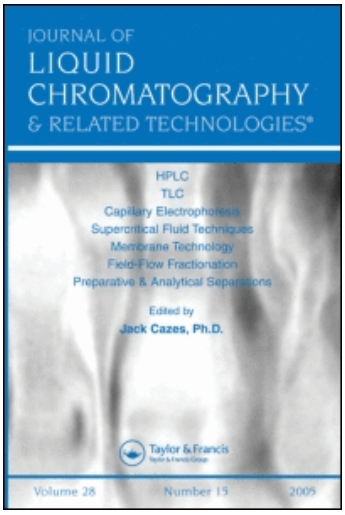

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High Performance Liquid Chromato-Graphic Assay For 5-Fluorouracil and 5-Fluorocytosine

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY FOR 5-FLUOROURACIL AND 5-FLUOROCYTOSINE

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ABSTRACT

A high performance liquid chromatographic assay for separating 5-fluorouracil and 5-fluorocytosine in the presence of potential decomposition products urea, barbituric acid, uracil 6-hydroxycytosine, and cytosine was developed.

It was found that ion-pair chromatography using a reverse-phase C18 column, 0.46 cm i.d. by 15 cm in length, employing gradient elution was satisfactory for separating the various compounds, however, isocratic elution was more appropriate for determining the concentration of the two drugs in the presence of their actual decomposition products.

The mobile phase for isocratic elution was 85% aqueous phosphate buffer pH 2.1 and 15% methanol containing 0.0025 M pentane and heptane sulfonic acid sodium salts delivered at 1.0 ml/min. The eluent was monitored at 254 and 285 nm as all compounds do not absorb strongly at a single wavelength. A linear relationship was obtained for the peak height ratio for both 5-fluorouracil and 5-fluorocytosine against 5-methylcytosine, the internal standard over the range 0.125 to 1.000 x 10^{-4} M.

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INTRODUCTION

In order to study the stability of 5-fluorouracil and 5-fluorocytosine, methods reported in the literature were considered for the high performance liquid chromatographic (HPLC) assay of 5-fluorouracil (1-10) and 5-fluorocytosine (11-14), however, these papers are mainly concerned with separating the drugs from other drugs in body fluids or the drugs from their metabolites.

The stability of 5-fluorouracil has been investigated, thus providing information on decomposition products which are non-chromophoric in nature (15). The potential decomposition products of 5-fluorocytosine may be postulated by considering the decomposition of other 5-halogenated cytosines or cytosine (16, 17, 18). The compounds in Table 1 were selected for HPLC separation study. All the compounds absorb in the UV spectrum except urea, which is frequently found when the pyrimidine ring is cleaved (20,23). There are other decomposition products from the degrada-

TABLE 1

Ionization Constants of Compounds and Wavelength of Maximum Absorbance in Acidic Medium

Abb. Code	Compounds	$pK_{a(1)}$	$pK_{a(2)}$	λ_{max}(acid) nm
I	5-Fluorouracil[d]	8[a]	13[a]	266
II	5-Fluorocytosine[f]	2.9[b]	10.71[a]	283-287
III	5-Methylcytosine[c]	4.6[b]	12.4[a]	283
IV	Cytosine[c]	4.5[b]	12[a]	276
V	6-Hydroxycytosine[e]	0.8[b]	---	260 expt.
VI	Uracil[c]	9.5[a]	>13[a]	260
VII	Barbituric Acid[c]	4.04[a]	---	205,253 expt.
VIII	Urea	---	---	nil expt.

a acidic dissociation b basic dissociation c Ref (19)
d Ref (20) e Ref (21) f Ref (22)

tion of 5-halogenouracils or cytosines, but none of them absorb in the UV portion of the spectrum (15, 17, 18). 5-methylcytosine was selected as the internal standard (13) as it resembles 5-fluorocytosine in many physico-chemical characteristics and is quite stable.

MATERIALS AND METHOD

Apparatus and Reagents

The HPLC was carried out on a system consisting of two pumps model 112, injector model 340, controller model 421, variable wavelength detector model 165, recorder Kipp and Zonen BD41 and C18 reverse phase column, Altex Ultrasphere ODS analytical 5 µm column and 10 µm precolumn (Beckman Instruments Inc., Berkley California).

