Journal of Chromatography, 530 (1990) 57–64
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 5347

High-performance liquid chromatography with chemiluminescence detection of serum levels of pre-column derivatized fluoropyrimidine compounds

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(First received November 17th, 1989; revised manuscript received April 2nd, 1990)

ABSTRACT

7-(Diethylamino)-3-[4-((iodoacetyl)amino)phenyl]-4-methylcoumarin (DCIA) and 4-(bromomethyl)-7-methoxycoumarin have been evaluated as fluoropyrimidine-derivatizing agents to be detected using peroxyoxalate chemiluminescence with high-performance liquid chromatography. The derivatization procedure required only one step. No chemiluminescence was observed from the bromo derivatives, and the detection limits of fluoropyrimidine compounds derivatized with the iodo compound and detected with peroxyoxalate chemiluminescence were in the low femtomole range.

INTRODUCTION

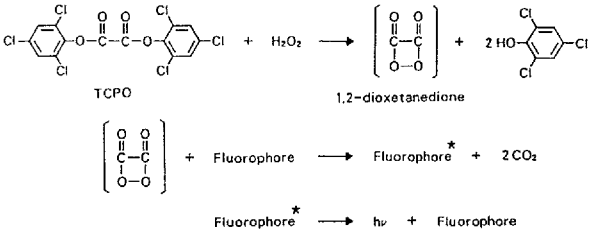
Fluoropyrimidine compounds, such as 5-fluorouracil (5-FU), 5-fluorouridine (FUrd), 5-fluoro-5′-deoxyuridine (FdUrd, a masked form, which is converted into 5-FU) and 1-(tetrahydro-2-furanyl)-5-fluorouracil (ftorafur, FT, a masked form), are widely used in the chemotherapy of a variety of human carcinomas [1,2]. The antitumour activity of these cytostatic agents is thought to be primarily due to inhibition of thymidine synthetase by 5-fluoro-deoxyuridine monophosphate (FdUMP), a common metabolite [3,4].

High-performance liquid chromatographic (HPLC) methods with UV detection have been reported for the determination of 5-FU, FUrd, FdUrd, their nucleosides and nucleotides [5,6]. In addition an HPLC analysis with pre-column derivatization and fluorescence detection has been published for 5-FU and FT [7]. More recently, newly developed spectroscopic analysis of fluoropyrimidine metabolism by ^{19}F nuclear magnetic resonance spectrometry was reported [8], but this assay is not suitable for routine use. For elucidation of the mechanism of their antitumour activity, it seemed important to develop a more sensitive method for the determination of fluoropyrimidine concentrations in serum.

Since the discovery of peroxyxalate chemiluminescence, much attention has been focused on developing an efficient chemical light source [9,10]. Like many chemiluminescent reactions, the overall process can be simplified into three basic

0378-4347/90/$03.50

steps. The first step involves the production of a key chemical intermediate containing the necessary excitation energy. The second step involves the conversion of this chemical energy into electronic excitation energy. The final step is the emission of light energy as the excited molecule returns to its ground electronic state. For the peroxyoxalate reaction, these steps are:



Here the postulated key intermediate is shown as 1,2-dioxetanedione, and the oxalate ester is bis(2,4,6-trichlorophenyl)oxalate (TCPO). One very important difference between the peroxyoxalate reaction and other efficient chemiluminescent reactions is that the chemical energy can be transferred to a variety of fluorescent molecules, whereas most chemiluminescent reactions involve emission from the key intermediate itself. It is this feature that allows this reaction to be applied to a wide variety of analytes.

Examples of fluorophores detected by using peroxyoxalate chemiluminescence are polycyclic and reduced nitropolycyclic aromatic hydrocarbons [11,12], polycyclic aromatic amines [13], fluorescamine-labelled catecholamines [14] and dansylated amino acids [15]. However, not all fluorophores are more efficiently detected by chemical excitation than by more conventional photoexcitation. Several characteristics of the fluorophore that contribute to efficient chemical excitation include high fluorescence efficiency, low oxidation potential and low singlet energy [13]. An important example is presented by Grayeski and DeVasto [16]. They reported the usefulness of 7-(diethylamino)-3-[4-((iodoacetyl)amino)-phenyl]-4-methyl-coumarin (DCIA) as a potential chemiluminescent derivatizing agent in the HPLC analysis of carboxylic acids. Coumarin compounds are widely used for pre- or post-column derivatization of, among others, OH [17], NH_2 and NHR groups [18].

