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High-performance liquid chromatography of 5-fluorouracil after derivatization with 4-bromomethyl-7-methoxycoumarin. Characterization of the derivative and the use of column switching for the improvement of resolution and the enhancement of sensitivity

CARL G. KINDBERG,* MILAN SLAVIK,† CHRISTOPHER M. RILEY*‡ and JOHN F. STOBAUGH*

*Center for Bioanalytical Research and Departments of *Pharmaceutical Chemistry and †Medicine, University of Kansas, Lawrence, KS 66045, USA*

Abstract: The derivatization of 5-fluorouracil with 4-bromomethyl-7-methoxycoumarin has been reported previously; however, the structure of the derivative was not confirmed. The synthesis and purification of the 5-FU derivative is described along with the spectroscopic (MS and NMR) determination that it is labelled at both heterocyclic nitrogens as expected. A column switching HPLC system is also presented which consists of primary separation on a cyanopropyl column followed by a final separation on an ODS column with fluorescence detection. This system removes all interferences from the derivatization system and has a limit of detection for the pure derivative of <50 fmol (injection volume = 100 μl).

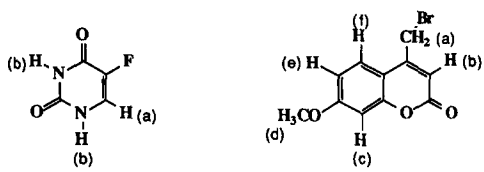
Keywords: *High-performance liquid chromatography; 5-fluorouracil; pre-column derivatization; 4-bromomethyl-7-methoxycoumarin; fluorescence detection; column switching.*

Introduction

5-Fluorouracil (5-FU, **I**) is an antineoplastic agent which has been in clinical use for many years [1]. Many quantitative methods for its determination in biological fluids are available, including microbial assays [2], high-performance liquid chromatography (HPLC) [3–14] and gas chromatography with mass spectroscopic detection (GC–MS) [15–18]. Despite the large number of methods that have been reported [2–18], only the GC–MS methods [15–18] have the sensitivities approaching those required for determining the pharmacokinetic parameters of 5-FU following continuous infusion of the drug. Low dose (<600 mg m^{-2} d^{-1}) infusion of 5-FU is being utilized more

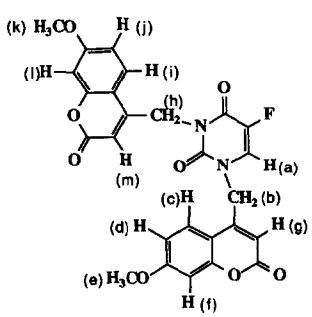
‡To whom correspondence should be addressed.

frequently and promising clinical results have been reported [19, 20]. After an intravenous (i.v.) bolus dose or the termination of a continuous i.v. infusion of 5-FU, the plasma concentration quickly decreases below the limit of detection of all assays (*ca* 5–10 ng ml^{-1}) with an elimination half-life of 6–12 min [14, 15]. Recent reports from a Japanese research group [10–13] have indicated that derivatization of 5-FU with 4-bromomethyl-7-methoxycoumarin (BrMmc, **II**) is simple and allows the use of HPLC with fluorescence detection to reduce the limit of detection. However, due to limitations of this procedure, related to interfering side products of the derivatization reaction, the potential of the method for the determination of 5-FU in biological fluids has not been realized. BrMmc was first synthesized by Secrist *et al.* [21] and used for the derivatization of thiouracil. Later [22] BrMmc was utilized for the derivatization of fatty acids. Iwamoto *et al.* [10, 11] reported the derivatization of 5-FU with BrMmc in acetone–acetonitrile with powdered K_2CO_3 as a catalyst and later [12, 13] in dimethylsulphoxide (DMSO) without any catalyst. Both derivatization schemes were followed by HPLC analysis with fluorescence detection. The reaction in DMSO proceeds quickly at room temperature and these workers [10] proposed that the highly fluorescent derivative was the bis-substituted compound, bis-*N*,*N*′-(4-methylene-7-methoxy-coumaryl)-5-fluorouracil (**III**). However, they neither isolated nor characterized the product. In the previous reports, impressive detection limits equivalent to about 10 pmol of 5-FU were demonstrated. However, these limits were achieved by serial dilutions of solutions containing relatively high concentrations of the derivative. These limits of detection cannot be achieved if 5-FU is reacted at low concentrations (<10 ng ml^{-1}), due to the presence of unreacted reagent, side products of the reaction and degradation products of BrMmc (**II**), all of which are fluorescent and interfere with the chromatography. Dilution of the reaction mixture reduces the size of the interfering peaks as well as the size of the peak for the derivative. As a result, the actual detection limits for 5-FU in plasma using pre-column derivatization with BrMmc are no better than



