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Assay of Flucytosine (5-Fluorocytosine) in Human Plasma by High-Pressure Liquid Chromatography

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The use of the antifungal agent flucytosine has been associated with dose-related hepatotoxicity and bone marrow depression, and measurement of plasma concentrations is useful in determining dosage schedules. The present study describes a rapid, simple, and sensitive high-pressure liquid chromatographic analysis of plasma flucytosine which may be completed within 30 min of receipt of a plasma sample. Sample preparation is minimal and merely involves precipitation of plasma protein before injection onto a reverse-phase column. The method is accurate and reproducible and yields results comparable to or perhaps slightly superior to those obtained by conventional microbiological assay. The value of the procedure for routine use is enhanced by the freedom from interference by a large range of medications administered concurrently with flucytosine.

INTRODUCTION

Flucytosine (5-fluorocytosine) is a fluorinated pyrimidine antimetabolite which is cytotoxic for certain pathogenic fungi but not for mammalian cells. It is a valuable antifungal agent in treatment of systemic *Candida albicans, Cryptococcus neoformans,* and *Torulopsis glabrata* (3, 11, 12) infections. However dose-related hepatotoxicity (12) and depression of the function of bone marrow (7, 8) have been observed in some patients. There is some evidence that flucytosine is metabolized to 5-fluorouracil to a small extent in humans (2), but about 80 to 95% of a given dose is excreted in the urine in unchanged form (5). As a result of the obligate renal excretion, dosage must be modified in patients with renal dysfunction. Measurement of plasma concentrations of flucytosine is useful in determining dosage schedules in such patients, but problems posed by existing methods have discouraged widespread use of this aid.

Previous analytical methods for flucytosine in plasma (or serum) have involved microbiological (4, 9) and fluorometric (13) procedures. A high-pressure liquid chromatographic (HPLC) assay using a cation-exchange column has been reported recently (1).

The present study describes a rapid, simple, and sensitive HPLC analysis of plasma flucytosine levels which may be completed within 30 min of the receipt of a plasma specimen. Sample preparation merely involves precipitation of plasma protein before injection onto a reverse-phase column. The results obtained by this assay in several patients are compared with those obtained by a microbiological procedure.

MATERIALS AND METHODS

Plasma samples (1 ml) were pipetted into tubes containing 50 μl of 4 M trichloroacetic acid. After mixing the acidfied samples for 20 s on a Vortex mixer, they were centrifuged for 10 min at 0°C to separate the precipitated protein. The supernatant was diluted (1:4) with distilled water, and 100-μl aliquots were injected into the chromatograph.

Analyses were performed on a reverse-phase column (Spherisorb, 10-μm octadecylsilane; 250 by 4.6 mm; Phase Separations Ltd., Queensferry, U.K.) at ambient temperature, using methanol–0.025 M KH_2PO_4, pH 2.5 (5:95) as the mobile phase. The flow rate was constant at 1.0 ml/min. A single-piston, high-pressure (Altex model 110), a sample injection valve containing a 100-μl loop (Chromatronix), and a fixed-wavelength ultraviolet detector with a 20-μl flow cell (Altex model 150) formed the basis of the chromatograph.

Absorbance of the effluent from the column at 280 nm was monitored at a sensitivity of 0.32 a.u.f.s. Peak heights were used for quantitation of the assay and quality control was achieved by external standardization.

To prevent contamination of the reverse-phase column by plasma constituents remaining after the protein removal step, a precolumn (50 by 4.6 mm) containing reverse-phase packing was incorporated into the system. Several hundred samples could be assayed before significant increases in perfusion pressure necessitated a change of precolumn (at 3,000 lb/in^2).

All chemicals used were analytical grade and water was doubly distilled. Flucytosine was donated by Roche Products Pty. Ltd., Dee Why, Australia.

Calibration curves were obtained by addition of pure flucytosine to pooled blood bank plasma followed by sample processing and chromatography as above.

