5-Bromomethyl Fluorescein (5-BMF) for Derivatization of Carboxyl Containing Analytes for Use with Laser-Induced Fluorescence Detection

Partha S. Mukherjee,[1] Kapila H. DeSilva,[1] and H. Thomas Karnes[1,2]

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Purpose. 5-Bromomethyl fluorescein (5-BMF) was evaluated in this work as a pre-column, off-line derivatizing reagent for analytes containing a free carboxyl group. The reagent possessed high molar absorptivity and quantum yield and it's excitation maximum matched the intense 488.0 nm emission line of an argon ion laser. The thermal and photo-stability of the reagent were also suitable for our work. ***Methods.*** A mixture of 5-BMF, palmitic acid, 18-crown-6 and potassium carbonate was reacted at 76°C for one hour. The reaction yield was maximized by a sequential single-factor optimization of the reaction variables. Spectral characterizations were accomplished using conventional instrumentation and detection limits were evaluated using a laboratory constructed laser-induced fluorescence (LIF) detector which was optimized for concentration detection. ***Results.*** Two apparent conjugates of palmitic acid were formed under the reaction conditions employed. The conjugate chosen for quantitative purposes was stable at room temperature over 24 hours and possessed high molar absorptivity (128,832 $M^{-1}cm^{-1}$) and quantum yield (0.82) under basic conditions. The excitation and emission maxima of the conjugate were 500 nm and 523 nm respectively which maintains suitability for argon-ion laser excitation. An argon-ion LIF detector suitable for use with conventional liquid chromatography was constructed. A detection limit of 7.56×10^{-10}M of palmitic acid was achieved at a signal to noise ratio of three, using the strong 488.0 nm laser emission line and a commercially available flow cell component. This corresponds to 38 femtomoles of palmitic acid on-column. ***Conclusion.*** The concentration detection limit was superior to literature reports for detection of fatty acids. The mass detection limit provided approximately an order of magnitude improvement over conventional fluorescence. The reagent is potentially useful for analysis of carboxyl containing analytes at low concentrations.

KEY WORDS: 5-bromomethyl fluorescein; palmitic acid; derivatization; high-performance liquid chromatography; laser-induced fluorescence.

INTRODUCTION

Approximately 8% of biologically interesting analytes including amino acids, peptides and fatty acids possess a carboxyl group (1). The analysis of such compounds is often complicated due to the fact that conventional separation and detection do not always provide sufficient selectivity or sensitivity for satisfactory low level quantitation. The absorbances of carboxyl group containing compounds are not adequate for acceptable sensitivity in many cases and carboxylic acids possessing native fluorescence are rare. Mass spectrometric detection of carboxylic acids has been shown to provide limited sensitivity (2). Application of chemiluminescence detection may be limited (3) and radioimmunological detection is time-consuming and laborious (4).

Derivatization of the carboxyl group with a suitable chromophore or fluorophore may often improve the selectivity and sensitivity of detection. The most commonly used derivatizing reagents for enhancement of the UV absorbance detection of carboxylic acids include 1-napthylamine (5) and (p-bromo) phenacylbromide (6). They have been employed in the analysis of short-chain and long-chain fatty acids, dicarboxylic acids, and prostaglandins. The resulting conjugates have high molar absorptivities at 254 nm and detection limits in the range of 1-10 ng on-column have been reported (6).

These detection limits, however, are not adequate for many analytes which require low level quantitation. Fluorescence detection has the potential for analysis in the low picomolar range while maintaining good ruggedness. Many fluorescence derivatization reagents have been developed for the determination of carboxylic acids by HPLC (7). 4-bromomethyl 7-methoxy coumarin (Br-MMC) and its structural analogs have been extensively employed to derivatize aliphatic monocarboxylic acids and dicarboxylic acids with detection limits in the low picomolar range (8). The excitation and emission maxima of most of these Br-MMC conjugates are in the range of 360-400 nm.

The detection limit can be further improved and the selectivity enhanced by the use of a laser excitation source. Laser light sources in conjunction with fluorescence detection have been utilized to achieve detection limits at low femtomolar and attomolar ranges for compounds of pharmaceutical and biomedical interest (9-12).

