oroacetyldesmethyldoxepin, and *N*-trifluoroacetylnortriptyline at *m/e* 280, 278, 362, and 360, respectively, were monitored.

Figure 4 shows the analysis in which the $[MH]^+$ ion and the $[MH - CF_3CONHCH_3]^+$ fragment ion of *N*-trifluoroacetyldesmethyldoxepin and *N*-trifluoroacetylnortriptyline at *m/e* 362, 360, 235, and 233 were monitored. The ratios of the $[MH]^+$ and $[MH - CF_3CONHCH_3]^+$ selected ion traces obtained from the analysis of a biological sample (Fig. 4) were 0.82 and 0.87 for the *N*-trifluoroacetyl derivatives of *cis*- and *trans*-desmethyldoxepin, respectively. These values are in close agreement with the theoretical values of 0.68 and 0.78 obtained from the mass spectra, which were recorded under the same experimental conditions (Table I), confirming as such the presence of the *N*-trifluoroacetyl derivatives of *cis*- and *trans*-desmethyldoxepin. Since *N*-trifluoroacetylnortriptyline overlapped with *cis*-(*N*-trifluoroacetyl)desmethyldoxepin on the SE-30 capillary column used in the selected ion monitoring experiments, the ^{13}C-isotope contributions of the nortriptyline signals were taken into account for calculating the true ratio of the $[MH]^+$ and $[MH - CF_3CONHCH_3]^+$ signals of *cis*-(*N*-trifluoroacetyl)desmethyldoxepin.

Preliminary results of the plasma levels obtained in two patients receiving chronic doxepin therapy are given in Fig. 5. The *trans*-isomer of doxepin was more prominent than its *cis*-form. For desmethyldoxepin, however, a considerable fraction occurred as the *cis*-form, although the product given to the patients contained only approximately 15% *cis*- *versus* 85% *trans*-isomer.

More patients are now being studied to evaluate if the levels of *cis*- and *trans*-doxepin and desmethyldoxepin can be correlated with clinical outcome.

REFERENCES

(1) R. M. Pinder, R. N. Brogden, T. M. Speight, and G. S. Avery, *Drugs*, **13**, 161 (1977).

(2) D. C. Hobbs, *Biochem. Pharmacol.*, **18**, 1941 (1969).

(3) J. E. O'Brien and O. N. Hinsvark, *J. Pharm. Sci.*, **65**, 1068 (1976).

(4) S. F. Reite, *Med. Norsk. Farm. Selskap.*, **37**, 141 (1975).

(5) A. Frigerio, C. Pantarotto, R. Franco, R. Gomeni, and P. L. Morselli, *J. Chromatogr.*, **130**, 354 (1977).

ACKNOWLEDGMENTS

The authors thank Dr. J. Gordts (Pfizer) for supplying doxepin and desmethyldoxepin and Dr. R. Deberdt and Dr. E. Suy for allowing them to study their patients.

Simple, Rapid, and Micro High-Pressure Liquid Chromatographic Determination of Plasma Griseofulvin Levels

ROGER L. NATION, GEOFFREY W. PENG, VICKY SMITH, and WIN L. CHIOU ˣ

Received July 25, 1977, from the *Clinical Pharmacokinetics Laboratory and the Department of Pharmacy, College of Pharmacy, University of Illinois at the Medical Center, Chicago, IL 60612.* Accepted for publication September 29, 1977.

Abstract □ A rapid high-pressure liquid chromatographic assay is described for the quantitative determination of griseofulvin in plasma. An aliquot (25–100 μl) of plasma was deproteinized by a simple procedure involving the addition of 2.5 volumes of acetonitrile, vortex mixing for a few seconds, and centrifugation for 1 min. The clear supernate, 50 μl, was injected into the high-pressure liquid chromatograph. A reversed-phase column was used with a mobile phase of distilled water–acetonitrile (1:1) at a flow rate of 2 ml/min and was operated at ambient temperature. A fluorescent detector with an excitation wavelength of 260 nm was employed to monitor the column effluent. Griseofulvin had a retention time of 3.8 min. This procedure yields reproducible results with high sensitivity; plasma concentrations as low as 50 ng/ml can be measured. Several commonly used drugs do not interfere. Analysis of plasma samples collected from a rabbit injected with griseofulvin indicated that the procedure is suitable for pharmacokinetic studies and clinical monitoring of plasma concentrations in patients. Assay turnaround time is less than 6 min. For clinical monitoring of plasma griseofulvin concentrations, a sample volume as small as 10 μl can be used.