The specific aim for the determination was to develop a more sensitive HPLC method for the determination of fluoropyrimidine concentrations in serum using chemiluminescence detection. Fluoropyrimidine compounds were quantitated by chemiluminescence detection after pre-column derivatization with DCIA. The method (extraction and derivatization procedure) was controlled by the addition of 5-chlorouracil (ClUra) to the serum samples, which served as an internal standard.

EXPERIMENTAL

Reagents and chemicals

4-(Bromomethyl)-7-methoxycoumarin (Br-Mmc) was purchased from Aldrich (Milwaukee, WI, U.S.A.) and was used without purification. DCIA was obtained from Sigma (St. Louis, MO, U.S.A.). Fluoropyrimidine compounds were purchased from Nakarai Tesque (Kyoto, Japan). TCPO was obtained from Tokyo Kasei (Tokyo, Japan). HPLC-grade methanol was obtained from Nakarai Tesque. 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 ZD20-11585).

Sample preparation

Human serum was used in the development of the HPLC method. Standards were prepared by the addition of known amounts of test compound to blank serum. The 0.5 ml of serum was centrifuged for 20 min at 1000 *g* and filtered through membrane filter (Millipore, UFC3TGCOO). A 0.1-ml aliquot of the membrane filtrate was used. ClUra (5 ng) and 100 μl of saturated ammonium sulphate solution were added to the filtrate. After vortex-mixing, 4 ml of ethyl acetate were added. After extraction and centrifugation (1000 *g*, 5 min), the organic layer was removed and evaporated under nitrogen. To the residue, 100 μl of anhydrous acetone were added.

Derivatization procedures

In a screw-cap mini-vial (1.0 ml) protected from light with aluminium foil and from moisture, fluoropyrimidine compounds were dissolved in 100 μl of anhydrous acetone. Then 50 μl of labelling reagent solution (150 μg/ml in acetone), 50 μl of 18-crown-6 solution (15 μg/ml in acetone) and 250 μg of freshly powdered anhydrous potassium carbonate were added. The mixture was sonicated for 30 min in a supersonic water-bath (USC-051A, Nippon Denshi Kagaku, Japan). A 4-μl aliquot was injected. By repeated chromatography of the same solution, it was shown that the DCIA derivatives were stable for at least several weeks in the reaction mixture.

Apparatus and HPLC conditions

Chromatography was performed using a Shimadzu (Kyoto, Japan) Model LC-4A chromatograph equipped with a Model SIL-1A injector. Separations were achieved with a Nucleosil 5 C_{18} column (20 cm × 4 mm I.D., 5 μm silica core, Macherey Nagel, Düren, F.R.G.). The column was packed using a balanced density slurry paking procedure. All chromatography was done at ambient temperature. The separation of fluoropyrimidine derivatives was carried out with the eluent composed of 50% (v/v) acetonitrile in 4 m*M* phosphate buffer (pH 7.5). The flow-rate of the mobile phase was 0.6 ml/min. A Jasco (Nihonbunkou, To-

kyo, Japan) dual-reagent, post-column mixing device was used to pump the TCPO and hydrogen peroxide solutions and combine them with the column effluent. The concentrations of TCPO in ethyl acetate and hydrogen peroxide in acetone were 4.4 m*M* and 1 *M*, and their flow-rates were both 0.7 ml/min. The generated chemiluminescence was monitored with a photon counter Model C-767 (Hamamatsu Photonics, Tokyo, Japan) equipped with a photomultiplier tube (R585, Hamamatsu Photonics). The photon counter was operated with a Toshiba L-42 prefilter (a 410-nm short-wavelength cut-off filter). A flow-cell (cell volume *ca.* 8 μl) was placed in front of the photomultiplier tube.

