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USE OF 4-BROMOMETHYL-7-METHOXYCOUMARIN FOR DERIVATIZATION OF PYRIMIDINE COMPOUNDS IN SERUM ANALYSED BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLUORIMETRIC DETECTION

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SUMMARY

Derivatization of the pyrimidine nucleobases and nucleosides with 4-bromomethyl-7-methoxycoumarin was studied with the aim of developing a sensitive and selective column liquid chromatographic method for these substances in serum. The labeling reactions and the nature of derivatives are discussed, together with the chromatographic properties of these derivatives. The derivatives are stable for at least several weeks. Typical detection limits are 50 pg for inosine, 150 pg for uridine, 50 pg for uracil, 50 pg for thymine and 100 pg for fluorodeoxyuridine, respectively. Within-day coefficients of variation averaged 5.0% for the stored-frozen serum pools; the mean day-to-day value was 5.2%. Thirty samples could be processed per working day.

INTRODUCTION

High-performance liquid chromatography (HPLC) has been widely used for the separation of nucleic acid bases and nucleosides. Although ion-exchange HPLC [1, 2] was used originally it was subsequently supplemented by reversed-

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phase HPLC [3, 4]. However, the direct analysis of serum nucleosides has, in our experience, been especially difficult because of the extremely low endogenous levels in the normal human population and possible interferences from other components found in serum.

Fluorescent derivatization of purine compounds has been used in combination with HPLC in nucleic acid research [5, 6]. However, the derivatization of pyrimidine nucleobases and nucleosides has not been studied extensively.

4-Bromomethyl-7-methoxycoumarin (Br-Mmc) [7, 8] was shown to form fluorescent esters with monocarboxylic acids. Reactions of Br-Mmc with other OH, NH_2 and NHR groups have been investigated in aqueous media, but with little success. Dünges and Seiler [9] described the derivatization with Br-Mmc in non-aqueous media of some barbiturates as well as pyrimidine nucleobases and nucleosides. Thus Br-Mmc appears to be useful for fluorescence labeling of pyrimidine compounds.

The aim of this paper is to demonstrate the applicability of Br-Mmc labeling in trace analysis of pyrimidine nucleobases, nucleosides and related compounds. A simpe and virtually quantitative esterification yields stable derivatives that are amenable to reversed-phase HPLC analysis.

EXPERIMENTAL

Reagents and chemicals

Br-Mmc, 18-crown-6, *p*-nitrobenzoic acid, hordenine and *n*-valeric acid were purchased from Nakarai (Kyoto, Japan) and were used without purification. 5-Fluorouracil (5FU), 1-(tetrahydro-2-furanyl)-5-fluorouracil (ftraful, FT), thymine (Thy), uracil (Ura), inosine (Ino), uridine (Urd), fluorodeoxyuridine (FdUrd), deoxyuridine (dUrd) and thymidine (Tdr) were obtained from commercial sources. All organic solvents (Nakarai) were commercial analytical reagent grade materials. The water used for the mobile phase was passed through an ion-exchange column (Millipore, Type ZD20-11583).

Labeling method

Mmc derivatives were prepared by adding Br-Mmc (10 mg) to a solution containing pyrimidine compounds (total weight, within 1 mg) and potassium carbonate (10 mg) in 5 ml of dimethyl sulphoxide (DMSO). The reaction was completed within 5 min at room temperature. The excess Br-Mmc was treated with *p*-nitrobenzoic acid, which gave a non-fluorescent Mmc derivative.

By repeated chromatography of the same sample solution, it was shown that the Mmc derivatives were stable for at least several weeks in the reaction mixtures.

HPLC analysis

The analyses were performed using a Shimadzu (Kyoto, Japan) Model LC-4A chromatograph equipped with a Model SIL-1A injector. The chromatographic column was a Nucleosil 5 C_{18} (20 cm × 4 mm I.D., 5 μm); (Macherey and Nagel, Düren, F.R.G.), packed using a balanced-density slurry-packing procedure similar to that described by Majors [10]. The mobile phase [methanol—acetonitrile—water (29:5:66, v/v/v)] was filtered and vacuum-

degassed before use. The flow-rate was 0.6 ml/min and the temperature was ambient.

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

RESULTS AND DISCUSSION

Reaction mechanisms

The structure of the alkylated derivatives has been confirmed by many workers [11, 12]. The possible labeling reactions of pyrimidine nucleobases and nucleosides are shown in Fig. 1.

