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# Extraction of Benzodiazepines from Whole Blood Using ISOLUTE® SLE+ Prior to GC/MS Analysis

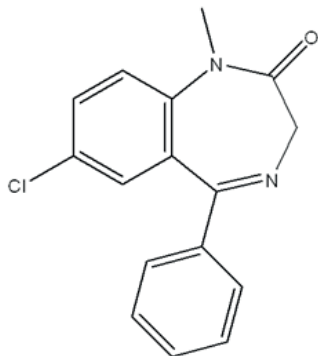


Figure 1. Structure of Diazepam

## Introduction

This application note describes the extraction of benzodiazepine compounds from whole blood, prior to GC/MS analysis. This protocol also allows the simultaneous extraction of various other drugs of abuse classes: amphetamines, barbiturates, cocaine and opiates.

ISOLUTE® SLE+ columns with 1 mL sample capacity are used to extract whole blood samples following a straightforward sample dilution. No protein precipitation or other pre-treatment is required prior to sample loading. The sample preparation procedure delivers clean extracts, good recoveries and RSD values and LLOQs from 10 ng/mL (analyte dependant).

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.

## Analytes

Diazepam-D5, Diazepam, Nordiazepam, Midazolam, Flunitrazepam, 7-amino-flunitrazepam, Bromazepam, Oxazepam, Nitrazepam, Flurazepam, Temazepam, 7-amino-clonazepam, Lorazepam, Clonazepam, 2-hydroxy-ethyl-flurazepam, Estazolam, Alprazolam, Triazolam, Alpha-hydroxy-alprazolam, Alpha-hydroxy-triazolam

## Sample Preparation Procedure

### Format:

ISOLUTE® SLE+ 1mL Sample Volume column, part number 820-0140-C

### Sample Pre-treatment

To 1 mL of whole blood, add 10 µL of ISTD (total 100 ng/mL). Allow to equilibrate and add 1 mL of 1% ammonium hydroxide (aq). Vortex.

### Sample Loading

Load 750 µL of the pre-treated whole blood onto the column and apply a pulse of vacuum or positive pressure (3–5 seconds) to initiate flow. Allow the sample to absorb for 5 minutes

### Analyte Extraction

Apply dichloromethane\* (DCM, 2.5 mL) and allow to flow under gravity for 5 minutes. Collect in an appropriate glass tube.

Apply a second aliquot of DCM\* (2.5 mL) and allow to flow under gravity for 5 minutes. Apply vacuum or positive pressure (5–10 seconds) to pull through any remaining extraction solvent into the collection vessel.

\* Note that MTBE can be used as an alternative extraction solvent if a non-chlorinated option is required. MTBE also suitable for extraction of other analyte classes (amphetamines, barbiturates, opiates). If simultaneous extraction of cocaine and metabolites is required, DCM should be used as extraction solvent.

### Post Elution and Reconstitution

Evaporate the extract in a stream of air or nitrogen using a TurboVap® LV (40 °C, 20 to 40 L/min).

Reconstitute the extracts with ethyl acetate (250 µL) and vortex for 20 seconds before transferring to high recovery GC vials. Evaporate the extract in a stream of air or nitrogen using a SPE Dry (40 °C, 20 to 40 L/min).

Reconstitute extracts with ethyl acetate (50 µL) and MTBSTFA (with 1% t-BDMCS) (50 µL), vortex and heat on a block for 30 minutes at 70 °C to complete derivatization.

## GC Conditions

### Instrument

Agilent 7890A with QuickSwap

### Column

Agilent J&W DB-5, 30 m x 0.25 mm ID x 0.25 µm

### Carrier

Helium 1.2 mL/min (constant flow)

### Inlet

260 °C, Splitless, purge flow: 50 mL/min at 1.0 min

### Injection

1 µL

### Wash Solvents

Acetone & ethyl acetate

### Oven

Initial temperature 60 °C, hold for 1 minute, ramp 12 °C/min to 330 °C, hold for 0.5 minutes

### Post Run

Backflush for 1.6 minutes (2 void volumes)

