High-Performance Liquid Chromatography of Benzodiazepines I: Stability-Indicating Assay of Diazepam Tablets

MARGARET EMERY ˣ **and JUNE KOWTKO**

Received January 17, 1979, from the *Quality Control Department, Hoffmann-La Roche Inc., Nutley, NJ 07110.* Accepted for publication March 20, 1979.

Abstract □ A high-performance liquid chromatographic procedure was developed for diazepam tablet analysis. This procedure separates the manufacturing intermediate, 7-chloro-1,3-dihydro-5-phenyl-2*H*-1,4-benzodiazepin-2-one, and the decomposition products, 3-amino-6-chloro-1-methyl-4-phenylcarbostyril and 2-methylamino-5-chlorobenzophenone, from diazepam. The liquid chromatographic procedure is superior to the USP UV assay because it is stability indicating. The method is simple, accurate, and fast, involving only one extraction step. A reversed-phase column and a methanol–water mobile phase were used. Under these conditions, 0.1% of the decomposition products was detectable. Typical samples of diazepam tablets showed no degradation.

Keyphrases □ Diazepam—analysis, high-performance liquid chromatography, stability, tablets □ High-performance liquid chromatography—analysis, diazepam tablets, stability indicating □ Sedatives—diazepam, high-performance liquid chromatographic analysis, tablets, stability indicating

1,4-Benzodiazepines are widely used as tranquilizers and muscle relaxants, and this wide use has prompted extensive literature on the analysis of these compounds. Diazepam[1] (I) is no exception. Most of the published literature on the use of high-performance liquid chromatography (HPLC) for I analysis describes methods for the quantitation and identification of I and its metabolites in biological samples for both pharmacological and clinical applications (1–5). Several liquid chromatographic separations of a benzodiazepine series including I (6–8) and of I from drugs of abuse (9, 10) have been reported. Liquid chromatography has also been used to quantitate I as a ketazolam decomposition product (11).

The current compendial (12) procedure for I analysis in tablets is a spectrophotometric method. In this procedure, the tablet mass is extracted three times with chloroform. The extracts are combined, brought to volume, and washed with a borate buffer solution. The chloroform layer is passed through anhydrous sodium sulfate; an aliquot is taken, evaporated to dryness, and dissolved in acidic alcohol. The absorbance of this solution is measured and compared to that of a USP standard.

This report describes the development of an HPLC procedure for the analysis of I in tablets. The liquid chromatographic procedure separates I from its manufacturing precursor, 7-chloro-1,3-dihydro-5-phenyl-2*H*-1,4-benzodiazepin-2-one (II), and the decomposition products 3-amino-6-chloro-1-methyl-4-phenylcarbostyril (III) and 2-methylamino-5-chlorobenzophenone (IV) and is both specific and stability indicating.

The sample workup is minimal, involving the addition of an internal standard to the weighed tablet mass, extraction of the active compound into methanol, and injection into the liquid chromatograph.

Table I—Retention Volume (Milliliters)

Compound	Water–Methanol 350:650	Water–Methanol 400:600	Water–Acetonitrile 350:650	Water–Acetonitrile 400:600	Water with 0.1% NH_4CO_3–Methanol, 300:700
I	8.3	11.7	5.7	6.6	8.3
II	7.4	9.8	4.8	5.3	7.3
III	14.8	23.3	7.6	9.1	13.6
IV	19.1	31.3	9.6	12.4	14.6

EXPERIMENTAL

Instrumental Conditions—A high-performance liquid chromatograph equipped with a 254-nm UV detector[2] and a reversed-phase C_{18} column[3] was used. Three-microliter injections were made with a 5-μl high-pressure syringe[4]. The flow rate was set at 0.5–0.6 ml/min, which