The compounds 5-fluorocytosine (Hoffman-LaRoche Ltd., Vaudreuil Quebec), 5-fluorouracil, 5-methylcytosine, 6-hydroxy-cytosine, barbituric acid, cytosine (Sigma Chemical Co., St. Louis Mo), urea (B.D.H. Laboratory Chemicals, Toronto, Ontario), uracil (Mann Research Lab., New York, N.Y.) were used as received. Methanol, 1-pentane sulfonic acid sodium salt and 1-heptane sulfonic acid sodium salt were HPLC grade.

Liquid Chromatography

Isocratic Elution. The mobile phase consisted of 85% of 0.05 M H_3PO_4 and 0.05 M KH_2PO_4 aqueous pH 2.1 buffer and 15% methanol; containing 0.0025 M pentane sulfonic acid and 0.0025 M heptane sulfonic acid as the sodium salts. All solvents were filtered through a 0.45 µm teflon filter Type HA (Millipore Corp., Bedford Ma) before use. Injections were made into a 20 µ liter fixed loop and the mobile phase was delivered at 1.0 ml/min which yielded a pressure of approximately 1500 psi. The column eluent was monitored at 254 nm and 285 nm. Chromatograms were obtained at an attenuation of 0.5 and 0.2 aufs respectively, at a chart speed of 0.5 cm/min. All chromatography was conducted at room temperature.

Gradient Elution. The aqueous mobile phase contained the same concentration of buffer salts and sulfonic acid salts and the methanolic phase contained the same concentration of sulfonic acids as described above. The gradient procedure at a flow rate of 1.0 ml/min was 100% aqueous solution for 2 min, a linear increase of methanolic solution to 30% of the mobile phase, and simultaneously a linear decrease of aqueous solution to 70% over a 1 min period. Then a 70% aqueous phase and a 30% methanolic phase was used thereafter. The other operating conditions, filtration, injection, monitoring wavelengths, attenuation, chart speed and temperature were the same as described for isocratic elution.

Analytical Procedure

The buffer-internal standard solution was prepared by diluting an aqueous stock solution of 5-methylcytosine ten-fold with a phosphate buffer solution to yield a concentration of internal standard of 1.5×10^{-4} M and a buffer which had the same concentration as the aqueous portion of the mobile phase. Each sample used in isocratic elution and gradient elution containing the seven compounds was diluted with the buffer internal standard 1 to 10, to yield a final concentration of 10^{-4} M for each of the compounds.

The calibration curves for the two main compounds of interest, 5-fluorouracil and 5-fluorocytosine were carried out along with the internal standard by using the 85% aqueous buffer phase and 15% methanolic phase described above. The calibration curves were prepared by repeating the HPLC analysis of the mixture ten times.

RESULTS AND DISCUSSION

In order to develop a suitable HPLC assay for 5-fluorouracil and 5-fluorocytosine and potential decomposition products, it was noted that several of the compounds are weak acids or bases (Table 1). Thus it was necessary to maintain the pH of the analytical sample and the eluting solvent so as to control the type and concentration of ionic species present in solution, assuring that their chroma-

tographic interactions and their absorption was constant during the assay. As a result, an acidic mobile phase pH 2-3 was used, this caused some of the compounds to remain neutral, namely barbituric acid, uracil, 5-fluorouracil and 6-hydroxycytosine, while other compounds 5-fluorocytosine, cytosine and 5-methylcytosine existed as cations. In order to increase the retention time of the ionic compounds at this pH on the reverse-phase column, a counter-ion was used. The elution patterns of the compounds were studied using various proportions of aqueous phosphate buffer pH 2.1 and methanol with 0.005 M pentane sulfonic acid or 0.005 M heptane sulfonic acid or a mixture of 0.0025 M pentane sulfonic acid and 0.0025 M heptane sulfonic acid prepared by using the sodium salts. It was found that pentane sulfonic acid would not allow complete separation of 5-fluorouracil and 5-fluorocytosine from the neutral compounds, whereas the heptane sulfonic acid and mixture of sulfonic acids effected the separation. The mixture of sulfonic acid salts was used as this decreased the analysis time. The eluting patterns of the compounds with various proportions of phosphate buffer pH 2.1 and methanol, containing 0.0025 M heptane sulfonic acid/0.0025 M pentane sulfonic acid were studied. Fig. 1 is a graph of the capacity factor