Keyphrases □ Griseofulvin—high-pressure liquid chromatographic analysis in plasma □ High-pressure liquid chromatography—analysis, griseofulvin in plasma □ Antifungal agents—griseofulvin, high-pressure liquid chromatographic analysis in plasma

Griseofulvin is administered orally for treatment of fungal infections of the skin, hair, and nails in humans; this drug treatment is sometimes required for many months. The incidence of relatively minor untoward effects associated with the use of griseofulvin may be as high as 15%, but more serious reactions occur less frequently (1).

BACKGROUND

A number of factors could possibly influence the time course of blood griseofulvin concentrations following multiple oral doses. For example, a reduction of the extent of systemic biological availability was demonstrated in human subjects administered single doses of certain griseofulvin formulations (2, 3). Repeated single oral doses of the same batch of some griseofulvin products resulted in extremely variable extents of absorption in some subjects (2). Additionally, the concurrent administration of phenobarbital decreased blood levels of griseofulvin by impairing its absorption (4, 5). The metabolic clearance of griseofulvin is subject to wide interindividual variability (2), and this factor alone may result in pronounced differences (probably severalfold) in blood levels of the drug among individuals receiving the same dosage regimen. In view of the chronic nature of griseofulvin therapy and the factors influencing its pharmacokinetic profile, it may be beneficial to patient care to monitor plasma drug concentrations, particularly if toxicity or therapeutic failure is suspected (6).

Spectrofluorometric and GLC methods were described previously for the quantitative determination of griseofulvin in plasma. The spectrofluorometric assay (2) and modifications of it (7, 8) require 0.5–1 ml of plasma, time-consuming extraction and evaporation steps, and the reading of fluorescence intensity of the final aqueous solution both before and after the addition of sulfuric acid. Moreover, griseofulvin metabolites interfere with the method (8). The lower detection limit of the spectrofluorometric procedures is approximately 0.1 μg of griseofulvin/ml of plasma.

GLC with an electron-capture detector was used for the estimation of griseofulvin in plasma (8). The preparation of plasma samples for analysis involved extraction and evaporation, dissolution of the residue in benzene, and injection into the gas chromatograph. Because of the nonlinear response of the detector, the standard curve was nonlinear above about

Table I—Standard Curve for Griseofulvin in Plasma [a]

Plasma Griseofulvin Concentration, μg/ml	Griseofulvin Peak Height [b]	Response Factor [c]
0.05	1.3	26.0
0.1	2.3	23.0
0.5	12.4	24.8
1.0	25.3	25.3
2.0	49.7	24.9
3.0	75.6	25.2

[a] Linear regression equation: $y = 25.1493x - 0.1288$, $r = 0.9999$. [b] Peak height in centimeters at detector sensitivity of 0.1 μamp with a 10-mv recorder. [c] Peak height divided by griseofulvin concentration.

1.5 μg/ml; therefore, for measurement of higher concentrations of griseofulvin, a smaller volume of plasma or a larger volume of benzene to make up the assay solution was needed. Variation in the response of the electron-capture detector made it necessary to run standard curves daily when plasma samples were analyzed. The assay sensitivity was 0.05 μg/ml using 1-ml plasma samples.

The present paper reports a very simple, rapid, sensitive, and specific micro high-pressure liquid chromatographic (HPLC) method for the determination of griseofulvin in plasma.

EXPERIMENTAL

Materials—Griseofulvin[1], glass-distilled acetonitrile[2] and ethanol[2], and distilled water were used.

Spectrofluorometric Studies—Excitation and emission spectra[3] of griseofulvin fluorescence in the HPLC mobile phase were obtained.