I

II



III

those that can be achieved with direct UV absorption. The purpose of the present study was to isolate, purify and characterize a laboratory standard of **III** and to investigate multidimensional chromatography as means of accessing the full potential of this highly fluorescent derivative.

Experimental

Chemicals and reagents

5-Fluorouracil was obtained from Sigma Chemical Co. (St. Louis, MO, USA). 4-Bromomethyl-7-methoxycoumarin, DMSO and NMR grade solvents were purchased from Aldrich Chemical Co. Inc. (Milwaukee, WI, USA). The HPLC grade solvents and potassium carbonate were from Fisher Scientific (Fair Lawn, NJ, USA). The potassium carbonate was powdered with a mortar and pestle and dried at 30°C in a vacuum oven before use. The distilled water was purified with a Milli-Q Water System (Millipore, Bedford, MA, USA).

Synthesis of III

5-Fluorouracil was derivatized with 4-bromomethyl-7-methoxycoumarin, on an analytical scale, using a modification of the procedure reported previously by Yoshida *et al.* [13]. For the large scale preparation of the derivative, BrMmc (100 mg) was first dissolved in DMSO (6 ml), 5-FU (20 mg) and K_2CO_3 (50 mg) were then added and the reaction mixture was stirred for 1 h at room temperature. The solvent was removed (at 30°C) with a rotary evaporator (Rotavapor, Buchi Scientific, Switzerland) attached to a vacuum pump (10 mmHg pressure). The residue was stored under N_2 at −20°C prior to further purification.

Analytical scale derivatizations of 5-FU were performed in 1.5 ml polypropylene microcentrifuge tubes (Fisher Scientific, Fair Lawn, NJ, USA) by mixing 5-FU (10 μg and 10 or 1 ng) in DMSO (100 μl) with a solution of BrMmc (400 μl of 2.5 mg ml^{-1} in DMSO) and 5 mg of K_2CO_3, and shaking for 15 min at room temperature. When 10 μg of 5-FU was derivatized, the reaction mixture was diluted 1:200 with mobile phase and then analysed by HPLC. The reaction mixtures containing 10 ng or less 5-FU were mixed with 500 μl distilled water and centrifuged at 15,600*g* (Centra-M Microcentrifuge, International Equipment Co., Needham Heights, MA, USA) for 5 min. The supernatant was then removed and analysed by HPLC.

Purification of III

A 15 mm × 150 cm burette was packed with 75 g of Kieselgel 60 (70–120 mesh, E. Merck, FRG) in ethyl acetate/hexane (9:1, v/v). The residue from the derivatization reaction was dissolved in 20 ml of ethyl acetate/hexane (9:1, v/v), layered onto the top of the silica column and eluted with 100 ml of ethyl acetate/hexane (9:1, v/v) followed by 100 ml of ethyl acetate and the eluent was collected as 7-ml fractions.

The presence of **III** in the fractions was monitored as follows. An aliquot of each fraction (100 μl) was dried under N_2, redissolved in 500 μl of DMSO/water (1:1, v/v) and 100 μl injected onto an ODS column (ODS–Hypersil, Shandon Southern Products Ltd, UK) eluted with methanol–water at 1 ml min^{-1}. The eluent was monitored by fluorescence measurements (λ_{ex} = 325 nm, λ_{em} = 395 nm). Those samples that contained the highest concentration of **III** with the least contamination were pooled, dried under N_2, and redissolved in DMSO.

The derivative was further purified by HPLC on the ODS column. Two volumes of the DMSO solution containing **III** was diluted with 1 vol of water and 200 μl was injected onto the HPLC column using the conditions described above. The fraction containing **III** was collected and the solvent was removed *in vacuo*.

Characterization of III

Mass spectra and the precise molecular weight of **III** were obtained using a Ribermag R 10-10 quadrupole mass spectrometer (Nermag, France). The sample was introduced by a direct probe heated to 300°C with ionization by electron impact (70 eV).

