

Simultaneously Analyze Benzodiazepines by HPLC

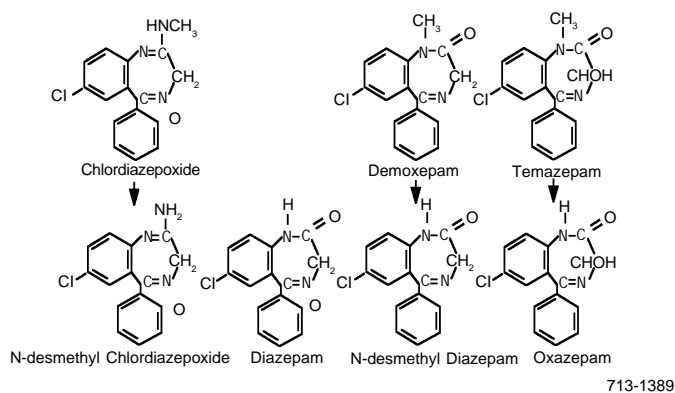
Benzodiazepines are encountered in a high percentage of drug screenings done in hospitals. These drugs are used widely to treat anxiety and insomnia. Patients must be monitored to evaluate compliance with a therapeutic regimen and to detect drug abuse. Chlordiazepoxide, diazepam, and five active metabolites often prescribed independently as anxiolytic drugs can be separated in only 12 minutes by HPLC. (ChromFax: 394013)

Key Words:

benzodiazepines • chlordiazepoxide • diazepam
• anxiolytic drugs

HPLC analyses for benzodiazepines are preferable to gas chromatography. GC analyses are complicated by the need to simultaneously quantify the ingested drug and several pharmacologically active metabolites (Figure A). Further complicating these analyses is the thermal instability of some of the compounds, particularly chlordiazepoxide and oxazepam.

Figure A. Chlordiazepoxide, Diazepam, and Pharmacologically Active Metabolites



The HPLC analysis described here includes chlordiazepoxide, diazepam, and five active metabolites prescribed independently as anxiolytic drugs. In Figure B, we separated these benzodiazepines and an internal standard (nitrazepam) on a 15cm x 4.6mm SUPELCOSIL™ LC-8 column. The separation took only 12 minutes.

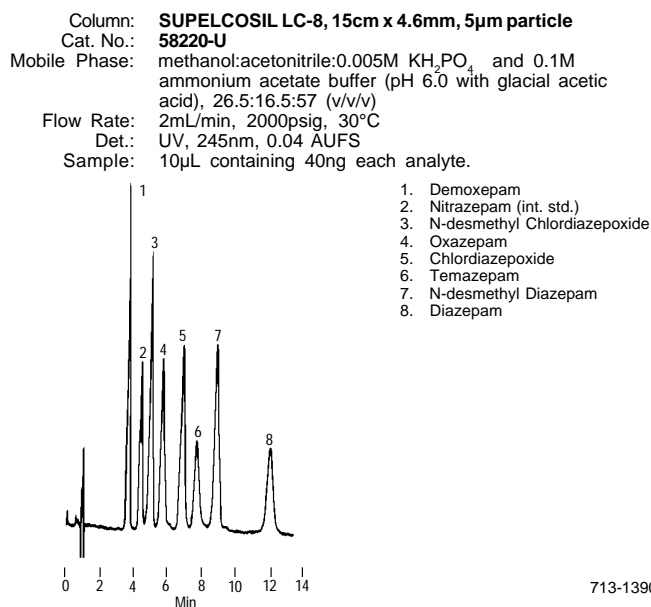
In this separation pH is not critical, since similar chromatograms can be obtained at pH 5 or 7. (At pH 3, however, chlordiazepoxide coelutes with oxazepam.) This procedure also eliminates the need for prolonged column equilibration, a characteristic of alternative analyses involving ion pairing reagents (1).

Separating benzodiazepines and their active metabolites is equivalent to monitoring seven separate anxiolytic drugs, and is thus unusual. As shown in Figure A, chlordiazepoxide is sequentially demethylated, deaminated, and reduced. Diazepam, on the other hand, may initially be either demethylated or hydroxylated. All of the metabolites are eventually converted to oxazepam. Although other investigators have separated similar combinations of benzodiazepines by HPLC (2-5) or GC (6), we believe this to be the first simultaneous separation of these seven drugs.

When separating benzodiazepines or other drugs in samples derived from physiological fluids, we recommend using a guard column to protect the analytical column.

Samples can be cleaned and concentrated by using 1mL Supelclean™ LC-18 solid phase extraction tubes. Using the following procedure, all eight benzodiazepines and metabolites

Figure B. Seven Benzodiazepines Analyzed Simultaneously



can be extracted from serum for the HPLC analysis, with average absolute recoveries ranging from 82% to 106%.

