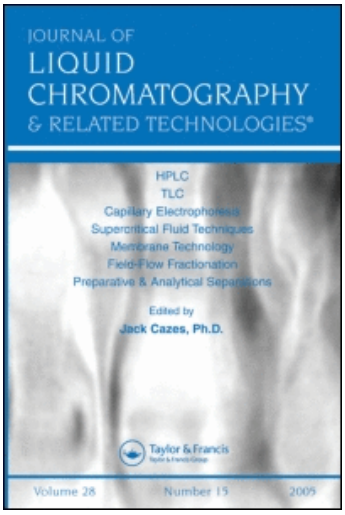

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF 5-FLUOROCYTOSINE IN HUMAN PLASMA.

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ABSTRACT

A high performance liquid chromatographic method has been developed for the determination of an antifungal drug : 5-Fluorocytosine (5-FC), in human plasma, using reversed-phase technique.

The rapid method involved single ethyl acetate extraction, in the presence of an internal standard (5-Fluorouracil). The eluent mixture was a pH 4.8 acetate buffer. A wavelength of 280 nm was used to monitor 5-FC and the internal standard.The limit of sensitivity of the assay was 0.6 µg/ml with a precision of $\pm$ 8 %. This method is used to quantitative 5-Fluorocytosine in human plasma from renal failure patients, with satisfactory accuracy and precision. Endogenous substances and a variety of drugs concomitantly used in (5-FC) therapy did not interfere with the assay.

INTRODUCTION

5-Fluorocytosine (5-FC), (4-Amino, 5-fluoro, 2-oxo, 1,2 dihydropyrimidine), is a derivative, with antifungal properties in several systemic mycotic infections including cryptococcal meningitidis, visceral candidiasis, torulopsis, chromomycosis (1, 2, 9).

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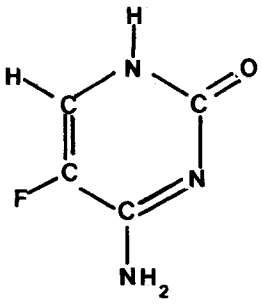
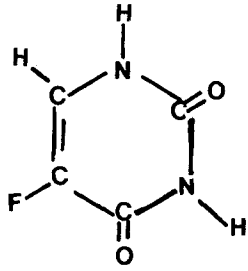


- 1 -

5-Fluorocytosine
(4-amino-5 fluoro-2 oxo-1-2 dihydropyrimidine)



- 2 -

5-Fluorouracil
(5-fluoro-2-4-dioxopyrimidique)

About 90 % of 5-Fluorocytosine is excreted unaltered in the urine (4, 7, 8, 12, 14, 16). The measurement of plasma 5-Fluorocytosine allows the dosage to be adjusted in patients suffering pathological conditions, e.g., when renal function is compromised by renal disease or from chronic drug administration. The determination of antifungal agents in biological fluids is often performed by microbiological assay procedures (11, 15). These assay techniques are long and not very specific, especially when the patients involved have received multiple antimicrobial agents.

A gas liquid chromatographic (G.L.C.) assay has been described (10). This method is not very rapid and has the disadvantage of low recovery (35 %).

Recently, two high performance liquid chromatographic (H.P.L.C.) procedures, with the use of thermostated cation exchange columns and direct injections of plasma onto column (3) deproteinization of plasma before assay (6), have been developed.

We report the development of a reversed-phase H.P.L.C. assay requiring only simple extraction with ethyl acetate, and using a structurally related agent (5-Fluorouracil) as the internal standard.

This method is rapid, selective and reproducible and has been used for plasma samples obtained following pharmacokinetic in renal failure patients.

METHODS

The procedure involves the addition of 5-Fluorouracil (5-FU) as the internal standard. After addition of 1.0 ml phosphate buffer (pH 7.0), plasma samples are extracted using ethyl acetate.

After evaporation of the organic solvent, the residue is dissolved in the mobile phase and the drug is analysed isocratically by reversed-phase liquid chromatography with pH 4.8 acetate buffer as eluant. The effluent is monitored by U.V. detection at 280 nm.

Apparatus

The H.P.L.C. system consists of a Model 6000-A solvent delivery system and a Model 710-A WISP sample injector (Waters Associates, Inc., Milford, Mass, 01757, U.S.A.).

