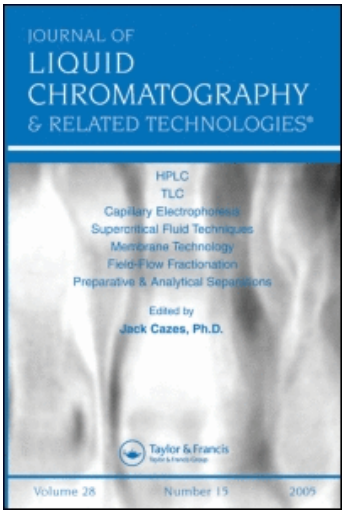

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APPLICATION OF HIGH PRESSURE LIQUID CHROMATOGRAPHY TO THE FORENSIC ANALYSIS OF SEVERAL BENZODIAZEPINES

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ABSTRACT

Eleven benzodiazepines are separated and measured by an HPLC adsorption system utilizing isocratic elution combined with dual wavelength detection at 254 nm and 280 nm. Powder and solid dosage forms of 10 benzodiazepines are simply ground and then extracted with chloroform and injected. Clorazepate dipotassium is not soluble in chloroform and is decarboxylated to N-desmethyldiazepam.

INTRODUCTION

The utility of HPLC in the analysis of the benzodiazepines is demonstrated by the variety of applications that have been published in the scientific literature. In one early study, Scott and Bommer (1) used bonded phase partition chromatography to analyze a pharmacological sample. Weber (2) used adsorption chromatography to analyze ketazolam, a benzodiazepine synthesized from diazepam. Rodgers (3) separated 4

benzodiazepines using adsorption chromatography. He also quantitatively analyzed Librium and Librax capsules. Greizerstein and Wojtowicz (4) used reverse phase chromatography in a metabolism study of chlordiazepoxide and its N-desmethyl metabolite in mouse blood. Zagar et al, (5) applied reverse phase chromatography to the analysis of chlordiazepoxide HCl and two related contaminants in bulk pharmaceutical powders. Perchalski and Wilder (6) used adsorption chromatography to analyze diazepam, clonazepam, and nordiazepam in plasma or whole blood. An internal standard was used. In a recent paper specifically concerning forensic applications, Noggle and Clark (7) described the analysis of 10 benzodiazepines. They discussed the ultraviolet absorption properties in acid and base for the 10 drugs and also provided infrared spectra in KBr. The benzodiazepines were so strongly retained in the first system described that diazepam, the eighth component, was not fully eluted in nearly one hour. A second solvent system was needed to elute two other, more strongly retained, benzodiazepines.

We present an isocratic adsorption system used in conjunction with the discriminating properties of dual wavelength ultraviolet (UV) detection to enable the forensic chemist to presumptively identify several

benzodiazepines. In addition, procedures for the quantitative analysis of the benzodiazepines are discussed.

EXPERIMENTAL

APPARATUS

A liquid chromatograph from Waters Associates, composed of an M6000A solvent delivery system, a UK6 injector, a M440 dual wavelength UV absorbance detector, and a 15 cm Micro Porasil column was used. This system was interfaced with a Perkin-Elmer M-1 computing integrator and a Houston Instruments Company strip chart recorder. The column eluate was passed first through the 254 nm detector and then through the 280 nm detector. The system was operated at ambient temperature with a flow rate of 2.0 ml per minute. The detector sensitivity was 0.1 AUFS and the recorder chart speed was 0.2 inch per minute.

Solvent System and Reagents

The solvent system was composed of 90 parts cyclohexane and 10 parts of the mixture NH_4OH-MeOH-W/W $CHCl_3$ (1 + 200 + 800). The chloroform was water washed and filtered through a medium sintered glass filter before use. Cyclohexane, chloroform, and methanol were obtained from Burdick and Jackson Laboratories, Muskegon, Michigan. All other reagents used were reagent grade. All drug standards were obtained from commercial sources.

Methodology

The simple extraction procedure used is applicable to powders and solid dosage forms of all benzodiazepines described except the dipotassium salt of clorazepate, which is insoluble in chloroform. An amount equivalent to 5 mg benzodiazepine, from a ground tablet, capsule, or composite, is accurately weighed into a 100 ml volumetric flask, and chloroform is added to volume, and an ultrasonic bath is used to help dissolve the benzodiazepine. Duplicate injections of the solution, free of any particulate material, are made and are then compared with duplicate injections of the appropriate benzodiazepine drug in chloroform at a concentration of 0.05 mg/ml to 0.10 mg/ml. Compounds are quantitated either by integrated peak areas or by peak height measurements.

