Fluorometric Measurement of 5-Fluorocytosine in Biological Fluids

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Abstract □ A spectrofluorometric method is described for the assay of 5-fluorocytosine in biological fluids. The method has the advantage of high specificity and has proved to be equally suitable for assaying the drug in human plasma and urine.

Keyphrases □ 5-Fluorocytosine—spectrophotofluorometric determination in biological fluids □ Spectrophotofluorometry—determination of 5-fluorocytosine in biological fluids

The concentration of 5-fluorocytosine in biological fluid can be measured by a microbiological technique involving the inhibition of the growth of *Candida albicans*[1]. Although this technique has high sensitivity (1–1.5 mcg./ml.), there is a problem of interference by unknown constituents in the urine and the specificity of the method may not allow measurement when there is simultaneous administration of other antifungal drugs.

The fluorometric method described here has the advantages of specificity and is equally applicable to plasma and urine. The method is less sensitive than the *C. albicans* assay but encompasses the therapeutic range and the very high plasma levels seen in some patients with renal insufficiency.

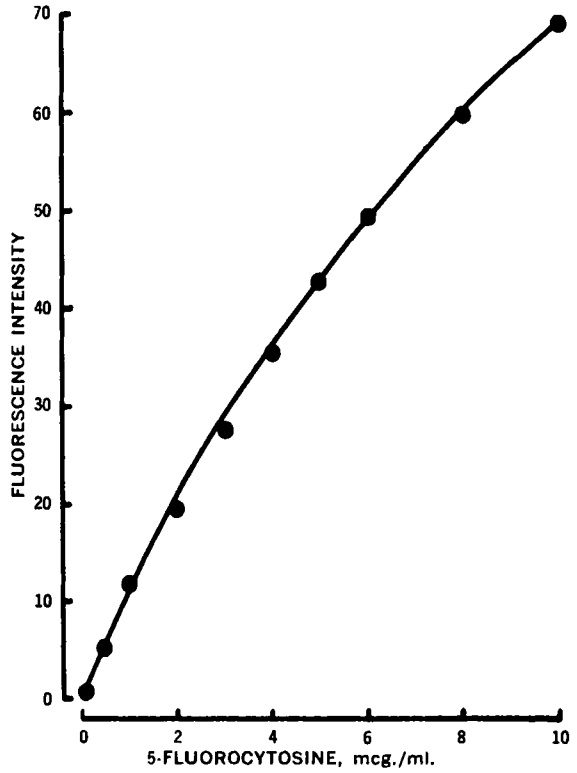

Figure 1—*Relationship between the concentration of 5-fluorocytosine in alkaline aqueous solutions and fluorescence intensity. Solutions were excited at a wavelength of 300 nm., and fluorescence was measured at 365 nm.*

[1] Method report: "Agar diffusion assay of 5-fluorocytosine in human serum," F. Hoffmann–La Roche & Co., Basel, Switzerland.

EXPERIMENTAL

Principle—5-Fluorocytosine is isolated from protein-free biological fluids by TLC. After elution from the chromatoplate, the fluorescence of the eluate is measured in alkaline solution.

Reagents—The following were used: (*a*) 5-fluorocytosine[2]; (*b*) *n*-butanol, analytical reagent grade; (*c*) glacial acetic acid, analytical reagent grade; and (*d*) 0.75 *M* NaOH. All solutions were made up in glass-distilled water.

Procedure—*Serum Estimations*—Two-milliliter serum samples are ultrafiltered through a pressure filtration funnel[3] using an ultrafiltration membrane[4] at 90 p.s.i. of oxygen. Twenty microliters of the first drop of the ultrafiltrates is spotted on thin-layer chromatoplates[5] alongside 5 mcg. of authentic 5-fluorocytosine as a standard. Chromatograms are then developed in one direction using the solvent system of butanol–acetic acid–water (6:1:2). Developed chromatoplates are dried and viewed under UV light at 254 nm. Squares, 2.54 cm. (1 in.), corresponding to the 5-fluorocytosine spots and serum blanks at an R_f of 0.59 are marked and cut out. The silica is scraped from these squares into centrifuge tubes, and the 5-fluorocytosine is eluted by thorough mixing with 2 ml. of distilled water. After centrifugation, the supernate is made alkaline with 2 drops of 0.75 *M* NaOH and the fluorescence is measured, exciting at a wavelength of 300 nm. and measuring emission at 365 nm.

