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A SENSITIVE ASSAY OF 5-FLUOROURACIL IN PLASMA BY GAS CHROMATOGRAPHY-MASS SPECTROMETRY

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1 A gas chromatographic-mass spectrometric method was developed for determining 5-fluorouracil in plasma, using methylated thymine as an internal standard.

2 5-fluorouracil was extracted from plasma by a novel procedure which removed plasma components interfering with the sensitivity of the assay. The method included heating the plasma, washing with ether and extracting the drug under optimum conditions.

3 The sensitivity of the assay was 10 ng/ml plasma, sufficient to determine the low concentrations of 5-fluorouracil found in plasma during continuous infusion of the drug in patients receiving chemotherapy for cancer.

Introduction

The drug, 5-fluorouracil (5-FU), is a synthetic pyrimidine which has been used in many types of cancer treatment (Heidelberger & Ansfield, 1963). To optimize chemotherapy with 5-FU, it is important to define the proper therapeutic regimen.

Arguments exist as to the most effective method of administering the drug. The chemotherapy with the drug is often accomplished in a once weekly push dose (Horton, Olson, Sullivan, Reilly & Schneider, 1970). However, the drug may be more effective when given by continuous infusion (Baker, Seifert, Reed & Vaitkevicius, 1973). If a correlation exists between the plasma level of the drug and the therapeutic response, then it may be possible to define the best therapeutic regimen based on this measurement. Furthermore, if an assay of sufficient simplicity and sensitivity exists, a therapeutic regimen could possibly be devised for individual patients.

Because the individualization of therapy is an important concept of clinical pharmacology, several assays for this drug have been developed. The gas chromatographic assay of Cohen & Brennan (1973) for 5-FU from plasma is sufficiently simple and sensitive for pharmacokinetic studies when the single dose regimen is used. However, we have found that the method is not sensitive enough to measure the low level of 5-FU during continuous infusion. Although gas chromatographic-mass spectrometric study on 5-FU in plasma was carried out by Pantarotto, Martini, Belvedere, Bossi, Donelli & Frigerio (1974), there is still a limit in sensitivity (100 ng/ml) because this method used only 0.1 ml of plasma. It is probable that extraction of plasma (1 ml) with a solvent as polar as butanol would extract enough material (King & Craig, 1962) to interfere seriously with the assay. For highly sensitive methods, a reasonably pure extract is needed. This is difficult to achieve by the usual

two-phase extraction with a water-immiscible organic solvent when dealing with polar molecules such as 5-FU.

We have developed a method for more efficient extraction from plasma which can be applied to a gas chromatographic-mass spectrometric assay. The use of thymine as an internal standard is also described. This obviates the necessity of synthesizing isotopically labelled derivatives of 5-FU for mass fragmentographic measurement.

Methods

Materials

5-FU and thymine were purchased from Sigma Chemical Company. [6-^{3}H]-5-FU (1.4 Ci/mmol) was obtained from New England Nuclear. Trimethylanilinium hydroxide was prepared according to Brochmann-Hanssen & Oke (1969). Normal plasma was obtained from McMaster University Medical Centre Blood Bank. Blood of patients was obtained during chemotherapy with 5-FU given as continuous 5-day infusion. The blood was anticoagulated with ethylenediaminetetraacetate and the plasma obtained was kept at -15°C until the analysis of 5-FU. The following reagents were used: anhydrous ethyl ether (Mallinckrodt Canada Ltd), *n*-propanol, K_2HPO_4 and KH_2PO_4 (Fisher Scientific Co), and absolute methanol (Matheson Coleman & Bell Manufacturing Chemists).