Clorazepate can be quantitated after decarboxylation to N-desmethyldiazepam. An accurately weighed amount of ground powder, equivalent to approximately 5 mg clorazepate dipotassium, is transferred to a 125 ml separatory funnel, and 25 ml of 0.1 N HCl or 0.1 N H_2SO_4 is added. After about 10 minutes, the N-desmethyldiazepam is extracted with three 30 ml portions of chloroform. The extracts are filtered directly into a 100 ml volumetric flask, and chloroform is added to volume. A procedural standard of

clorazepate dipotassium is prepared either by weighing 5 mg on a micro balance or by dilution from a stock solution in water at pH 8. The standard is transferred to a 125 ml separatory funnel and extracted as above. The sample solution and the procedural standard are then chromatographed and quantitated as previously described.

RESULTS AND DISCUSSION

Table 1 shows the retention data for 11 benzodiazepines, expressed relative to flurazepam, and also the UV absorbance ratios, 254 nm/280 nm, for each drug. Baker et al, (8) assessed the extra discriminatory power of absorbance ratios used in conjunction with relative retention. Cyprazepam and diazepam are not resolved by the chromatography, but they have widely different absorbance ratios. Thus, if only one compound is present it could be presumptively identified. If both were present one would not be able to determine the identity of the substance(s), because the altered absorbance ratio would not correspond to either cyprazepam or diazepam.

Figure 1 shows the chromatogram of a synthetic mixture of 11 benzodiazepines at 254 nm. The structural formulas of those 11 benzodiazepines plus clorazepate dipotassium are shown in Figure 2. The compounds that have absorbance ratios less than 2

TABLE 1

Retention Expressed Relative to Flurazepam.
Retention Time of Flurazepam = 308 seconds.
Absorbance Ratios 254 nm/280 nm.

	Benzodiazepine	RRT	A254/A280
1.	Medazepam	0.40	3.77
2.	Prazepam	0.53	4.47
3.	Cyprazepam	0.61	1.15
4.	Diazepam	0.62	4.62
5.	Flurazepam	1.00	4.97
6.	Chlordiazepoxide	1.14	1.32
7.	N-Desmethyldiazepam	1.19	4.58
8.	Nitrazepam	2.07	1.38
9.	Clonazepam	2.57	1.79
10.	Demoxapam	4.18	2.98
11.	Oxazepam	4.71	4.20

either have an oxide at N-4 or they are 7-nitro instead of 7-chloro. All of the 2-ones have absorbance ratios near 4.5 or higher. Demoxapam is interesting because it possesses both a 2-one and a 4-N oxide moiety and the resultant absorbance ratio of 2.98 is near the middle of the absorbance range of the 4-N oxide and the 2-one benzodiazepines.

Quantitative Analysis

Standard solutions of diazepam were prepared in chloroform in the concentration range of 0.020-0.098 mg/ml, and 5.0 microliter aliquots were injected. The response was linear at 0.1 AUFS over that range. Ten 5.0 microliter injections of the 0.080mg/ml standard gave a standard deviation of 1.9 mm and a