Plasma concentrations of flucytosine were also measured by a well diffusion microbiological method, based on that of Kasper and Drutz (4), using a flucytosine-susceptible laboratory strain of *C. albicans*.

5-Fluorouracil, which has been described as a metabolite of flucytosine (2), did not interfere with either the HPLC or the microbiologically based assay.

RESULTS

Typical chromatograms of plasma samples (Fig. 1) show that control samples are free from contaminating peaks. Flucytosine was eluted in 5 min, immediately before elution of endogenous compounds in plasma. Calibration curves for flucytosine passed through the origin and were linear up to 160 μg/ml, the maximum concentration used. Less than 1 μg of flucytosine per ml in plasma was easily detectable. Recovery of flucytosine added to plasma was 103% in comparison with aqueous standards. At a concentration of 40 μg/ml, the intra-assay coefficient of variation was 1.3% ($n = 10$). The day-to-day precision of the assay determined over 5 weeks is shown in Table 1.

To demonstrate the effectiveness of the assay in clinical situations, blood samples were taken at various intervals from four hospitalized patients being treated with flucytosine. Plasma concentrations of flucytosine in 28 samples were determined in duplicate by the HPLC and microbiological methods. There was close overall agreement between levels determined by the two procedures. The correlation coefficient was 0.9548 (Fig. 2), and differences between the results were not statistically significant ($2 P > 0.10$; paired t-test).

In one patient who had received 3.75 g of flucytosine daily for 3 weeks, the decline of plasma concentrations with time was monitored by using both chromatographic and microbiological methods after the final dose (1.25 g) in the course of treatment (Fig. 3). In this particular subject, concentrations obtained with the microbiological assay were significantly higher ($2 P < 0.05$; paired t-test) than those determined by the chromatographic procedure. Both methods indicated that absorption is essentially complete within 1 h, and the fall in concentration with time thereafter was found to be monoexponential when analyzed by the Autoan suite of computer programs (10). It is apparent from Fig. 3 that the results with the HPLC procedure show less scatter about the calculated line, although the correlation coefficients, −0.9931 and −0.9993 for microbiological and HPLC assay data, respectively, are not significantly different when tested statistically using significance limits of Fisher's Z-transformation. The systematically higher levels obtained by the microbiological method during the elimination phase resulted in significantly different elimination rate constants ($2 P < 0.05$) corresponding to half-lives of 8.06 h for microbiological data and 6.54 h for HPLC data.

TABLE 1. *Day-to-day reproducibility of the assay over 5 weeks*

Plasma flucytosine concn (μg/ml)	No. of determinations	Coefficient of variation (%)
12	4	5.7
30	4	1.2
75	4	1.5

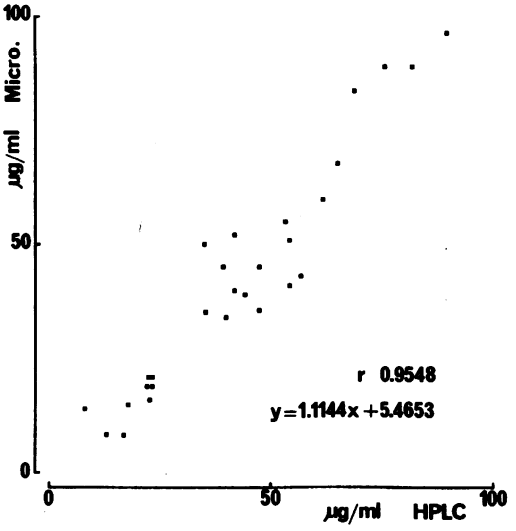

FIG. 2. *Comparison of flucytosine concentrations in 28 plasma specimens determined by microbiological and HPLC assays.*