Extraction procedure

Plasma (1 ml containing a known amount of 5-FU as a standard) was diluted to 4 ml with phosphate buffer, pH 6.8, to give a final buffer concentration of 0.25M. The mixture was heated at 100°C for 5 min and the precipitate formed was removed by centrifugation at 20,000 *g* for 15 minutes. The supernatant was washed with an equal volume of ether to remove materials soluble in ether. The washed sample (2 ml) was extracted with 30 ml of 16% *n*-propanol in ether. After shaking the mixture for 5 min, 25 ml of the upper layer was mixed with water (1.5 ml) and evaporated at about 70°C under dry nitrogen. Addition of water to the *n*-propanol-ether solution made *n*-propanol (boiling point, 97°) an azeotrope with water (boiling point, 88°) which reduced evaporation time. The dry residue obtained was dissolved in methanol (2.5 ml), and 2 ml of the methanol solution trasferred to a 4.5 ml Reacti-Vial containing thymine (200 ng) and the solution evaporated to dryness again as described above. To the residue, 0.2M trimethylanilinium hydroxide in methanol (30 µl) was added for derivatization and 0.5 µl of the solution injected onto the gas chromatographic column. The concentrations of 5-FU and thymine used as standards were determined by the absorbance at 265 mµ in 0.1N HCl (Garrett, Seydel & Sharpen, 1966) and at 265 mµ at pH 7.0 (Shugar & Fox, 1952).

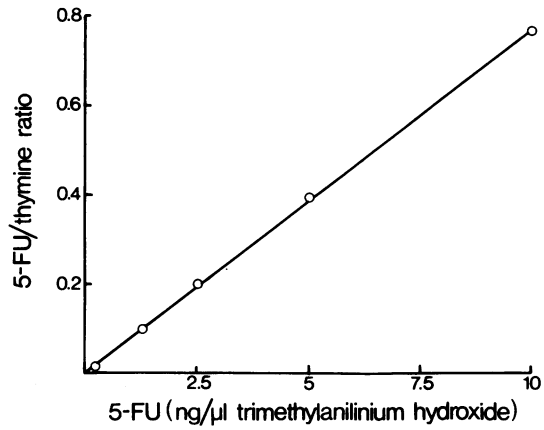

Figure 1 Calibration for pure 5-FU. Trimethylanilinium hydroxide solutions (0.2M) containing 5-FU at the various concentrations indicated, and thymine at 10 ng/l were prepared. The solution was injected onto the gas chromatography column and the ratio of peak height of 5-FU to thymine was measured.

Gas chromatography-mass spectrometry (GC-MS)

The gas chromatograph-mass spectrometer system was a Varian, Model 2700 gas chromatograph, and Model CH-7 mass spectrometer, linked by a Watson-Bieman separator.

The gas chromatographic conditions were as follows: the coiled glass column (180 cm long x 4 mm inner diameter) was packed with 3% OV-17 on chromosorb W 80-100 mesh (Chromatographic Specialties), which had been conditioned at 280°C overnight, injection port 300°C, detector oven 300°C, column oven 200°C, carrier gas–helium, flow-rate 20 ml/minute.

The experimental conditions used for the mass spectrometry were as follows: ionization energy, 70 eV and emission current, 300 µA. The mass spectra of 5-FU and of thymine as their dimethyl derivatives are shown in Figure 3. The base peaks at m/e 158 (dimethyl 5-FU) and at 154 (dimethyl thymine) are due to the molecular ions and were used for mass fragmentography.

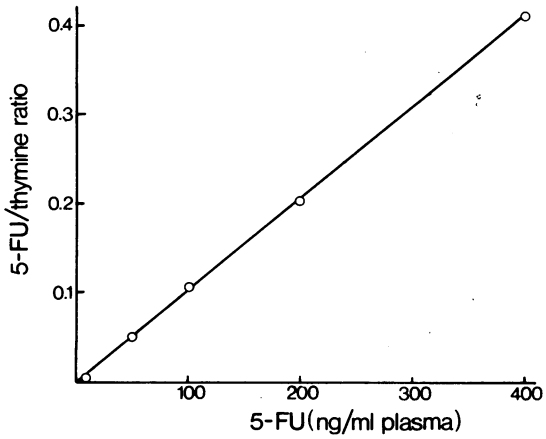

Figure 2 Calibration for 5-FU extracted from plasma. A known amount of 5-FU was dissolved in normal plasma. 5-FU was extracted from the plasma and measured as described in text.

Quantitative determination

Standard curves were prepared from chromatograms of extracts of plasma and of a trimethylanilinium hydroxide solution, each containing known amounts of 5-FU. The ratio of the peak height of the compound to that of thymine as an internal standard was plotted against concentration as illustrated in Figures 1 and 2. In both, a linear relationship was obtained. By comparing the results in Figures 1 and 2, a recovery of 80% 5-FU from plasma was again confirmed.

