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FLUORESCENCE DETERMINATION OF 5-FLUOROURACIL AND 1-(TETRAHYDRO-2-FURANYL)-5-FLUOROURACIL IN BLOOD SERUM BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Fluorescence derivatization of 5-fluorouracil (5-FU) and 1-(tetrahydro-2-furanyl)-5-fluorouracil (ftorafur, FT) with 4-bromomethyl-7-methoxycoumarin using 18-crown-6 as a catalyst is studied with aim of developing a sensitive and selective liquid chromatographic method. 5-FU and FT form virtually substituted derivatives which possess maxima in their fluorescence emission spectra near 400 nm. These derivatives are separated by thin-layer chromatography and high-performance liquid chromatography to confirm the completion of reaction. For the determination of 5-FU and FT in serum, the reversed-phase high-performance liquid chromatographic separation of the derivatives is studied with a C_{18} column. This chromatography is of importance for the accurate determination of 5-FU and FT, which are, respectively, an important antitumour agent for the treatment of solid tumours in clinical medicine and a masked form of 5-FU generated in vivo.

INTRODUCTION

5-Fluorouracil (5-FU), an antimetabolite of uracil, has been in clinical use for many years and plays an important role in the chemotherapy of certain forms of cancer [1]. It may undergo anabolism to form ribo- and deoxyribonucleosides, and their mono-, di- and triphosphates. Among these, 5-fluorodeoxyuridine monophosphate which inhibits thymidylate synthetase and DNA synthesis, and 5-fluorouridine triphosphate which is incorporated to form fraudulent RNA, are believed to be responsible for the cytotoxicity of 5-FU [2].

1-(Tetrahydro-2-furanyl)-5-fluorouracil (ftorafur, FT), a masked form of 5-FU, is an effective antitumour agent. FT can be given orally and has a lower toxicity than 5-FU [3]. The mechanism of conversion of FT to 5-FU remains unknown. Several reports support the hypothesis that 5-FU formation may not

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be the only mechanism of FT activation and that a microbiologically active metabolite fraction of FT in addition to 5-FU may be present [4, 5].

For elucidation of their mechanism of action, it seemed important to establish a quantitative method for the determination of FT and 5-FU in serum. Several methods are reported for measuring FT and 5-FU in serum: bioassay to determine FT and 5-FU separately [6] and high-performance liquid chromatography (HPLC) with a reversed-phase chromatographic system for the simultaneous determination of FT and 5-FU [7, 8]. Most frequently in these HPLC separations detection of the eluate is achieved using an ultraviolet (UV) detector. However, the direct analysis of serum samples has been especially difficult with low concentrations of FT and 5-FU.

Fluorescence labelling in conjunction with HPLC is attractive since the detection limits are quite low. Fluorescence labelling of FT, 5-FU, pyrimidine bases and nucleosides has not been used extensively. An important example is presented by Dünges and Seiler [9]. They reported the use of 4-bromomethyl-7-methoxycoumarin (Br-Mmc) as fluorescence labelling reagent in the HPLC analysis of imide compounds.

The purpose of this paper is to demonstrate the applicability and scope of Br-Mmc labelling in the trace analysis of FT and 5-FU. A rapid, simple and virtually quantitative esterification method yields stable derivatives that are amenable to HPLC using a reversed-phase system.

EXPERIMENTAL

Reagents and chemicals

All organic solvents (Nakarai Chemicals, Kyoto, Japan) were commercial analytical-reagent grade materials. The distilled water used for the mobile phase was passed through an ion-exchange column (Millipore, type ZD20-11583). Br-Mmc, 18-crown-6 and *n*-valeric acid were purchased from Nakarai Chemicals and were used without purification. 5-FU and FT were obtained from commercial sources.

