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Note

4-Bromomethyl-6,7-dimethoxycoumarin as a fluorescence reagent for precolumn derivatization of 5-fluorouracil compounds in high-performance liquid chromatography

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5-Fluorouracil (5-FU) and its nucleoside analogue 5-fluoro-2′-deoxyuridine (FdUrd) are antimetabolites used in the treatment of malignant neoplasma [1]. In the body FdUrd is extensively metabolized to 5-FU in reactions catalysed by at least two different nucleoside phosphorylases [2]. 1-(Tetrahydro-2-furyl)-5-fluorouracil (Ftorafur, FT), a masked form of 5-FU, is also an effective antitumour agent. FT can be given orally and has a lower toxicity than 5-FU [3]. The mechanism of conversion of FT into 5-FU remains unknown.

Although it is widely used as a single agent and in combination chemotherapy, 5-FU has several shortcomings as an antitumour agent [4]. Accordingly, the measurement of serum levels can be important to ensure that they are sufficient to prevent developed resistance and yet not so high that they cause dose-dependent leukopenia and thrombocytopenia. Early studies on fluoropyrimidine determination were based on microbiological [5] or combined anion-exchange and paper chromatographic [6] methods, which lacked specificity or sensitivity. Brown and co-workers [7, 8] described a sensitive high-performance liquid chromatographic (HPLC) method with UV detection.

Formation of various fluorescent derivatives for HPLC determination has been popular when high-sensitivity measurements are essential. Although a number of fluorescent derivatization procedures are available for acids, primary amines, or even carbonyl compounds, there has been a conspicuous lack of similar techniques for imide compounds. Dünges and Seiler [9] and afterwards others [10, 11] reported the use of 4-bromomethyl-7-methoxycoumarin (Br-Mmc) as a fluorescence labelling reagent in the HPLC analysis of imides.

Farinotti et al. [12] reported that fatty acid esters formed with 4-bromome-

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thyl-6,7-dimethoxycoumarin (Br-Mdmc) have higher quantum yields than Br-Mmc [12]. This new derivatization reaction was applied to 5-FU compounds and optimum conditions for the reaction of Br-Mdmc with samples were thoroughly investigated.

EXPERIMENTAL

Chemicals

Br-Mdmc was purchased from Aldrich (Milwaukee, WI, U.S.A.) and was used without purification. 5-FU, FT and FdUrd were purchased from Nakarai Chemicals (Kyoto, Japan). HPLC-grade methanol was obtained from Nakarai. All other organic solvents were of analytical-reagent grade. The water used for the mobile phase was passed through an ion-exchange column (Millipore, Type ZD20-11583).

High-performance liquid chromatography

The chromatographic system consisted of a Shimadzu Model LC-4A solvent-delivery pump (Shimadzu, Kyoto, Japan), a Model SIL-1A syringe-loading injection valve and a Shimadzu Model RF-530 fluorescence spectrophotometer operated at $\lambda_{ex}=340$ nm and $\lambda_{em}=420$ nm. Chromatography was performed on a reversed-phase C_{18} analytical column (Macherey-Nagel, Düren, F.R.G., Nucleosil 5 C_{18}, 5 μm, 200 mm $\times$ 4 mm I.D.). The mobile phase, methanol–water (60:40, v/v), was filtered and vacuum-degassed before use. The flow-rate was 0.8 ml/min and the temperature was ambient. Standard curves were plotted as the pyrimidine peak height versus the concentration of standard samples; the linear regression line was calculated by the method of least squares.

Sample preparation

Human serum was used in the development of the HPLC method. Standards were prepared by the addition of various known amounts of test compound to blank serum. The sample (250 μl) was loaded onto an anion-exchange column (Seikagaku Kogyo, Tokyo, Japan, DEAE-Cellulofine AM, 20 mm $\times$ 7 mm I.D.). The column was washed with 3.5 ml of 10^{-3} *M* hydrochloric acid; the initial 0.5 ml of eluate was discarded and the remaining 3 ml were collected. The eluate was condensed to 0.5 ml in vacuo. The later procedure are summarized in Fig. 1.

Derivatization procedures

In a 10-ml flask protected from light with aluminium foil and from moisture with anydrous calcium chloride, fluoropyrimidine compounds were dissolved in 800 μl of anhydrous acetone. The 100 μl of Br-Mdmc solution (750 μg/ml in acetone), 100 μl of 18-crown-6 solution (250 μg/ml in acetone) and 1.5 mg of anhydrous potassium carbonate were added. The flask was heated at 70 °C for 15 min. The solution was then cooled and an aliquot was injected in the chromatograph. By repeated chromatography of the same solution, it was shown that the methyl-6,7-dimethoxycoumarin (Mdmc) derivatives were stable for at least several weeks in the reaction mixture.