RESULTS AND DISCUSSION

Application to HPLC

The iodoacetyl-containing aminocoumarin was treated with 5-FU, FUrd, FdUrd and FT, and the chromatograms were monitored by chemiluminescence. The electron-donating amine functional group on DCIA would contribute to lowering the oxidation potential and singlet energy as well as to increasing the fluorescence efficiency [13,19]. Since the emission wavelength of the fluorophore is a general indication of relative singlet energies, a comparison was made of the spectral maxima values of the coumarin compounds (Br-Mmc: λ_{ex} = 325 nm, λ_{em} = 390 nm; DCIA: λ_{ex} = 375 nm, λ_{em} = 470 nm). These results indicate that addition of the amine should contribute to increased chemical excitation efficiency.

Reactivity of DCIA

The barbiturates can be alkylated by alkyl halide using the crown ether–potassium complex as a catalyst [20]. They 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.

In the procedures for the derivatization of pyrimidine compounds with DCIA, the anhydrous acetone reacted rapidly and efficiently as well as the crown ether. With 18-crown-6 as the catalyst in the presence of powdered potassium carbonate, formation of the FT-DCIA derivative was complete under reflux after 10 min. More than 20 min were needed when the mixture was sonicated in acetone, and at room temperature the reaction was not complete after 30 min (Fig. 1). The reaction of DCIA with other pyrimidine compounds proceeded rapidly compared with FT.

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 fluoropyrimidine compounds in blood serum. Therefore, the present procedure is suitable for quantitation.

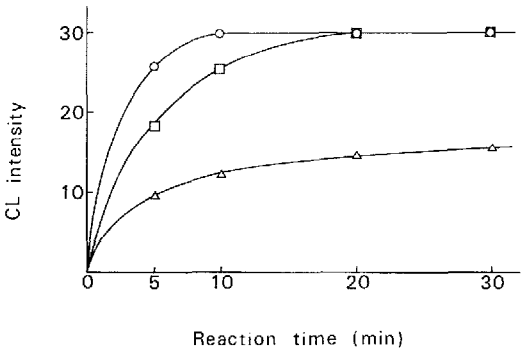


Fig. 1. Reaction profiles of the derivatization of FT with DCIA under various conditions: (○) reflux; (□) sonication; (△) room temperature. For the reaction, 25 ng of FT, 10 μg of DCIA, 1 μg of 18-crown-6 and 500 μg of K_2CO_3 were dissolved in 0.2 ml of acetone.

Structures of the derivatives

The structures of the alkylated derivatives have been confirmed by many workers [20,21], and one possible equation for the labelling reaction of FT with DCIA is shown in Fig. 2.

Determination of fluoropyrimidine compounds in serum

The chromatograms of blank serum samples contained several unidentified peaks, but none of these interfered with the fluoropyrimidine-DCIA derivatives at the concentrations used. FT, 5-FU, FUrd and FdUrd derivatives were well separated from each other. Using the procedure described above, five samples were routinely filtered and analysed simultaneously for FT, 5-FU, FUrd and FdUrd in 3 h. The chemiluminescence chromatograms of a standard mixture and a serum extract are shown in Fig. 3. The problem of decomposition of FdUrd to 5-FU, observed during gas chromatographic analysis, did not occur with the chemiluminescence HPLC method.

Recovery

The recovery of the fluoropyrimidine compounds after pretreatment was tested by determining pooled serum samples containing known amounts of four components. The recoveries were calculated by comparison of peak heights obtained with standard solutions and serum samples with added fluoropyrimidine compounds. The recoveries ranged from 75.2 to 80.6% with ethyl acetate as the extraction solvent (Table I).

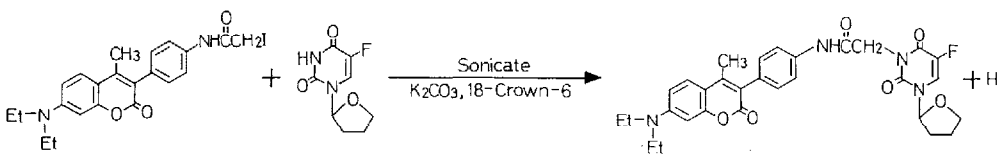


Fig. 2. Fluorescent labelling reaction of FT with DCIA.

