Application Note: 20451

Analysis of Benzodiazepines in Serum

C .E. Blythe, S. Aspey, L. Pereira, D. Milton, R. Lewis, Thermo Fisher Scientific, Runcorn, UK

Key Words

- HyperSep SPE
- BETASIL
- Benzodiazepines
- Serum

This application note details a method for the extraction and isolation of benzodiazepines from a biological matrix (serum) and their subsequent HPLC analysis. Benzodiazepines are psychoactive drugs utilized primarily for their tranquilizing and sedative effects. The most notable and commonly recognized examples of the drug are diazepam and nitrazepam, marketed under the trade names of Valium® and Mogadon®, respectively.

Benzodiazepines can be divided into two classes, anxiolytics (used in treatment of anxiety disorders) and hypnotics (used to combat sleep disorders, e.g., insomnia). Both classes work similarly on the brain by inducing a calming effect. All benzodiazepines act by inhibiting the transmission of nerve signals in the brain and central nervous system by binding to gamma-aminobutyric acid (GABA) receptors, the most prolific inhibitory receptors. Benzodiazepines are also used as anticonvulsants, in treatment of muscular disorders and alcohol dependence, and increasingly in veterinary practice as an alternative to ketamine (which has many unwanted side effects).

Similar to barbiturates (formerly extensively used for treating anxiety and sleep disorders) the therapeutic use of benzodiazepines can result in side effects and dependency. Unlike barbiturates, however, benzodiazepines are relatively non-toxic and the risk of overdose is minimal.

The inherent properties of benzodiazepines can be exploited in less than desirable ways. For example, the drug can be used as a secondary drug of abuse when taken with euphoria inducing drugs, reducing the 'down'/drug withdrawal effect. More prevalent in recent years, benzodiazepines have been used as a date-rape drug where the chemical's amnesia and sedative properties are key.

In clinical settings, the concentration of benzodiazepines in biological matrices (blood, serum, urine etc.) is often monitored in order to determine the efficacy of therapeutic doses. Increasingly, for legal/criminal investigations, there is a requirement for robust and reliable separation methods for this class of drugs.

Psychoactive drugs are efficiently extracted from serum using a HyperSep™ C18 SPE (Solid Phase Extraction) column. The extraction method described here uses only water and methanol and does not require the time consuming preparation and pH modification of buffer solutions.

After SPE extraction, the separation of ten benzodiazepines was achieved using a simple three-solvent isocratic mobile phase method using a BETASIL™ Phenyl/Hexyl HPLC column. The BETASIL Phenyl/Hexyl chemistry facilitates mixed mode separation through a mixture of C6 alkyl chains together with phenyl groups. The phenyl groups provide alternative selectivity for moderately polar compounds whereas the C6 chains result in classical reverse phase retention.

Experimental Conditions

SPE

Compounds:	Benzodiazepines in horse serum (alprazolam, bromazepam, chlordiazepoxide, clobazam, clonazepam, diazepam, flunitrazepam, nitrazepam, nordiazepam, oxazepam)
Phase:	HyperSep C18
Volume:	3 mL
Bed Weight:	200 mg
Part Number:	60108-303
Vacuum:	5 mm Hg
Conditioning:	6 mL MeOH, 6 mL H_2O
Application:	1 mL Serum
Washing:	50 µL MeOH, 12 mL H_2O
Elution:	2 mL MeOH, 1 mL MeOH (x 2)

The sample was dried under nitrogen and re-dissolved in 1 mL LC mobile phase

LC

Column:	BETASIL Phenyl/Hexyl, 5 µm, 150 x 4.6 mm
Part Number:	73005-154630
Mobile Phase:	H_2O/ACN/MeOH, 65/32/3
Flow Rate:	1.5 mL/min
Temperature:	30 °C
Injection Volume:	10 µL
Detection:	UV @ 254 nm

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Results and Discussion

Figure 1 shows a typical trace for the separation of nine benzodiazepines (plus internal standard) using the experimental conditions described. Separation is achieved in less than fourteen minutes with all analytes possessing excellent peak shape (greatest asymmetry at 10% value recorded at 1.10 for chlordiazepoxide).

A series of experiments was performed in order to assess the suitability of the method for quantitative analysis. First, the linearity of response for the analytes was investigated. Figure 2 shows the calibration curve for the benzodiazepines over a concentration range of 0.5 to 100 µg/mL. The R^2 coefficient value for all analytes was > 0.995, indicative of a true linear response.