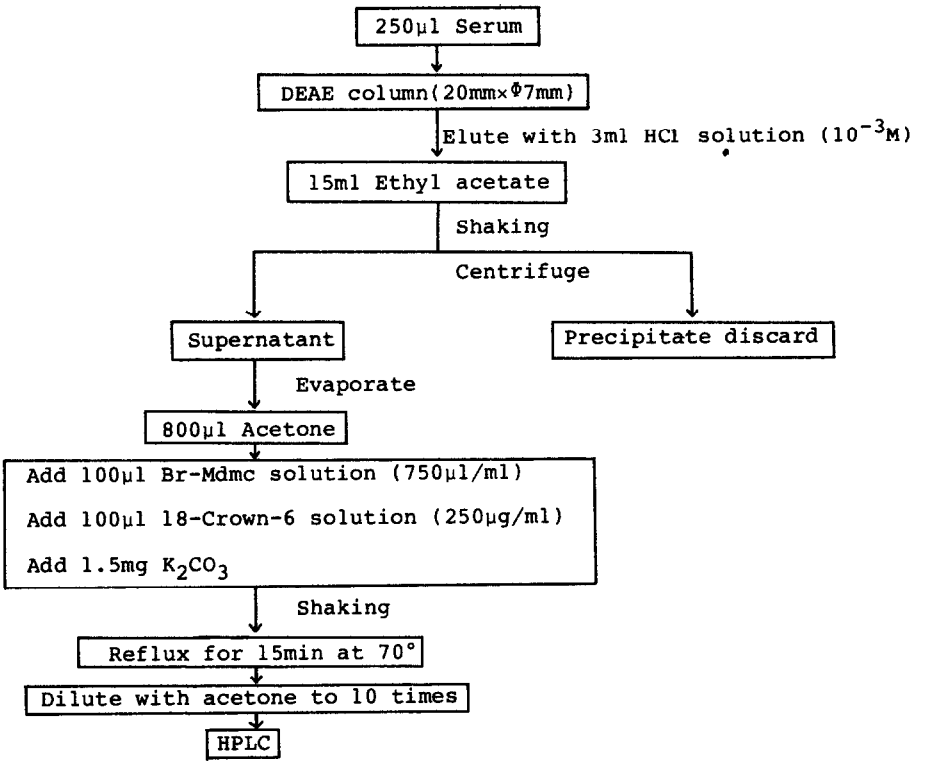

Fig. 1. Schematic diagram of the analytical procedure for the determination of fluoropyrimidine in serum.

RESULTS AND DISCUSSION

Structure of fluorescent derivatives

The barbiturates can be alkylated by alkyl halide [9], and the crown ether–potassium complex is used as a catalyst. The structure of the alkylated derivatives has been confirmed by many workers [13, 14]. The barbiturates usually contain two acidic imide groups, each of which can be alkylated at the nitrogen atom. The pyrimidine compounds, as well as the barbiturates, contain an acidic imide group. The possible labelling of FT and 5-FU are shown in Fig. 2.

Optimum conditions for the derivatization

Under optimum conditions a standardized procedure was devised for the determination of 5-FU compounds, as described in Experimental. The superiority of the present method is demonstrated by the fact that only a slight excess of the expensive reagent is required. The short reaction times and small amounts of Br-Mdmc used eliminate interferences in the chromatogram due to excess of derivatizing agent and decomposition products that might be formed during longer reactions. As an example, Fig. 3 shows the HPLC separation of FT and 5-FU.

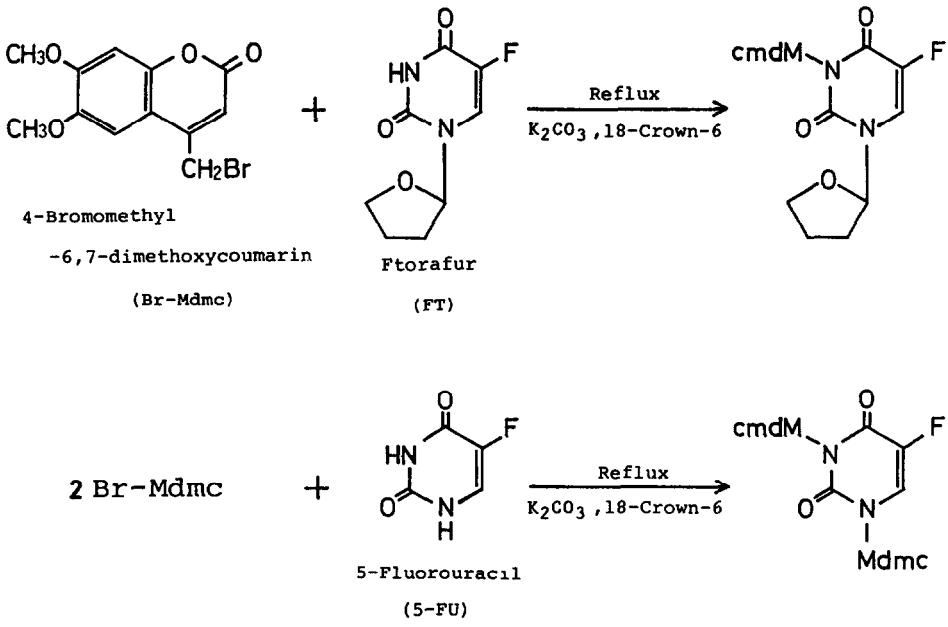

Fig. 2. Scheme for the reaction of Br-Mdmc with Ftorafur and 5-fluorouracil.