The proton NMR spectra of 5-FU (**I**), BrMmc (**II**), and **III** were obtained by means of an XL300 NMR spectrometer (Varian Associates, Palo Alto, CA, USA) operated at 299.9 MHz with a probe temperature of 20°C. 5-Fluorouracil was dissolved in DMSO-d_6 (5 mg ml^{-1}), and 1.5 mg of BrMmc and **III** were each dissolved in 0.7 ml $CDCl_3$ containing 0.03% v/v TMS.

UV–vis absorption spectra were obtained on an HP 8451A diode array spectrophotometer (Hewlett–Packard, Palo Alto, CA, USA) over the range 190–800 nm. Spectra of **III** were obtained at a concentration of 37 μg ml^{-1} in methanol, methanol/water (1:1, v/v), acetonitrile, and acetonitrile/water (1:1). The molar extinction coefficient (ε) was calculated for **III** in methanol.

Fluorescence spectra were obtained using a Perkin–Elmer 650–40 fluorescence spectrophotometer (Norwalk, CT, USA). A stock solution of **III** (10 μl; 0.94 mg ml^{-1} in DMSO) was added to 2.0 ml of methanol, acetonitrile, water, and various proportions of the organic solvents (25, 50, 75%, v/v) in water. The emission wavelength was maintained at 395 nm while scanning the excitation wavelengths from 250 to 380 nm. The emission spectra from 340 to 550 nm were obtained with an excitation wavelength of 325 nm. A 5-nm slit width was used for both excitation and emission.

High-performance liquid chromatography

The chromatographic system consisted of two Model 2150 HPLC pumps, a Model 2151 variable wavelength UV detector, and a Model 2152 HPLC controller (LKB Instruments, Inc., Gaithersburg, MD, USA); a Perkin–Elmer 650-10S fluorescence specrophotometer (Norwalk, CT, USA); a 6-port switching valve (Valco Instrument Co. Inc., Houston, TX, USA); and a 100 μl fixed volume (Rheodyne, Cotati, CA, USA) or U6K (Waters Associates, Milford, MA, USA) injectors. The columns used were 4.6 mm (i.d.) × 15 cm (length) packed with 5 μm silica particles bonded with cyanopropyl (CPS-Hypersil, Shandon Southern Products Ltd, UK) or ODS (ODS–Hypersil) side chains. The columns were packed in the authors' laboratory using the upward slurry technique [23].

The CPS column was jacketed and thermostatted at 35°C with a circulating water bath (Haake, Saddlebrook, NJ, USA) to minimize fluctuations in retention due to periodic changes in the ambient temperature, and was eluted with methanol/water (1:1, v/v). The ODS column was eluted with methanol/water (6:4, v/v) at ambient temperature. The flow rate for both columns was 1 ml min^{-1}.

A schematic representation of the HPLC system when configured for column switching is given in Fig. 1. Samples (100 μl) were injected onto the CPS column and a 2-min heart cut containing the derivative peak was switched onto the ODS column. The time of the cut was determined by injecting a standard solution of **III** onto the CPS column and monitoring the eluent with the UV detector set at 325 nm. The switching

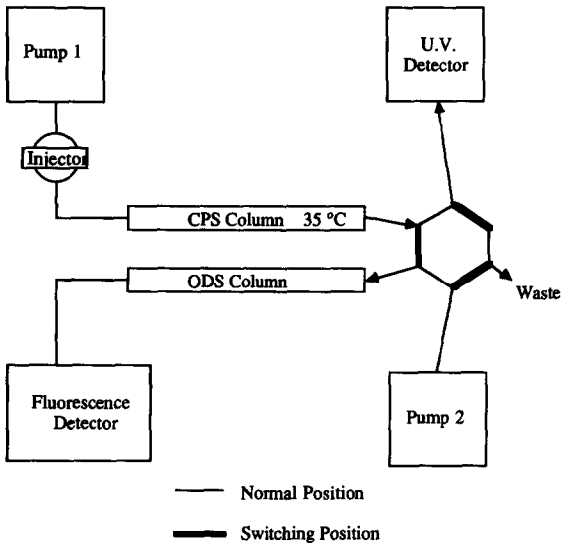


Figure 1
Schematic representation of the column switching system.

valve was actuated by the system controller. The eluent from the ODS column was monitored with the fluoroscence detector (λ_{ex} = 325 nm, λ_{em} = 395 nm).

