Gas-Chromatographic Determination of 5-Fluorocytosine in Human Serum

Sally A. Harding, George F. Johnson, and Harvey M. Solomon

We describe a sensitive and precise gas-chromatographic method, in which cytosine is used as the internal standard, for determination of an antifungal agent, 5-fluorocytosine, in serum. The trimethylsilyl derivative of this drug is well separated from the internal standard and from normal serum constituents. Amphotericin B does not interfere with the determination of 5-fluorocytosine. The lower limit of detection for 5-fluorocytosine is 1 mg/liter when 200 μl of serum is analyzed. Within-run precision (CV), established by analysis of 10 replicates, was 4.5% at a concentration of 19.9 mg/liter. Twenty-five serum samples were analyzed for 5-fluorocytosine by a microbiological assay and by the gas-chromatographic method. Mean value observed with the bioassay was 78.5 mg/liter and with our procedure was 69.4 mg/liter. When values for our assay were regressed against values for the bioassay, slope of the least-squares line was 0.85, intercept was 2.7 mg/liter, and r was 0.93.

Many clinical microbiologists still use biological assays to estimate various antibiotics in physiological fluids, even though such procedures are inherently less precise, more difficult to control, and subject to greater interferences than are modern chemical assays. The first chemical method for quantitative determination of sulfonamides was introduced in 1939 by Bratton and Marshall (1) and this colorimetric procedure is still widely used to measure these compounds. Moreover, when an initial hydrolysis step is included, the biologically inactive N-acetyl metabolite of various sulfonamides can also be determined; hence, it is possible to determine whether patients acetylate these drugs slowly or rapidly (2). Although chemical and immunochemical methods for the determination of antibiotics have improved greatly in accuracy, precision, sensitivity, and specificity (3, 4) since the introduction of the Bratton-Marshall technique for sulfonamides, some clinical microbiologists (5) still claim that chemical assay methods for antibiotics are nonspecific, detecting (e.g.) metabolites of these drugs that possess little if any biological activity.

Patients with serious bacterial and fungal infections are frequently treated concurrently with several chemotherapeutic agents. When the antibiotics involved in such therapy cannot be separated by relatively simple physical techniques and when assay organisms possessing selective sensitivity to individual drugs in such therapeutic regimens are not available, it is difficult if not impossible to determine drug concentrations meaningfully by biological assay.

5-Fluorocytosine is an antifungal agent frequently utilized in combination with amphotericin B for treatment of deep-seated mycotic infections (6, 7). Monitoring of serum concentrations of 5-fluorocytosine has been recommended to provide optimum therapeutic benefit and avoid toxicity (8). To our knowledge, such determinations have been performed only by bioassay. The yeasts utilized in these bioassays for 5-fluorocytosine are, however, also inhibited by amphotericin B, and various techniques—including ultra-filtration (8) and differential diffusion through yeast nitrogen base agar from filter paper disks—have been used to separate these drugs (9).

5-Fluorocytosine, which has a relatively simple chemical structure, is readily converted to a volatile trimethylsilyl derivative. We describe an accurate and precise gas-chromatographic method that is specific for 5-fluorocytosine and is suitable for the routine determination of this drug in serum.
Materials and Methods

Reagents

5-Fluorocytosine was kindly provided by Hoffmann-La Roche, Inc., Nutley, N. J. 07110; cytosine was purchased from Aldrich Chemical Co., Milwaukee, Wis. 53223. We prepared stock solutions (100 mg/liter) of each of these compounds by dissolving 10 mg in 100 ml of ethanol. A working solution of 5-fluorocytosine (10 mg/liter) was prepared by diluting the stock solution 10-fold with ethanol.

Amphotericin B was purchased from E. R. Squibb and Sons, Inc., Princeton, N. J. 08540. A stock solution (100 mg/liter) was prepared by suspending 50 mg of the drug in 10 ml of distilled water and then diluting this solution 50-fold with distilled water.

n-Butanol and ethanol were purchased from Fisher Scientific Co., Fair Lawn, N. J. 07410. Regis silylation mix was obtained from Regis Chemical Co., Morton Grove, Ill. 60053.