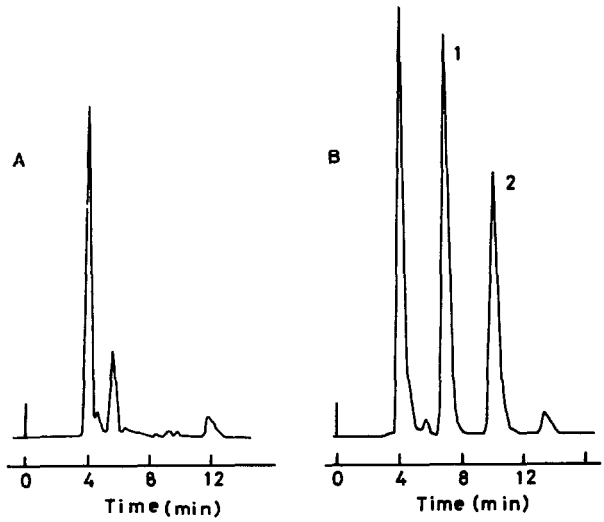

Fig. 3. Chromatographic separation of Mdmc-FT and Mdmc-5-FU derivatives. Column temperature, ambient; flow-rate, 0.8 ml/min; mobile phase, methanol–water (60:40, v/v). (A) Blank; (B) sample. Peaks: 1 = Ftorafur; 2 = 5-FU.

Clean-up and extraction efficiency

In this fluorescent labelling method for the determination of 5-FU compounds in serum, an important and unique feature is the pre-analysis ion exchange of the sample, which effectively removes proteins. We found that the readily available, inexpensive DEAE-Cellulofine ion exchanger gave excellent adsorption of pro-