HPLC System—Analyses were performed at ambient temperature with a system consisting of a mobile phase delivery pump[4], a sample injection apparatus[5], a 30-cm microparticulate reversed-phase HPLC column[6], a liquid chromatographic fluorescence detector[7], and a potentiometric 25.4-cm recorder[8]. The mobile phase was prepared by mixing equal volumes of acetonitrile and distilled water, and this solution was passed through the HPLC system at 2.0 ml/min. Unless otherwise specified, the fluorescence detector was operated with the excitation wavelength set at 260 nm, and an interference filter (KV 389) was used to select the fluorescence emission for detection. Recorder chart speed was 4 mm/min.

Preparation of Standard Curves—Plasma samples were spiked with griseofulvin by adding 5–7.5 μl of ethanolic griseofulvin stock solution to 500-μl plasma aliquots. The analysis of rabbit plasma samples containing griseofulvin gave the same results when the samples were analyzed with and without the addition of the equivalent proportion of ethanol used for spiking standards. Griseofulvin concentrations in the spiked plasma samples were 0.05, 0.1, 0.5, 1.0, 2.0, and 3.0 μg/ml.

Aliquots of 100 μl of spiked plasma samples were pipetted into 13 × 100-mm screw-capped culture tubes. After the addition of 250 μl of acetonitrile to each tube, the mixture was vortex mixed for a few seconds and centrifuged at 2000 rpm for approximately 1 min. Most of the clear supernate was poured into another culture tube, and 50 μl of this solution was then injected. A plasma sample, without the addition of griseofulvin, also was treated in the same way. Similarly, another batch of pooled human plasma and of plasma obtained from six patients receiving other drugs was analyzed without the addition of griseofulvin.

Reproducibility Studies—Two 3-ml aliquots of pooled plasma were spiked such that the plasma griseofulvin concentrations were 0.5 and 1.0 μg/ml. Each plasma sample was assayed for griseofulvin concentration in 20 replicates on the same day using 0.1-ml plasma aliquots and the procedure described under *Preparation of Standard Curves*. Additionally, the plasma sample spiked with 0.5 μg of griseofulvin/ml was assayed once a day on 4 days over 1 week.

Drug Interference Study—Thirteen drugs and drug metabolites were tested for potential interference of the griseofulvin assay by using ethanolic stock solutions of the compounds for direct chromatography or by spiking blank plasma with the compounds, adding 2.5 volumes of acetonitrile, vortexing, centrifuging, and injecting 50 μl of the supernate into the liquid chromatograph.

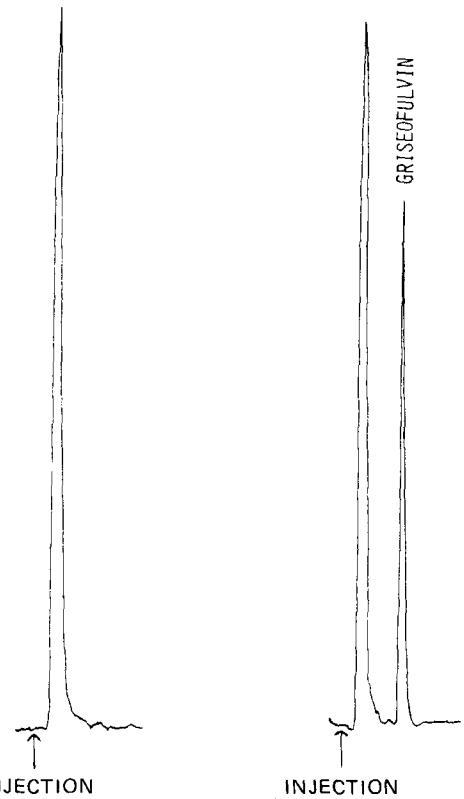

Figure 1—*Chromatograms of blank human plasma (left) and human plasma spiked with 0.5 μg of griseofulvin/ml (right).*

Preliminary Study in a Rabbit—Griseofulvin, 16 mg in 3.2 ml of polyethylene glycol 400, was infused over 1 min into the medial vein of one ear of a 4-kg albino rabbit. Blood samples (0.5 ml) were collected from the marginal vein of the contralateral ear prior to drug administration and at intervals for 6 hr. Blood samples were placed in heparinized tubes which were centrifuged immediately to obtain plasma. The separated plasma samples were stored at −20° until analysis.