Procedure for Extraction and Silylation

Serum (200 μl) was combined in a 16 × 125 mm test tube (with a Teflon-lined screw cap) with 800 μl distilled water, 30 μl HCl (2 mol/liter) and 1 ml of phosphate buffer (0.1 mol/liter, pH 3.0) containing 10 mg/liter of the internal standard, cytosine. The buffer solution was stored at 4 °C and was dispensed from an automatic pipet (Repipet; LabIndustries, Berkeley, Calif. 94710). The contents of the tubes were mixed on a vortex-type mixer for 30 s and 5 ml of butanol was then added to each tube with a Repipet. The tubes were shaken for 10 min in an Eberbach shaker at 250 oscillations per minute and centrifuged at 2000 rpm for 10 min. The butanol layers were aspirated and discarded. One milliliter of phosphate buffer (1 mol/liter, pH 7.9) was added to the aqueous phase to adjust the pH to 7.4, 5 ml of butanol was added with the Repipet, and the contents of the tubes were mixed and extracted as just described. The phases were separated by centrifugation as described above. The butanol layers were transferred to 7-ml screw-capped silylation vials and evaporated under nitrogen in a water bath at 55 °C. Regis Silylation mix, 50 μl, was added to each vial and the contents of the vials were mixed thoroughly on a vortex-type mixer. Two-microliter samples were injected into the gas chromatograph.

Standard curves were prepared from pooled normal human serum that contained known amounts of 5-fluorocytosine. These serum standards (10, 25, 50, 75, and 100 mg/liter) were prepared by evaporating appropriate amounts of the working solution of 5-fluorocytosine under nitrogen and adding 200 μl of drug-free serum to the residue.

Gas Chromatography

A Model 5711A dual-column gas chromatograph equipped with flame-ionization detectors (Hewlett-Packard, Avondale, Pa. 14311) was used. The glass columns (122 cm × 2 mm i.d.) were packed with 3% OV-17 on 100/120 Gas Chrom Q (Applied Science Labs., Inc., State College, Pa. 16801). Injector port temperature was 250 °C, detector temperature was 300 °C, and oven temperature was programmed from 100 to 240 °C at 16 °C/min. The nitrogen flow rate was 50 ml/min.

Microbiological Assay for 5-Fluorocytosine

Specimens were analyzed for 5-fluorocytosine according to a modification of the bioassay procedure of Block and Bennett (8). In the original procedure standard solutions of the drug, prepared in pooled, normal human serum, were not ultrafiltered through collodion membranes while serum samples obtained from patients treated concurrently with 5-fluorocytosine and amphotericin B were ultrafiltered. In the modified procedure both standard solutions and patient samples were ultrafiltered. All bioassays for 5-fluorocytosine were performed in the laboratory of Dr. John E. Bennett, Clinical Mycology Section, Laboratory of Clinical Investigation, National Institute of Allergy and Infectious Diseases, Bethesda, Md. 20014.

Results

When sera from normal individuals were extracted and derivatized according to the procedure we have described, several chromatographic peaks were observed; however, none interfered with either 5-fluorocytosine or cytosine (Figure 1A). Normal sera and sera from patients treated with 5-fluorocytosine did not contain endogenous cytosine. The chromatogram presented in Figure 1B was obtained from a normal serum to which 5-fluorocytosine and cytosine were added. A single chromatographic peak was observed for each compound, with a retention time of 2.65 min for 5-fluorocytosine and 3.62 min for cytosine. No interference from amphotericin B was noted with specimens obtained from any of the patients on combined drug therapy or with normal serum samples to which were
added amphotericin B in final concentrations of 12.5, 25.0, or 50.0 mg/liter and 5-fluorocytosine in final concentrations ranging from 20 to 100 mg/liter. Figure 1C shows a chromatogram obtained with serum from a patient on combined therapy with 5-fluorocytosine and amphotericin B. The concentration of 5-fluorocytosine in this clinical specimen was 48.4 mg/liter.