A barbiturate ester of Br-Mmc was derivatized by refluxing an acetone solution of the reaction components in the presence of crystalline water-free potassium carbonate with crown ethers as catalysts [8, 13, 14]. The barbiturates usually contain two acidic imide groups, each of which can be alkylated at the nitrogen atom. The pyrimidine nucleobases and nucleosides, as well as the barbiturates, can also be alkylated by alkyl halide, and the crown

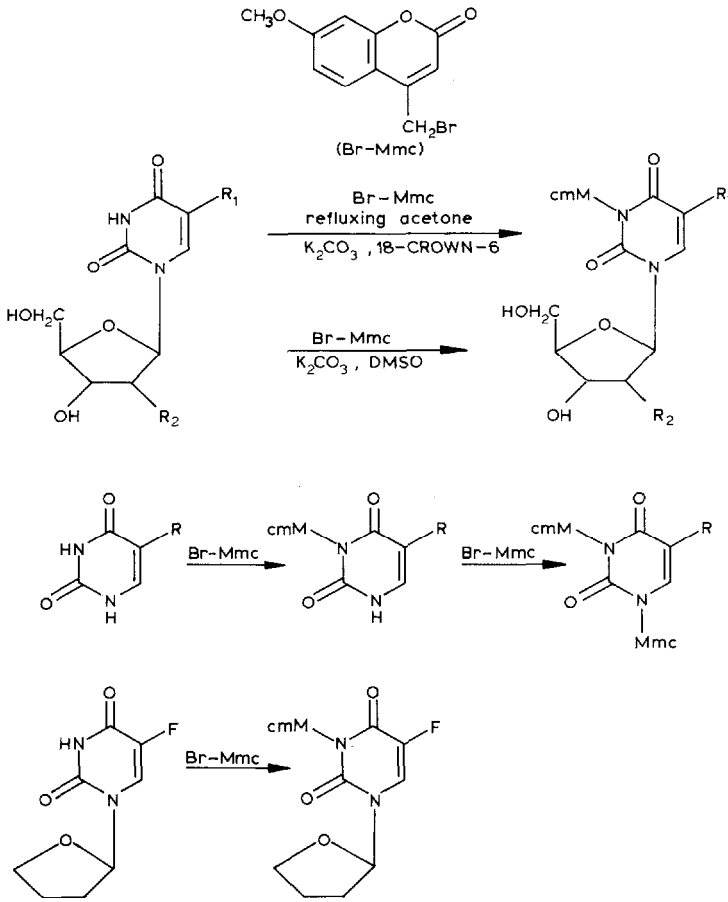
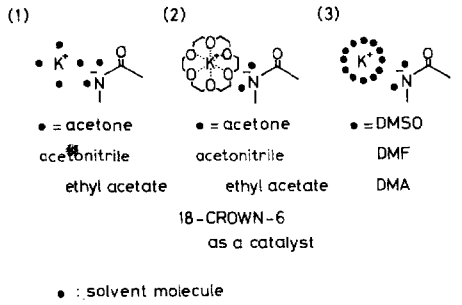


Fig. 1. Possible labeling reactions of pyrimidine nucleobases and nucleosides.



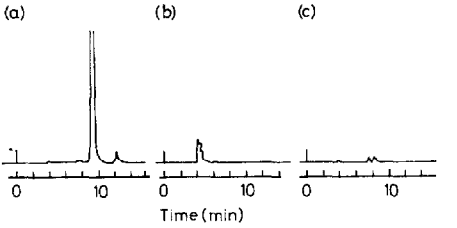


Fig. 2. Effect of solvated ion.

Fig. 3. Treatment of excess Br-Mmc. (a) With *n*-valeric acid; (b) with hordenine; (c) with *p*-nitrobenzoic acid. Mobile phase, 70% (v/v) methanol in water; flow-rate, 0.6 ml/min.

ether–potassium complex provides increased anion reactivity, as shown in Fig. 2-2. The crown ether (18-crown-6) is used as a catalyst because it is inexpensive.