Preparation of derivatives for chromatographic studies

A ten-fold excess of Br-Mmc, a two-fold excess of 18-crown-6 and 25 mg of crystalline water-free potassium carbonate were added to 0.5 mg each of 5-FU and FT in 20 ml of acetone—acetonitrile mixture (1:2, v/v) (protected from light by wrapping the flask in aluminium foil). The mixture was allowed to reflux for 45 min in a water bath. After cooling, 0.2 ml of *n*-valeric acid was added to the mixture for the esterification of excess Br-Mmc. The mixture was refluxed again for 5 min. About 10 μl of this solution were applied to a thin-layer chromatography (TLC) plate. For HPLC injection, the mixture was diluted 10^2- to 10^5-fold with acetone.

Preparation of derivatives for serum analysis

Serum samples (0.5 ml) were adjusted with physiological saline to a total volume of 1 ml, and 0.1 ml of 0.5 *M* sodium dihydrogen phosphate buffer and 8 ml of ethyl acetate were added. After extraction and centrifugation, the organic layer was removed [4] and evaporated by aspirator vacuum. To the

residue, 1 ml of acetone—acetonitrile mixture containing 0.5 mg/ml Br-Mmc, 0.1 mg/ml 18-crown-6 and 1 mg of potassium carbonate were added and the mixture was refluxed as above. The resulting Mmc esters were utilized for HPLC.

Thin-layer chromatography

A 5—10 μl sample solution was spotted on a Kieselgel 60 plate, F-254 (Merck). Chromatography was performed in the solvent system ethyl acetate—methanol—10% (v/v) formic acid in water (9:1:1). The chromatogram was developed by the ascending technique for 15 cm and dried in a stream of cold air for a few minutes. The separated Mmc esters were identified by viewing the plate under a UV lamp giving maximum transmission at about 254 nm and 365 nm.

HPLC analysis

Analysis was performed using a Shimadzu (Kyoto, Japan) Model LC-4A chromatograph equipped with a Model SIL-1A injector. Separations were achieved with a column (20 cm × 4 mm I.D.) with C_{18}-brushes on a 5-μm silica core (Nucleosil 5, C_{18}; Macherey, Nagel & Co., Düren, F.R.G.). The column was packed using a balanced density slurry packing procedure similar to that described by Majors [10]. All chromatography was done at ambient temperature. The separation of 5-FU and FT was carried out with the eluent 70% methanol in water.

The column effluent was monitored by fluorescence detection with excitation and emission wavelengths of 346 nm and 395 nm, respectively. The fluorescence intensity was measured by a Shimadzu Model RF-530 fluorescence spectrophotometer.

RESULTS AND DISCUSSION

Reactivity of Br-Mmc

The reactivity of Br-Mmc in non-aqueous media was investigated in series of experiments using comparative TLC [11]. Lam and Grushka have studied various derivatization schemes of monocarboxylic [12] and dicarboxylic acids [13] with Br-Mmc. They have shown that the use of potassium carbonate to form the salt prior to the phase transfer yields an excellent compromise between the ease of the procedure and the rate of derivatives formation, and that the use of crown ether as a catalyst drastically reduces the reaction time [14].

In the procedures for the 5-FU-Mmc and FT-Mmc derivatives, the acetone—aetonitrile (1:2) reacted rapidly and efficiently as well as the crown ether. Fig. 1 shows the amount of 5-FU-Mmc derivative formed versus the reaction time for three different derivatization procedures. The rate of FT-Mmc derivative formation in the optimum conditions is also shown in Fig. 1.

The reaction rates were dependent on the 18-crown-6 concentration, the volume ratio of acetonitrile in acetone and the temperature. With 18-crown-6 as the catalyst in the presence of powdered potassium carbonate, formation of the 5-FU-Mmc derivative proceeded to completion at 75°C in 45 min. More