Condition a 1mL Supelclean LC-18 tube with 2mL of methanol, followed by 2mL of deionized water, then 500µL of 0.001M KOH in water. The packing should remain wet during conditioning. Interrupt the flow and add 500µL of deionized water, followed by 100µL of 5% ethanol in water, then 500µL of the serum sample. The order of addition is important to maximize recoveries. Resume flow and draw the sample mixture through the tube in a slow dropwise manner, using a Visiprep™ Solid Phase Extraction Vacuum Manifold. Wash the packing with 500µL of 0.01M H₃PO₄. This step should be completed as quickly as possible; a prolonged wash can reduce recoveries. Wash the packing with 1mL deionized water, then dry for 5-10 minutes under nitrogen, using a Visidry™ Drying attachment. Elute samples with 400µL of methanol, then evaporate the eluant to dryness under nitrogen. Reconstitute with 200µL of mobile phase prior to analysis.

Table 1. Benzodiazepines Recovered from Serum by Solid Phase Extraction

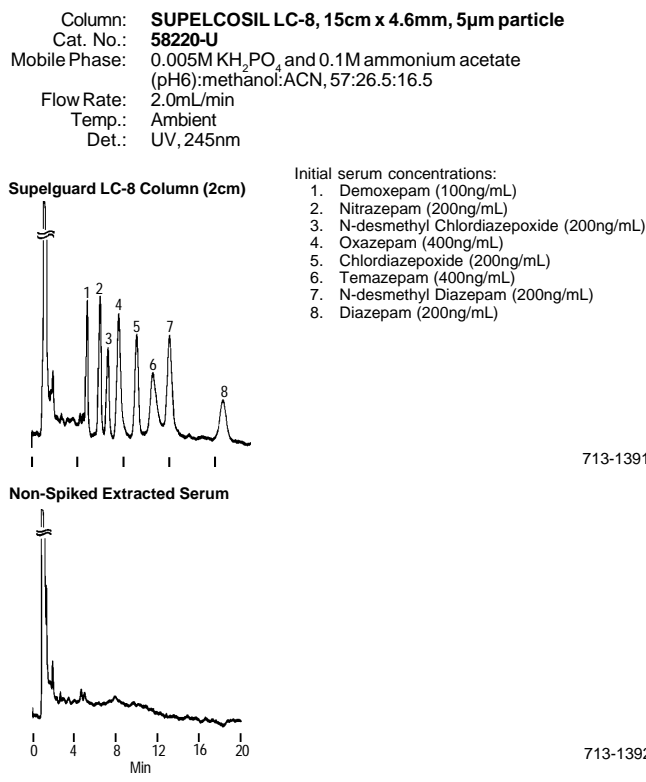
	Absolute Recovery (%)			Mean (%)	Coefficient of Variation
	1	2	3		
Demoxepam (100ng/mL)	92	91	90	91	1.1
Nitrazepam (200ng/mL)	107	105	104	105	1.5
N-desmethyl Chlordiazepoxide (200ng/mL)	97	93	95	95	2.1
Oxazepam (400ng/mL)	88	84	84	85	2.7
Chlordiazepoxide (200ng/mL)	98	96	98	97	1.2
Temazepam (400ng/mL)	83	81	86	83	3.0
N-desmethyl-Diazepam (200ng/mL)	108	104	106	106	1.9
Diazepam (200ng/mL)	88	88	93	90	3.2

This extraction provides clean samples with good recovery and reproducibility that can be analyzed with the HPLC system. Table 1 shows the recoveries at levels that might result from routine dosage administration. Minimum levels quantitated for chlordiazepoxide and diazepam were 100ng/mL. Figure C shows a representative extracted sample and a serum blank.

- SUPELCOSIL LC-8 Column, 15cm x 4.6mm **58220-U**
- SUPELCOSIL LC-8, 25cm x 4.6mm **58297**
- 2cm Supelguard™ LC-8 Columns, pk. of 2 (5µm packing) **59562**
- Supelguard LC-8 Kit **59052**
- 2cm Pelliguard™ LC-8 Columns, pk. of 4 (40µm packing) **58953**
- Pelliguard LC-8 Kit **59643**
- Supelclean LC-18 Solid Phase Extraction Tubes, 1ml, pk. of 100 **57002**
- Visiprep Solid Phase Extraction Vacuum Manifold **57030-U**
- Visidry Vacuum Manifold Drying Attachment **57100-U**

- US Pat. Nos. D.289,861; 4,810,471; other patents pending.
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Figure C. Benzodiazepines Recovered from Serum by Solid Phase Extraction



References

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