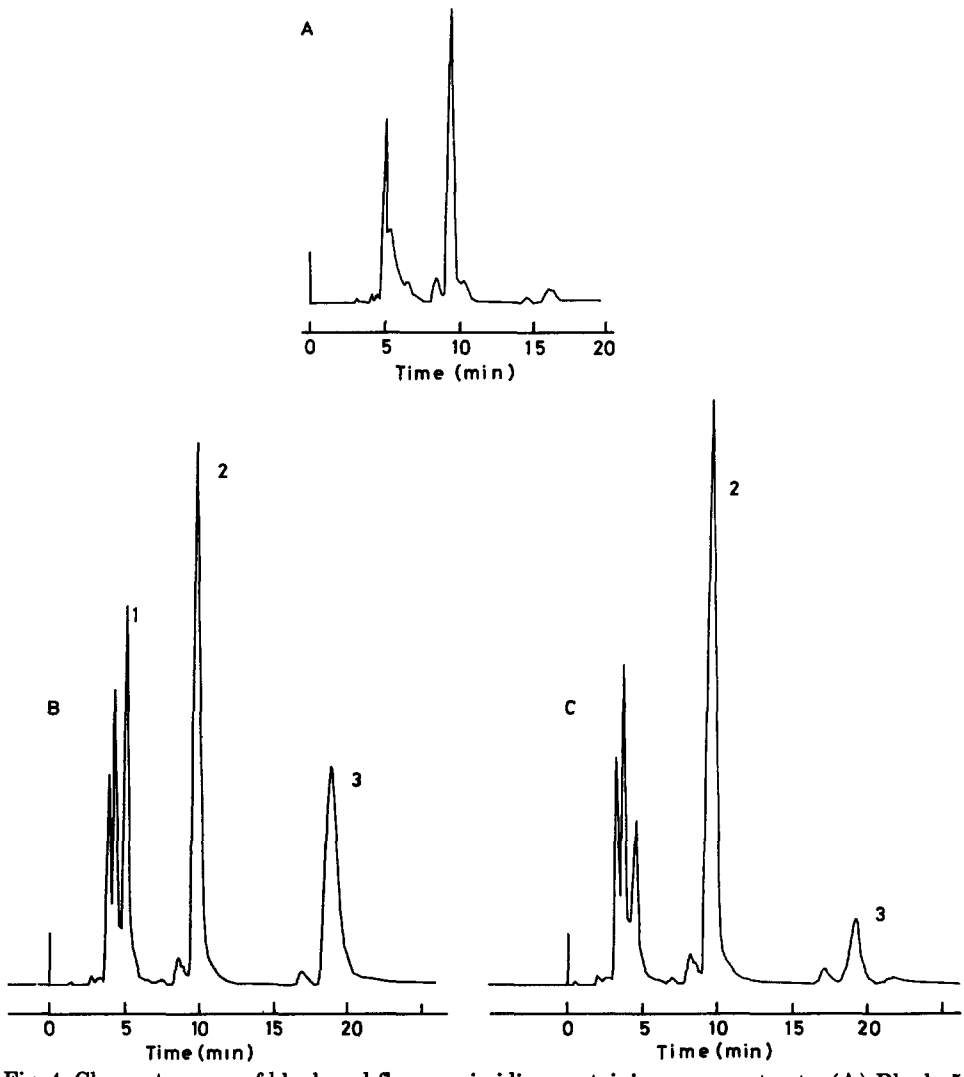

Fig. 4. Chromatograms of blank and fluoropyrimidine-containing serum extracts. (A) Blank, 5 μl injected; (B) serum containing 1 μg/ml FdUrd, 1 μg/ml FT and 1 μg/ml 5-FU, 5 μl of each injected; (C) serum from a patient treated with FT for colorectal carcinomas. Peaks: 1 = FdUrd; 2 = FT; 3 = 5-FU.

teins. The absorbance (at 280 nm) of the column eluate decreases if 10^{-3} *M* hydrochloric acid is present in the eluent. The 5-FU compounds were eluted quantitatively in the column eluate. A 3-ml aliquot of the column eluate was concentrated to 0.5 ml in vacuo and extracted with ethyl acetate. Data from three spiked samples indicated a recovery (mean $\pm$ S.D.) of $90 \pm 0.5\%$ for the 5-FU compounds. The excellent clean-up efficiency and quantitative analytical recovery of the sample preparation procedure ensures optimum day-to-day instrument performance and analytical precision.

Fluorescence properties

The excitation maximum was at 340 nm and maximum emission at 420 nm (uncorrected values). There was no shift in excitation or emission spectra in the range 60–70% methanol. However, a 70% decrease in the quantum yield was found below 50% methanol.

Chromatographic separations

Chromatograms of blank and fluoropyrimidine-containing serum extracts are shown in Fig. 4. Blank serum contained several unidentified peaks but none interfered with the fluoropyrimidine derivatives at the concentrations employed in these studies. FT, 5-FU and FdUrd derivatives were well separated from each other. Using the procedure described above, five samples were routinely extracted and analysed simultaneously for FT, 5-FU and FdUrd in 2 h. The problem of decomposition of FdUrd to 5-FU, observed during gas chromatographic analysis [15], did not occur with the HPLC method.

Quantitation and detection limits

There was a linear relationship between peak height and serum concentration for each compound. Concentrations as low as 0.1 μg/ml for FdUrd, 0.06 μg/ml for 5-FU and 0.006 μg/ml for FT were readily quantified (5 μl injected). In routine experiments, ten-point standard curves were generated from serum samples containing 0.1–10 μg/ml FdUrd, 0.06–10 μg/ml 5-FU and 0.006–10 μg/ml FT. The equations obtained from ten replicate curves with each compound were (in units of μg/ml): FdUrd, $y=0.7410x+0.4886$ ($r=0.9942$); 5-FU, $y=0.4060x+0.1533$ ($r=0.9918$); and FT, $y=1.4335x+1.8025$ ($r=0.9932$).

Applications

To demonstrate the potential application of this method for biological samples, the deproteinized serum of a patient treated with FT for colorectal carcinomas was analysed. The chromatogram (Fig. 4C) shows that the 5-FU compounds could be separated and no interfering peaks occurred. Precision studies were performed with Tri-level patient sera. As shown in Table I, the intra-assay coefficients of

TABLE I

PRECISION STUDIES WITH THE FT DETERMINATION

Sample	Concentration (mean ± S.D.) (ng/ml)	Coefficient of variation (%)
Intra-assay (n=5 each)		
1	21.5 ± 1.6	7.6
2	58.0 ± 3.1	5.4
3	170.0 ± 10.5	6.2
Inter-assay (n=3 each)		
1	21.0 ± 0.8	3.9
2	68.0 ± 5.9	3.6
3	179.0 ± 3.0	1.7

variation (C.V.) for FT concentrations of 21.5, 58 and 170 ng/ml were 7.6, 5.4 and 6.2%, respectively. Inter-assay C.V. for the same sera were 3.9, 8.6 and 1.7%, respectively.

The advantages of our method are the increased sensitivity and, in comparison with the other fluorimetric derivatization method [10, 11], the absence of significant interfering peaks.

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