### Transfer Line

280 °C

## MS Conditions

### Instrument

Agilent 5975C

### Source

230 °C

### Quadrupole

150 °C

### MSD mode

SIM

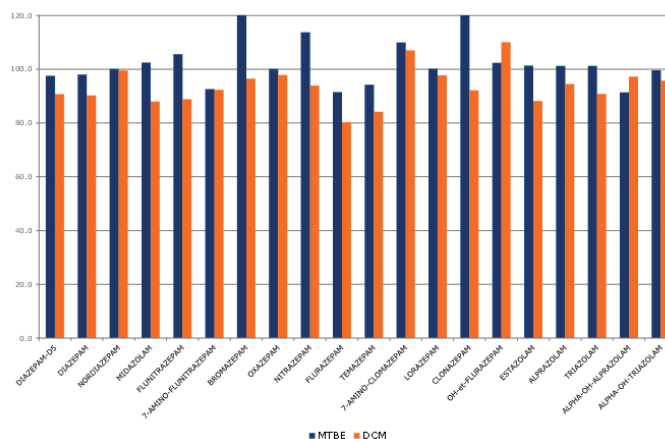
## SIM Parameters

**Table 1.** Ions acquired in the Selected Ion Monitoring (SIM) mode.

SIM Group	Analyte	Target (Quant) Ion	Qual Ion
1	Diazepam-D5	261	289
1	Diazepam	256	284
2	Nordiazepam	327	329
3	Midazolam	310	312
3	Flunitrazepam	312	285
3	7-amino-flunitrazepam	255	283
3	Bromazepam	372	374
4	Oxazepam	457	513
4	Nitrazepam	338	291
4	Flurazepam	86	99
5	Temazepam	357	359
6	7-amino-clonazepam	342	344
6	Lorazepam	491	513
6	Clonazepam	372	374
6	2- hydroxy-ethyl-flurazepam	389	390
6	Estazolam	294	205
7	Alprazolam	279	273
8	Triazolam	313	238
9	Alpha-hydroxy-alprazolam	381	383
10	Alpha-hydroxy-triazolam	415	417

## Results

Blank whole blood was spiked at 100 ng/mL for recovery testing; the typical recovery data is shown in **Figure 2**. Both MTBE and DCM protocols gave reproducible data with RSD values <10%.



**Figure 2.** Typical benzodiazepine recoveries using MTBE or DCM as extraction solvent.

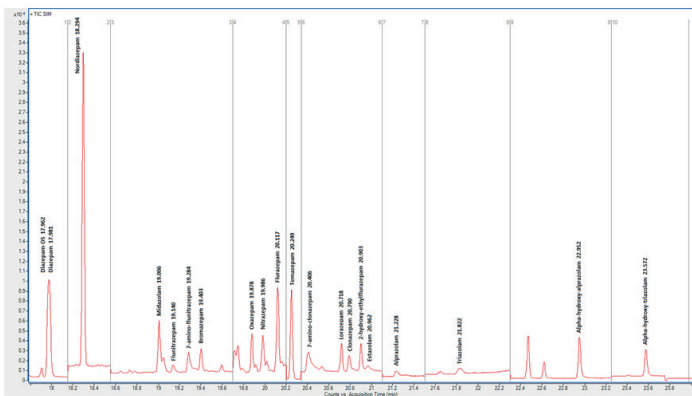


Figure 2. Total Ion Chromatogram of application analytes at 100 ng/mL using the DCM extraction protocol

### Calibration Curves

Whole blood was spiked prior to extraction at concentrations of 10, 20, 50, 75, 100, 200 and 500 ng/mL for each analyte to create calibration curves. Diazepam-D5 was spiked at 100 ng/mL for each level. The curves are shown in Figure 4.