Results

Extraction of plasma should not only give a high yield of the substance extracted, but should also remove interfering components of the plasma. Such components can interfere in two ways: first by interfering with the signal due to the compound being measured, especially when methods other than GC-MS are used (e.g. flame ionization detection); secondly, simply by fouling the detector, thus restricting the number of assays which can be performed in sequence. This is particularly crucial in GC-MS analysis since cleaning of the ion source is a long and complex procedure.

Extraction properties of 5-FU from plasma are shown in Figures 3 and 4 and Tables 1 and 2. First, precipitable materials, mainly plasma proteins, were removed with heat in 0.25M phosphate buffer, pH 6.8. The effects of pH and concentration of buffer on the precipitation of

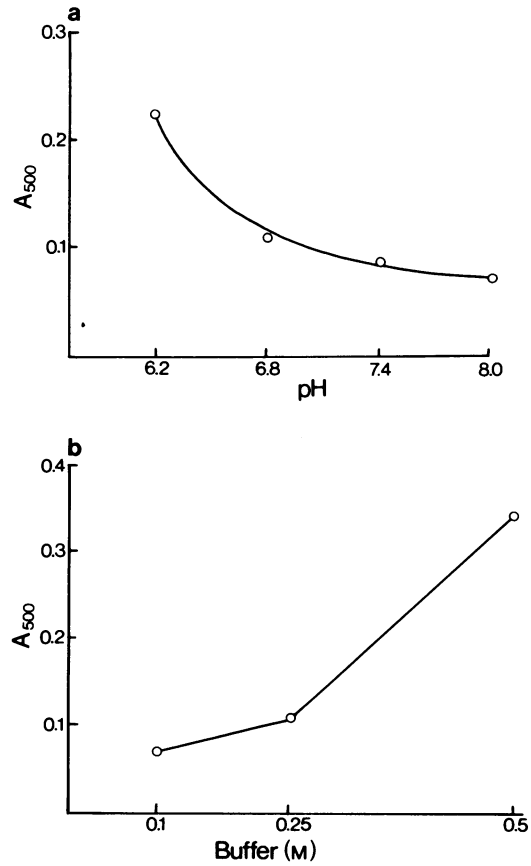

Figure 3 The effects of (a) pH and (b) concentration of buffer on the precipitation of plasma residue by heat treatment.

A solution (2 ml), containing plasma (0.5 ml) at the various pH values and concentrations of phosphate buffer, was heated at 100° C for 5 minutes. The concentration of buffer was 0.25M in (a) and pH was 6.8 in (b). The suspension obtained was diluted with water (5 ml) and uniformly dispersed by sonication for the measurement of absorption at 500 mμ.

plasma components were measured by absorbance of visible light (500 mμ) as an index of amount of precipitate (Figures 3 and 4). At the lower pH, more precipitable materials could be removed. However, using low pH is not recommended since extraction of 5-FU is dependent on pH. At a constant pH, the precipitate was obtained at higher buffer concentration. Again there was a limit to increasing the buffer concentration since KH_2PO_4 was oversaturated in the process of the extraction with ether which follows this process. The yield on extraction of 5-FU with 16% propanol in ether at different pH values and with