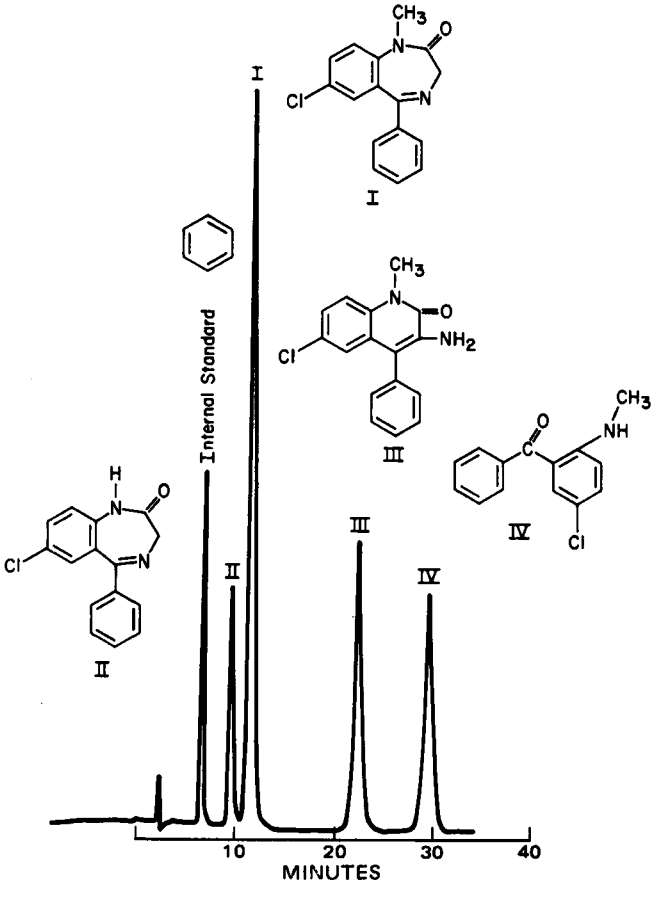

Figure 1—*Chromatogram of a synthetic mixture of the internal standard and I–IV.*

[1] Valium, Hoffmann-La Roche Inc.

[2] Model 830, du Pont Instrument Co., Wilmington, Del.
[3] μBondapak C_{18}, Waters Associates, Milford, Mass.
[4] Model HP305N, Hamilton Co., Reno, Nev.

0022-3549/79/0900-1185$01.00/0

Table II—Second-Day Reproducibility Data

	2-mg Tablets		5-mg Tablets		10-mg Tablets	
	Day 1, mg/ tablet	Day 2, mg/ tablet	Day 1, mg/ tablet	Day 2, mg/ tablet	Day 1, mg/ tablet	Day 2, mg/ tablet
Mean	2.01	2.02	4.97	4.99	10.01	10.08
SD	0.02	0.03	0.05	0.04	0.03	0.1
CV, %	1.3	1.3	0.9	0.7	0.3	1.0
USP	2.00		4.95		10.1	

required pressures of 600–1000 psi, depending on the age of the column. The column was at ambient temperature, the electrometer setting was 8, and the recorder chart speed was 3.9 min/cm. Quantitation was by peak height measurement.

Mobile Phase—Various mixtures of water and methanol or water and acetonitrile were tried to optimize the separation. The quantitative results were obtained with a 65:35 methanol–water mobile phase.

Internal Standard—The internal standard stock solution was prepared by pipetting 25 ml of benzene into a 1000-ml volumetric flask and bringing to volume with methanol.

Standard Diazepam Solutions—The standard stock solution was prepared by accurately weighing ~100 mg of I into a 100-ml volumetric flask and diluting to volume with methanol. The standard for the 2-mg diazepam tablet was made by pipetting 2 ml of the standard stock solution and 2 ml of the internal standard solution into a 10-ml volumetric flask and diluting to volume with methanol. The standard solution for the 5-mg diazepam tablets was prepared by pipetting 5 ml of the standard stock solution and 5 ml of the internal standard solution into a 25-ml volumetric flask and diluting to volume with methanol. For the standard solution for the 10-mg diazepam tablets, 10 ml of the standard stock solution and 10 ml of the internal standard solution were pipetted into a 50-ml volumetric flask and diluted with methanol to volume.

Preparation of Sample Solutions of Diazepam Tablets—*2-mg Tablets*—Approximately 170 mg of ground tablet mass was weighed accurately into a 10-ml volumetric flask, and 2 ml of internal standard solution was added. The flask was half filled with methanol and shaken for 0.5 hr on a wrist-action shaker. The contents were brought to volume with methanol, shaken, transferred to a plastic syringe, and filtered without vacuum through a 0.6-μm polyvinyl chloride filter[5].