A model 440 absorbance detector (280 nm wavelength) (Waters Associates, Inc., Milford., Mass, 01757, U.S.A.) was used at a sensitivity of 0.01 absorbance units full scale (a.u.f.s.) for plasma samples. The chromatograms were recorded on an Omniscribe (Houston Instruments, Gistel, Belgium).

The mobile phase : acetate buffer (pH 4.8) and methanol (99: 1, v/v) was filtered through a 0.45 µm membrane filter type GS-cellulose ester (Millipore Corp., Bedford, Mass, 01730) and carried through an octadecylsilane µ-Bondapak C_{18} column (30 cm x 3.9 mm, particle size 10 µm, Waters Assoc.) at 1 ml/min. and ambient temperature.

Under these conditions, 5-Fluorocytosine and the internal standard (5-FU) were eluted with retention times of 5.4 and 6.5 min ., respectively, as illustrated in Figure 1.

Chemical and reagents :

5-Fluorocytosine (5-FC) and 5-Fluorouracil (5-FU) were both supplied by Roche Laboratories (Neuilly - France). Water was doubly distilled and filtered through a 0.22 µm (type GS-cellulose ester) Filter (Millipore, Corp., Bedford, Mass, 01730).

Standard stock solutions of 5-FC and internal standard were prepared by dissolving solid standards in bidistilled water at concentrations of 400 µg/ml and 100 µg/ml respectively and could be stored at 4°C for two weeks in amber glass containers. Standard concentrations of 5-Fluorocytosine in plasma ranging from 0.625 to 20 µg/ml were made by appropriate dilution of the stock solution.

- All organic solvents (methanol, ethyl acetate) were H.P.L.C. grade (Carlo Erba, Milan, Italy).

- Acetic acid (Art. 63, Merck, Darmstadt G.F.R.).

-Sodium acetate (R.P. Prolabo, France).

- Phosphate buffer, pH 7.0, was prepared by dissolving 7.72 g of disodium hydrogen phosphate. 2 H_2O (Merck) and 3.18 g of potassium dihydrogen-phosphate in 1000 ml of bidistilled water.

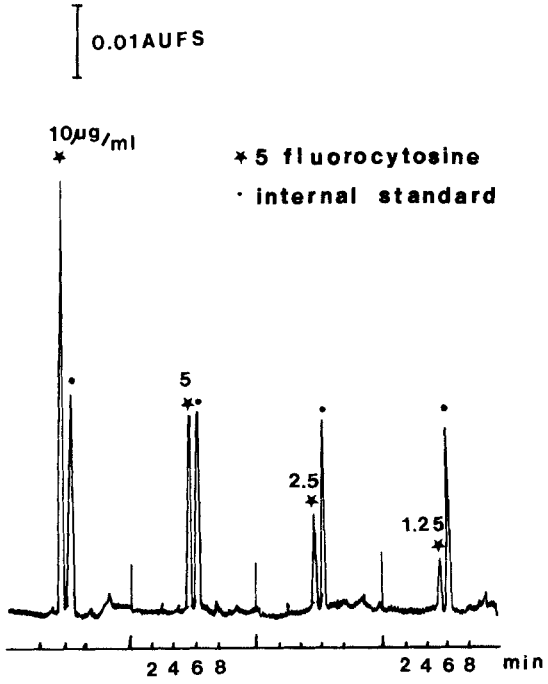


Figure 1 : Chromatogram of 5-Fluorocytosine.

- Acetate buffer (pH 4.8) was prepared by mixing 10 ml of a 0.2 M acetic with 15 ml of 0.33 M sodium acetate and 975.0 ml of bi-distilled water.

Extraction procedure :

In a 10 ml screw-capped tube, one milliliter of plasma (sample to be assayed or standard) was supplemented with 50 µl of an aqueous solution (5 µg/ml) of the internal standard and 1.0 ml of pH 7 phosphate buffer, then homogenized by slow rotation.

The drug was extracted with 6.0 ml of ethyl acetate by shaking mechanically for 10 min. The two phases were separated by centrifugation for 15 min. at 3500 rpm. An aliquot of the upper organic layer was transferred to another clean glass tube and evapored to dryness under a stream of nitrogen at 40°C.

The dry residue was redissolved into 200 µl of mobile phase and an aliquot of 20 µl was injected into the H.P.L.C. system.