RESULTS AND DISCUSSION

Chromatograms resulting from acetonitrile treatment and HPLC of blank human plasma, together with similarly treated plasma previously spiked with griseofulvin, are shown in Fig. 1. Griseofulvin eluted from the reversed-phase column as a symmetrical peak with a retention time of 3.8 min and was well resolved from the more polar endogenous fluorescing compounds of plasma.

Standard curves based on the peak height of griseofulvin from the HPLC of acetonitrile-treated human plasma samples spiked with various drug concentrations were linear over the range of plasma drug concentrations from 0.05 to 3.0 μg/ml and had an intercept close to the origin. Plasma concentrations in patients receiving griseofulvin orally in clinical practice would be expected to fall within the range of concentrations spanned by the standard curve (3). Data used to establish a typical standard curve are summarized in Table I. The correlation coefficient of 0.9999 and the constancy of response factors (peak height divided by concentration) both indicate good linearity.

The reproducibility of the method on a given day was very good. The coefficient of variation ($n = 20$) of the procedure at 0.5 and 1.0 μg of griseofulvin/ml in plasma was 0.82 and 1.9%, respectively. Additionally, the day-to-day reproducibility of the method was good; the coefficient of variation for the analysis of the same plasma sample on 4 days was 3.4%. However, since the analysis is carried out without the use of an internal standard, at least one standard sample should be assayed on those days when unknown plasma samples are analyzed.

The analysis of various batches of pooled human plasma and of plasma obtained from a number of individual patients, without the addition of griseofulvin, indicated the existence in some samples of a very small peak

[1] Sigma Chemical Co., St. Louis, Mo.
[2] Burdick and Jackson Laboratories, Muskegon, Mich.
[3] Aminco-Bowman spectrophotofluorometer, American Instrument Co., Silver Spring, Md.
[4] Model M-6000A, Waters Associates, Milford, Mass.
[5] Model U6K, Waters Associates, Milford, Mass.
[6] μBondapak C_{18}, Waters Associates, Milford, Mass.
[7] Model FS 970, Schoeffel Instrument Corp., Westwood, N.J.
[8] Laboratory Data Control, Riviera Beach, Fla.

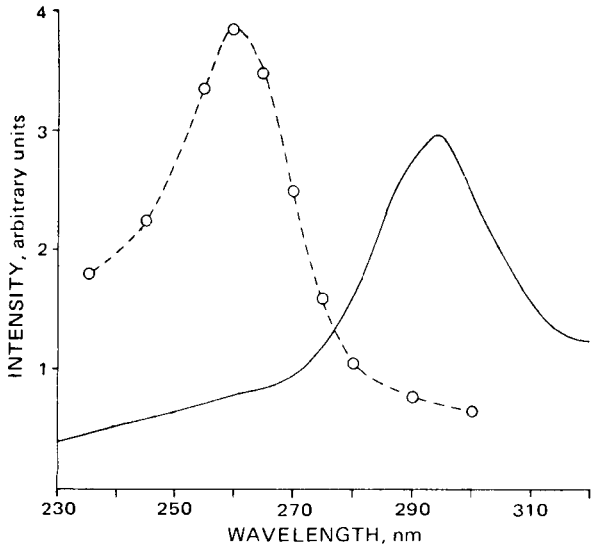

Figure 2—*Fluorescence excitation spectrum of griseofulvin recorded using a spectrophotofluorometer (—) and peak height of griseofulvin from HPLC analysis as a function of the excitation wavelength setting of the flowthrough fluorescence detector (- - -).*

with a similar retention time to griseofulvin. The peak was too small to estimate its contribution accurately, but it was probably only 5–15 ng/ml. Therefore, this factor should not reduce the usefulness of this analytical method for the clinical monitoring of plasma griseofulvin concentrations.

