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Improved Procedure for Determination of Flucytosine in Human Blood Plasma by High-Pressure Liquid Chromatography

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Several high-pressure liquid chromatography procedures for the determination of flucytosine in serum or plasma have appeared. Some of these suffer from significant disadvantages, and none was applicable in our routine clinical therapeutic-drug-monitoring laboratory. A new high-pressure liquid chromatography assay for flucytosine was therefore developed. A 100-μl sample of plasma was treated with an aqueous 5-iodocytosine internal-standard solution, and the mixture was deproteinized with trichloroacetic acid. A portion of the protein-free supernatant was diluted with 0.1 M ammonium phosphate, and an aliquot of the resulting solution was injected into the high-pressure liquid chromatography system. Chromatography was performed on a strong-cation-exchange column with a mobile phase containing aqueous ammonium phosphate, phosphoric acid, methanol, and acetonitrile. Detection was at 254 nm. The assay was shown to be linear in the 10 to 200-μg/ml drug-concentration range. Forty other drugs were tested for potential interference with the assay, and none was found. For routine use, a single-point working standard containing 75 μg of flucytosine per ml was used, giving intraassay coefficients of variation at 50 and 150 μg/ml of 1.8 and 2.3% respectively, whereas the day-to-day coefficient of variation at 50 μg/ml was 10.0%. Advantages of the procedure include the small sample size, the use of a convenient and reliable internal standard, speed, and simplicity. The assay is highly suitable for routine clinical drug-analysis laboratories.

Flucytosine (5-flurocytosine) is an antifungal agent used in the treatment of several systemic mycotic infections (1). Toxic effects of the drug include dose-related hepatotoxicity and depression of bone marrow function (1, 9). About 80 to 95% of a given dose of the drug is excreted unchanged in the urine (10), and the dosage must be modified in patients with impaired renal function. The importance of monitoring flucytosine concentrations in serum to minimize toxicity and optimize the therapeutic effect has been pointed out (9, 13).

Methods used for the determination of flucytosine in biological fluids include microbiological procedures (8), fluorescence (12), gas-liquid chromatography (6), and high-pressure liquid chromatography (HPLC) (2, 3, 5, 11, 14). Flucytosine is a polar, water-soluble molecule with good UV light absorption, and therefore analysis of this drug in biological fluids is best performed with aqueous HPLC systems. Such techniques are faster and more specific than microbiological methods and have important advantages over other chemical methods (13). Some of the published HPLC methods appear useful, but all have significant disadvantages, and none was suitable for our clinical therapeutic-drug-monitoring laboratory. For example, only one (11) of the published methods includes the use of an internal standard. Other disadvantages include a long (1-h) chromatographic run (2), relatively large (0.5- to 1.0-ml) sample sizes (5, 14) unsuitable for some pediatric specimens, heated columns (2, 5), direct injection of serum (2), and no information on potential interference by other drugs (2). Interference by 5-fluorouracil was reported in one of the described methods (11). This was of concern to us, as the biotransformation of flucytosine to 5-fluorouracil and its derivatives—originally thought to occur only in susceptible fungi (7, 10)—also appears to take place in humans (4). The reported interference by 5-fluorouracil in the determination of flucytosine could be eliminated by altering the assay conditions, but the procedure was not evaluated in the modified form (11).

In summary, then, at the outset of this work we felt that despite the availability of published HPLC methods for the determination of flucytosine, a different procedure was needed, one which would not suffer from the above-described shortcomings and which would be suitable for use in a routine, high-volume clinical therapeutic-drug-monitoring laboratory. In this communication we describe the procedure we developed.

MATERIALS AND METHODS

Chemicals. Flucytosine and 5-iodocytosine were obtained from Sigma Chemical Co., St. Louis, Mo. Trichloroacetic acid and monobasic ammonium phosphate were purchased from J. T. Baker Chemical Co., Phillipsburg, N.J., and phosphoric acid was purchased from Fisher Scientific Co., Pittsburgh, Pa. Methanol and acetonitrile were of the distilled-in-glass grade and were provided by Burdick and Jackson Laboratories, Inc., Muskegon, Mich. Water used throughout the procedure was double distilled. Human blood plasma was obtained from the Belle Bonfils Memorial Blood Bank of the University of Colorado Health Sciences Center.