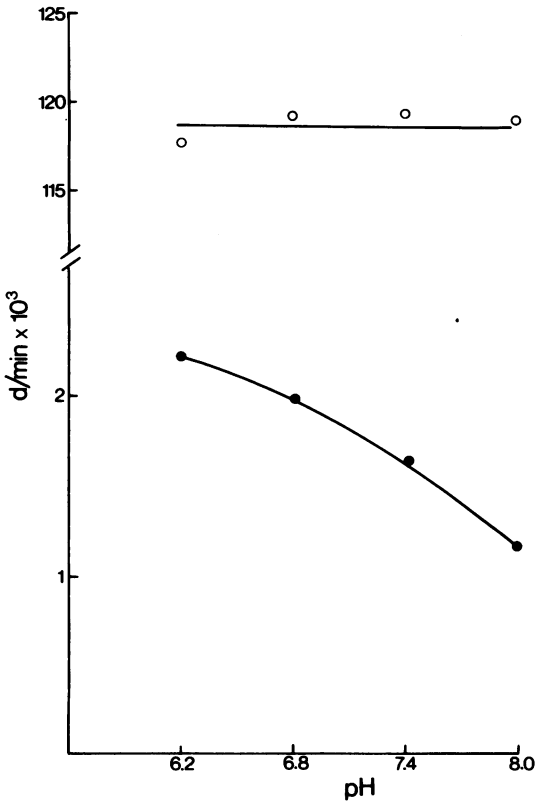

Figure 4 The effect of pH on the recovery of 5-FU in supernatant of plasma treated with heat and on the extraction of 5-FU into ether. Plasma (0.5 ml) containing 7.7×10^{-7}M [6-^{3}H]-5-FU (1.4 Ci/mmol) was mixed with phosphate buffer at various pH values to give a final concentration of buffer 0.25M in 2 ml total mixture. After treatment with heat as described in the text, the radioactively of [6-^{3}H]-5-FU in 0.2 ml of the supernatant (o) added to 10 ml of scintillation fluid was counted in a Nuclear-Chicago Scintillation Counter. Extraction of 5-FU into ether was measured by counting the radioactivity in 0.2 ml ether phase after washing the supernatant with an equivalent amount of ether (•). The control (100% recovery = 1.25×10^5 d/min) employed water instead of plasma.

various concentrations of buffer was determined (Table 1). The lower extraction of 5-FU at high pH would result from the higher solubility of 5-FU in water due to ionization of the hydroxy group. Considering the limitation mentioned obove, optimum conditions were pH 6.8 with 0.25M buffer in the final plasma-buffer mixture. At this pH, the buffer action was strong enough (pK_2-*circa* 7) to adjust the pH of plasma specimen. This is important because the pH of plasma is not always constant and depends on the individual and the method of preparation of the plasma specimen. Such differences in pH can cause variation in the yield of extracted 5-FU.

When we tested the recovery of 5-FU following extraction with propanol in ether at various ratios, the most effective concentration of propanol in ether was 15-16% as recommended by Cohen & Brennan (1973). As seen in Table 2, ether alone could extract only 15% of 5-FU. When a volume of ether equal to the plasma sample was used at pH 6.8, the yield was found to be only *circa* 2%. Because of this property, the plasma sample was washed with ether, after heat treatment, to remove ether soluble materials. Since 5-FU is extracted at a higher efficiency into ether at lower pH during this step (Figure 4), we kept the pH at 6.8. The final recovery of 5-FU after both steps was 80% (coefficient of variation = 4%). Of the 20% loss, *circa* 2% was due to the heat treatment, *circa* 2% to the ether washing, and 15% to the extraction with propanol-ether. The recovery after extraction was also assessed by measuring 5-FU and thymine, using a modified gas chromatographic method of Cohen & Brennan (1973) and yielded the same results.

The development of a chromatographic procedure requires the choice of an internal standard and an appropriate derivatization procedure. Since the base peak of the mass spectrum of dimethylthymine (1,3,5-trimethyluracil) is at m/e 154, 4 less that that of

Table 1 Effect of pH and concentration of phosphate buffer on the extraction of 5-FU from plasma.

pH	*Concentration of buffer in plasma-buffer mixture (M)*	*Recovery of 5-FU in extract (%)*
6.0	0.25	86.1
6.4	0.25	85.3
6.8	0.25	84.5
7.2	0.25	82.8
7.6	0.25	79.8
6.8	0.5	84.5
6.8	0.25	84.5
6.8	0.125	83.7
6.8	0.025	79.9
6.8	0.0125	71.2

5-FU was extracted with 16% *n*-propanol in ether at various pHs and concentrations of phosphate buffer. Plasma containing 7.7×10^{-7}M [6-^{3}H]-5-FU (1.4 Ci/mmol) was mixed with buffer and treated with heat as described in the text. The supernatant (1 ml) was extracted with 16% *n*-propanol in ether (15 ml) and then radioactivity in the extract was determined.