An excitation wavelength in the visible region of the spectrum (> 400 nm) is preferable in bioanalysis due to the fact that excitation in this region is relatively free from many interferences from endogenous compounds present in biological matrices (serum, plasma etc) (4). In this respect, argon ion lasers are useful for the quantitation of analytes in biological matrices because of the high power output and the multiple emission lines available in the blue-green region of the spectrum. In the work presented here, a new derivatization reagent, 5-bromomethyl fluorescein (5-BMF) has been evaluated for carboxylic acids which is suitable for argon ion laser-induced fluorescence detection. Palmitic acid was chosen as a model analyte for this purpose.

MATERIALS AND METHODS

Materials

5-bromomethyl fluorescein (5-BMF, purity 99%, mol wt. 425) was purchased from Molecular Probes Inc. (Eugene, OR). Palmitic acid (purity approx. 99%), potassium carbonate and 18-crown-6 were obtained from Sigma Chemical Co. (St. Louis, MO). All reagents were of ACS grade. HPLC grade acetonitrile (ACN) and acetone were purchased

[1] Department of Pharmacy & Pharmaceutics, Box 533, Medical College of Virginia, Virginia Commonwealth University, Richmond, Virginia 23298-0533.

[2] To whom correspondence should be addressed.

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from Baxter Healthcare Corp. (McGaw Park, IL). Distilled deionized water was prepared in the laboratory using a Corning megapure distillation unit.

Methods

Liquid Chromatography System

A reciprocating pump (model 302, Gilson Medical Electronics Inc., Middleton, WI) in conjunction with a manometric module (Gilson model 802 B) was used to deliver the mobile phase (85:15 ACN:H_2O) to a Spherisorb ODS-2 column (250 × 3.2 mm, 5 μ particle size, Alltech Assoc., Deerfield, IL) at 1 ml/min. A guard column (10 × 4.6 mm) packed with 50 μ Corasil™ particles (Milipore Corp., Marlborough, MA) was placed before the analytical column. A Rheodyne injector model 7125 was used to load and inject the samples. The mobile phase was filtered through a 0.45 μ membrane filter and deaerated by helium sparging for 2-3 minutes. Detection was performed at 250 nm using an absorbance detector (model SPD 6A, Shimadzu Corp., MD). Chromatograms were integrated using a Hewlett-Packard integrator model 3396 A.

Electrospray Ionization/Mass Spectrometry (ESI/MS)

ESI-MS was performed on an Extrel 4000 AMU quadrupole mass spectrometer. The samples were trapped from the chromatographic eluate corresponding to the conjugates of interest and dried under nitrogen. The solid residues were dissolved in 50 μl of 1% acetic acid/methanol (50/50) solution. Samples were infused into the ESI source at 2 μl/min using a syringe infusion pump. An optimum voltage of 4.5 kV was maintained on the ESI-MS electrode to form protonated ions of the samples. The mass spectrometer was scanned from 0 to 700 mass/charge (m/z) units and 23 scans were averaged to obtain the mass spectra.

Derivatization Procedure

The derivatization reaction mixture consisted of 250 μl of 5-BMF (125 μg/ml in ACN), 188 μl of palmitic acid (100 μg/ml in acetone), 20-25 mg of anhydrous solid potassium carbonate and 48 μl of 700 μg/ml of 18-crown-6 (in ACN). The ability of crown ethers to form complexes with metal ions is well known (13). 18-crown-6 was chosen as catalyst based on its specificity for K ion. All the solvents were dried over molecular sieves (4-8 mesh beads, Fisher Scientific, St. Louis, MO). The stock solutions of 5-BMF and 18-crown-6 were stored at 4°C, while the palmitic acid solution was stored at room temperature. All the stock solutions were stored in amber glass vials. The reaction components were mixed in a 3 ml amber Reacti-vial™ (Pierce, Rockford, IL) for 10 seconds and refluxed at 76°C for one hour, using a micro-reflux condensor (Aldrich Chemical Co., Milwaukee, WI). The derivatization reaction was carried out without protection from ambient light.