Reagents and standards. A series of plasma samples containing flucytosine at 10, 50, 100, 150, and 200 μg/ml were prepared by diluting a 1.0-mg/ml stock solution. For routine use of the assay, a single-point working standard solution containing flucytosine at 75 μg/ml in normal saline was prepared. The working internal-standard solution contained 5-iodocytosine at 500 μg/ml in water. The working standard and internal-standard solutions were stored at −20°C and remained stable for at least 6 months. Trichloroacetic acid was used as a 10% (wt/vol) solution in water. Ammonium

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phosphate buffer (0.1 M) was used in the sample preparation. Plasma controls (25 and 150 μg/ml) were prepared by diluting the stock flucytosine solution with plasma. The control samples were stored at −20°C and remained stable for 6 months.

Chromatography. The equipment used was a Waters Associates (Milford, Mass.) system consisting of a model 6000 pump, a model U6K injector, and a model 440 absorbance detector. The mobile phase was prepared as follows: after each solvent was vacuum-filtered separately, 900 ml of water, 900 ml of methanol, and 300 ml of acetonitrile were mixed, 400 μl of phosphoric acid and 2.3 g of ammonium phosphate were added, and the mixture was stirred with a magnetic stirrer until homogeneity was obtained. The mobile phase was pumped at 1.0 ml/min. Chromatographic separation was achieved on a strong-cation-exchange column (150 by 4.6 mm; SCX; Analytichem International, Harbor City, Calif.), and the absorbance of the effluent was monitored at 254 nm. A strip-chart recorder (model 56; The Perkin-Elmer Corp., Norwalk, Conn.) was used to record the chromatograms.

Assay procedure. A 100-μl sample was placed in a 1.5-ml microcentrifuge tube, 100 μl of internal-standard solution was added, and the mixture was blended for 5 s with a Vortex mixer. Trichloroacetic acid solution (100 μl) was added, and the tube was capped and blended with the Vortex mixer for 30 s. The tube was then centrifuged (Eppendorf model 5412; Brinkmann Instruments, Inc., Westbury, N.Y.) for 2 min. A 100-μl aliquot of the supernatant was transferred to another tube, 100 μl of 0.1 M ammonium phosphate was added and the mixture was blended for 10 s with the Vortex mixer. A 10-μl aliquot was injected into the HPLC system.

Calculations. To obtain the concentration of flucytosine in a sample, we compared the flucytosine/internal standard peak-height ratio in the sample with the flucytosine/internal standard peak-height ratio in the working standard.

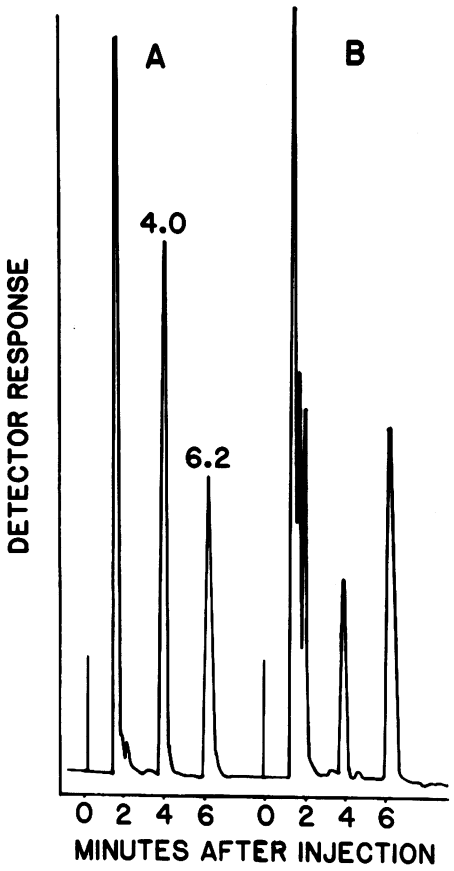

FIG. 1. HPLC elution pattern of flucytosine and the 5-iodocytosine internal standard. (A) Authentic standards. (B) Human plasma containing 50.0 μg of flucytosine per ml carried through the assay procedure. The rentention time of flucytosine was 4.0 min, and that of the internal standard was 6.2 min.

RESULTS

Chromatography. Figure 1 shows the chromatogram of authentic flucytosine and 5-iodocytosine and the results of the analysis of human plasma containing flucytosine.

Assay linearity and precision. A series of spiked plasma samples containing flucytosine at 10 to 200 μg/ml were analyzed. The data were linear over the range studied. A typical standard curve is shown in Fig. 2.

Intraassay precision was determined by analyzing a set of 10 replicate plasma samples containing flucytosine at 50 μg/ml and a set containing flucytosine at 150 μg/ml; the coefficients of variation (CV) were 1.8 and 2.3%, respectively. The day-to-day variation was determined by analyzing plasma samples containing 50 μg of flucytosine per ml on 10 separate days during a 3-week period; the CV was 10.0%.