The apparent difference in the fluorescence excitation spectra of griseofulvin as recorded using a conventional spectrophotofluorometer compared to the flowthrough fluorescence detector, which was used to monitor the effluent from the HPLC column, is of interest. Using the spectrophotofluorometer and an emission wavelength of 425 nm, griseofulvin dissolved in the HPLC mobile phase had an excitation maximum of 295 nm; but upon repeated HPLC of a standard griseofulvin solution, the peak height was at a maximum at an excitation wavelength of 260 nm when the KV 389 filter was used to control fluorescence emission in the fluorescence detector (Fig. 2).

Difference in the relative spectral distribution of source intensity in the two instruments is the most likely explanation for the apparent difference in the excitation spectra. The deuterium source in the HPLC detector has peak radiant intensity at a considerably lower wavelength than the xenon source in the spectrophotofluorometer. This finding indicates that with griseofulvin, and probably other compounds, the excitation spectrum obtained with a spectrophotofluorometer may be of limited value for predicting the optimum excitation wavelength to be used in the fluorescent detection of the compound in the effluent from an HPLC column. This situation probably would not exist if both instruments contained the same type of source.

The deproteinization of plasma samples with acetonitrile proved to be a simple, rapid, and effective method of preparing samples for HPLC analysis. The addition of 2.5 volumes of acetonitrile to 1 volume of plasma, followed by brief vortexing and centrifugation, resulted in a clear supernate with the protein material forming a solid mass, which adhered to the base of the culture tube. Therefore, the supernate could be poured off to another tube, although this step is not necessary since an aliquot of the supernate can be injected directly into the liquid chromatograph.

This method of deproteinizing plasma samples with acetonitrile has been employed successfully in this laboratory for the HPLC analysis of several compounds including creatinine (9), aspirin, salicylic acid, salicyluric acid, theophylline, procainamide, and *N*-acetylprocainamide. Other ratios of acetonitrile to plasma may be satisfactory for preparation of plasma samples. Indeed, an acetonitrile to plasma ratio of 1:1 was reported for the denaturation of plasma protein prior to the HPLC determination of theophylline in plasma (10). However, the larger the ratio of acetonitrile to plasma, the more complete may be the deproteinization step, with the less likelihood of blockage of HPLC columns. Addition of trichloroacetic acid to the supernate of the mixture of 1 volume of plasma and 2.5 volumes of acetonitrile does not result in further precipitation, whereas this may not be the case at lower ratios of acetonitrile to plasma.

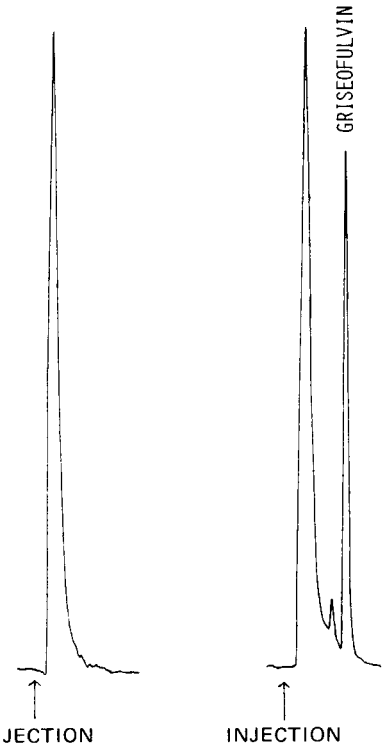

Figure 3—*Chromatograms of blank rabbit plasma (left) and plasma collected from a rabbit 125 min after intravenous administration (right). Griseofulvin concentration was 0.48 μg/ml.*

Other water-miscible organic solvents such as methanol or acetone also can be used to deproteinize plasma. When these solvents are employed, the resulting precipitate is very fine. The supernate may contain particulate matter, which would necessitate a filtration step. For example, an HPLC method for determination of amphotericin B in plasma involved the addition of methanol to plasma, followed by periods of vortexing (15 sec), standing (10 min), and centrifugation (10 min at 2000×*g*) (11). The supernate obtained after centrifugation was filtered, and an aliquot of the filtrate was injected into the liquid chromatograph (11).

