Application info 55

Automated HPLC analysis of Benzodiazepines in serum using On-line Solid Phase Extraction

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Introduction

Benzodiazepines (BZD) are widely prescribed and used for an array of medical and psychiatric maladies. In fact, benzodiazepines are the most frequently prescribed class of drugs for adults (i.e. Valium). The benefits of benzodiazepines as effective therapies are well documented. However, as with most drugs, benzodiazepines can produce toxic side effects when misused or abused by the patient. Consequently, knowledge of the benzodiazepines plasma levels is helpful in therapeutic drug monitoring and in the control of overdosed patients.

Several methods for BZD analysis in biological samples have been described in the literature, employing such diverse techniques as thin-layer chromatography (TLC), gas chromatography (GC) and high performance liquid chromatography (HPLC). TLC is a commonly used technique for the detection of drugs, but the detection systems lack sensitivity and selectivity. GC procedures require the conversion of BZD into benzophenones, or derivatization, or the use of capillary column chromatography. HPLC is the best way to determinate BZD in biological samples.

In this application note a method is described in which benzodiazepines can be determined directly in pure serum using on-line SPE-HPLC.

Experimental

Instrumentation

HPLC experiments were carried out with a Midas autosampler, a Prospekt on-line SPE unit and Varian 's HPLC system consists of a 9012 pump and a Varian 9050 UV detector. For data handling Varian's system STAR was used.

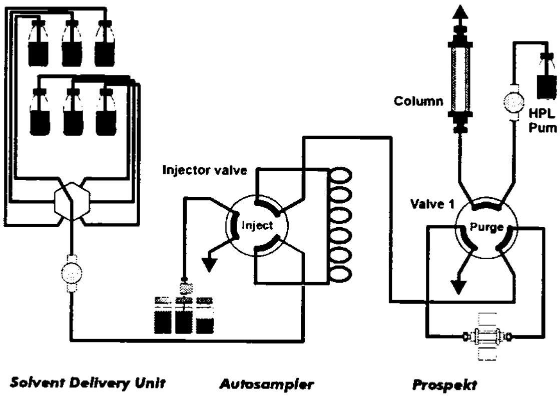

Figure 1 On-line SPE-HPLC system

Chromatographic conditions

Anal. Column:	*Varian RES ELUT-bd C8, 250 x 4.6 mm ID*
SPE-cartridge:	*C2 Analytichem 10 x 2 mm ID*
Mobile Phase:	*Methanol; Acetonitrile; 0.1M ammonium acetate pH = 6, (40:25:35 v/v%)*
Flow rate:	*1.2 ml/min*
Detection:	*240 nm UV*
Injection loop:	*50 µl (plasma)*

	SDU		**Prospekt**						
TIME H:MM:SS	Solv.	Purge flow mL/min	Change Cartridge F1: Execute	Valve 1 F1: Elute F2: Purge	Auxiliaries			End Time	Comment
					1	2	3		
0:00	1	3.0	F1	F2					Activation
1:00	2								Conditioning
2:00		0.5							Equilibrate
4:00					Off				Load/wash sample
6:30	0	0.0					On		Start analysis, start data
6:31							Off		
23:00						On			Start next injection Midas.
23:01					On	Off			
24:00								END	

Solvent 1: Methanol, Solvent 2: Water pH=9
AUX1: Freeze marker, AUX2: next injection marker, AUX3: data marker

Sample preparation program

- *Activate cartridge with 3 ml Methanol at flowrate 3 ml/min*
- *Condition cartridge with 3 ml H_2O pH = 9 (adjusted with NaOH)at flowrate 3 ml/min*
- *Equilibrate system with 1 ml H2O pH = 9 at flowrate 0.5 ml/min*
- *Inject 50 µl sample (flushed loop)*
- *Load sample/clean up sample with 1.5 ml H_2O pH = 9 at flowrate 0.5 ml/min*
- *Switch valve to elute position and start analysis*

Results and discussion

Results

During the development of the on-line SPE method various silica-bonded phases were investigated. C8 and C18 SPE phases give quantitative recovery but also retain impurities, which disturbed the chromatogram. Variations in the clean-up solvent and volume didn't give much improvement. Best results were obtained with a C2 phase, which sufficiently traps the benzodiazepines but does not retain the interfering compounds. Among the compounds that show no retention on C2 are barbiturates and caffeine, often present in human biological samples.

A chromatogram of a processed sample is shown in figure 2. It can be seen that clean up has been very effective.

To get a better separation between Temazepam and Lormetazepam 5% H2O was added to the mobile phase.

Multiple experiments were carried out with the developed assay in order to investigate its reproducibility, linearity, recovery and robustness. Analytical recoveries between 87 and 107 % were found (table 1) with RSD values between 0.4 and 6.6. The linearity of the assay was found to be good with correlation coefficients between 0.9998 and 0.9999.

Chromatogram

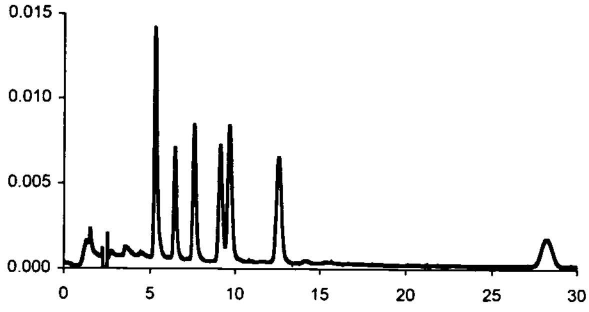

Figure 2: Chromatogram of a spiked serum sample (1mg/ml for each drug)

Table 1: Recoveries, reproducibility and linearity of Benzodiazepine (n = 5)

Compound	Recovery*	RSD	Corr. Coef.
Bromazepam	87 %	2.8 %	0.9998
Clonazepam	96 %	2.1 %	0.9998
Oxazepam	92 %	1.4 %	0.9999
Temazepam	101 %	0.4 %	0.9999
Lormetazepam	107 %	1.2 %	0.9999
Diazepam	105 5	2.1 %	0.9999
Medazepam	99 %	6.6 %	0.9999

**: Recoveries were derived from results obtained after on-line SPE-HPLC of standard solutions.*

In Table 1 the detection limits are shown for each component. The detection limit was based on a signal-to-noise ratio of 3:1. The sensitivity of each benzodiazepine can be increased when measurements are carried out with the components UV_{max} instead of 240 nm.

Table 2: Detection limits measured at 240 nm

Compound	LOD
Bromazepam	25 µg/L
Clonazepam	50 µg/L
Oxazepam	50 µg/L
Temazepam	50 µg/L
Lormetazepam	50 µg/L
Diazepam	50 µg/L
Medazepam	100 µg/L

Conclusions

- *A simple and fast on-line SPE-HPLC assay for benzodiazepines in serum has been developed.*
- *The recovery and the reproducibility of the assay are good.*
- *The assay is robust and is suited for routine analysis.*
- *The sample turn-over time is approximately 20 min.*

References

O. Halmingh,
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