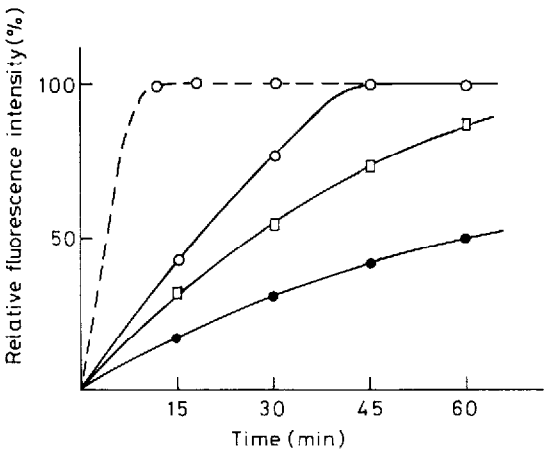


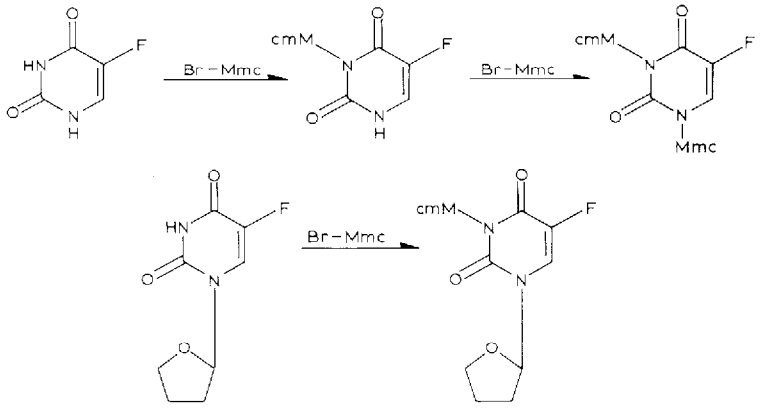
Fig. 1. Study of the rate of derivative formation. •, 0.5 mg of 5-FU in 20 ml of acetone is derivatized with 5 mg of Br-Mmc and 1 mg of 18-crown-6 in the presence of potassium carbonate at room temperature. □, 0.5 mg of 5-FU in 20 ml of acetone is derivatized in the above conditions at 75°C. ○——○, 0.5 mg of 5-FU in 20 ml of the solvent mixture acetone—acetonitrile (1:2) is derivatized in the above conditions, ○ - - ○, 0.5 mg of FT is derivatized in the above optimal conditions.

than 60 min were needed when the mixture was reacted in acetone at 75°C, and at room temperature the reaction was not complete after 2 h. The reaction of Br-Mmc with FT proceeded rapidly compared with 5-FU and only 10 min were needed when FT was derivatized under the optimal conditions for 5-FU.

The yield of the reaction was not investigated. However, as will be discussed in a later section, a linear relationship exists between the reaction yield and the amount of 5-FU and FT present in blood serum. Therefore, the present procedure is suitable for purposes of quantitation.

Structure of the derivatives

The structure of the alkylated derivatives has been confirmed by many workers [15—18], and it can be concluded that the possible equations for the labelling reaction of 5-FU and FT are



The separation of N-mono-Mmc and N,N′-bis-Mmc derivatives of 5-FU is shown in Fig. 2. The fluorescence intensity differs markedly between the mono and the bis derivative; i.e. the quantum yield of the bis derivative is higher than that of the mono derivative. The chromatogram of the Mmc-FT derivative showed a peak not containing other undesirable components. These labelling reactions were also studied in detail in conjunction with TLC.