A typical standard curve obtained with our procedure is shown in Figure 2. Linearity was observed over the range of 0–200 mg of 5-fluorocytosine per liter. The lower limit of detection for 5-fluorocytosine, based on twice the standard deviation of baseline noise, was 1 mg/liter when the sample volume was 200 μl.

Recovery of 5-fluorocytosine from serum was measured in the following way: a solution of 30 μg of the compound in methanol was evaporated and to the residue was added 1 ml of drug-free serum. This sample was extracted as described in the Methods section; however, volume transfers were quantitated. A second (non-extracted) standard was prepared concurrently by evaporating 30 μg of the same compound to dryness, and both sample and standard were derivatized by addition of 50 μl of silylating reagent. Two microliters of each sample were chromatographed. The peak height of the serum sample was corrected for solvent transfers. Absolute recovery of the drug from serum was calculated by comparing the ratio of corrected peak height of the extracted serum sample to that of the non-extracted standard. The absolute recoveries of 5-fluorocytosine and cytosine determined by this procedure were 30.3% and 30.9%, respectively. When concentrations of 5-fluorocytosine ranging from 25–150 mg/liter were prepared in pooled normal sera and assayed according to our procedure, the mean recovery of this drug relative to the internal standard was 95.9% (n = 12).

Within-run reproducibility (CV) for our assay was 4.5% at a concentration of 19.9 mg/liter (n = 10). Between-run reproducibility was 7.5% as determined by the use of paired duplicates according to the following formula: SD = \( \sqrt{\frac{\sum d^2}{2n}} \), where d = difference between duplicate pairs. The mean value for this group of samples was 48.1 mg/liter, with a range of 4.1 to 187.8 mg/liter (n = 23).

Twenty-five serum samples were analyzed for 5-fluorocytosine by the microbiological assay and with the gas chromatographic method we have described (Figure 3). Seventeen of these samples were prepared by adding various amounts of 5-fluorocytosine to pooled normal sera and eight were obtained from patients receiving both amphotericin B and 5-fluorocytosine. The mean value for the bioassay was 78.5 mg/liter (range, 5.0 to 194.0 mg/liter). The mean value by the gas chromatographic procedure was 69.4 mg/liter (range, 4.5 to 156.0 mg/liter). When values obtained by the gas-chromatographic assay were regressed against those obtained with the bioassay, the slope of the linear least-squares line was 0.85, the intercept was 2.7 mg/liter, and the coefficient of correlation was 0.93.

Discussion

The incidence of systemic fungal infections has risen progressively with increased use of such drugs as steroids, antibiotics, and immunosuppressants, and as a result of larger numbers of renal-transplant recipients and more prolonged survival of patients with neoplastic diseases (10, 13). Systemic yeast infections are the most frequently encountered of these opportunistic mycoses and the prognosis in such systemic infections is poor, the overall mortality being 75% (14).

In vitro and clinical studies indicate that 5-fluorocytosine is effective in treating infections from sensitive strains of Candida, Torulopsis, and Cryptococcus (15, 17).

In man, about 90% of the administered dose of 5-fluorocytosine is excreted unchanged in the urine and the remainder appears in the feces (18). Concentrations of this drug in serum are greater in patients with impaired renal function and clearance is prolonged compared to that observed in patients with normal renal function (8, 15, 19). Toxic effects of 5-fluorocytosine on the bone marrow and liver have been observed more commonly in patients with renal insufficiency treated with usual therapeutic doses of 5-fluorocytosine (19, 20), which suggests that rational dosage regimens in such patients should be based on measurements of serum concentrations. Block and Bennett (8) indicate that peak serum concentrations of 5-fluorocytosine as great as 120 mg/liter can be attained without risk of drug-induced toxicity. Concentrations of at least 25 mg/liter

![Fig. 2. Standard curve for 5-fluorocytosine in serum](image_url)

![Fig. 3. Comparison of a microbiological assay and gas chromatographic assay for 5-fluorocytosine in serum](image_url)
of serum should be attained during therapy, because many resistant mutants emerge at lower concentrations (21).