A standard curve of the relationship between 5-fluorocytosine concentration and fluorescence intensity is established using freshly prepared standard solutions of 0.2, 0.5, and 1 mcg./ml. Since the ultrafiltrate is diluted by a factor of 100, these standards correspond to 20, 50, and 100 mcg./ml. of 5-fluorocytosine in serum, respectively.

Urine Estimations—5-Fluorocytosine in urine is measured using the same method. Two-milliliter urine samples are filtered through Whatman No. 1 filter paper, and 2-μl. aliquots of the filtrate are spotted on the chromatoplates. Standard solutions of 0.1, 0.5, 1, and 1.25 mcg./ml. are used to establish the standard curve from which the 5-fluorocytosine concentration in the eluting solution is read. These standards correspond to urine 5-fluorocytosine levels of 100, 500, 1000, and 2000 mcg./ml., respectively, since a dilution factor of 1000 is involved.

Calculation—The concentration of 5-fluorocytosine in the eluting solutions is established from their fluorescence measurements by reference to the standard curves. Since these standard curves are obtained from solutions in water and the recovery of 5-fluorocytosine from serum and urine averages approximately 80%, each value obtained for 5-fluorocytosine concentration is corrected accordingly.

RESULTS

Fluorescence of 5-Fluorocytosine—5-Fluorocytosine fluoresces strongly in alkaline solution. The emission maximum is at 365 nm. when excited at 300 nm. The fluorescence of alkaline solutions of 5-fluorocytosine increases with concentration up to 25 mcg./ml. and decreases at higher concentrations due to quenching. The relationship is sufficiently linear up to 10 mcg./ml. (Fig. 1) for the assay of solutions containing from 0.05 to 10 mcg./ml. of 5-fluorocytosine.

5-Fluorocytosine in Plasma—There is good linear correlation between fluorescence and concentration up to 250 mcg./ml. of 5-fluoro-

[2] Ro 29915 E/265'601, Roche Products Pty Ltd., Sydney, Australia.
[3] Gelman.
[4] Diaflo type UM-20E.
[5] Kieselgel F_{254}, Merck.

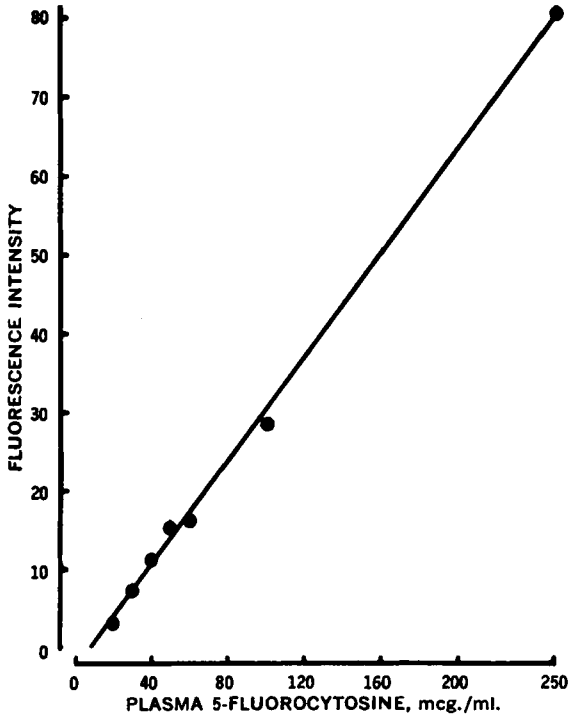

Figure 2—*Relationship between fluorescence intensity of the chromatoplate eluate and the concentration of authentic 5-fluorocytosine added to normal human plasma.*

cytosine in plasma (Fig. 2). Concentrations up to 1000 mcg./ml. can be read if standards are applied to the chromatogram to correct for altered recovery in this range. The upper and lower limits of the linear region lie outside the therapeutic blood levels and those high levels that may develop in patients with renal insufficiency (1).

5-Fluorocytosine in Urine—There is good linear correlation between fluorescence and concentration up to 5000 mcg./ml. of 5-fluorocytosine in urine. Since 5-fluorocytosine in the urine can be up to 100 times more concentrated than in the plasma, the fluorescence of eluates from undiluted urine samples may fall outside the linear region of the standard curve.