Reversed-phase high-performance non-fluorescent TLC (UNIBOND™, Analtech Chemical Co., Newark, DE) was used for confirmation of the presence of the palmitic acid conjugate, using a mobile phase of 100% ACN. Visualization of fluorescence was achieved under UV radiation at 254 nm using a Spectroline model ENF-280C portable UV lamp (Analtech Chemical Co.). The percent yield of the palmitic acid conjugate was calculated based on the ratio of the peak height of the conjugate to the total peak heights of all the peaks corresponding to 5-BMF, using absorbance detection. The assumption involved was the molar absorptivities of the conjugate and the reagent were equal in the mobile phase at 250 nm. The following variables were optimized by a sequential single-factor approach (i.e., - changing one of the variables while keeping the other factors constant) to maximize the yield of the intended conjugate: (a). Molar ratio of 5-BMF : palmitic acid, (b). Molar ratio of 5-BMF : 18-crown-6, (c). Reflux time, and (d). Amount of the potassium carbonate base.

Laser-induced fluorescence (LIF) instrumentation

The instrument for the laser-induced fluorescence is shown in Figure 1. An argon ion laser (Lexel laser model 95-5, Lexel Laser Corp., Fremont, CA) with a maximum power output of 5 watts in the multi-line emission mode was used as the excitation source. The laser was tuned at 488.0 nm (maximum power output 1.7 watts, 34% of the total emission power in the blue-green region of the spectrum) and operated in the light stabilization mode. The power output was adjusted to 25 mW. The laser beam was passed through a microscope objective (model 13570, Oriel Corp., Stratford, CA, 5 ×, N.A = 0.12) and focussed onto a 5 μl flow cell, the latter being an accessory of a fluorescence detector (model 470, Waters™, Milford, MA). The flow cell was mounted on a micropositioner (Oriel model 16021) for precise focussing of the laser beam onto the flow cell. The analytical column was connected to the flow cell and fluorescence was collected at 90° via a camera lens (F/# 0.95), passed through a long-pass colored glass filter (Oriel model 51294, %T 64% and 0% at 523 nm and 488 nm respectively) and imaged onto the photomultipler tube (PMT) model R 928 (Hamamatsu Corp., Bridgewater, NJ). The PMT was encased in an Oriel model 70680 housing. An adjustable iris diaphragm (Oriel

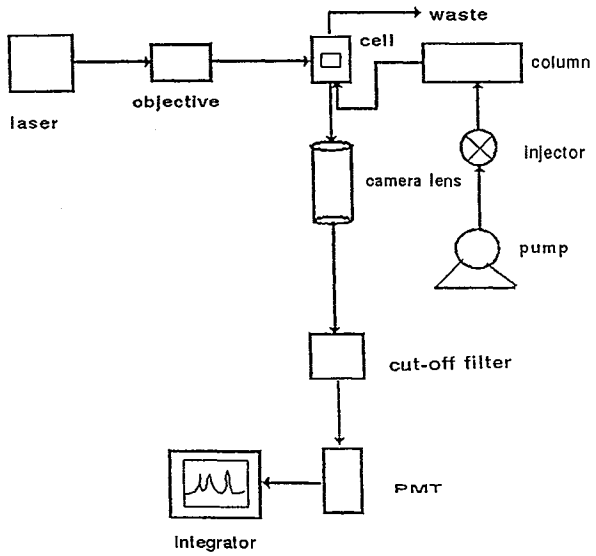

Fig. 1. LIF instrument for quantitation of the palmitic acid conjugate.

model 62030) was placed before the PMT to regulate the amount of scattered light and fluorescence radiation reaching the PMT. The current from the PMT was fed to a low noise multimeter (model 2833, B&K Precision, Chicago, IL) and finally displayed on an integrator (model 3396 A, Hewlett-Packard Co., Avondale, PA). The entire instrumental set-up was placed inside a laboratory constructed light-opaque box to prevent interferences from ambient light.

Determination of Molar Absorptivities and Quantum Yields

The molar absorptivities of 5-BMF and the palmitic acid conjugate were calculated according to Beer's law, using the corresponding absorbances and a 1.8×10^{-7} molar solution of 5-BMF prepared in 0.1 N sodium hydroxide. The molar concentration of the palmitic acid conjugate (trapped and diluted in the mobile phase) was determined based on the amount of 5-BMF in the reaction mixture, the % yield of the conjugate, and the eluent volume. The absorbances of both solutions were determined at their respective absorption maxima using an LKB Ultraspec II UV/Visible spectrophotometer.