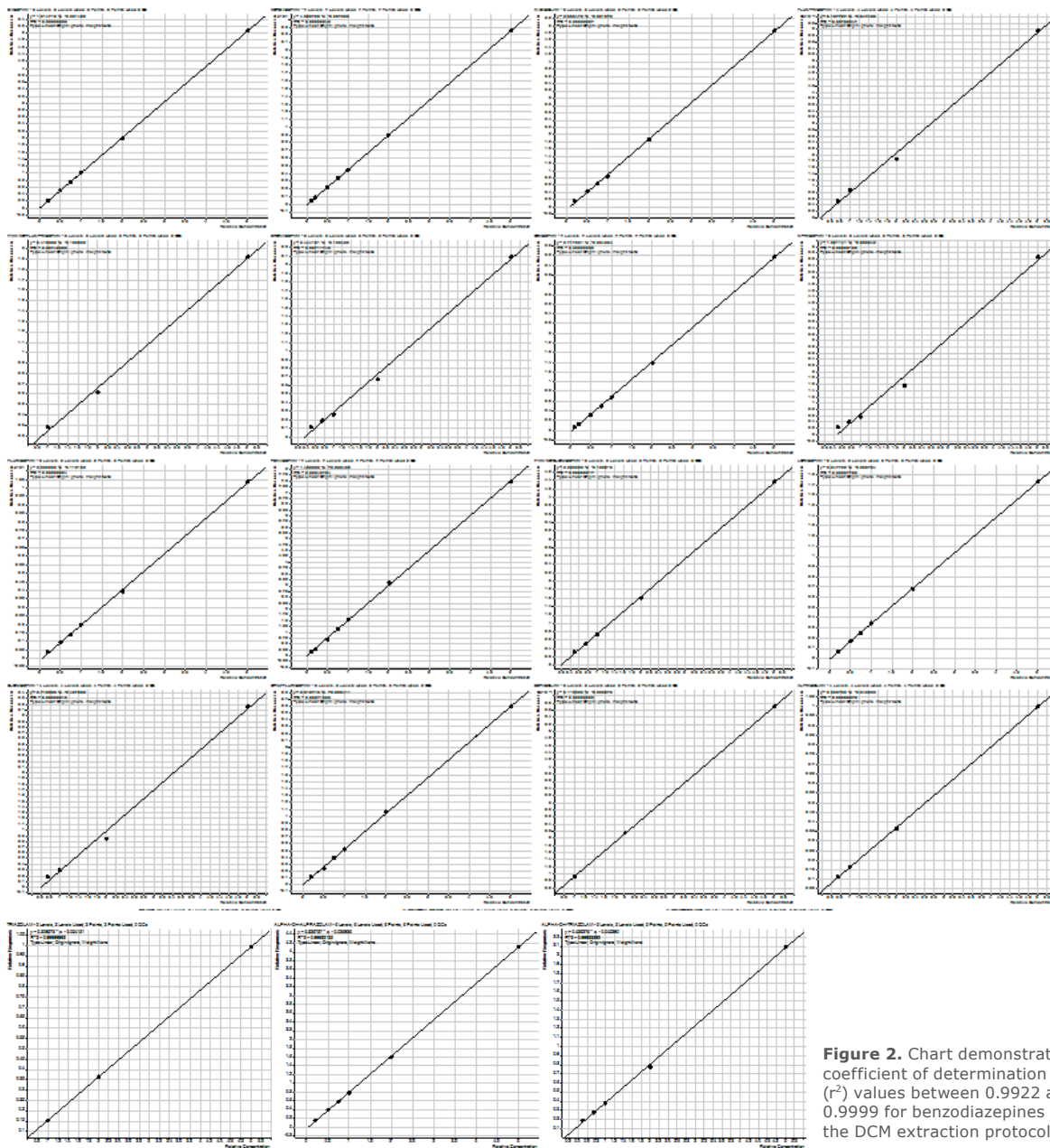


Figure 2. Chart demonstrating coefficient of determination ( $r^2$ ) values between 0.9922 and 0.9999 for benzodiazepines using the DCM extraction protocol.

**Table 3.**  
Lower Limits of Quantitation (LLOQ) using ISOLUTE® SLE+ procedure

Drug Analyte	LLOQ (ng/mL) DCM Extraction	LLOQ (ng/mL) MTBE Extraction
Diazepam	20	10
Nordiazepam	<10	<10
Midazolam	20	20
Flunitrazepam	75	50
7-amino-flunitrazepam	100	100
Bromazepam	50	50
Oxazepam	10	20
Nitrazepam	50	50
Flurazepam	20	20
Temazepam	10	20
7-amino-clonazepam	50	100
Lorazepam	20	20
Clonazepam	75	75
2-hydroxy-ethyl-flurazepam	20	50
Estazolam	100	100
Alprazolam	75	75
Triazolam	100	100
Alpha-hydroxy-alprazolam	20	20
Alpha-hydroxy-triazolam	50	50

## Additional Notes

### Solvents and reagent preparation:

- » All solvents were HPLC grade.
- » 1% ammonium hydroxide (aq): Add concentrated ammonium hydroxide (28–30%) (1 mL) to HPLC grade water (99 mL).

**Column loading:** ISOLUTE® SLE+ columns are underloaded (750 µL sample on a 1 mL capacity column) to avoid breakthrough of whole blood matrix.

**Non-chlorinated extraction solvent alternative:** MTBE can be used as an alternative extraction solvent if a non-chlorinated option is required. MTBE also suitable for extraction of other analyte classes (amphetamines, barbiturates, opiates). If simultaneous extraction of cocaine and metabolites is required, DCM should be used as extraction solvent.

## Ordering Information

Part Number	Description	Quantity
<b>820-0140-C</b>	ISOLUTE® SLE+ 1 mL Sample Volume Column*	30
<b>820-0140-CG</b>	ISOLUTE SLE+ 1 mL Sample Volume Column (tabless)	30
<b>PPM-48</b>	Biotage® PRESSURE+ 48 Positive Pressure Manifold	1
<b>SD-9600-DHS-EU</b>	Biotage® SPE Dry Sample Concentrator System 220/240 V	1
<b>SD-9600-DHS-NA</b>	Biotage® SPE Dry Sample Concentrator System 100/120 V	1

\*ISOLUTE SLE+ 1 mL Sample Volume columns are available in the tabless (or flangeless) format for compatibility with the Biotage® Extrahera™ and other sample processing platforms. Bulk packs are also available, visit [www.biotage.com](http://www.biotage.com) for further information.

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