The limit of detection (LOD) for pure **III** with the column switching system was determined by dissolving a known quantity of **III** in DMSO. This solution was diluted with water (1:1), followed by serial dilutions (1:1) with DMSO/water (1:1, v/v) and injection onto the complete system until the signal to noise ratio (S/N) was <2.

Results and Discussion

Characterization of the derivative (III)

The purified **III** was obtained as a white powder that melted at 264°C with decomposition. Purity of **III** was determined to be >99% by HPLC on CPS and ODS columns with both UV and fluorescence detection. The mass spectra and proton NMR spectra confirmed that no detectable impurities were present. An adequate quantity (*ca.* 4 mg) was isolated for future use as an analytical standard and for the confirmation of structure. The derivative was characterized by high resolution MS, ^{1}H NMR (field strength 300 MHz), UV–vis spectroscopy, fluorescence spectroscopy and HPLC using two different stationary phases (CPS and ODS).

The spectroscopic data on **III** were consistent with the proposed structure shown in the Introduction. High resolution mass spectrometry of **III** gave a molecular weight of 506.1134 which compares with a calculated value of 506.1124 and corresponds to an error of 0.000198%. The fragmentation pattern for **III** included major peaks at *m/e* = 506 (M^+), 318, 190, 188, 161 and 147. The peak at *m/e* = 318 is due to the loss of one coumarin moiety, which itself appears at 188 or 190, depending on the loss or gain of a hydrogen atom, respectively. The coumarin fragment can undergo further fragmentation producing the peaks at *m/e* = 161 and 147.

The proton NMR results are presented in tabular form (Table 1) for 5-FU, BrMmc and **III**. The chemical shifts for 5-FU and BrMmc are in agreement with those previously

Table 1
Chemical shifts for **I**, **II** and **III**

Proton*	Chemical shift ppm	Multiplicity†
I		
a	7.77	d
b	11.15	s (broad)
II		
a	4.53	s
b	6.39	s
c	6.86	d
d	3.90	s
e	6.94	dd
f	7.68	d
III		
a	7.97	d
b	5.24	s
c	7.59	d
d	6.96	d
e	3.94	s
f	6.89	d
g	5.92	s
h	5.36	s
i	7.71	d
j	6.97	d
k	3.94	s
l	6.90	d
m	6.18	s

*See structures in Introduction.
†d = Doublet; s = singlet; dd = doublet of doublets.

reported [24] and peak integrations for all three compounds were in good agreement with the predicted number of protons. It was interesting to note that the additional carbonyl group has a profound effect on the chemical shifts for all the protons of the coumarin moiety attached to the N-3 of 5-FU except for the protons of the methoxyl group.

Since the two nitrogens of 5-FU are the only sites available for labelling, one can propose that the derivative would be the mono-*N*- or the bis-*N*,*N*'-coumarin. The high resolution molecular weight obtained by mass spectrometry is in excellent agreement with the predicted molecular weight of the bis-derivative. The ^{1}H NMR data also suggest that BrMmc reacts with 5-FU at two locations, forming bis-*N*,*N*'-(4-methylene-7-methoxycoumaryl)-5-fluorouracil. This is in agreement with the kinetics of the reaction observed by HPLC in this study and previously by Iwamoto *et al.* [10], in which the appearance of a peak with the same retention as **III** is preceded by the appearance of an earlier eluting peak. As the reaction proceeds, the peak attributed to **III** reached a maximum and the earlier eluting peak, presumably the mono-derivative, increased and then declined.

The absorption spectrum of **III** is shown in Fig. 2 and was found to be independent of the solvent. The spectrum shows two maxima at 282 (ϵ = 17,000) and 322 nm (ϵ = 27,700) with no absorbance above 380 nm. There was a single minimum around 295 nm. The fluorescence emission and excitation spectra (Fig. 3) were qualitatively the