The quantum yield of 5-BMF (Φ_{BMF}) in 0.1 N sodium hydroxide solution was determined with laser grade fluorescein 548 (Eastman Kodak Co., Rochester, NY) as the reference. The reported quantum yield of fluorescein (Φ_F) in 0.1 N sodium hydroxide solution was 0.92 at an excitation maximum of 491 nm (14). The experiment was carried out at room temperature and without deoxygenation of the solvent. The Φ_{BMF} was determined according to the following equation:

$$\Phi_{BMF} = \Phi_F{*}(AUC_{BMF}/AUC_F){*}(A_F/A_{BMF})$$

where AUC_F and AUC_{BMF} refer to the integrated areas under the emission curves of equal concentrations for fluorescein and 5-BMF respectively. A_{BMF} and A_F were the absorbances of equal concentrations of 5-BMF and fluorescein solutions at their respective absorption maxima.

The quantum yield of the palmitic acid derivative (Φ_D) in the mobile phase was determined by first determining the quantum yield of fluorescein in the mobile phase (Φ_{Fmp}), using fluorescein in 0.1 N sodium hydroxide solution (Φ_F) as a reference. The calculated Φ_{Fmp} was then used as the reference for the determination of Φ_D.

Fluorescence Characteristics of 5-BMF and the Palmitic Acid Conjugate

A luminescence spectrofluorometer (model LS-50, Perkin Elmer Ltd., Beaconsfield, England) was used for acquisition of the excitation and fluorescence emission spectra of 5-BMF in different solvents. The effect of pH on the excitation and emission spectra of 5-BMF in ACN was studied. The apparent pH of the 5-BMF solution was adjusted to highly acidic (by addition of few drops of tricarboxylic acid) and highly basic (by addition of 1.0 N sodium hydroxide solution) conditions. The excitation and emission spectra of the conjugate were obtained by trapping the corresponding peak eluting from the absorbance detector. The effect of highly basic pH on the excitation and emission spectra of the conjugate was studied by the addition of 1.0 N sodium hydroxide solution to a solution of the conjugate in the mobile phase. The influence of elevated temperature on the fluorescence intensity of 5-BMF was also investigated by comparing the relative fluorescence intensity of a 1.25×10^{-8} molar solution of 5-BMF in 0.1 N sodium hydroxide solution at room temperature vs. heating the same solution at 76°C for one hour.

Stability Studies

The stability of 5-BMF in ACN was investigated at room temperature and at 4°C. Fifty microliters of the stock solution (125 μg/ml) was injected onto the analytical column and compared to the peak height for 5-BMF stored at room temperature for two weeks. In order to investigate the thermal stability of 5-BMF, the peak height for 50 μl of the stock solution, heated at 76°C for one hour, was compared with an equal on-column amount of 5-BMF at room temperature. The stability of the palmitic acid conjugate solution in the mobile phase was also studied. The peak height of 50 μl of a 8.6×10^{-7} molar solution of the conjugate was monitored by the laser-induced fluorescence detector described earlier. The derivative solution was kept at room temperature for 24 hours without protection from light and the peak height for 50 μl of this solution was compared to the previous case.

Detection Limits of Palmitic Acid Using Conventional Fluorescence and LIF

A Waters™ scanning fluorescence detector model 474 equipped with a 18 μl rectangular flow cell was used for the determination of the detection limit of palmitic acid. The excitation and emission bandpass were set at 18 nm and 30 nm respectively. The detection was carried out at excitation and emission wavelengths of 501 nm and 523 nm respectively. Fifty microliters of the refluxed reaction mixture was progressively diluted in the mobile phase to achieve a final concentration of 2.6×10^{-8} M palmitic acid in the solution. Fifty microliters of this solution was injected on column and the peak height corresponding to the derivative was monitored. The limit of detection for palmitic acid by LIF was calculated based on the observed peak height when 50 μl of a solution of the conjugate (representing 2.26×10^{-9} molar concentration of palmitic acid) was injected onto the column.

RESULTS AND DISCUSSION

Confirmation of the Palmitic Acid Conjugate by TLC and HPLC

Two bright green fluorescent spots (r_f 0.65 and 0.77) distinct from excess unreacted 5-BMF (r_f 0.92) were reproducibly observed under UV radiation at 254 or 365 nm. The two spots were confirmed to be due to the reactions between the reagent and the analyte by carrying out the following elimination reactions under identical conditions: (a) Refluxing a mixture of 18-crown-6, potassium carbonate, and palmitic acid at 76°C for one hour. (b) Refluxing a mixture of 18-crown-6, potassium carbonate, and 5-BMF at 76°C for one hour. Both reaction mixtures failed to generate spots at

r_f 0.65 and 0.77. The two spots appeared only when all the reaction components were present in the reflux mixture.