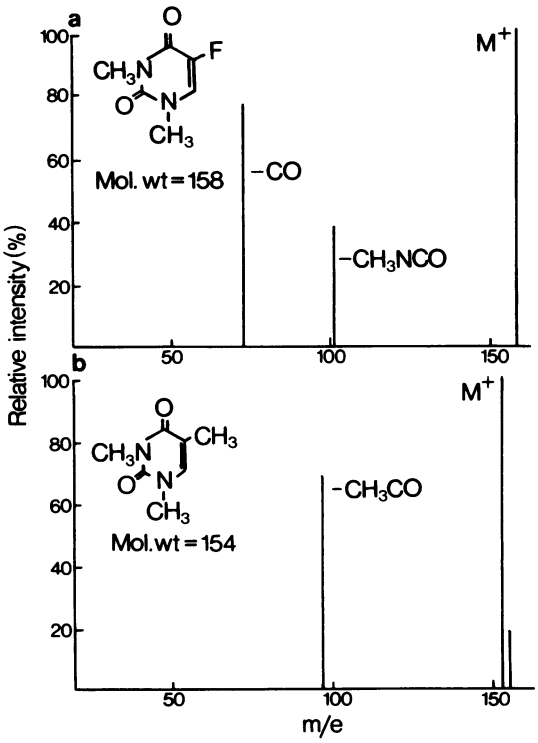

Figure 5 Mass spectra (a) of dimethyl-5-FU and (b) dimethylthymine. 5-FU and thymine were methylated in trimethylanilinium hydroxide (0.2M) in methanol. Mass spectra were obtained using the gas chromatographic input monitored and generated by computer after calibration with perfluorokerosene.

dimethyl-5-fluorouracil (Figure 5), we used the thymine as an internal standard for the multiple ion detection method. An additional advantage in the use of thymine is that the dimethyl derivatives of thymine and 5-FU are poorly resolved on GC so that dimethylthymine acts as a carrier for the dimethyl 5-FU during the GC-MS analysis and probably adds to the sensitivity of the assay. Thymine and 5-FU were converted to their dimethyl derivatives by the on-column methylation technique of Pantarotto *et al.* (1974). We chose methylation as derivatization opposed to silylation because the derivatization procedure does not require the extra manipulations involved in preparing silyl compounds. Also, silylated compounds are susceptible to hydrolysis by water vapour. Futhermore, when we followed Cohen & Brennan's (1973) method, we found that the flame ionization detector quickly became covered with a white inorganic precipitate from repeated use of the silylating reagent. Cohen & Brennan (1973) described this problem and how to keep the detector clean. Using the on-column methylating procedure, there is the added advantage that trimethylanilinium hydroxide is converted to the volatile low molecular weight derivative, anisole (Williams & Halpern, 1974), which would not be expected to contaminate the detector any more than the usual solvents.

Table 2 The effect of concentration of *n*-propanol on the extraction of 5-FU

n-propanol (% in ether)	*% extraction*
0	15
5	18
10	61
15	83
20	54

The method was the same as in Table 1 except that 5-FU was extracted with ether (15 ml) containing various concentrations of *n*-propanol.

Figure 6 shows the gas chromatograph monitored by a total ion current (TIC) of extract of plasma obtained as described above. The chromatogram showed only two main peaks resulting from materials extracted from plasma. This is contrasted to the TIC trace of a 16% propanol/ether extract (Figure 7). Both traces were obtained at the same sensitivity. The use of heat to remove precipitable material prior to extraction for drug analysis has not been reported to date. It is apparent, however, that in the extraction of polar compounds, this step removes much endogenous interfering material and may represent an important step in the analysis of such drugs.

In Figure 6, peaks of 5-FU and internal standard are not observed because of their low concentration. The chromatograms of the dimethyl-5-FU and thymine of high concentration at a lower column temperature is given in Figure 9 as reference. The retention times of pure 5-FU and thymine at 200°C, determined by mass fragmentography, were 110 s and 120 s, respectively. The fragmentograms of the extracts (Figure 8) show that only one peak at the mass of each molecular ion was obtained at the corresponding retention time for each compound. No other peak was observed at that mass through the chromatographic procedure.