$$(k = \frac{t_r - t_o}{t_o}, \; t_r = \text{retention time of compound}, \; t_o = \text{retention time of solvent front})$$

plotted against the percent of methanol in the eluent. It can be seen that the compounds which remain neutral elute as resolved bands only when the mobile phase is very weak. In order to effect a reasonable separation of the neutral compounds and to maintain a suitable elution time for the paired ionic compounds, gradient elution was used, employing a high water content at the beginning of the elution and then increasing the percent of methanolic phase to remove the paired ionic compounds from the column. The most appropriate conditions are those described above in the experimental section. The compounds are clearly separated one from the other, and the eluent was analysed at two separate wavelengths, since all

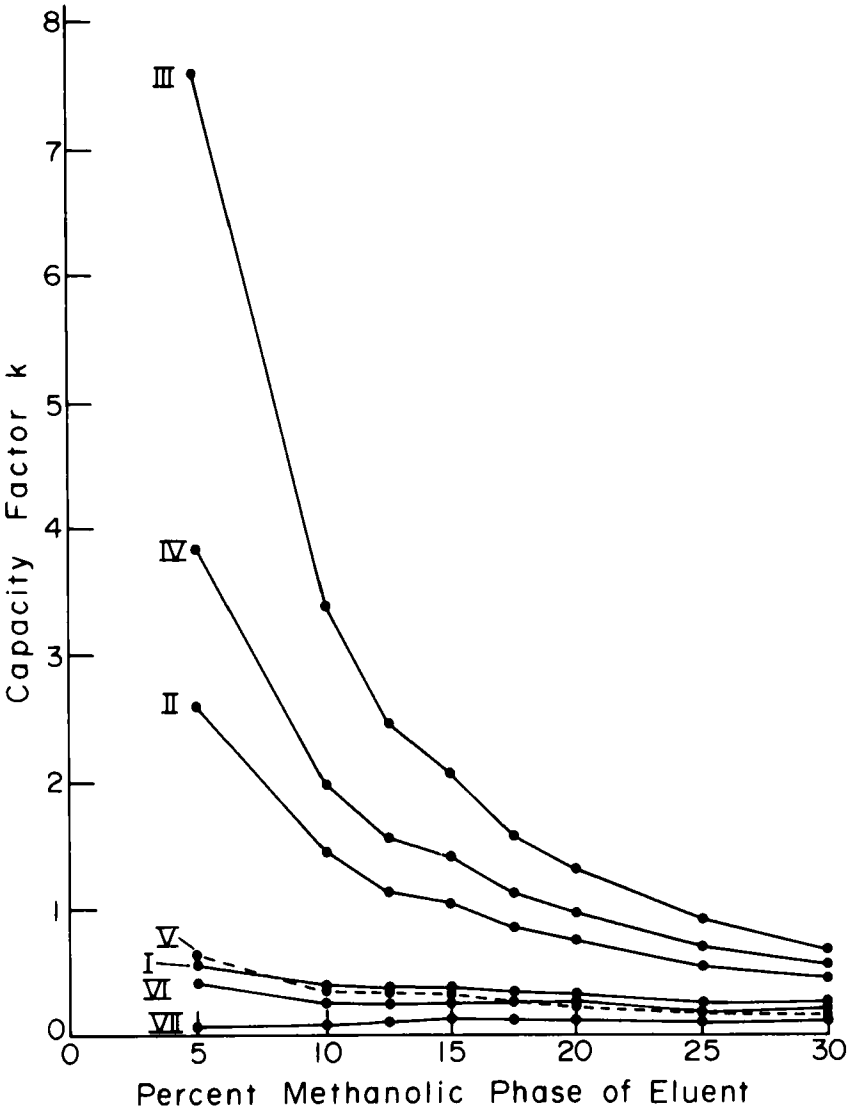

Figure 1 - Capacity Factor as a Function of Percent Methanolic Phase of the Eluent.
Key: see Table 1