5-mg Tablets—Approximately 170 mg of ground tablet mass was weighed accurately into a 25-ml volumetric flask, 5 ml of internal standard solution was added, and the procedure for the 2-mg tablets was followed.

10-mg Tablets—Approximately 170 mg of ground tablet mass was weighed accurately into a 50-ml volumetric flask, 10 ml of internal standard solution was added, and the procedure for the 2-mg tablets was followed.

RESULTS AND DISCUSSION

Several mobile phases can be employed to obtain the desired separation. Mixtures of water–acetonitrile, water–methanol, and methanol–water with 0.1% ammonium carbonate were tried. Table I gives the retention volumes of the four experimental compounds with the different mobile phases.

A 350:650 mixture of water–methanol was chosen because the analysis time was reasonable and the solvents are readily available. The preliminary work was done with a mobile phase of water with 0.1% ammonium carbonate and methanol. However, since it was thought that the ammonium carbonate might contribute to column deterioration and since the ammonium carbonate was not necessary, a change was made to water–methanol.

Figure 1 shows the separation of a synthetic mixture of benzene, II, I, III, and IV. Benzene is used as the internal standard for the quantitative work, II is the manufacturing intermediate, and III and IV are the I decomposition products.

Quantitative Determination of Diazepam—All variables in the quantitative procedure were validated to ensure that errors could not be introduced inadvertently. The linearity of peak height ratios of I *versus* two possible internal standards, benzene and *p*-xylene, was verified over the range of possible concentrations of I. Benzene was the internal standard used for the quantitative work. However, *p*-xylene elutes just after I and before II and is an alternative.

[5] Type BD filter, Millipore Corp., Bedford, Mass.

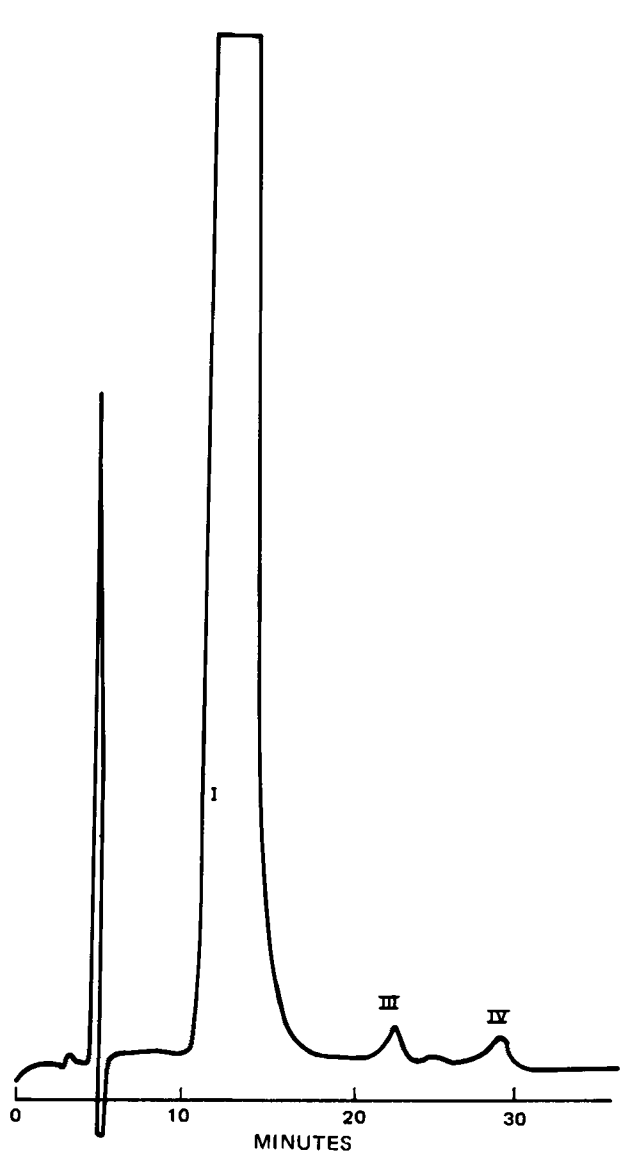

Figure 2—*Chromatogram of a synthetic mixture representing 0.1% decomposition of I to III and IV.*

The linear relationship of the amount of I *versus* sample weight was verified also. These measurements, made in triplicate, show that the method is valid for a large range of sample sizes.