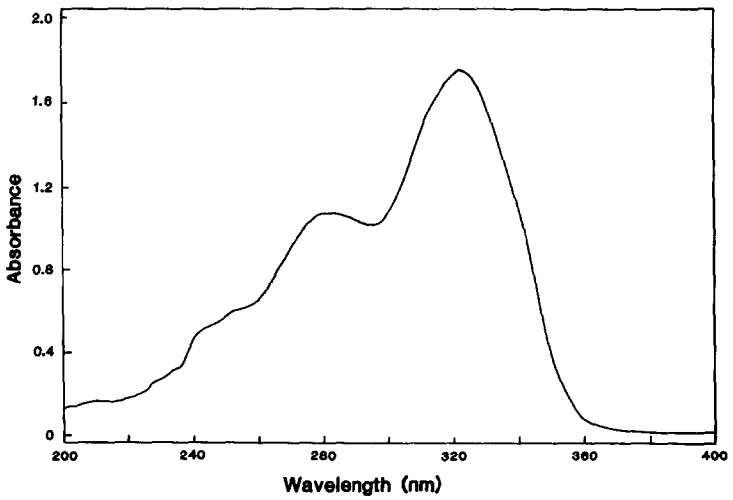


Figure 2
Absorption spectrum of **III** in methanol; 185 μg of **III** in 100 μl of DMSO diluted to 5 ml with methanol. The absorption spectrum has two maxima at 282 (ε = 17,000) and 322 nm (ε = 27,700), and was the same when measured in methanol or acetonitrile.

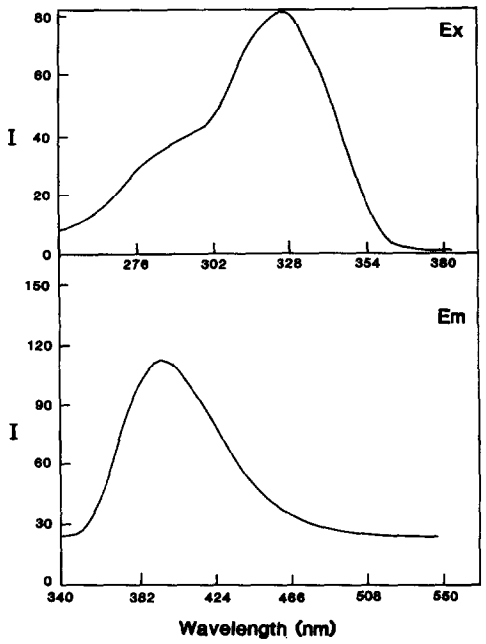


Figure 3
Fluorescence spectra of **III** in methanol. The excitation spectrum of **III** was obtained with the emission wavelength set at 395 nm. The excitation wavelength was 325 nm while scanning the emission spectrum.

same when measured in methanol, acetonitrile, or combinations of either of these two solvents with water. The excitation spectra for **III** had more fine detail than those that have been reported previously [10–13] and were consistent with the absorption spectrum shown in Fig. 2. The maximum fluorescence intensity for all solvent mixtures tested, occurred at an excitation wavelength of 325 nm with emission at 395 nm and is in

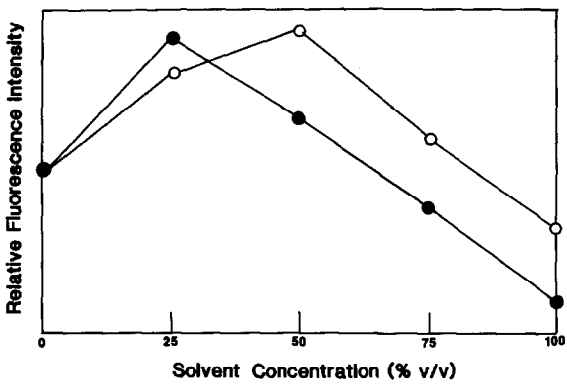


Figure 4
Maximum fluorescence intensity of **III** in solvent–water mixtures. The fluorescence intensity of **III** was measured at λ_{ex} = 325 nm and λ_{em} = 395 nm in methanol (○) and acetonitrile (●) with varying concentrations of water.

agreement with earlier reports [10–13]. However, the fluorescence intensity was highly dependent upon the solvent composition (Fig. 4) and it was found that the maximum intensity in both acetonitrile and methanol occurred at a somewhat lower water concentrations than had been reported previously [13]. It was fortunate that the optimum solvent composition (60% methanol) used for the HPLC in the present study coincided with the region of maximum fluorescence intensity (Fig. 4).