Specificity—*Normal Human Plasma and Urine*—The method appears to be highly specific for 5-fluorocytosine in plasma. Control estimations on the plasma from eight drug-free normal individuals gave fluorescence readings corresponding to only 1.6–3.8 mcg./ml. of 5-fluorocytosine in plasma, with a mean and standard deviation of 2.6 ± 0.82 mcg./ml., well below the minimum therapeutically useful plasma level of the drug.

A similar high degree of specificity is seen with estimations in human urine. Control estimations on the urine from eight drug-free normal individuals gave fluorescence readings corresponding to 13–75 mcg./ml. of 5-fluorocytosine in urine, with a mean and standard deviation of 35.7 ± 24.5 mcg./ml. This again represents levels well below minimum values seen with therapeutic use of the drug.

Possible Metabolites of 5-Fluorocytosine—It was shown previously (1) that little, if any, 5-fluorocytosine is metabolized in normal subjects and that 90% of a single dose is excreted as unchanged drug in the urine within 48 hr. If any 5-fluorocytosine (I) was converted to 5-fluorouracil (II), this would not interfere with the assay because the two compounds are widely separated by the chromatography method described (R_f I 0.59; R_f II 0.77).

Other Drugs—In patients receiving no other drugs, 5-fluorocytosine may be assayed directly in the plasma ultrafiltrate without the chromatography step. Ultrafiltered plasma blanks from drug-free individuals showed no significant interfering fluorescence; a standard curve showing a linear relationship between 5-fluorocytosine added to plasma and fluorescence in the ultrafiltrate was established over the range 10–200 mcg./ml. of plasma. However, aspirin and its metabolite, salicylic acid, fluoresce strongly at the wavelengths used in the assay and show significant interference with this direct assay at therapeutic blood levels. Chromatography using the solvent system described separated 5-fluorocytosine (R_f 0.59) from salicylate (R_f 0.88) which thus does not interfere with the assay. Other drugs likely to be given simultaneously with 5-fluorocytosine, including amphotericin B, nystatin, and azathioprine, do not interfere with the assay.

Sensitivity—The effective and safe range of blood levels probably depends in part on the particular organism and the site and extent of the infection. This range is not yet known with certainty, but this assay is clearly sufficiently sensitive to measure below the minimum therapeutically effective blood level. The relative sensitivity limit (RSL) was calculated from the blank values of plasma and urine samples from eight drug-free normal individuals using the formula:

$$\text{RSL} = t_{0.05} \cdot s \sqrt{1 + \frac{1}{N}} \qquad \text{(Eq. 1)}$$

where s = standard deviation of the readings, $t_{0.05}$ = one-sided t-deviate for the probability of error $P = 5\%$ and $N - 1$ degrees of freedom, and N = number of observations.

In human plasma and in human urine, the relative sensitivity limit had a value of 1.65 and 49.1 mcg./ml. of 5-fluorocytosine, respectively.

Reproducibility—The method gives good reproducibility both in plasma and urine. The reproducibility was tested by eight analyses of the same sample of human plasma containing 80 mcg./ml. of 5-fluorocytosine. A value of 68.01 ± 2.28 mcg./ml. (mean and standard deviation) was obtained.

In the case of human urine, the reproducibility was determined from 10 analyses of the same urine sample containing 2000 mcg./ml. of 5-fluorocytosine. A value of 1902 ± 106 mcg./ml. (mean and standard deviation) was obtained.

Recovery—The recovery from human plasma was determined from seven analyses of samples containing known 5-fluorocytosine concentrations in the 20–100-mcg./ml. range. It varied between 78 and 89.5%, with a mean and standard deviation of 81.72 ± 4.07%. The recovery from human urine was determined from 10 analyses of samples containing known 5-fluorocytosine concentrations in the 500–5000-mcg./ml. range. It varied between 70 and 92%, with a mean and standard deviation of 80.2 ± 7.7%. Recovery from plasma did not vary systematically with concentration, but recovery from urine did decrease with increasing concentration.

SUMMARY

A specific spectrofluorometric method is described for the assay of 5-fluorocytosine in plasma and urine. The method has sufficient sensitivity to cover the therapeutic range and higher values occasionally seen with cumulative overdose. The drugs likely to be used in association with 5-fluorocytosine do not interfere with the method, which would recommend it for the monitoring of blood levels in patients on long-term therapy.

REFERENCE

(1) D. N. Wade and G. Sudlow, *Aust. N. Z. J. Med.*, **2**, 153 (1972).

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