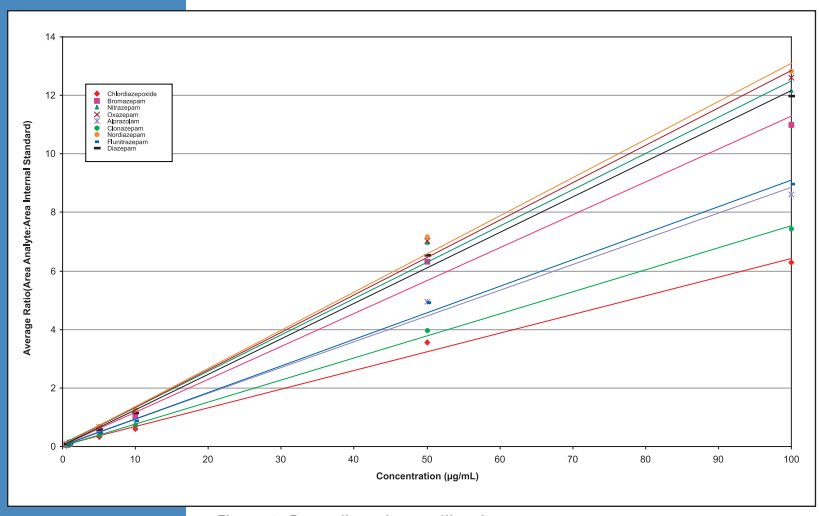

Figure 2: Benzodiazepines calibration curve

Analyte	Equation	R^2 Value
Chlordiazepoxide	y = 63.946x + 0.0395	0.9959
Bromazepam	y = 112.22x + 0.0659	0.9945
Nitrazepam	y = 124.05x + 0.0911	0.9953
Oxazepam	y = 127.9x + 0.0709	0.9970
Alprazolam	y = 87.955x + 0.0571	0.9949
Clonazepam	y = 75.169x + 0.0195	0.9988
Nordiazepam	y = 130.21x + 0.072	0.9987
Flunitrazepam	y = 90.687x + 0.0494	0.9978
Diazepam	y = 121.4x + 0.0392	0.9981

Data table for Figure 2

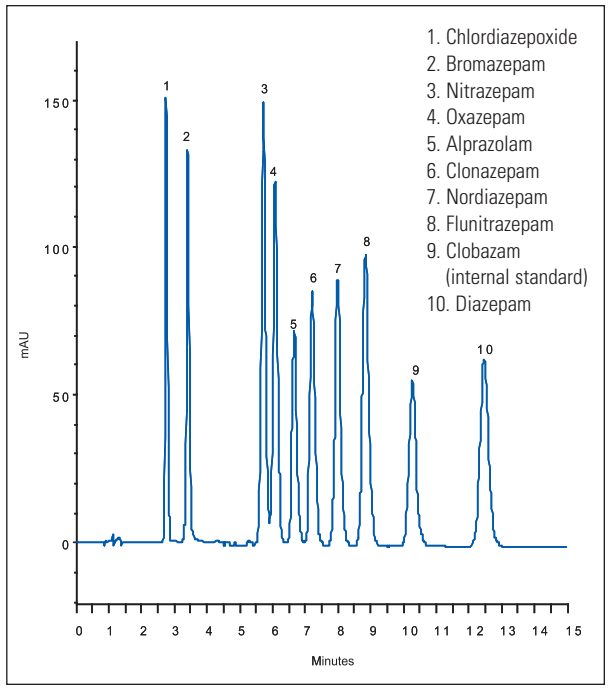

Figure 1: Benzodiazepines separation – analytes at 10 µg/mL

Second, the reproducibility of the method was investigated. Figures 3 to 5 highlight the excellent reproducibility of the separation in terms of peak area, retention time and ratio of response to the internal standard (clobazam). Over 20 injections (equating to 300 column volumes), the retention time reproducibility was found to be better than 0.6% RSD with the exception of the retention time reproducibility for the internal standard (1.3% RSD). The reproducibility of the peak area and ratio of peak area to internal standard was found to be less than 0.6% RSD for all analytes.

