

Extraction of Benzodiazepines From Human Urine Using ISOLUTE® SLE+ 96-Well Plates and Columns Prior to LC-MS/MS Analysis

Introduction

This application note describes the extraction of a range of benzodiazepines from human urine using ISOLUTE SLE+ supported liquid extraction plates (96-well) and columns prior to LC-MS/MS analysis.

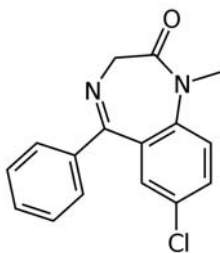


Figure 1. Structure of Diazepam

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 extraction plates (96-well) and 1 mL sample volume columns to extract a range of benzodiazepines from human urine. This simplified and efficient extraction method has analyte recoveries from 85% with LOQs as low as 100 pg/mL.

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

Midazolam, Flurazepam, Bromazepam, α -hydroxyalprazolam, Nordiazepam, Nitrazepam, Oxazepam, Estazolam, Alprazolam, Lorazepam, Triazolam, Diazepam, Temazepam and Flunitrazepam.

ISOLUTE SLE+ procedure

Sample Pre-treatment: To 1 mL of urine add 100mM ammonium acetate pH5 (950 μ L) and β -glucuronidase (50 μ L) enzyme ($\sim$ 4500 U/mL). Hydrolyze in a water bath at 60°C for 2 hours. 50 μ L of enzyme is required per mL of urine used.

For a 200 μ L sample: **ISOLUTE SLE+ 200 Supported Liquid Extraction plate, part number 820-0200-P01**

Sample Load: Load pre-treated sample (200 μ L) to plate followed by a pulse of vacuum to initiate flow and leave for five minutes to completely adsorb.

Analyte Elution: Elute with dichloromethane (1 mL). Leave to flow under gravity for 5 minutes, then apply short pulse of vacuum.

Post extraction: Evaporate to dryness at room temperature (80 L/min) and reconstitute in HPLC grade water/acetonitrile (70/30, v/v) (200 μ L). Cap with sealing mat and vortex gently (60 sec).

For a 1 mL sample: **ISOLUTE SLE+ 1 mL sample volume column, part number 820-0140-C**

Sample Load: Load pre-treated sample (1 mL) to plate followed by a pulse of vacuum to initiate flow and leave for five minutes to completely adsorb.

Analyte Elution: Elute with dichloromethane (2.5 mL). Leave to flow under gravity for 5 minutes, then follow with a further aliquot of dichloromethane (2.5 mL), apply a short pulse of vacuum.

Post extraction: Evaporate to dryness at room temperature (80 L/min) and reconstitute in HPLC grade water/acetonitrile (70/30, v/v) (1 mL). Vortex gently (60 sec).

Reagents

Formic Acid concentration 98%-100%.

100mM NH₄OAc pH 5. Dissolve 7.708 g of ammonium acetate in 950mL of H₂O. Adjust pH to 5 with formic acid then make up to 1000 mL.

HPLC Conditions

Instrument:	Waters Alliance 2795.
Column:	Phenomenex Kinetex C18 100 Å column (2.6µ, 50 x 2.1 mm id)
Mobile phase:	0.1% formic acid (aq) / acetonitrile (70/30, v/v) (isocratic)
Flow rate:	0.30 mL/min.
Injection:	25 µL (partial loop).
Sample Temperature:	20 °C.
Column Temperature:	Ambient.

Mass Spectrometry Conditions

Instrument: Waters Ultima Pt triple quadrupole mass spectrometer equipped with an electrospray interface for mass analysis.

Source temp: 100 °C.

Desolvation temp: 350 °C.

Table 1. MRM transitions in positive mode for a range of benzodiazepines.