Liquid chromatography

Chromatograms of 10 μg of 5-FU derivatized with 1.0 mg BrMmc in DMSO and diluted 1:200 to give a final concentration equivalent to 50 ng ml^{-1} of 5-FU, are shown in Fig. 5. It can be seen that the derivative, **III**, is well separated from the other potentially interfering peaks. However, when the derivatization is performed on smaller quantities of 5-FU (1 ng), the interferences seriously inhibit the detection of **III** (Fig. 6A,B). The problem of interfering peaks arising from the reaction system is a significant limitation for the use of BrMmc as a derivatization reagent for HPLC analysis and has been observed previously [10–13].

The problem of interfering peaks has been addressed by Yoshida *et al.* [13] who used a thin-layer chromatography (TLC) step prior to analysis by HPLC. While successful, this procedure is tedious and a simpler alternative using automated column switching is proposed in this study. The derivative is partially separated from the other components of the reaction mixture on a CPS column. An automated switching valve then directs the derivative onto the ODS column where the separation is completed. This produces a chromatogram with **III** well separated from any other components in the matrix (Fig. 6C).

Automated column switching produces a satisfactory chromatogram without a need for preliminary sample clean up with TLC. This reduces the losses and labour associated with manual sample handling. Also, it has the added advantage of increased sample throughput and higher sensitivity. It is interesting to note that the band broadening that occurred on the first column, did not contribute to the overall band width of the peak that finally eluted from the second column. This can be attributed to compression of the

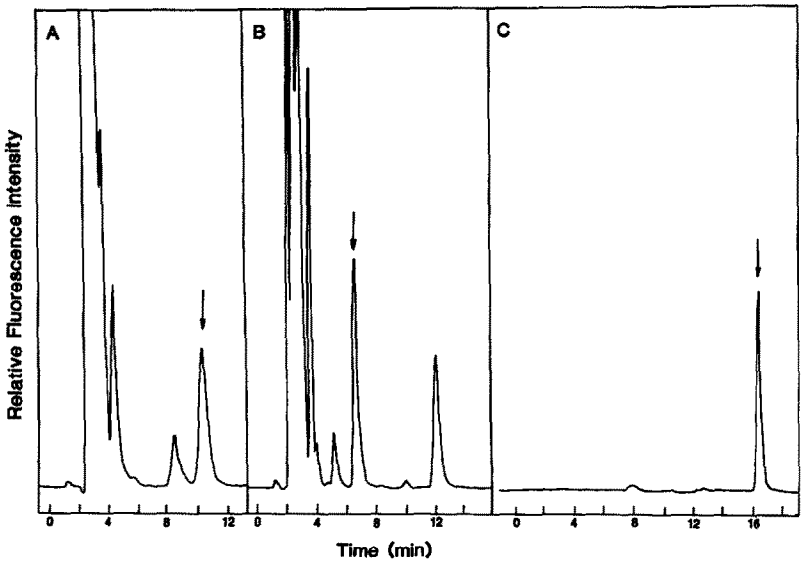


Figure 5
Chromatograms of 10 μg 5-FU derivatized with BrMmc and diluted to 50 ng ml^{-1}. The eluents were monitored with a fluorescence detector (λ_{ex} = 325 nm and λ_{em} = 395 nm). (A) The reaction mixture (100 μl) was injected onto a CPS (CPS-Hypersil) column and eluted with methanol/water (1:1, v/v) at 1 ml min^{-1}. The CPS column was thermostatted at 35°C. (B) The reaction mixture was injected onto a ODS (ODS-Hypersil) column. The mobile phase was methanol/water (6:4, v/v) with a flow rate of 1 ml min^{-1} at ambient temperature. (C) The reaction mixture was injected onto (A) and a 2-min cut (9.5–11.5 min) was switched to (B). The arrows mark the elution positions of **III**.