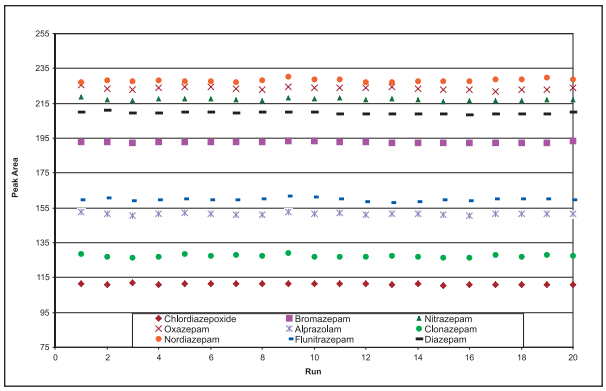

Figure 3: Peak area reproducibility

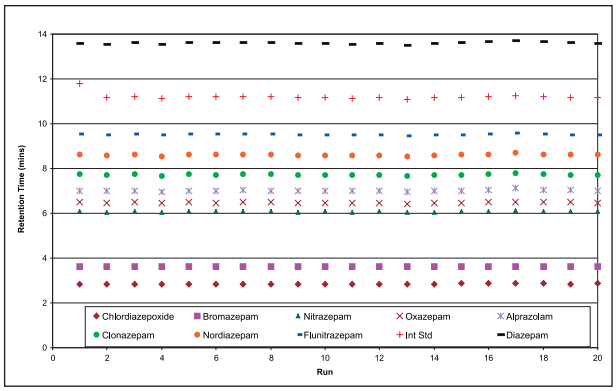

Figure 4: Retention time reproducibility

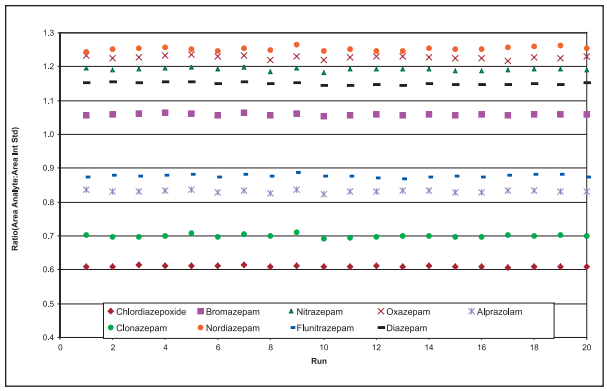

Figure 5: Reproducibility, ratio of area to internal standard

	Chlordiazepoxide	Bromazepam	Nitrazepam	Oxazepam	Alprazolam	Clonazepam	Nordiazepam	Flunitrazepam	Diazepam
Mean	111.2	192.8	217.1	223.6	151.5	127.5	228.2	159.8	209.5
Std Dev	0.3	0.4	0.6	0.8	0.5	0.7	0.9	0.9	0.7
RSD (%)	0.3	0.2	0.3	0.4	0.3	0.6	0.4	0.5	0.3

Data table for Figure 3

	Chlordiazepoxide	Bromazepam	Nitrazepam	Oxazepam	Alprazolam	Clonazepam	Nordiazepam	Flunitrazepam	Int Std	Diazepam
Mean	2.85	3.62	6.07	6.47	7.01	7.72	8.61	9.52	11.2	13.60
Std Dev	0.01	0.01	0.02	0.02	0.03	0.03	0.03	0.03	0.1	0.04
RSD (%)	0.2	0.2	0.3	0.3	0.4	0.4	0.4	0.3	1.3	0.3

Data table for Figure 4

	Chlordiazepoxide	Bromazepam	Nitrazepam	Oxazepam	Alprazolam	Clonazepam	Nordiazepam	Flunitrazepam	Diazepam
Mean	0.610	1.058	1.192	1.228	0.831	0.700	1.252	0.878	1.150
Std Dev	0.003	0.003	0.004	0.005	0.003	0.004	0.005	0.004	0.003
RSD (%)	0.4	0.3	0.4	0.4	0.4	0.6	0.4	0.5	0.3

Data table for Figure 5

Third, the method recovery was evaluated. Figure 6 shows the resultant chromatogram for all analytes after doping at 1 µg/mL and subsequent extraction from blank horse serum. Good recoveries were observed for all analytes, as detailed in Table 1, which shows the average recoveries for the analytes from serum over six runs at this concentration.

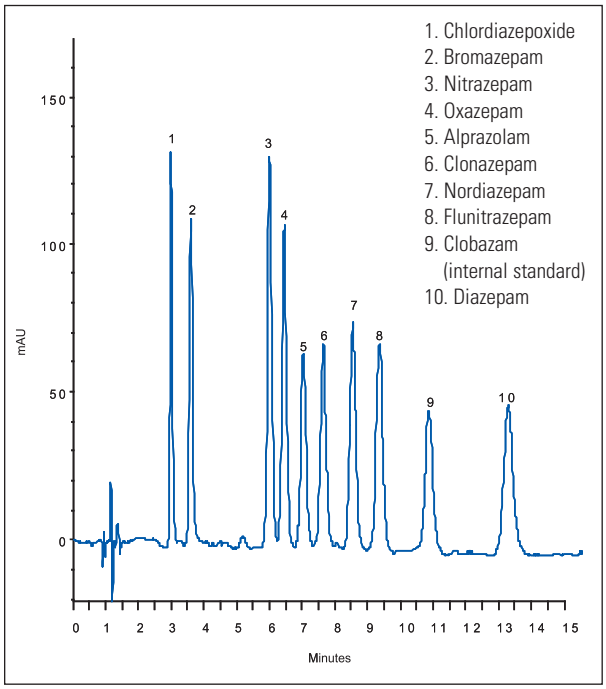

Figure 6: LC trace for recovery of analytes at 1 µg/mL

Analyte	Average Recovery (%)
Clobazam (internal standard)	92
Chlordiazepoxide	97
Bromazepam	91
Nitrazepam	89
Oxazepam	90
Alprazolam	90
Clonazepam	96
Nordiazepam	90
Flunitrazepam	87
Diazepam	100

Table 1: SPE recoveries at 1 µg/mL (average over six runs)

Conclusion

The suitability of the Thermo Scientific HyperSep C18 SPE columns and BETASIL Phenyl HPLC columns products for the extraction and subsequent analysis of psychoactive drugs has been demonstrated. The methods possess high reproducibility and linearity and necessitate only the use of simple solvent systems, thus reducing preparation time for the analyst.

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