Scan function	Compound	MRM transition	Cone voltage	Collision energy (ev)
1	Bromazepam	316.0 > 182.0	35	25
2	Midazolam	326.1 > 291.1	35	25
3	Flurazepam	388.2 > 315.0	35	21
4	Nordiazepam	271.0 > 140.0	35	25
5	Nitrazepam	282.0 > 236.0	35	22
6	Oxazepam	287.0 > 241.0	35	20
7	Estazolam	295.0 > 267.0	35	23
8	Alprazolam	309.0 > 281.0	35	24
9	Lorazepam	321.0 > 275.0	35	20
10	α-hydroxy-alprazolam	325.0 > 297.0	35	25
11	Triazolam	343.0 > 308.0	35	25
12	Diazepam	285.0 > 154.0	35	25
13	Temazepam	301.1 > 255.0	35	20
14	Flunitrazepam	314.0 > 267.9	35	25

Results

Figure 2 shows the mass chromatograms for all the extracted benzodiazepines spiked at 10 ng/mL. Figure 3 shows average analyte recoveries for the 96 fixed-well plate method at 10 ng/mL, with RSDs <5 %.

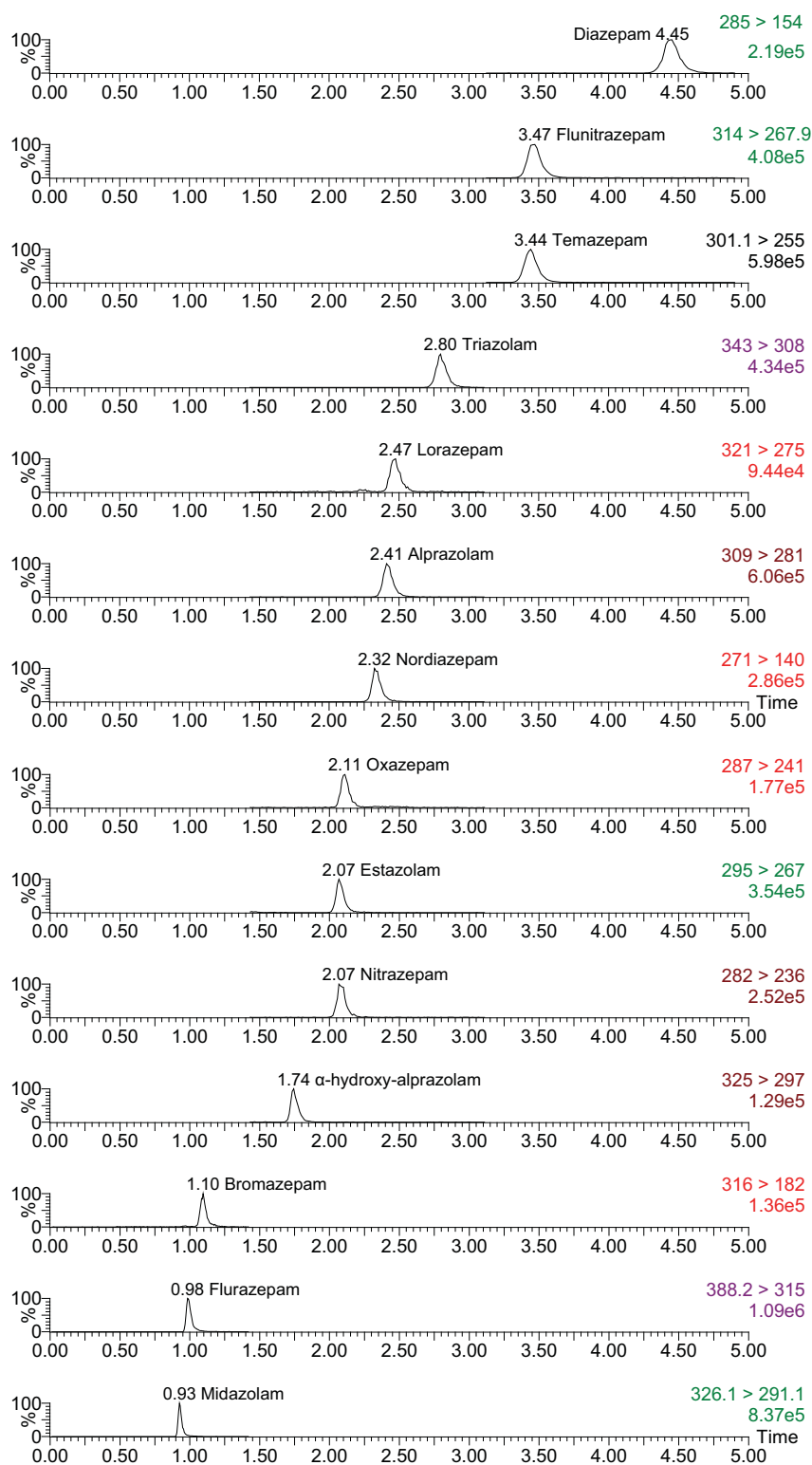


Figure 2. Typical mass chromatograms for all benzodiazepines at 10 ng/mL

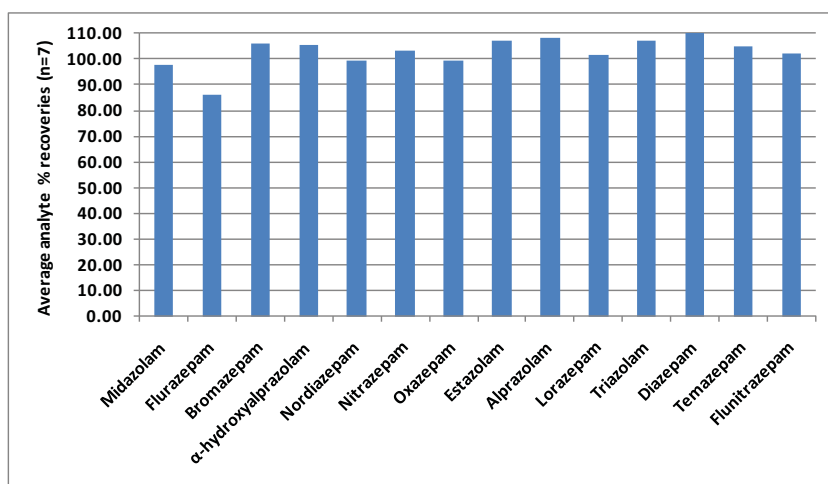


Figure 3. Average analyte recoveries of benzodiazepines analytes on 96 fixed well plates at 10 ng/mL (n=7)

Table 1. LOQ for each analyte using the ISOLUTE SLE+ 200 µL supported liquid extraction plate.

Analyte	LOQ
Bromazepam	10 ng/mL