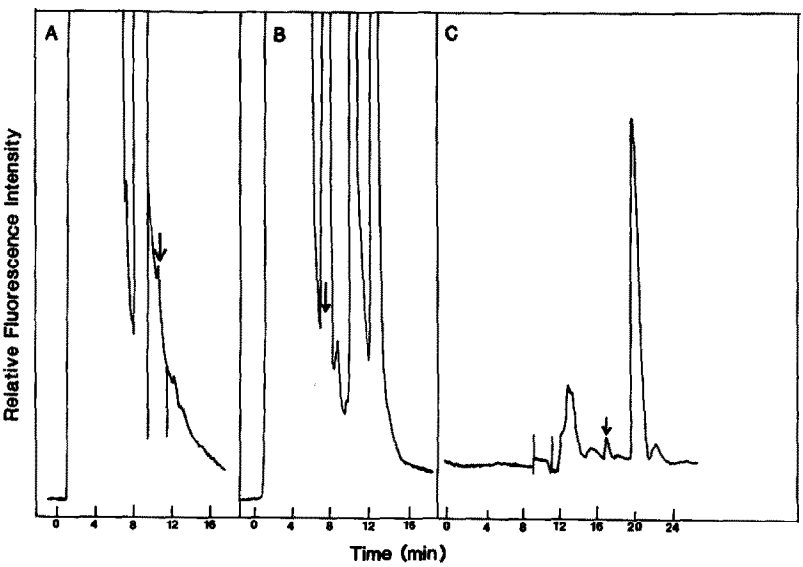


Figure 6
Chromatograms of the reaction mixture when 1 ng 5-FU was derivatized. The arrows mark the elution positions of **III**. The reaction mixture was injected onto (A) CPS column, (B) ODS column, and (C) column switching set-up. Conditions as described in Fig. 7. The by-products of the derivatization reaction prevent quantification of **III** unless column switching is used. The peak in (C) corresponds to the derivative from 0.1 ng 5-FU.

chromatographic zone as it was transferred from the first column to the second. The peak width of **III** as it eluted from the CPS column was 1.75 ml, yet it was only 0.8 ml as it eluted from the ODS column into the detector. Zone compression was achieved by transferring **III** from the first column, using a mobile phase of 50% methanol in water, and then eluting from the second column with a stronger eluent of 60% methanol in water (Figs 5 and 6). The potential benefits of zone compression on the overall sensitivity of the assay are clearly indicated. Figure 6 shows that, when column switching is used, the peak height of **III** is only about 10% less than the peak height obtained when the ODS column is used alone. Another advantage of column switching for the analysis of 5-FU is that it eliminates the need to quench the reaction and remove the excess reagent [10–13]. Previous methods [10–13] have advocated the addition of *n*-pentanoic or *p*-nitrobenzoic acid to remove the excess reagent, once the reaction is completed. In the present method, the excess reagent was removed chromatographically without the need of an additional chemical reaction.

The limit of detection (LOD) for this method (S/N = 2) using column switching is 20 pg of **III** injected onto the column (Fig. 7); this LOD appears to be limited by the detector noise rather than interferences from the matrix. Assuming a reaction efficiency of >90%, which is reasonable, this translates into a theoretical LOD of 5 pg or 40 fmol of 5-fluorouracil injected. It remains to be seen whether this LOD can be improved by the use of more powerful detector systems, such as those based on laser induced fluorescence (LIF) which are currently under development.

Conclusions

In this study, the structure of **III** has been characterized and confirmed. In addition, it has been demonstrated that the use of column switching enables the full potential of this highly fluorescent derivative to be realized. The technique should be adaptable to a wide

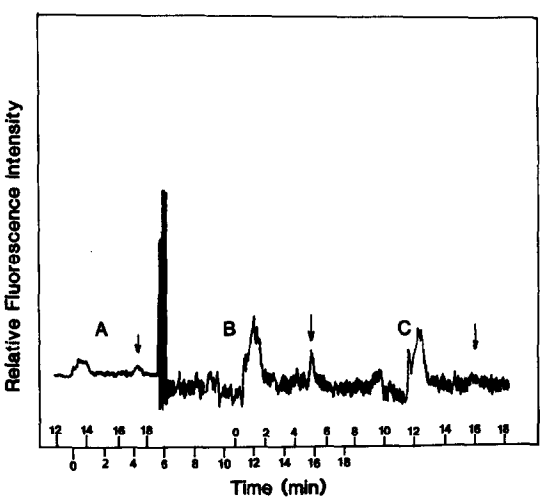


Figure 7
Chromatogram of 6.0 pg of **III** with column switching, (A) and (B). Although the S/N ratio is about 2, a peak is still evident. The peak was not present when a mobile phase blank was injected (C). Conditions as described in Fig. 5C.

variety of biological applications and investigations into the pharmacokinetics of 5-FU following low dose infusions into animals and humans using this analytical methodology are currently in progress.

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