Discussion

We have achieved a significant increase in the sensitivity and specificity of GC-MS method for

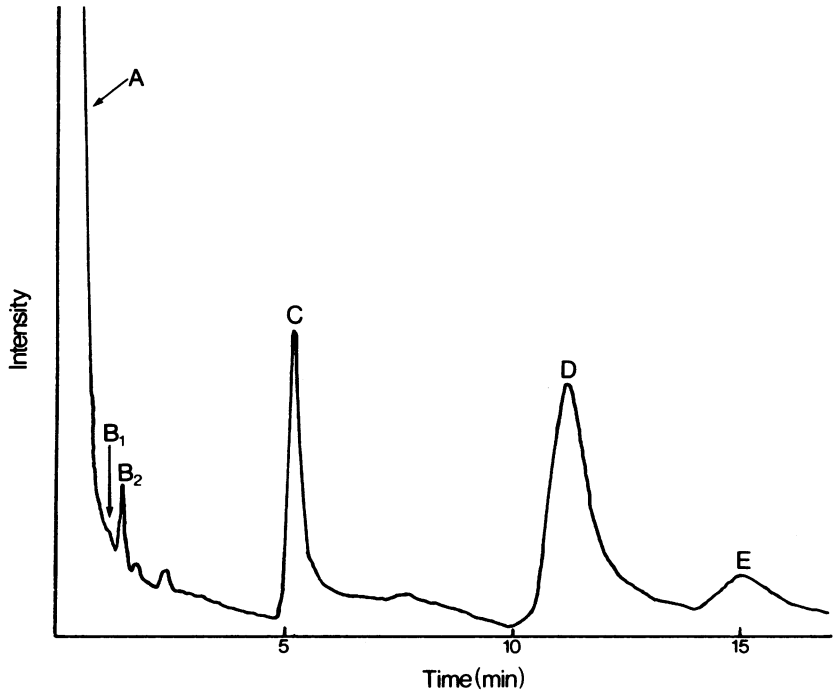

Figure 6 Gas chromatogram monitored by total ion current detection. A, solvent peak; B_1, retention time for 5-FU; B_2, retention time for thymine; other peaks (C–E), unknown components extracted from plasma following the method in text.

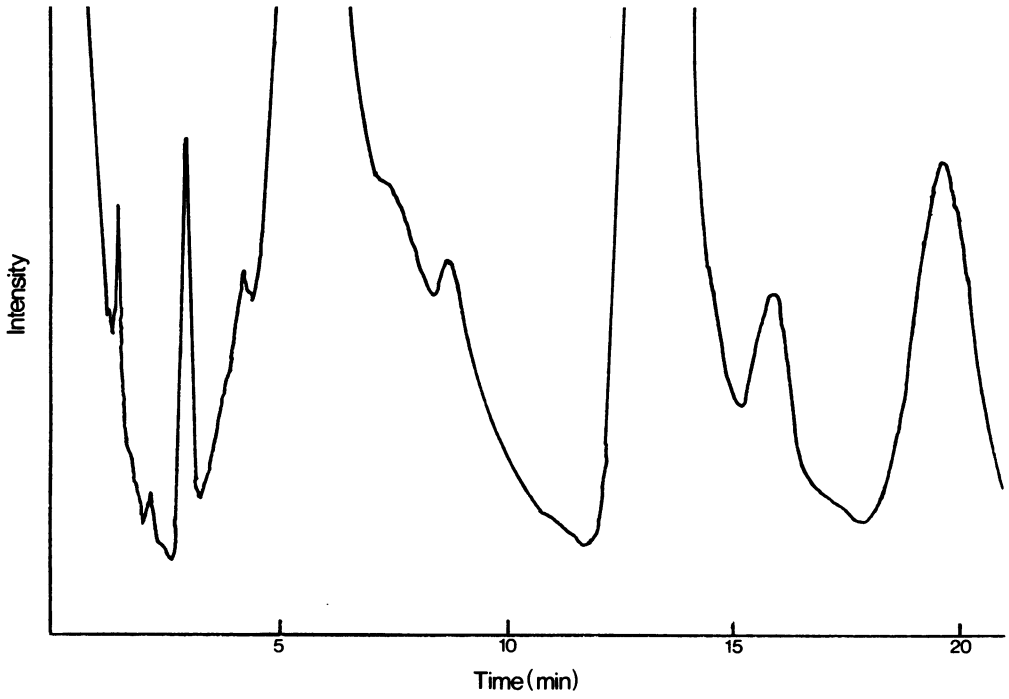

Figure 7 Gas chromatogram monitored by total ion current detection. The peaks represent unknown components extracted from plasma with 16% *n*-propanol in ether.