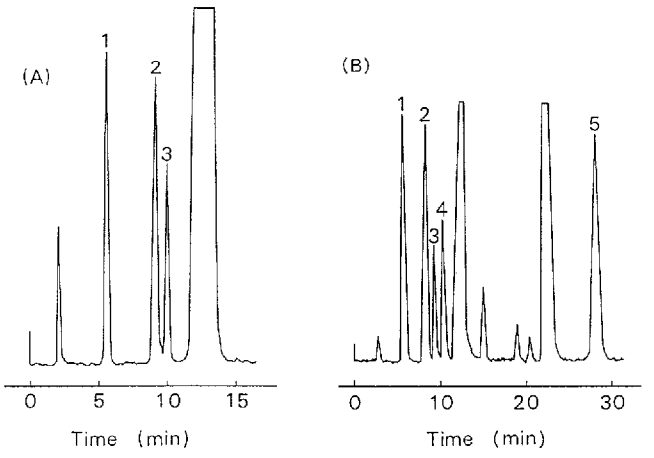


Fig. 3. HPLC profiles of pyrimidine standards and serum extract with chemiluminescence detection. (A) Standard mixture; (B) serum extract. Peaks: 1 = 5-FU; 2 = FUrd; 3 = FdUrd; 4 = ClUra; 5 = FT. A 50-ng amount of each compound, 10 μg of DCIA, 1 μg of 18-crown-6 and 500 μg of K_2CO_3 were dissolved in 0.2 ml of acetone, and a 4-μl aliquot was injected into the chromatograph. Mobile phase, methanol–4 m*M* phosphate buffer (50:50, v/v).

Calibration curves and detection limit

For the method to be useful in quantitative analysis, the amount of product formed in the derivatization reaction should be related to the amount of fluoropyrimidine compound. Serum samples were spiked with increasing amounts of fluoropyrimidine compounds (final concentrations 1.25, 2.5, 5.0 and 250 ng of each fluoropyrimidine per ml of serum). The samples were extracted and calibration curves were generated for each series of determinations by plotting relative peak height (sample/internal standard) against known fluoropyrimidine concentration (pg per injection). The linear relationships [$y = 0.0177x - 0.0460$ ($r = 0.9998$) for FT, $y = 0.0171x - 0.0583$ ($r = 0.9999$) for 5-FU, $y = 0.0130x -$

TABLE I

RECOVERY OF ADDED FLUOROPYRIMIDINE COMPOUNDS FROM SERUM

A 10-mg amount of each fluoropyrimidine compound was added to 1 ml of serum, and 4 μl were injected. Solvent, ethyl acetate.

Compound	Recovery (mean ± S.D., $n = 5$) (%)
5-FU	76.7 ± 1.8
FUrd	75.2 ± 2.3
FdUrd	80.6 ± 1.7
FT	75.8 ± 3.2
ClUra	78.2 ± 2.7

TABLE II

COMPARISON OF DETECTION LIMITS OBTAINED WITH FLUORESCENT AND CHEMILUMINESCENT METHODS

Compound	Detection limit[a] (fmol)		
	Chemiluminescence method	Fluorescence method 1[b]	Fluorescence method 2[c]
5-FU	40	95 000	2300
FUrd	20		
FdUrd	20	62 500	2000
FT	20		125

[a] Injected amounts.
[b] See ref. 22.
[c] See ref. 7.

0.0760 ($r = 0.9992$) for FdUrd and $y = 0.0109x - 0.0479$ ($r = 0.9996$) for FUrd] indicate that this procedure can be used to quantitate fluopyrimidine compounds in serum.

The detection limits were found to be a function of the column condition, the mobile phase composition and the flow-rate. Under the present separation conditions, a typical detection limit was 40 fmol for 5-FU, and 20 fmol for FUrd, FdUrd and FT per 4-μl injection. The detection limits obtained by the fluorescent and chemiluminescent methods are compared in Table II.

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