Treatment of a number of fungal diseases is frequently undertaken with 5-fluorocytosine and amphotericin B, because this combination is synergistic and reduces the development of resistance to 5-fluorocytosine by the infecting organism (6, 7). The usual therapeutic doses of amphotericin B often produce nephrotoxicity; hence serum concentrations of 5-fluorocytosine should also be monitored in this population to provide safe and effective therapy with the drug.

Although several bioassay procedures for estimating 5-fluorocytosine in serum have been reported, it is difficult to determine their precision because no meaningful information has been published concerning within- or between-run reproducibility (8, 9). It is likely, however, that the precision of these assays is rather limited, because one procedure involves processing all specimens in triplicate and the other in quadruplicate. The within-run coefficient of variation of our assay for 5-fluorocytosine was 4.5% at a concentration of 19.9 mg/liter and the between-run precision (CV) was 7.5 percent. These data suggest that our gas-chromatographic assay has adequate precision to permit determinations on single samples rather than on replicates as required by the bioassays. The relatively good reproducibility of this procedure is due primarily to use of cytosine, a closely related compound, as the internal standard in the assay. When normal human serum was extracted and derivatized according to our procedure without addition of this internal standard, a chromatographic peak with the retention time of the trimethylsilyl derivative of cytosine was not observed. This is consistent with the observations of Bishop et al. (22), who did not detect free purines or pyrimidines in normal human blood. Our procedure is not interfered with by normal constituents in serum or by amphotericin B, which suggests that it also has requisite specificity for 5-fluorocytosine.

Although chemical methods for analysis of 5-fluorocytosine in biological fluids have not been previously described, several procedures have been reported for the quantitative determination of 5-fluorouracil, an antimetabolite used in cancer chemotherapy (23, 25). This drug, a weak acid (pKₐ = 8.0), can be separated from plasma by various techniques, including dialysis, anion-exchange chromatography, and extraction with a mixture of n-propanol in ether. The trimethylsilyl derivative of 5-fluorouracil has good chromatographic characteristics on OV-1 and SE-30 and the drug can also be derivatized by on-column alkylation with trimethylsilylnitrohydride. Recently Pantarotto et al. (26) determined by gas chromatography–mass spectrometry that the product of this alkylation is N₅,N₁-dimethyl-5-fluorouracil.

The double-extraction procedure we use involves an initial extraction with butanol at pH 3.0 to remove interfering neutral and acidic compounds. 5-Fluorocytosine is not extracted into butanol at this pH. The pH is then adjusted to 7.4 and the drug and internal standard are extracted into butanol. The extraction efficiency of 5-fluorocytosine is only 30.3% and that of the internal standard is 30.9%, which indicates that these compounds have similar partition characteristics. The trimethylsilyl derivatives of 5-fluorocytosine and cytosine prepared in our assay have excellent chromatographic characteristics and are stable for as long as 24 h if they are protected from moisture. We have also established that 5-fluorocytosine can be alkylated with trimethylsilylnitrohydride; however, this derivative is absorbed to the column and is therefore not suitable for the quantitative determination of the drug.

Because of the variation inherent in the bioassay it is difficult to know whether the slope (0.85) that we found for the regression line is statistically different from unity.

Safe and effective treatment with many potent drugs having narrow margins of safety depends in part on measurements of their concentrations in serum (27). Therapeutic monitoring of antiarrhythmic drugs, anticonvulsants, cardiac glycosides, and the bronchodilator theophylline are increasingly utilized to ensure optimum therapeutic benefit and to avoid drug toxicity. Rapid, specific, accurate, and precise chemical methods are available for these compounds, and it is mandatory that similar techniques be developed for the quantitative determination of antibiotics.

We thank John E. Bennett, M.D., Head, Clinical Mycology Section, LCl, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Md. 20014, for providing the microbiological assay for 5-fluorocytosine used in this study.

References