Specificity. Potential interference by a wide variety of drugs was evaluated; these drugs were as follows (concentrations [in micrograms per milliliter] in parentheses): acetaminophen (25.0), *N*-acetylprocainamide (4.0), amikacin (15.0), amitriptyline (1.0), amoxicillin (5.0), amphotericin B (200), ampicillin (200), carbamazepine (5.0), carbenicillin (200), cephalothin (100), chloramphenicol (30.0), chlordiazepoxide (1.0), chloroquine (10.0), chlorpheniramine (30.0), cimetidine (1.0), codeine (1.0), diazepam (1.0), ephedrine (1.0), 5-fluorouracil (1.0), flurazepam (1.0), gentamicin (8.0), imipramine (1.0), isoniazid (5.0), ketoconazole (5.0), mercaptopurine (10.0), methaqualone (20.0), metronidazole (10.0), miconazole (5.0), nitrofurantoin (10.0), oxacillin (40.0), oxazepam (1.0), penicillin G (30.0), phenobarbital (30.0), phenytoin (20.0), procainamide (6.0), quinidine (5.0), salicylate (300), sulfamethoxazole (50.0), theophylline (20.0), and tobramycin (8.0). No interference was found. Many of the compounds were not retained by the ion-exchange column used; some were strongly retained, and no peak was observed for these.

DISCUSSION

The first question addressed in developing the assay for flucytosine was the identity of a suitable internal standard. With one exception (11), previously published HPLC procedures did not use an internal standard. Although it appears that the use of an internal standard in nonextraction, direct-injection HPLC procedures is often considered unnecessary, we feel that an internal standard should be included in our flucytosine assay. The presence of the internal standard eliminates potential errors which may result from variable volume delivery caused by a faulty injector or from incorrect volume transfers during sample preparation (after addition of the internal standard). After evaluating several cytosine derivatives, we selected 5-iodocytosine to serve as the internal standard. This compound is chemically very similar to flucytosine, is readily available at low cost, and has suitable properties for the assay conditions used.

In the assay procedure, the sample is treated with the internal-standard solution, the proteins are precipitated with

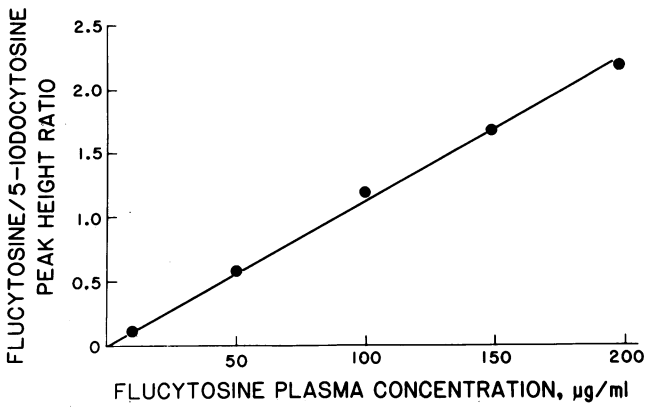

FIG. 2. Linearity of the procedure. Plasma samples containing known amounts of flucytosine were analyzed as described in the text. Least-squares linear regression analysis gave the following equation: drug/internal-standard peak-height ratio = 0.0109 (flucytosine) + 0.0246; $r = 0.9987$.

trichloroacetic acid, and the supernatant is diluted with a buffer before injection into the HPLC system. The retention times were highly sensitive to small variations in the composition of the mobile phase, and it is therefore recommended that the same batch of mobile phase be used during the analysis of a batch of samples. The small sample size, 100 μl, is convenient, and may be suitable for most pediatric specimens. Furthermore, because only a small portion of the sample is actually used for injection into the HPLC system, the sample size may be further reduced if necessary. The selection of the concentration range, 10 to 200 μg/ml, for construction of the standard curve was based on observations that blood levels of flucytosine in patients with normal renal function range between 50 and 100 μg/ml when normally recommended doses are administered and that prolonged blood levels in excess of 100 to 125 μg/ml are frequently associated with significant toxicity (1).

Chromatographic separation is achieved on a microparticulate-silica-based strong-cation-exchange column, a significant improvement over the previously used pellicular ion-exchange columns (2, 5). The mobile phase chosen for the analysis of flucytosine is also used in our laboratory for the determination of several other drugs, thus obviating the need for the preparation of an additional, different mobile phase. Detection is at 254 nm, but experiments at 280 nm (data not shown) have demonstrated that detection at that wavelength may be used, with essentially identical results. The assay is rapid, and analysis of a working standard, a control, and a patient sample can be easily performed in 1 h. The assay is free from interference by a large number of other drugs, including 5-fluorouracil, and is highly suitable for use in the clinical drug-analysis laboratory.

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