Two peaks were reproducibly observed at 18 min. and 28 min. respectively under the HPLC conditions employed for the separation of the palmitic acid derivative from reaction byproducts. These two peaks were confirmed to be the reaction products between 5-BMF and palmitic acid by carrying out the same elimination reactions as performed for TLC (Figure 2). Several small peaks were observed in the chromatogram of the refluxed reaction mixture and were assumed to be due to minor side reactions/degradation products.

Postulated Reaction Scheme for 5-BMF and Palmitic Acid

The ESI-MS spectra of the two conjugates show that a base peak corresponding to the protonated molecular ion species was observed at m/z of 601. The doubly charged species were present at approximately 70-80% abundance relative to the base peak at m/z of 302 for both spectra. Small peaks were observed at m/z of about 156, 360 and 537 respectively, which were assumed to be due to impurities. The kinetic profile of the derivatization reaction as monitored over a period of 20-80 minutes is shown in Figure 3. The peak at 28 min. appeared simultaneously with the peak at 18 min. when the time course of the reaction was monitored. Both peak heights were observed to increase at about the same rate as the reflux time was increased from 20 minutes to 60 minutes and declined when the reflux time was further increased to 80 minutes. It was, therefore, improbable that this later eluting peak was an intermediate or a degradation product of the conjugate eluting at 18 min. or vice versa. Based on these observations, a proposed reaction scheme for 5-BMF and palmitic acid is shown in Figure 4. The peak at 18 min. was due to the product formed by the displacement of the bromine atom by the carboxylate group of potassium palmitate. The conjugate eluting at 28 min. was formed by the reaction of the COOH group of the palmitic acid with the phenolic group of 5-BMF, to form an ester. Under the reaction conditions employed, the $-CH_2Br$ moiety on this ester derivative was converted to a hydroxymethyl group. The conjugate eluting at 18 min. was more polar due to the free phenolic hydroxyl group and had a r_f value of 0.77 in reversed phase TLC. The percent yields of the peaks eluting at 18 min. and 28 min. were 10% and 30% respectively.

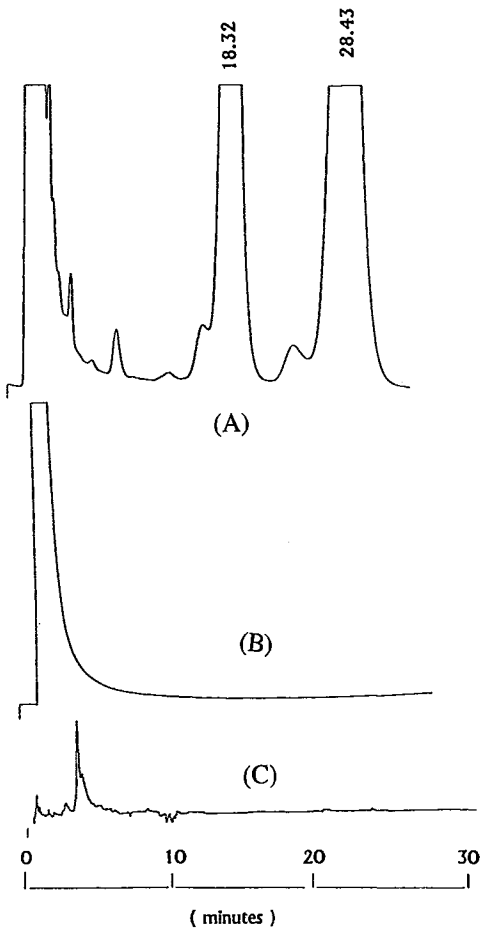

Fig. 2. Confirmation of palmitic acid conjugate formation by HPLC-(A). 5-BMF, palmitic acid, 18-crown-6 and base refluxed at 76°C for 60 min. (B). 5-BMF, 18-crown-6 and base refluxed at 76°C for 60 min. (C). Palmitic acid, 18-crown-6 and base refluxed at 76°C for 60 min.