the compounds do not absorb strongly at a single wavelength as can be seen from Fig. 2. The wavelength of 285 nm was selected because it is the λmax for 5-fluorocytosine and close to the λmax for 5-methylcytosine and cytosine. All the other compounds have appreciable absorbance at 254 nm. The retention times are given in the figure legend from which the capacity factor k and the selectivity $\alpha = \frac{k_b}{k_a}$ can be determined, where k_a and k_b are the capacity factors for two compounds. The selectivity factor had a value greater than 1 for all adjacent peaks.

Because of the changing solvent conditions and the need for re-equilibration before starting a new elution, it was decided to determine the reproducibility of the assay for 5-flurocytosine by gradient elution. A sample of 5-fluorocytosine and 5-methylcytosine prepared as described in the method section was chromatographed a number of times. The coefficients of variation (percent) for the peak height ratio of the two was 3.97% (n = 12).

In order to study the kinetics of decomposition of a drug, high reproducibility for the drug peaks is important in order to quantify the results, and the peaks in the degradation products should be separated from the drug peak. A typical isocratic elution chromatogram, along with the retention times, is given in Fig. 3. Under these conditions uracil, 6-hydroxycytosine and 5-fluorouracil were not completely resolved, however, they were distinguishable at 254 nm.

Quantitation of 5-fluorouracil and 5-fluorocytosine was obtained from calibration curves in which the peak height ratio drug/internal standard was plotted against the drug concentration. There is a linear relationship between the peak height ratios of 5-fluorouracil to 5-methylcytosine over the range 0.125 to 1.00×10^{-4} M. The least-square regression for the curve is $y = 0.3076 x - 0.001200$ and the correlation coefficient is 0.9995. The coefficients of variation (percentages) at 0.125, 0.25, 0.5, 0.75 and 1.00×10^{-4} M are 3.99, 2.34, 1.47, 2.78 and 0.90 respectively. There is also a linear relationship between the peak height ratios of 5-fluorocytosine and 5-methylcytosine over