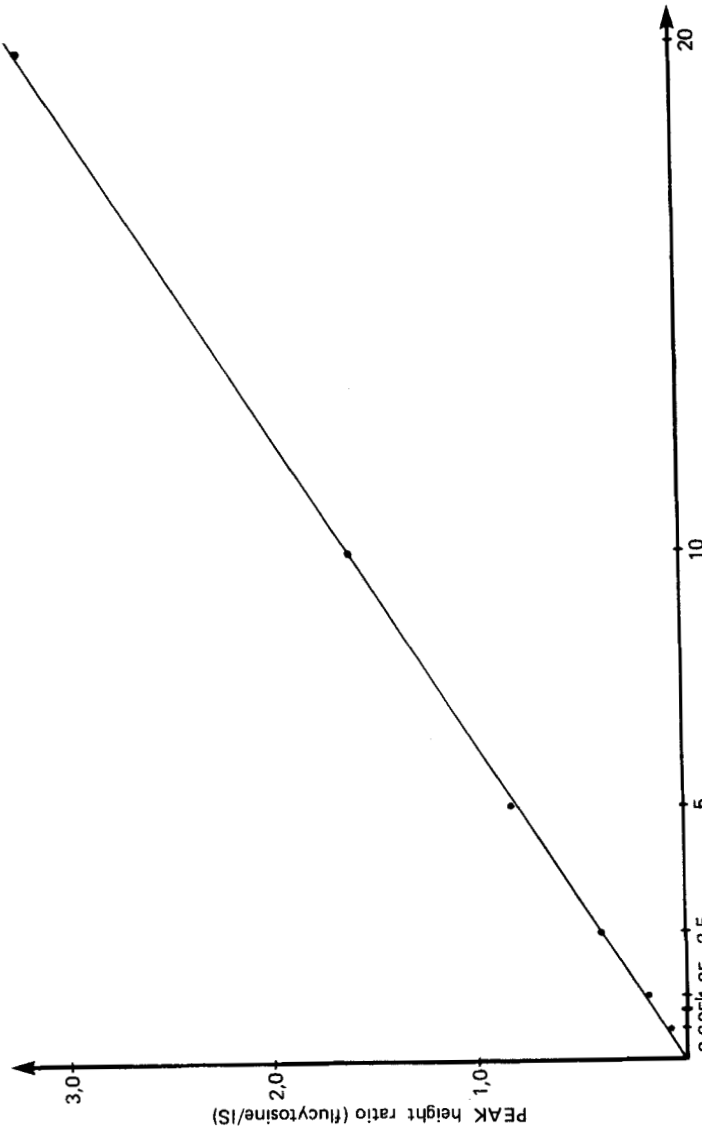


Figure 2 : Linear regression analysis of 5-Fluorocytosine.

Table I : INTRA-ASSAY PRECISION 5-FLUOROCYTOSINE IN HUMAN PLASMA.

Spiked concentration (µg/ml)	Measured concentration (n = 10) Mean ± s.d. (µg/ml)	C.V. (%)
1.0	0.94 ± 0.07	8.1
20.0	20.1 ± 0.80	4.1

Calibration :

A standard concentration curve (Fig. 2) was obtained by adding 5-Fluorocytosine at concentrations of 0.625, 1.25, 2.5, 5.0, 10, 20 µg/ml in control plasma under the same experimental conditions as used for sample analysis.

RESULTS AND DISCUSSION

We found a linear correlation between the concentration of 5-FC and the ratio of peak heights : 5-FC/i.s, in the range 0.625 to 20 µg/ml.

Each point of Figure 2 represents an average of three determinations. The line drawn is the least-squares regression line of equation.

$y = 0.1608\ x - 0.00696$ (x = 0.625 to 20 µg/ml, $n = 6$, $r = 0.9998$).

The intra-assay precision for 5-FC was assessed by repeated analysis on fresh drug-free human specimens spiked with known concentrations of 5-FC. As shown in Table I, within-day precision of the method, the coefficient of variation was 4.1. and 8.1 for 20.0 and 1.0 µg/ml respectively. The inter-assay precision, evaluated by analysing spiked plasma samples on different days over one week ($n = 5$), was found to be ± 5.8 % for samples to concentration 10 µg/ml.

Figure 4 illustrates the chromatographic profile of a human plasma from a patient with renal failure receiving daily infusion administration 1.25 g of 5-FC (15 th hour sample after end of infusion). The stability of samples was tested from spiked human plasma. The samples were stored deep-frozen. Frozen samples remained stable for at least two months.