Small "closed-shell" cations, such as Na^+ and K^+, are more solvated by dimethylacetamide (DMA), dimethylformamide (DMF) and DMSO than by acetone [15]. A number of investigators have used a solution of Na^+ and K^+ in dry DMSO in order to permethylate acidic compounds. The strongly basic methylsulphinyl carbanion, which is formed in the solvent, deprotonates even very weak acids, which are then able to react with methyl iodide. The reaction is fast and quantitative at room temperature. This procedure has enabled successful gas chromatographic determinations of bile acids [16], nucleotides [17], 5FU [11] and 5-fluorouridine [18]. When Br-Mmc is used instead of methyl iodide, the same solvation effects are expected to label pyrimidine bases and nucleosides in the solvent containing Na^+ and K^+, as shown in Fig. 2-3. The labeling reaction is fast at room temperature.

Treatment of excess Br-Mmc

A critical part of this labeling method is the interfering peak of unchanged Br-Mmc, which can be eliminated by derivatizing excess Br-Mmc after the labeling reaction. For easy esterification of excess Br-Mmc, *n*-valeric acid, hordenine and *p*-nitrobenzoic acid can be used; *p*-nitrobenzoic acid gives an Mmc derivative which does not fluoresce (Fig. 3).

Fluorescence quantum yields

Quantum yields of fluorescence are estimated by the comparative method of Parker and Ree [19], using tryptophan (20%) [20] as the comparison standard of known quantum yield.

$$Q = Q_0 (A_0/F_0) (F/A)$$

where Q and Q_0 denote the quantum yield of the unknown and of the standard (tryptophan), respectively, A and A_0 are absorbances of the unknown and standard solution used for fluorescence assay, and F and F_0 denote the areas under the fluorescence spectra of the unknown and standard solution.

TABLE I

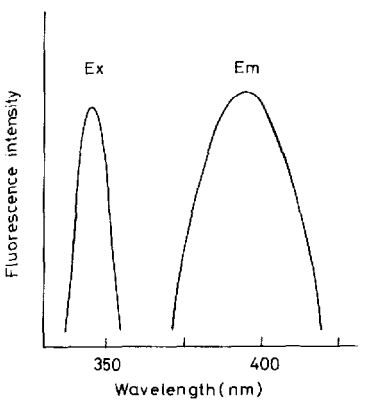
UV ABSORPTION AND FLUORESCENCE DATA FOR SOME PYRIMIDINE NUCLEOBASES AND NUCLEOSIDES IN 70% (v/v) METHANOL IN WATER

	Ex_{max} (nm)	Em_{max} (nm)	Q	UV_{max} (nm)	$\epsilon \times 10^2$ ($cm^{-1} M^{-1}$)
Mmc-Ura	333	400	0.13		
Mmc-5FU	333	396	0.14		
Mmc-Thy	333	397	0.14		
Mmc-Urd	330	396	0.11	329	127
Mmc-dUrd	329	398	0.11	329	125
Mmc-FdUrd	334	397	0.12	335	135
Mmc-Tdr	329	396	0.10	328	129
Mmc-Ino	330	399	0.10	329	129
Br-Mmc	325	396	0.02	326	120

UV absorption and fluorescence spectral data and quantum yields for some esters including the Br-Mmc derivatives of pyrimidine nucleobase and nucleoside in 70% (v/v) methanol are given in Table I. The uncorrected fluorescence excitation and emission spectra of the Mmc-5FU derivative in 70% (v/v) methanol are shown in Fig. 4.

Spectral characteristics and solvent effects

The effect of the variation of methanol concentration in water on fluorescence intensity of the compounds listed in Table I, excited at 346 nm and monitored at 395 nm, is shown in Fig. 5. On addition of methanol to the aqueous solution of the derivatives, an initial rapid increase of fluo-



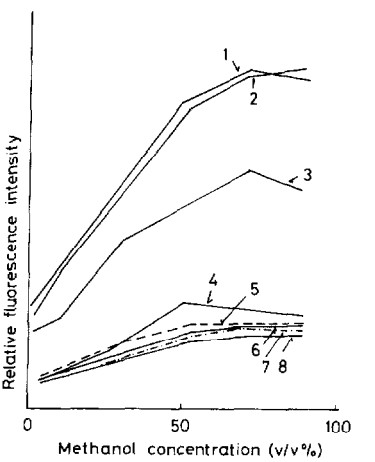


Fig. 4. Fluorescence spectra (uncorrected) of Mmc-5FU derivative. Ex, excitation; Em, emission. Solvent, 70% (v/v) methanol.