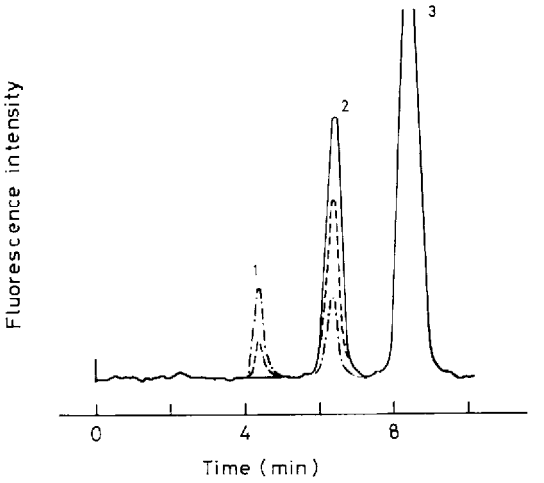


Fig. 2. Separation and formation process of N-mono-Mmc and N,N′-bis-Mmc derivatives of 5-FU. [Br-Mmc]/[5-FU] = 10. 1 = N-mono-Mmc derivative of 5-FU, 2 = N,N′-bis-Mmc derivative of 5-FU, 3 = Mmc derivative of *n*-valeric acid. — • — • —, after 15 min reaction time; - - - - -, after 30 min reaction time; ——, after 45 min reaction time. After each reaction time, 0.2 ml of *n*-valeric acid was added to the reaction mixture and the solution refluxed for 5 min at 75° C. Mobile phase = 70% methanol; flow-rate = 0.8 ml/min.

Separation by TLC

With the solvent system ethyl acetate—methanol—10% formic acid, a satisfactory separation was obtained with R_F values of 0.89 for the N,N′-bis-Mmc derivative of 5-FU, 0.87 for the Mmc derivative of FT and 0.65 for the N-mono-derivative of 5-FU. The Mmc derivative of *n*-valeric acid (esterification of excess Br-Mmc) had an R_F value of 0.93. The spot of the N-mono-Mmc derivative of 5-FU gradually disappeared according to the progress of reaction and was not seen 45 min after the start of the reaction. Other fluorescent spots, which could represent by-products of Br-Mmc and other undesirable components, were well separated in both systems.

The spots of the Mmc derivatives on the silica gel plates were stable without spraying or other special protective procedures for several days. By repeated chromatography of the same sample solution it could be shown that the Mmc derivatives are stable for at least several weeks in the reaction mixtures.

Fluorescence properties

The fluorescence spectrum of the FT-Mmc derivative was obtained in a solvent system of 70% methanol (Fig. 3). The excitation maximum appeared at 346 nm and the emission maximum at 395 nm. The fluorescence spectrum of the 5-FU-Mmc derivative was similar to that of the FT-Mmc derivative.