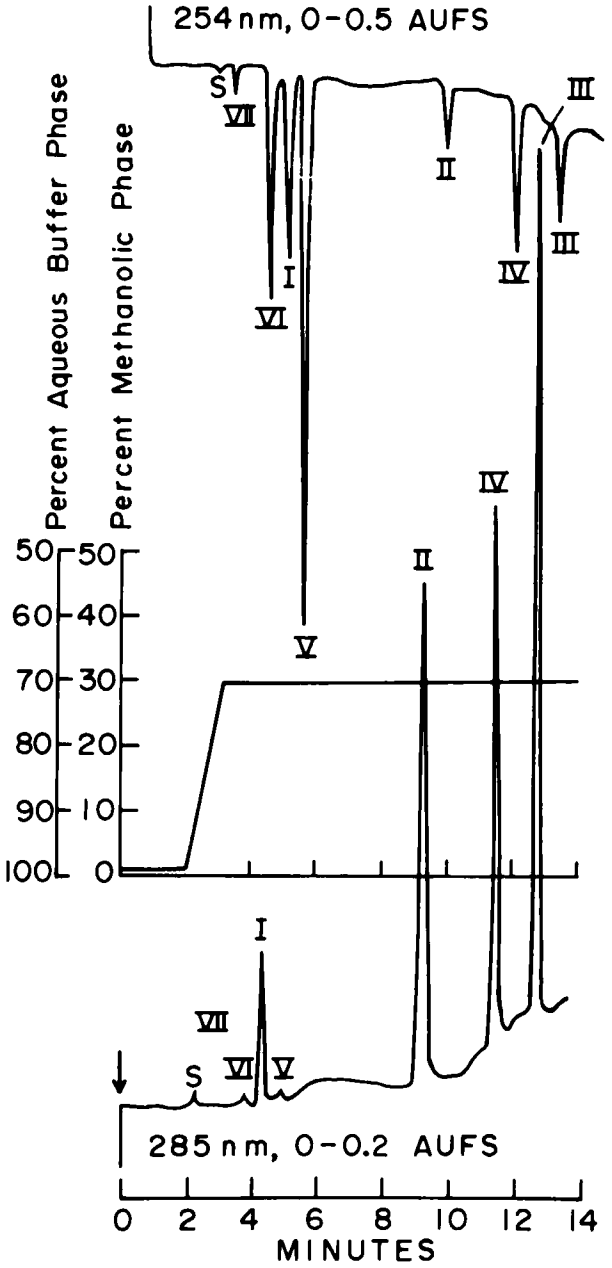

Figure 2 - Representative Chromatogram for Gradient Elution. S, solvent front, Injection event, arrow. Retention times, min, t_s,2.16; t_{VII},2.66; t_{VI},3.82; t_I,4.36; t_V,4.82; t_{II},9.20; t_{IV}11.36; t_{III},12.62.
Key: see Table 1

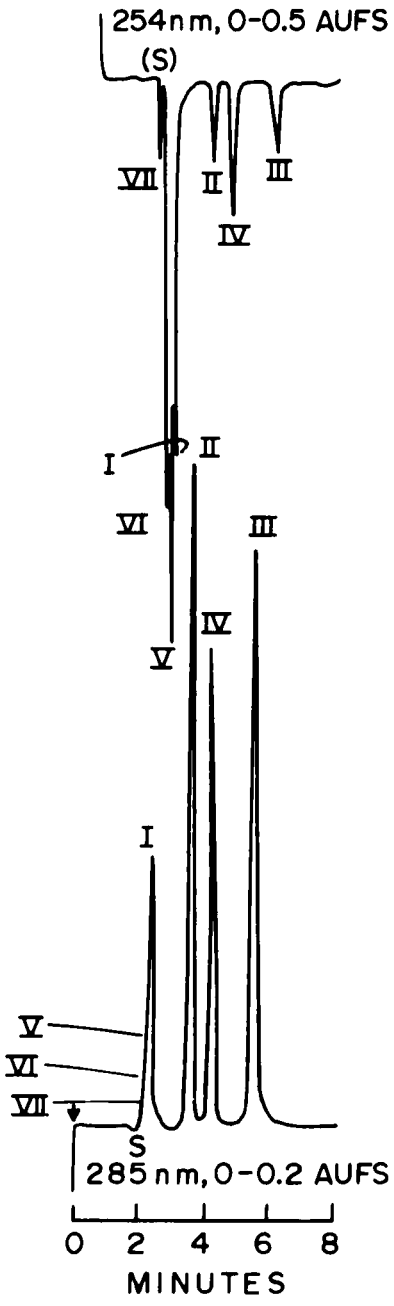

Figure 3 - Representative Chromatogram for Isocratic Elution. S, solvent front, Injection event, arrow. Retention times, min, t_S,1.84; t_{VII},2.04; t_{VI},2.38; t_V,2.44; t_I,2.52; t_{II},3.76; t_{IV},4.40; t_{III},5.66. Key: see Table 1

the range 0.125 to 1.00 x 10^{-4} M. The least-square regression for the curve is y = 0.9868 x + 0.002312 and the correlation coefficient is 0.9999. The coefficients of variation (percentages) at 0.125, 0.25, 0.5, 0.75 and 1.00 x 10^{-4} M are 1.16, 1.03, 0.59, 0.82, 0.35 respectively.

It has been suggested that an internal standard is not needed in some cases if a fixed loop is used for the injection of the samples. It was found that the coefficients of variation for the individual compound's peak height were greater than that of the corresponding ratio of drug to internal standard peak height listed above, which indicates the value of using an internal standard to minimize variations during the analysis.

In conclusion, the above chromatographic system can be employed to separate and identify the two drugs and potential decomposition products using gradient elution.

Stability studies of 5-fluorocytosine in acidic media employing the above analytical procedure, show that the only chromophoric product is 5-fluorouracil. In neutral and basic media, 5-fluorocytosine breaks down to give 5-fluorouracil and non-chromophoric products (24). Consequently, isocratic elution can be used to separate and quantify the two compounds in the presence of their decomposition products.

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