As can be seen from Figure 3, no plasma constituent peak extracted from the blank interferes with that of 5-Fluorocytosine and i.s., which is well below the detection limit.

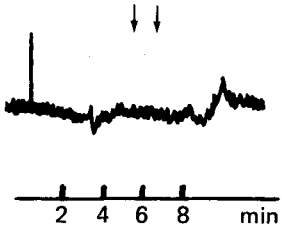


Figure 3 : Chromatograph of human drug free plasma extract.

The limit of detection of this method was 0.6 µg/ml, allowing a signal-to-noise ratio of 3, when 1.0 ml of plasma was used (Figure 5).

The sensitivity of this H.P.L.C. procedure is thus found to be much higher than obtained previously (1.0 µg/ml), using a cation-exchange chromatographic system (3,6).

The buffer and extraction solvent chosen here were, however, found to lead to a minimum of interference in the analysis. The pH and the type of eluent are important when analyzing plasma samples. Using acetate buffer at pH of 4.8, no endogenous peak is detected.

No interfering peak were observed in the plasma of patients receiving 5-Fluorocytosine in combination with drugs such as : amphotericin B, miconazole, carbenicillin, ticarcillin, piperacillin, penicillin G, ampicillin, amoxicillin, cefadroxyl, cefazolin, cefotaxim, moxalactam, cefoxitin, vidarabin.

The life time of the column appears to be very good, as it is still in excellent condition after 6 months use.

The G.L.C. assay (10) requires a total clean up at least 2 h, due to a multitude of diverse extraction, reextraction and centrifugation steps. The H.P.L.C. method described here involves a single extraction procedure. A.D. Blair et al (3) reported a greater precision and speed of the H.P.L.C. method comparatively with the microbiological assay (11, 15). However, we have not compared the present H.P.L.C. assay to the microbiological method. 5-Fluorouracil (5-FU) was retained as internal standard, because 5-Fluorocytosine was reported not to be metabolized to 5-Fluorouracil in mammalian tissues (5).

In conclusion, the H.P.L.C. procedure proposed here is a rapid, sensitive and reproducible technique for the determination of 5-FC.

The sensitivity and rapidity are better than that reported previously with microbiological assay (11, 15) and earlier H.P.L.C. methods (3,6) using an ion-exchanged column with heating.

The chromatograms are interference-free of normal components of fresh human plasma as well as from drugs simultaneously administered. We

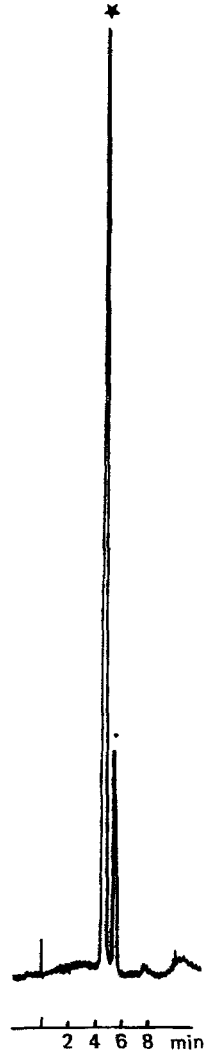


Figure 4 : Chromatogram of plasma extract from a patient receiving 1.25 g of 5-Fluorocytosine.
(15 th hour sample after end of infusion).

loop: 25 µl, S=0.01 a.u.f.s.
✱ 5-Fluorocytosine .(i.s)
21.6 µg/ml of plasma

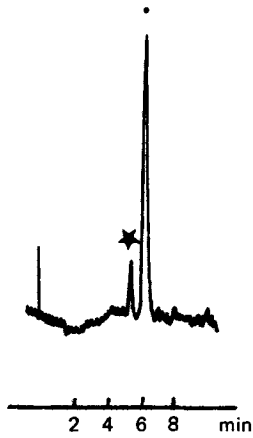


Figure 5 : Chromatogram from a spiked plasma sample (O.6 µg/ml) showing the detection limit of the method.

loop: 20 µl, S=0.01 a.u.f.s.
✱5-Fluorocytosine .(i.s.)

have found that this H.P.L.C. procedure is particulary valuable where rapid determination of 5-FC has been necessary, such as in patients with renal failure.

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