The required extraction time was determined also. Samples of 2-mg tablets were analyzed in triplicate using 10, 20, 30, and 40 min on the shaker. There was no difference within the experimental error for samples shaken for different times. This finding indicates that the timing of the shaking is not critical. The results of a 2-day reproducibility study for each strength tablet as well as the USP spectrophotometric method are given in Table II. The mean value is for 10 samples.

Determination of Degradation Products—A synthetic mixture of I, III, and IV representing the analysis of a diazepam tablet with 0.1% decomposition is shown in Fig. 2. This figure indicates that if the decomposition products were present, the limit of detection would be 0.1%. However, expired manufactured lots of diazepam tablets were analyzed and no decomposition was detected.

REFERENCES

(1) A. Bugge, *J. Chromatogr.*, **128**, 111 (1976).

(2) M. D. Osselton, M. D. Hammond, and P. J. Twitchett, *J. Chromatogr. Pharmacol.*, **29**, 460 (1977).

(3) P. M. Kabra, G. L. Stevens, and L. J. Marton, *J. Chromatogr.*, **150**, 355 (1978).

(4) R. R. Brodie, L. F. Chasseaud, and T. Taylor, *ibid.*, **150**, 361 (1978).

(5) R. J. Perchalski and B. J. Wilder, *Anal. Chem.*, **50**, 554 (1978).
(6) C. G. Scott and P. Bommer, *J. Chromatogr. Sci.*, **8**, 446 (1970).
(7) D. H. Rogers, *ibid.*, **12**, 742 (1974).
(8) K. Macek and V. Rehak, *J. Chromatogr.*, **105**, 182 (1975).
(9) M. L. Chan, C. Whetsell, and J. D. McChesney, *J. Chromatogr. Sci.*, **12**, 512 (1974).
(10) I. Jane, *J. Chromatogr.*, **111**, 227 (1975).
(11) D. J. Weber, *J. Pharm. Sci.*, **61**, 1797 (1972).
(12) "The United States Pharmacopeia," 19th rev., Mack Publishing Co., Easton, Pa., 1975.

ACKNOWLEDGMENTS

Presented in part at the Pharmaceutical Analysis and Control Section, APhA Academy of Pharmaceutical Sciences, Orlando meeting, November 1976.

The authors thank Dr. S. A. Moros for many helpful discussions.

Quantitative Flash-Methylation Analysis of Phenobarbital

KOUICHI KURATA ˣ, MASASHI TAKEUCHI, and KEIKO YOSHIDA

Received October 18, 1978, from the *Department of Neuropsychiatry, School of Medicine, Kanazawa University, 13-1 Takara-Machi, Kanazawa 920, Japan.* Accepted for publication February 26, 1979.

Abstract □ In phenobarbital measurement by GLC with the flash-methylation technique, using trimethylanilinium hydroxide as a methylating reagent, a small amount of water decomposed phenobarbital and interfered with the quantitative analysis. Thus, both the sample and the methylating reagent must be sufficiently dehydrated to attain quantitative analyses. The hydrolysis decomposition product of phenobarbital was *N*-methyl-2-phenylbutyramide, as shown by its mass spectrum. The sum of methylated phenobarbital and *N*-methyl-2-phenylbutyramide (if observed in the spectrum) can be used for an accurate phenobarbital assay in the present flash-methylation technique.

Keyphrases □ Phenobarbital—analysis, flash-methylation GLC, degradates □ GLC, flash methylation—analysis, phenobarbital in biological fluids, degradates □ Mass spectrometry—analysis, phenobarbital in biological fluids, degradates

GLC determination of anticonvulsant drugs in biological fluids has been widely adopted in clinical laboratories. Phenytoin and phenobarbital have usually been assayed with the flash-methylation technique, using trimethylanilinium hydroxide as a methylating reagent. Some authors (1, 2) described the common errors in the assay of phenobarbital due to its decomposition by hydrolysis, and the GLC peak derived from the decomposition product has been called "early phenobarbital" (3); its structure has been speculated to be *N*-methyl-2-phenylbutyramide (III) (4, 5).