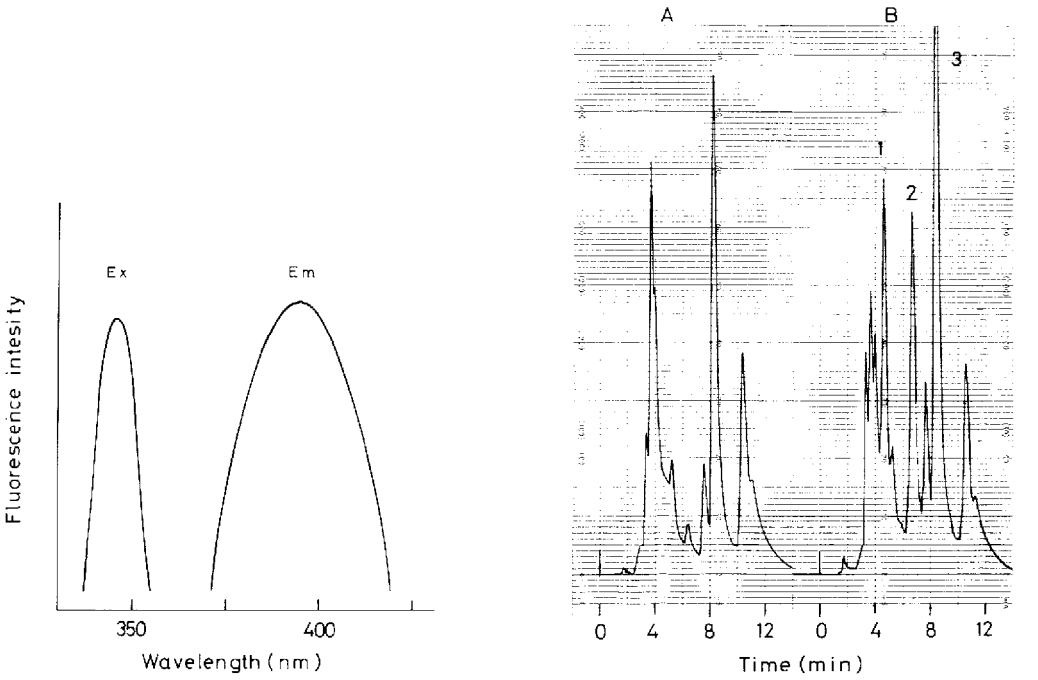


Fig. 3. Fluorescence spectra of FT-Mmc derivative. Fluorescence spectra are not corrected. Ex = excitation, Em = emission. Solvent = 70% methanol.

Fig. 4. Separation of 5-FU-Mmc and FT-Mmc derivatives in a serum sample. A, Serum blank. B, Serum spiked with 10 μg each of 5-FU and FT per ml. Mobile phase = 70% methanol; flow-rate = 0. 8 ml/min. 1 = Ft-Mmc derivative, 2 = 5-FU-Mmc derivative, 3 = *n*-valeric-Mmc derivative.

The fluorescence properties of the Mmc derivatives are very favourable for reversed-phase work as a signal can be observed in an aqueous mobile phase.

Separation by HPLC

Fig. 4B shows a chromatogram of the 5-FU-Mmc and FT-Mmc derivatives in serum and Fig. 4A shows the separation of a serum extract free from 5-FU and FT (blank sample). The blank sample shows that the serum constituents do not interfere with the separation of the 5-FU and FT derivatives. The identification of each compound was made by cochromatography with pure derivatives and comparing retention times. The blank peaks in Fig. 4 increased when using Br-Mmc stock solution that was exposed continuously to light. When the derivatizing reagent was protected from light, the 5-FU-Mmc and FT-Mmc derivatives obtained were almost completely separated from blank peaks as can be seen in Fig. 4A and B. It should be emphasized that the derivatization reaction was carried out under protection from light.

Calibration curves and detection limit

To be useful in quantitative analysis, the amount of ester formed in the derivatization reaction should be related to the amount of FT and 5-FU. Serum samples were spiked with increasing amounts of FT and 5-FU (final concentrations 0.02, 0.05, 0.1—10 μg of each drug per ml of serum). The samples

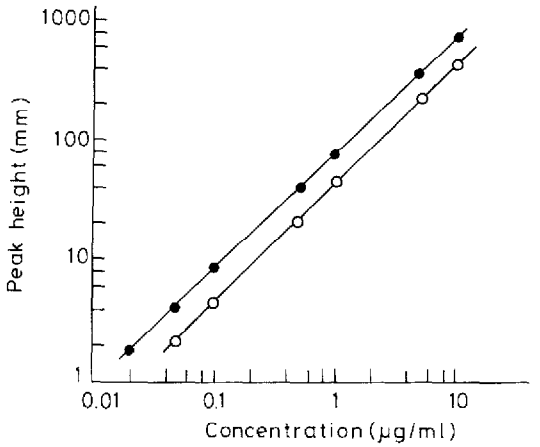


Fig. 5. Calibration curves of Mmc-derivatized 5-FU (•) and FT (○) extracted from serum.

were submitted to the extraction procedure described and standard curves were generated for each series of determination by plotting peak height against known drug concentration (Fig. 5). The linear relationships indicate that the procedure described here can be used to quantitate FT and 5-FU in serum. Recoveries for the method including extraction, Mmc derivatization and quantitation averaged 85 ± 5% for FT and 55 ± 5% for 5-FU.

The detection limits were found to be a function of the column condition, the mobile phase and the flow-rate. Under the present separation conditions, typical detection limits were 384 fmol for FT and 100 fmol for 5-FU.

Since the derivatization reaction can be scaled down to a reaction volume of 10 μl [19], it is possible to determine a few fmoles of the derivatized compounds.

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