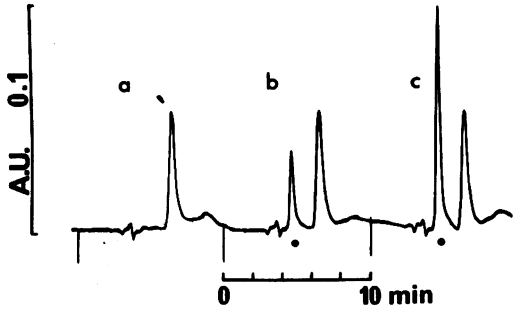

FIG. 1. *Typical chromatograms of deproteinized plasma. (a) Blank; (b) 15 μg of flucytosine per ml (●); (c) 40 μg of flucytosine per ml (●). The ordinate represents absorbance units (AU).*

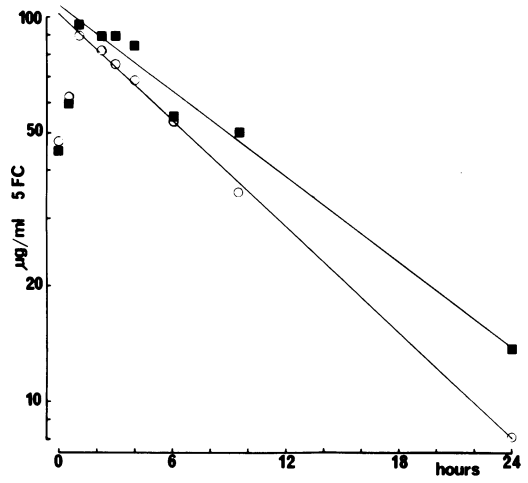

FIG. 3. *Plasma concentration-time profiles of flucytosine (5-FC) in a patient after the last dose (1.25 g) in the course of treatment, using concentrations determined by microbiological (■) and HPLC (○) methods.*

No interference with the chromatographic measurement of flucytosine was observed in any patient, despite concurrent medication with a wide range of drugs. These included amphotericin B, ampicillin, aspirin, atropine, cephalexin, chloral hydrate, cimetidine, codeine, dextropropoxyphene, digoxin, framycetin, heparin, imipramine, morphine, nitrazepam, nystatin, paracetamol, pethidine, prednisolone, sulfamethoxazole, trimethoprim, and vancomycin.

DISCUSSION

Until recently, most assays of antimicrobial agents in clinical use have been based on microbiological techniques using selected strains of organisms, but there are now increasingly numerous reports of chemical methods of analysis. The latter must be designed carefully to avoid interference from inactive metabolites or other substances with physicochemical properties similar to those of the assayed drug. On the other hand, antimicrobial activity not due to the drug being measured may interfere with bioassay. These possibilities of confusion caused by different specificities of assay must be remembered in assessing results. Agreement between chemical and biological assay data should be mandatory before either method is accepted as fully validated for research or routine applications.

The reverse-phase HPLC determination of flucytosine in plasma represents a desirable alternative to conventional microbiological assay. The HPLC method is accurate and reproducible and yields results comparable to or perhaps slightly superior to those from the microbiological assay. Furthermore, the procedure described in this report is simpler and faster than existing microbiological (1, 9) and chemical methods (4, 13) and requires only relatively inexpensive HPLC equipment. Its value for routine use is enhanced by the lack of interference by a large number of other drugs and their circulating metabolites. Obviously, the method is free from interference by growth-inhibiting factors for *Candida* that have been reported to occur in serum (6).

The differences between the results obtained by the HPLC and microbiological assays are generally of insufficient magnitude to alter clinical interpretation of the data. However, in the case of significant discrepancy between the concentrations estimated by the two methods (Fig. 3), the HPLC data appeared to be more in keeping with expected pharmacokinetic behavior of the drug; e.g., there is no apparent explanation for the failure of the plasma concentrations to decline from 2.25 to 3.0 h, as indicated by the microbiological assay.

Using the HPLC method described in this paper, the concentration of flucytosine in plasma can be estimated in less than 30 min compared with 8 to 24 h for the conventional microbiological assay. Adjustments in flucytosine therapy can now be planned before the succeeding dose in the event of rapidly changing renal function.

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