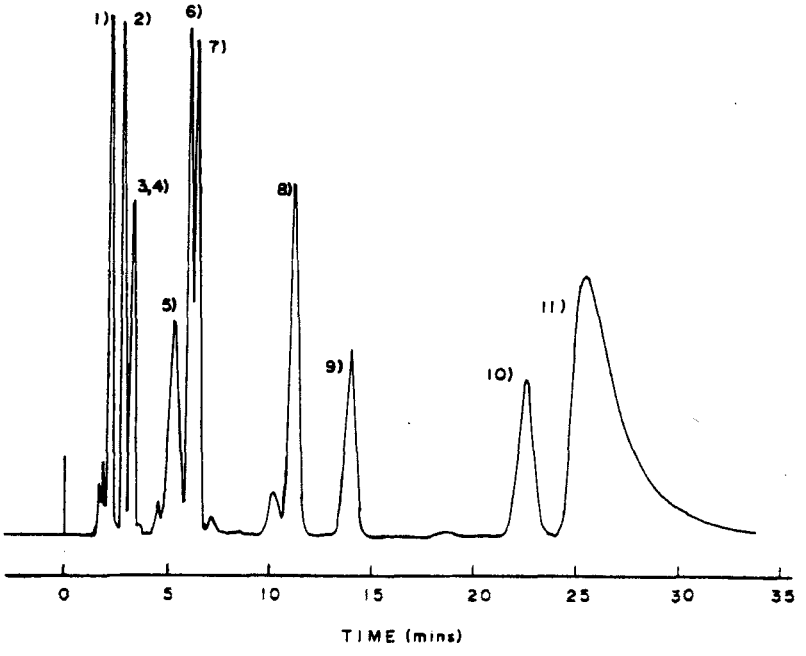

FIGURE 1. Chromatogram of a mixture of 11 benzodiazepines determined at 254 nm wavelength. 1 = Medazepam; 2 = Prazepam; 3 = Cyprazepam; 4 = Diazepam; 5 = Flurazepam; 6 = Chlordiazepoxide; 7 = N-Desmethyldiazepam; 8 = Nitrazepam; 9 = Clonazepam; 10 = Demoxapam; 11 = Oxazepam.

coefficient of variation of 1.7% for peak height measurements and a standard deviation of 346 and a coefficient of variation of 0.49% for peak area measurements (See Table 2).

The application of the extraction procedure is shown in the analysis of a Valium (diazepam) tablet. Figure 3 shows chromatograms of the sample and standard diazepam injections. The results of duplicate analyses

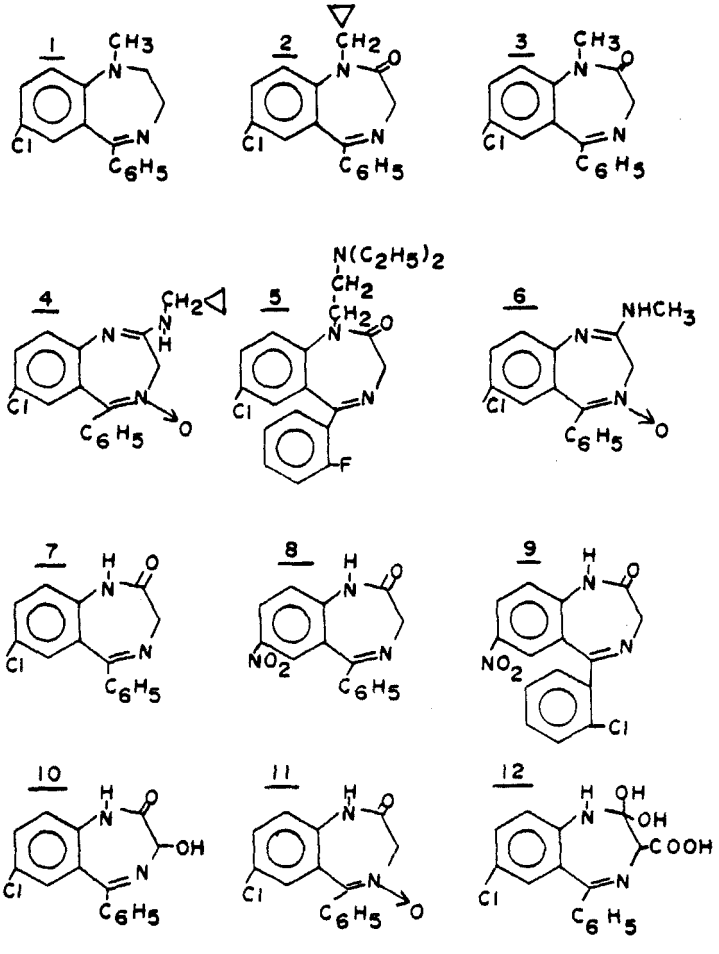

FIGURE 2. Structural Formulas. 1 = Medazepam; 2 = Prazepam; 3 = Diazepam; 4 = Cyprazepam; 5 = Flurazepam; 6 - Chlordiazepoxide; 7 = N-Desmethyldiazepam; 8 = Nitrazepam; 9 = Clonazepam; 10 = Demoxapam; 11 = Oxazepam; 12 = Clorazepate.