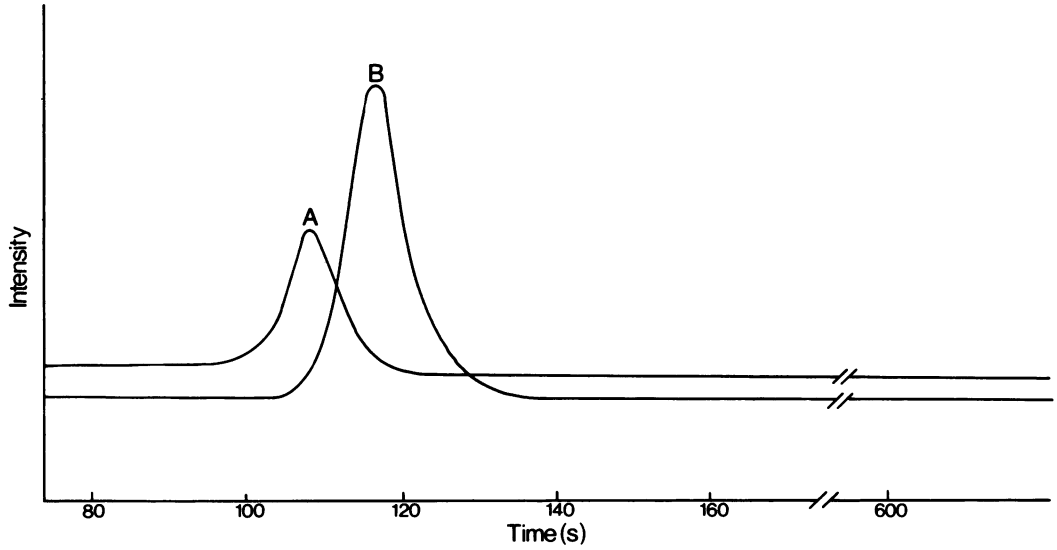

Figure 8 Mass fragmentograms of a plasma extract prepared by the method described in text. A, 1,3-dimethylthymine monitored at m/e 154; B, 1,3-dimethyl-5-FU at m/e 158.

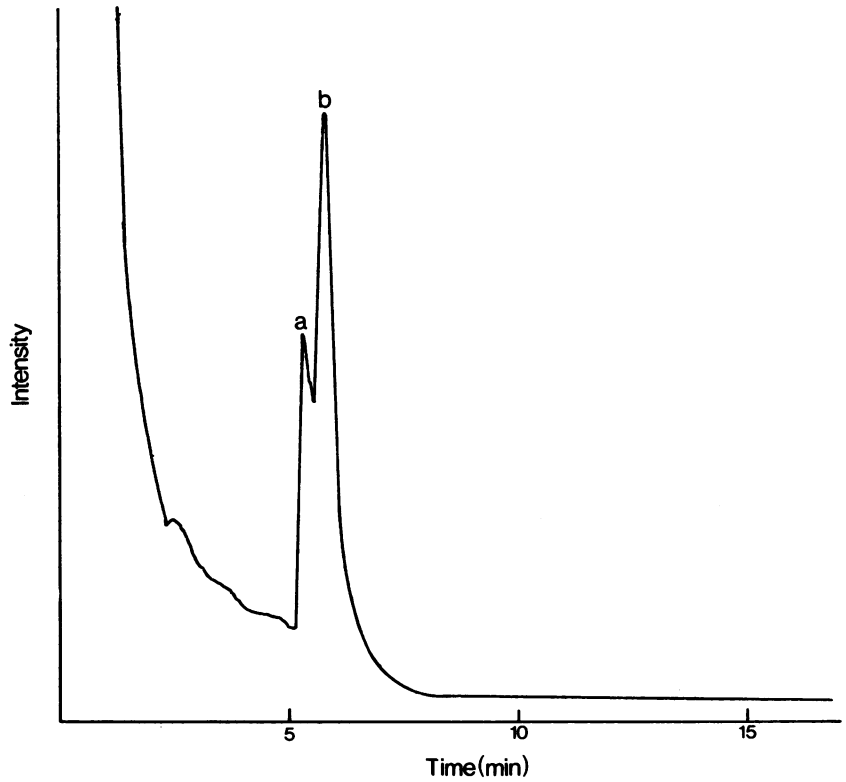

Figure 9 Gas chromatogram of (a) 5-FU and (b) thymine as their dimethyl derivatives at low column temperature. 5-FU (1 μg) and thymine (as solution in trimethylanilinium hydroxide) was injected. The column temperature was 170° C.