Fig. 5. Variation of fluorescent intensity as a function of methanol concentration. Curves: 1 = Mmc-5FU; 2 = Mmc-Thy; 3 = Mmc-Ura; 4 = Mmc-FdUrd; 5 = Mmc-Ino; 6 = Mmc-Urd; 7 = Mmc-dUrd; 8 = Mmc-Tdr.

rescence intensity of pyrimidine nucleobases is observed compared with nucleosides. The maximum fluorescence intensity for the nucleobase derivatives is at a methanol content of 70% (v/v). Above this level, a slow decrease of intensity occurs. The results are similar for other nucleosides. It is well known that the fluorescence of many compounds depends on the water content of the solvent [21].

Thus the HPLC separation employing mobile phases of higher water content will not give the high sensitivity typical of fluorescence detection. However, the reversed-phase separations of the higher-molecular-weight derivatives can be performed using mobile phases containing approximately 70% (v/v) methanol. This affords high fluorescence intensities.

Determination of nucleobases and nucleosides in serum

The internal standard for the assay of some nucleobases and nucleosides was chlorodeoxyuridine (CldUrd) (1.0 μg per 100 μl). The extraction and derivatization procedures are summarized in Fig. 6. The reaction mixture was first purified by thin-layer chromatography (TLC), and the known spots were isolated, extracted and subsequently analysed by HPLC. Recoveries of

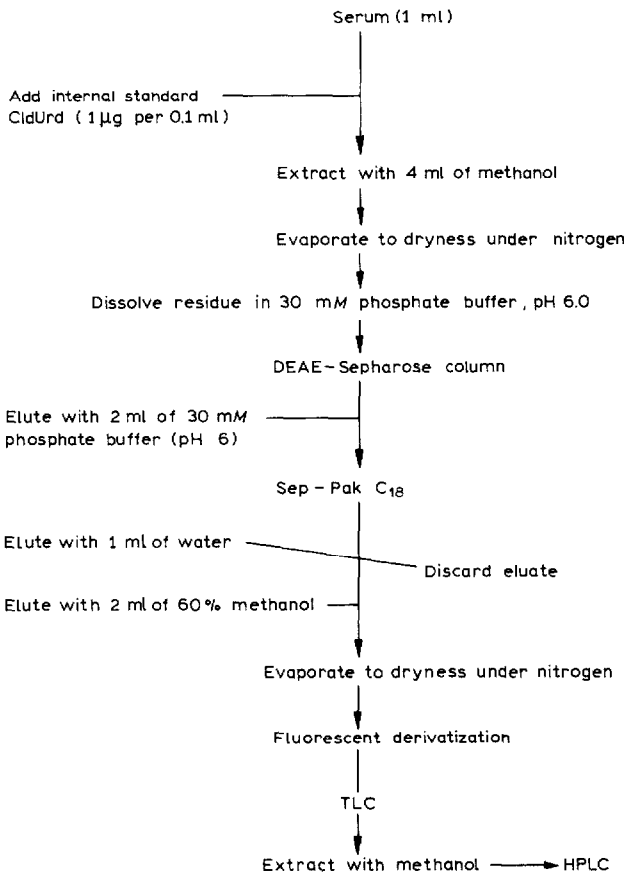


Fig. 6. Schematic diagram of the analytical procedure for the quantitative determination of FdUrd in serum.