TABLE 2

Precision of 10 Replicate Injections of 0.40 microgram Diazepam.

Injection	Area	Peak Height (mm)
1	69702	105
2	69712	111
3	69645	109
4	70101	108
5	69635	109
6	70378	111
7	70437	110
8	70166	111
9	70213	109
10	70506	108
Av.	70050	109
S. D.	346	1.9
C. V. (%)	0.49	1.7

were 5.01 and 5.02 mg/tablet by area integration and 5.02 and 4.96 by peak height measurement.

With the exception of oxazepam which, as shown in Figure 1, tails badly, the other benzodiazepines give symmetrical peaks, so peak area and peak height measurements should be equally suitable.

Clorazepate dipotassium has limited solubility in organic solvents, so it cannot be analyzed by the HPLC system described. Noggle and Clark (7) discuss the decarboxylation of clorazepate to N-desmethyldiazepam at pH less than 8. To avoid the possibility of decarboxylation during chromatography, they adjusted the pH of the reverse phase system to about 8.0. They

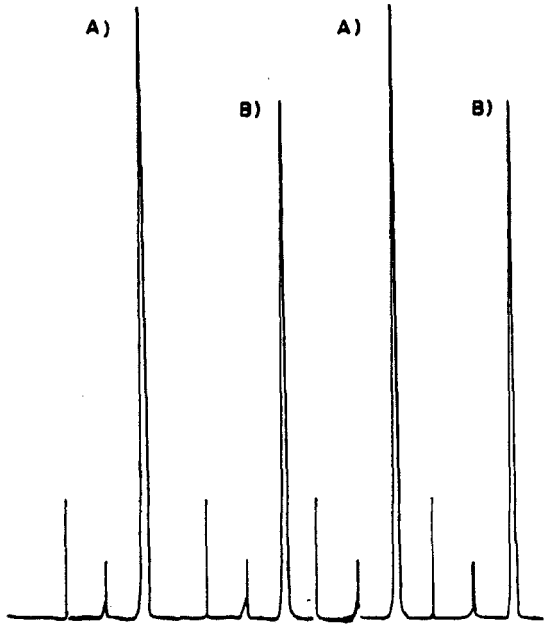

FIGURE 3. Analysis of a Valium tablet. A = Diazepam standard; B = Valium tablet.

report complete decarboxylation at pH 4.6 within 30 minutes.

To determine recovery of N-desmethyldiazepam 10 ml aliquots from a stock solution in chloroform of about 0.5 mg N-desmethyldiazepam/ml were transferred to separatory funnels. An additional 20 ml of chloroform and either 25 ml 0.1N HCl, 25 ml 0.1N H_2SO_4, or 50 ml 0.1N HCl were added. The N-desmethyldiazepam was extracted with a total of three 30 ml portions of chloroform. The extracts were filtered directly into

a 100 ml volumetric flask and chloroform was added to volume. These solutions were chromatographed along with a standard solution of N-desmethyldiazepam and the per cent recoveries were calculated. The partition of N-desmethyldiazepam between 0.1N acid and chloroform, using equal volumes of acid and solvent, was roughly determined to be about 70-75% with a single extraction. Table 3 shows that the recovery of N-desmethyldiazepam, using 3 extractions of 30 ml chloroform, is about the same for 0.1N H_2SO_4 or HCl. When the volume of acid was doubled to 50 ml and the volume of chloroform held to 30 ml, the partitioning was less favorable and the recovery was lower. The use of a procedural standard under these conditions would be a necessity.

TABLE 3

Recoveries of Standard N-Desmethyldiazepam

	25 ml 0.1N H_2SO_4 3-30 ml $CHCl_3$	25 ml 0.1N HCl 3-30 ml $CHCl_3$	50 ml 0.1N HCl 3-30 ml $CHCl_3$
1	95.3%	95.4%	90.9%
2	95.9%	95.6%	
3	95.6%		
Av.	95.6%	95.5%	90.9%

CONCLUSION

The chromatographic system coupled with the UV absorbance ratios is a useful qualitative tool for the presumptive identification of benzodiazepines. The analysis of the chloroform-soluble benzodiazepines was demonstrated and a novel approach to the analysis of clorazepate dipotassium was proposed.

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