For the setting up of standard curves, 100-μl aliquots of plasma were deproteinized with acetonitrile. Smaller volumes of plasma also may be used if there is a shortage. The treatment of 25- and 100-μl aliquots of two spiked plasma samples (griseofulvin concentration of 0.5 and 2.0 μg/ml) with 2.5 volumes of acetonitrile and injection of 50 μl yielded the same value for the griseofulvin concentration in the samples. For the analysis of small volumes of pediatric plasma samples, even smaller volumes of plasma can be used (*e.g.*, 10 μl) with a corresponding reduction in injection volume and sensitivity.

The injection volume of 50 μl was chosen as being adequate to achieve the sensitivity required for clinical monitoring and pharmacokinetic studies. Increasing the injection volume resulted in a less than proportional increase in griseofulvin peak height. For example, increasing the injection volume from 50 to 100 μl resulted in only a 78% increase in peak height. This effect was probably due mainly to a peak broadening resulting from a slower transfer of total sample from the injection loop onto the head of the column as the sample size increased.

None of the compounds tested for potential interference (acetaminophen, methotrexate, ampicillin, aspirin, caffeine, ephedrine, phenobarbital, phenytoin, salicylic acid, salicyluric acid, tetracycline, theobromine, and theophylline) interfered with the analysis. Additionally, no interference from drugs was observed when plasma from patients receiving multiple-drug therapy was assayed.

To investigate the suitability of the griseofulvin analysis for monitoring plasma drug levels, a rabbit was given griseofulvin intravenously and blood samples were collected. Figure 3 shows chromatograms resulting from acetonitrile treatment and HPLC of predose blank rabbit plasma and similarly treated plasma obtained from the rabbit after griseofulvin administration. In addition to the griseofulvin peak, a smaller peak with a retention time of 2.95 min was present in the samples collected after drug administration. This additional peak was not present when stock solutions of griseofulvin or acetonitrile-treated spiked plasma were in-

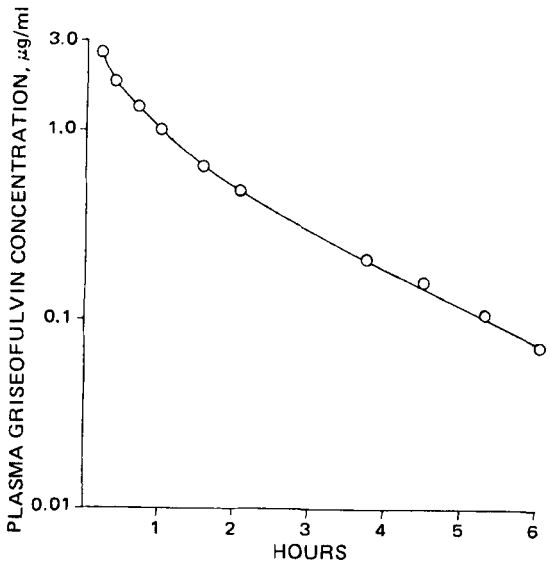

Figure 4—*Plasma griseofulvin concentration as a function of time in a 4-kg rabbit given 16 mg of griseofulvin intravenously over 1 min.*

jected into the liquid chromatograph, indicating that the peak was not due to another component or an impurity in the griseofulvin. It seems most likely that the peak was due to a metabolite of griseofulvin, although the structure of this compound is unknown. The time course of plasma griseofulvin concentrations in the rabbit is shown in Fig. 4.

The described method for quantitative determination of griseofulvin in plasma offers several advantages over the spectrofluorometric (2, 7, 8) and GLC (8) methods. The HPLC procedure is simple and rapid, with a turnaround time of 5–6 min required for each sample. The analysis uses only a small volume of plasma and is specific and reproducible; commonly used drugs do not interfere. The method is well suited for the clinical monitoring of plasma griseofulvin concentrations or for pharmacokinetic studies.

REFERENCES

(1) "The Pharmacological Basis of Therapeutics," L. S. Goodman and A. Gilman, Eds., Macmillan, New York, N.Y., 1975.

(2) M. Rowland, S. Riegelman, and W. L. Epstein, *J. Pharm. Sci.*, **57**, 984 (1968).

(3) W. L. Chiou and S. Riegelman, *ibid.*, **60**, 1376 (1971).