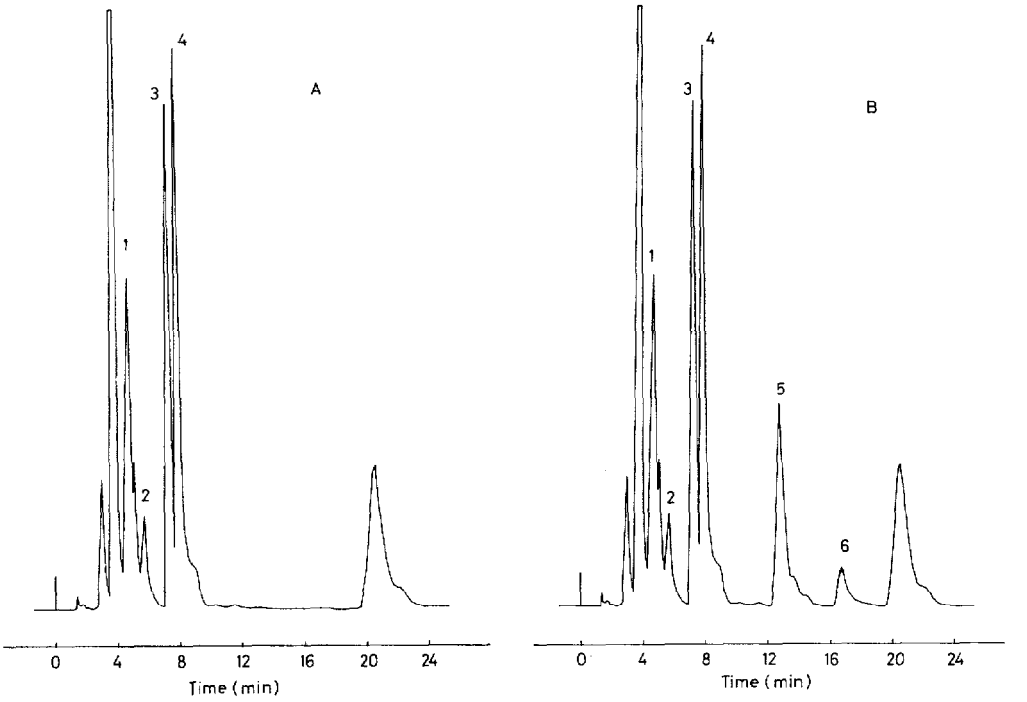


Fig. 7. Chromatogram of a serum sample spiked with FdUrd. (A) Serum blank; (B) serum spiked with 10 μg each of FdUrd and CldUrd. Column, Nucleosil 5 C_{18} (20 cm × 4 mm I.D.); mobile phase, methanol—acetonitrile—water (29:5:66, v/v/v); flow-rate, 1.0 ml/min; detection, excitation 346 nm, emission 395 nm. Peaks: 1 = Mmc-Ino; 2 = Mmc-Urd; 3 = Mmc-Ura; 4 = Mmc-Thy; 5 = Mmc-FdUrd; 6 = Mmc-CldUrd (internal standard).

FdUrd for the method, including extraction, Mmc derivatization and quantitation, averaged 75 ± 5% (μmol/l FdUrd). The HPLC profile is shown in Fig. 7.

Fig. 7B shows a chromatogram of the Mmc-nucleobase and Mmc-nucleoside derivatives in serum and Fig. 7A shows the separation of a serum extract free from FdUrd and CldUrd (blank sample). The blank sample shows that the

TABLE II

DETERMINATION OF INOSINE, URIDINE, URACIL AND THYMINE IN NORMAL SERUM SPECIMENS

Results are expressed as mean ± S.D. (n = 5).

	Inosine (μmol/l)	Uridine (μmol/l)	Uracil (μmol/l)	Thymine (μmol/l)
1	4.03 ± 0.201	1.52 ± 0.084	7.14 ± 0.371	9.44 ± 0.425
2	8.59 ± 0.386	2.44 ± 0.119	8.18 ± 0.401	3.81 ± 0.191
3	3.59 ± 0.197	1.65 ± 0.082	8.89 ± 0.489	5.03 ± 0.277
4	7.70 ± 0.400	1.98 ± 0.102	7.01 ± 0.351	8.89 ± 0.436
5	8.15 ± 0.399	2.57 ± 0.116	6.50 ± 0.292	2.44 ± 0.127
Mean	6.41	2.03	7.54	5.92

serum constituents do not interfere with the separation of the FdUrd and CldUrd derivatives. 5-FU and FT have been in clinical use for many years. They may undergo anabolism to form ribo- and deoxyribonucleoside, and their mono-, di- and triphosphates. The determination of FdUrd is particularly interesting, because it may change biochemically and pharmacologically in the concentration. Compounds were identified by cochromatography with pure derivatives and comparison of retention times. Calibration plots (peak height versus concentration) were linear up to 150 pg for each compound. Typical detection limits were 50 pg for Ino, 150 pg for Urd, 50 pg for Ura, 50 pg for Thy and 100 pg for FdUrd injected, respectively, at a signal-to-noise ratio of 2:1. The results for the determination of Ino, Urd, Ura and Thy in serum from normal subjects are presented in Table II. Within-day coefficients of variation averaged 5.0% for the stored-frozen serum pools; the mean day-to-day value was 5.2%. Thirty samples could be processed per working day.

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