In the present work, the authors identified the decomposition product as III by mass spectrometry and found that its formation was due to the basic phenobarbital hydrolysis caused in pretreating the sample with basic methylation reagent and the subsequent methylation of the resulting 2-phenylbutyramide (I) in the GLC unit. Several problems in the assay of the anticonvulsant drugs by flash methylation are discussed here also.

EXPERIMENTAL

Methylation—Various concentrations of trimethylanilinium hydroxide in methanol were prepared as described previously (6) and stored at 4°. A 10-μg sample of phenobarbital was dissolved in 30 μl of trimethylanilinium hydroxide–methanol solution (the reagent must be used within 2 weeks of preparation), and the mixture was kept standing for 5 min. Each 3-μl sample obtained was then subjected to GLC.

GLC—The gas–liquid chromatograph[1] was equipped with a dual flame-ionization detector and columns for linear temperature programming. The glass columns, 200 cm long × 4.0 mm i.d., were packed with an equal amount of either 1.5% QF-1 on 60–80-mesh Chromosorb W or 3% OV-17 on 80–100-mesh Shimalite[2]. The analysis was performed under the following conditions for 1.5% QF-1: injection temperature, 245°; detector temperature, 245°; and column temperature, initial 150° and programmed to 215° at 6°/min. For 3% OV-17, the injection temperature

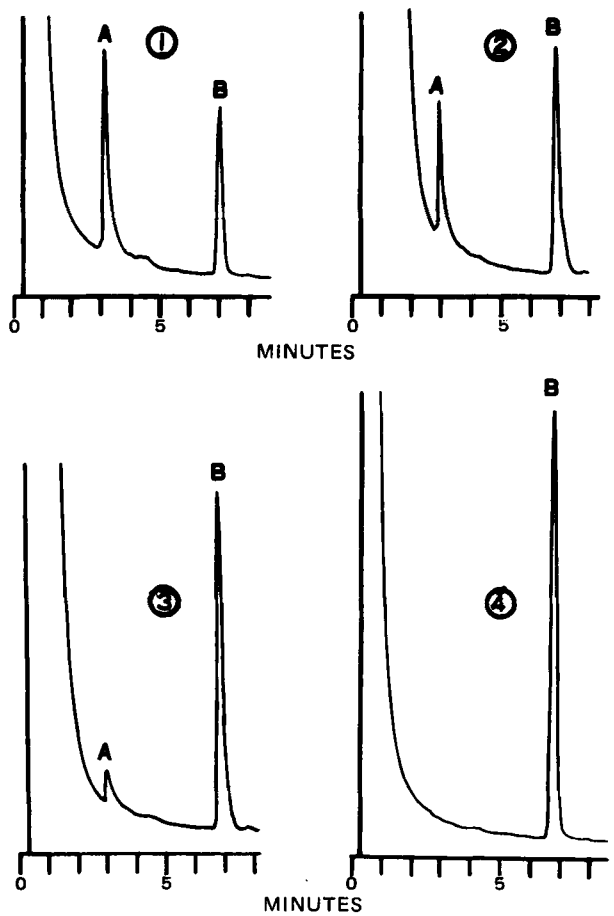

Figure 1—*Gas chromatograms of phenobarbital treated with various trimethylanilinium hydroxide concentrations. Phenobarbital was analyzed with 1.5% QF-1 in 0.20 (①), 0.10 (②), 0.05 (③), and 0.025 (④) M trimethylanilinium hydroxide solution. A 10-μg sample of phenobarbital was dissolved in 30 μl of trimethylanilinium hydroxide solution; after 5 min, a 3-μl sample was injected into the chromatograph.*

[1] Shimadzu GC-4CMPF, Shimadzu Ltd., Japan.

[2] Wako Chemical Industries Ltd., Japan.

0022-3549/79/0900-1187$01.00/0