(4) S. Riegelman, M. Rowland, and W. L. Epstein, *J. Am. Med. Assoc.*, **213**, 426 (1970).

(5) D. Busfield, K. J. Child, R. M. Atkinson, and E. G. Tomich, *Lancet*, **2**, 1042 (1963).

(6) S. Riegelman and W. Sadee, "Clinical Pharmacokinetics: A Symposium," APhA Academy of Pharmaceutical Sciences, Washington, D.C., 1974, p. 169.

(7) V. P. Shah, S. Riegelman, and W. L. Epstein, *J. Pharm. Sci.*, **61**, 634 (1972).

(8) H. J. Schwarz, B. A. Waldman, and V. Madrid, *ibid.*, **65**, 370 (1976).

(9) W. L. Chiou, M. A. F. Gadalla, and G. W. Peng, *ibid.*, **67**, 182 (1978).

(10) J. W. Nelson, A. L. Cordry, C. G. Aron, and R. A. Bartell, *Clin. Chem.*, **23**, 124 (1977).

(11) I. Nilsson-Ehle, T. T. Yoshikawa, J. E. Edwards, M. C. Schotz, and L. B. Guze, *J. Infect. Dis.*, **135**, 414 (1977).

Stability of Furosemide in Aqueous Systems

A. G. GHANEKAR, V. DAS GUPTA ˣ, and CHARLES W. GIBBS, Jr. *

Received February 28, 1977, from the *College of Pharmacy, University of Houston, Houston, TX 77004.* Accepted for publication September 28, 1977. *Present address: Harris County Hospital District Pharmacy, Taub Loop, Houston, TX 77030.

Abstract □ A stability-indicating assay for furosemide based on high-pressure liquid chromatography was developed. The method is sensitive, accurate, and precise. The standard deviation based on six injections of the standard solution was ±1.37%. This method was used to study furosemide stability in various aqueous solutions and dosage forms. Stability tests were conducted at room temperature as well as at higher temperatures (45, 65, and 85°) at various pH values and with different vehicles. Some decomposition products were identified.

Keyphrases □ Furosemide—high-pressure liquid chromatographic analysis, stability in aqueous solutions and dosage forms □ High-pressure liquid chromatography—analysis, furosemide in aqueous solutions and dosage forms □ Stability—furosemide in aqueous solutions and dosage forms, high-pressure liquid chromatographic analysis □ Diuretics—furosemide, high-pressure liquid chromatographic analysis, stability in aqueous solutions and dosage forms

Furosemide is extensively used as a diuretic. Very little information is available concerning its stability, possibly because of the lack of a stability-indicating assay. The USP method (1) for furosemide in injections and tablets is based on UV absorption. A fluorometric method for furosemide in urine and serum also was reported (2), but any other fluorescent substances interfere with the assay. Furosemide was reported to be unstable in acidic media (3). The USP (1) indicates that the pH of the injection should be between 8.9 and 9.3.

The present investigations were undertaken to develop a stability-indicating assay for furosemide using high-pressure liquid chromatography (HPLC) and to study furosemide stability in aqueous systems.

EXPERIMENTAL

Chemicals and Reagents—All chemicals and reagents were ACS, NF, or USP quality and were used without further purification.

Preparation of Solutions and Dosage Forms—The solutions and dosage forms prepared are given in Table I.

Apparatus—A high-pressure liquid chromatograph[1] equipped with a UV detector (254 nm), a recorder[2], and an integrator[3] was used.

Column—A column[4] (30 cm × 4 mm i.d.) of a very nonpolar packing material, consisting of octadecyltrichlorosilane permanently bonded by silicon–carbon bonds, was used.

Chromatographic Conditions—The chromatographic mobile phase was 0.01 *M* $(NH_4)_2HPO_4$ in water containing 25% (v/v) methanol. The

[1] Model ALC 202, equipped with U6K universal chromatograph injector, Waters Associates, Milford, Mass.
[2] Omniscribe 5213-12, Houston Instruments, Austin, Tex.
[3] Autolab Minigrator, Spectra-Physics, Santa Clara, Calif.
[4] μBondapak C_{18}, Catalog No. 27324, Waters Associates, Milford, Mass.