Fluorescence Characteristics of 5-BMF and the Palmitic Acid Conjugate

The effect of different solvents on the excitation and emission spectra of 5-BMF were studied. The solvents investigated included tetrahydrofuran, acetone, acetonitrile, methanol, isopropanol and water. A red shift in both the excitation and emission wavelengths of 5-BMF were observed with increasing solvent polarity. The excitation and emission maxima were 437 nm and 477 nm respectively in tetrahydrofuran. The excitation and emission maxima were shifted to 494 nm and 519 nm respectively when water was used as the solvent for 5-BMF. In a manner analogous to the solvent dependency of coumarin (15), it was assumed that the 5-BMF molecules possessed higher dipole moment in the excited state compared to the ground state. This led to a higher electronic stabilization and lowering of the energy of the Franck-Condon excited state in a polar solvent. This

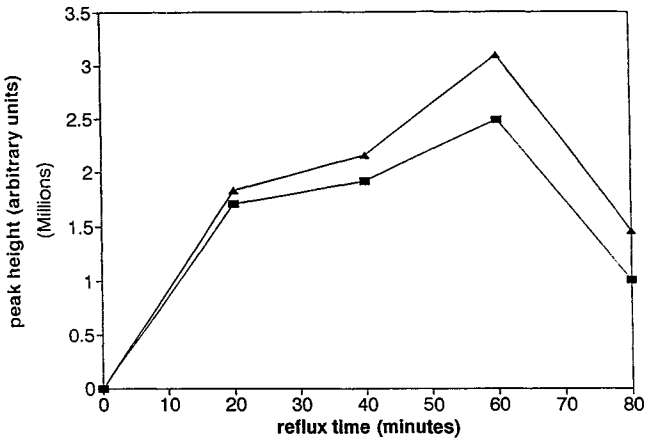

Fig. 3. Kinetic profile of the derivatization reaction for palmitic acid. The apparent conjugate eluting at 28 min. (triangles). Apparent conjugate eluting at 18 min. (squares).

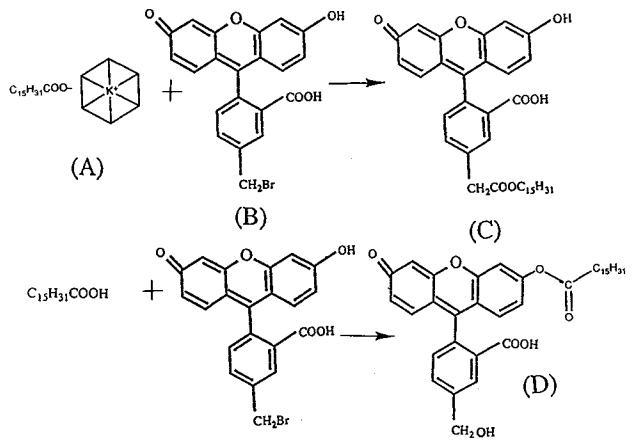

Fig. 4. Postulated reaction scheme between 5-BMF and palmitic acid- (A). Adduct formed between the cationic group of palmitic acid and 18-crown-6. (B). 5-Bromomethyl fluorescein. (C). Apparent conjugate eluting at 18 min. (D). Apparent conjugate eluting at 28 min.

solvent relaxation phenomenon resulted in a shift to longer wavelengths with increasing solvent polarity.

Under highly acidic conditions, the excitation and emission maxima of 5-BMF in ACN were unchanged at 441 nm and 481 nm respectively. The excitation and emission maxima were red shifted to 490 nm and 516 nm respectively when the apparent pH of the solution was adjusted to basic conditions. As expected with fluorescein and its analogs, the relative fluorescence intensity of 5-BMF solution increased with increasing pH and the maximum fluorescence intensity was observed under highly basic conditions (pH 13.5).

The effect of temperature on the relative fluorescence intensity of the 5-BMF solution was investigated and a 3.0% decrease was observed in the relative fluorescence intensity over a temperature increase of 51°C. This change was not significant considering the high molar absorptivity and quantum yield of the reagent.

The fluorescence intensities of the apparent conjugates were compared with an equivalently absorbing solution of pure 5-BMF in the mobile phase. The conjugate which eluted at 18 min. retained only 43% of the original fluorescence, while the peak at 28 min. retained 94% of the relative fluorescence intensity of 5-BMF following the derivatization reaction.

