater with RSDs <10% (n=3).

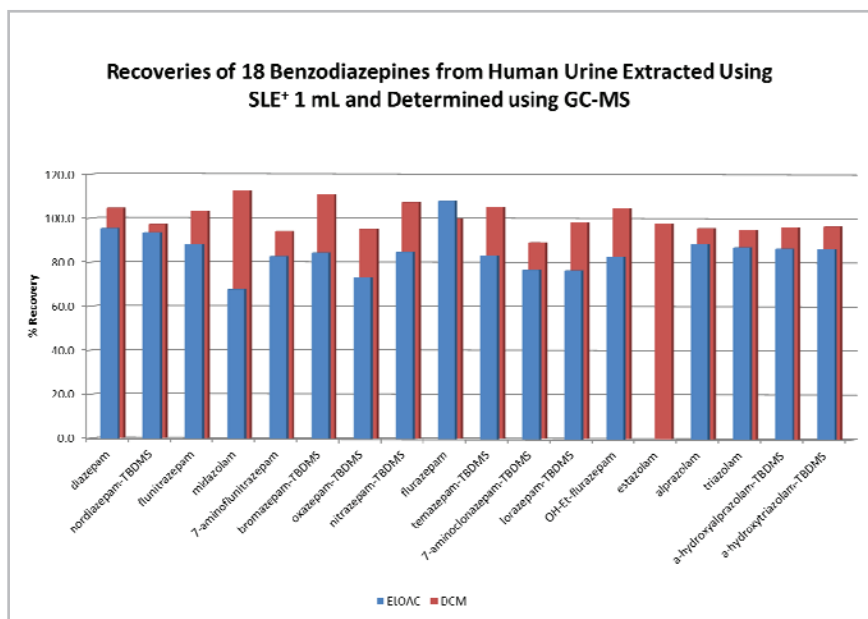


Figure 3. Analyte % recoveries for the full range of extracted benzodiazepines, comparing two different extraction solvents, halogenated and non-halogenated

Ordering Information

Part Number	Description	Quantity
820-0140-C	ISOLUTE® SLE+ 1 mL Sample Volume Columns	30
121-1016	Biotage® VacMaster™-10 Sample Processing Manifold	1

For the latest application notes and more information about ISOLUTE® SLE+ visit www.biotage.com

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