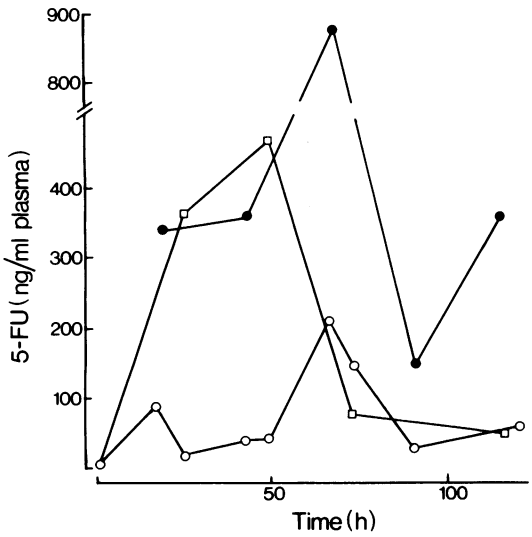

Figure 10 Plasma levels of 5-FU in patients receiving continuous infusions of the drug. Plasma samples were taken from three patients at times shown after beginning the infusion and processed as described in the text. Dosage of 5-FU was 30 mg kg^{-1} body weight 24 h^{-1} for 5 days.

the measurement of 5-FU in human plasma. The practical limit of sensitivity was found to be 10 ng/ml which proved to be sufficient to measure the level of the 5-FU found during treatment with continuous infusion. The standard deviation was 4% of the mean value above a concentration of 50 ng/ml plasma. Below a concentration of 10 ng/ml measured by using the highest sensitivity of the instrument, the error increased. At such concentrations, an error of 20% would have to be allowed because of the reliance on peak heights as a measure for quantitation. However, it would be possible to measure as low as 1 ng/ml with less error if the peak area is measured with an integrator and by increasing the amount of sample injected into the gas chromatograph.

Cohen *et al.* (1974) studied the pharmacokinetics of 5-FU in patients after single oral or intravenous administration of the drug, with analytical techniques of sufficient sensitivity to monitor plasma levels for a period of only several hours after oral or intravenous treatment. The plasma level of the drug fell quickly, and a half-life time for the drug of 10 min was found.

The concept of continuous infusion for therapy is to keep the plasma level of 5-FU more or less constant throughout. We found, during this type of treatment, the plasma levels of 5-FU fluctuate and futhermore, that the level of drug is much lower than with oral or intravenous administration, particularly when monitored over a period of five days. Figure 10 shows the plasma levels of 5-FU in patients with adenocarcinoma of gastro-intestinal tract, receiving 30 mg/kg body weight of the drug per day for five days by continuous infusion. Compared to the plasma level achieved with a single dose of 15 mg/kg (100 μg/ml plasma shortly after the injection, having a half-life time of *circa* 10 min) (Cohen, Irwin, Marshall, Darvey & Bateman, 1974), the plasma level of the drug given by infusion is low. Such low plasma levels of the drug could be measured by the more sensitive method we describe here.

The analytical method described offers a number of advantages over existing procedures. The extraction technique, in particular, could be readily adapted to the study of 5-FU in other biological material since it shows that it is possible to remove much extraneous biological substances under very mild conditions which do not destroy 5-FU.

Addendum

During the preparation of this report, Finn & Sadée (1975) presented a mass fragmentographic assay for 5-FU using highly enriched [^{14}C]-5-FU as an internal standard. This requires the unnecessary manipulation of radioactive material. Furthermore, the [^{14}C]-5-FU is only 81% enriched thus giving a high background which necessitates the use of small amounts of internal standard (3 ng/ml). This would limit the dynamic range of the assay and should be avoided if possible. Lastly, the authors used the extraction and derivatization techniques of Cohen & Brennan (1973) which were discussed in the text.

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