Midazolam	100 pg/mL
Flurazepam	200 pg/mL
Nordiazepam	500 pg/mL
Nitrazepam	500 pg/mL
Oxazepam	1 ng/mL
Estazolam	200 pg/mL
Alprazolam	200 pg/mL
Lorazepam	500 pg/mL
α-hydroxy-alprazolam	500 pg/mL
Triazolam	100 pg/mL
Diazepam	100 pg/mL
Temazepam	200 pg/mL
Flunitrazepam	100 pg/mL

Ordering information

Part number	Description	Quantity
820-0200-P01	ISOLUTE SLE+ 200 µL supported liquid extraction plate	1
820-0140-C	ISOLUTE SLE + 1 mL sample volume column	30
121-5203	Deep well collection plate 2 mL	50
121-9600	VacMaster-96 Sample processing manifold complete (without vacuum control)	1
SD2-9600-DHS-UK	SPE Dry 96 Dual, 240 V UK	1

NORTH AMERICA

Main Office: +1 704 654 4900
Toll Free: +1 800 446 4752
Fax: +1 704 654 4917
Order Tel: +1 704 654 4900
press (4) at the auto attendant
Order Fax: +1 434 296 8217
ordermailbox@biotage.com
1-pointsupport@biotage.com

EUROPE

Main Office: +46 18 56 5900
Fax: +46 18 59 1922
Order Tel: +46 18 56 57 10
Order Fax: +46 18 56 57 05
order@eu.biotage.com

Japan

Tel: +81 3 5627 3123
Fax: +81 3 5627 3121
jp_order@biotage.com

Distributors

To locate a distributor please visit our
Web site at www.biotage.com