The later eluting conjugate had a higher % yield and retained most of the fluorescence of 5-BMF after derivatization, it was decided therefore that this conjugate would be more suitable for quantitation. The fluorescence characterizations of the conjugate and the detection limit of palmitic acid were, therefore, based on the peak which eluted at 28 min. without modification of the chromatographic conditions.

The excitation and emission maxima of 5-BMF trapped and diluted in the mobile phase were 500 nm and 522 nm respectively. Under basic conditions, the intended conjugate had excitation and emission maxima of 500 nm and 520 nm respectively (Figure 5). Under highly acidic conditions, the excitation maximum of the conjugate was blue shifted to 443 nm while the emission maximum was red shifted to 523 nm. The relative fluorescence intensity of the apparent conjugate

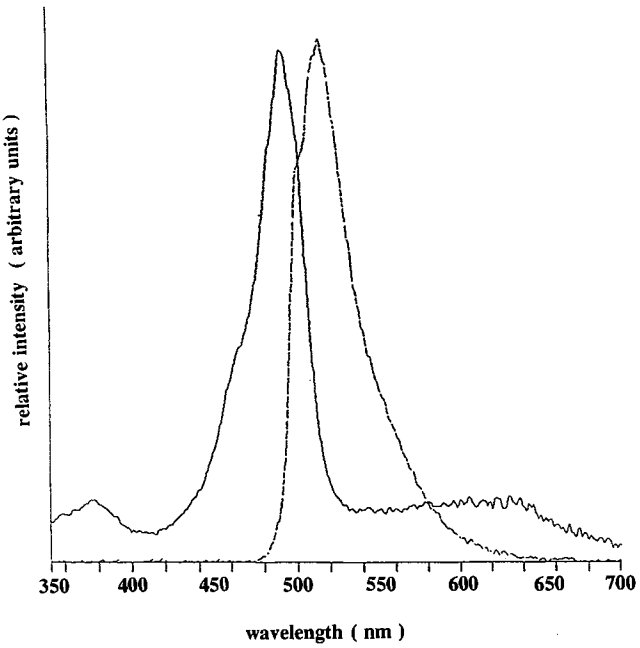

Fig. 5. Excitation (solid line) and fluorescence emission spectra (broken line) of the conjugate eluting at 28 min. under basic conditions.

under basic conditions was approximately 10 fold greater when compared to an equal concentration of the conjugate under acidic conditions. Also, no emission line was available at 443 nm from the argon ion laser being used in our laboratory. It was, therefore, decided to quantitate the conjugate under basic conditions using the strong 488.0 nm laser line, which provided approximately 73 percent of maximum excitation.

Molar Absorptivities and Quantum Yields

The molar absorptivity and quantum yield of 5-BMF were found to be 77,022 $M^{-1}cm^{-1}$ and 0.90 respectively. The high molar absorptivity and quantum yield indicated that under basic conditions, the reagent possessed good fluorescence. The molar absorptivity of the intended conjugate in the mobile phase was 128,832 $M^{-1}cm^{-1}$ and the quantum yield was determined to be 0.82.

Optimization of Derivatization Conditions

The conjugate eluting at 28 min. was more suitable for analytical monitoring and attempts were made to maximize the peak height of this conjugate by optimizing several reaction variables. The molar ratio of 5-BMF to palmitic acid in the reaction mixture was varied in the range of 0.5:1-12:1 (n = 5) and the optimum was found to be 1:1. The molar ratio of 5-BMF to 18-crown-6 was varied in the range of 0.25:1-5.5:1 (n = 5) and the maximum peak height was observed at 0.45:1. Reaction time was varied in the range of 20-80 minutes (n = 5) to investigate the influence of reflux time on the yield of the intended conjugate. The maximum peak height of the conjugate was obtained at reflux time of 60 minutes which was consistent with what has been observed for other fatty acid derivatization reagents (8). The amount of base can also significantly affect the yield of derivatization reac-

tions involving carboxylic acids (16). The amount of potassium carbonate base added was varied from 2.5 mg to 45 mg in the reaction mixture (n = 5). The maximum peak height was obtained with 20-25 mg of base in the reaction mixture. The conjugate eluting at 28 min. had a peak height approximately 2 fold greater compared to the conjugate eluting at 18 min. under the optimal conditions for all of the variables tested.

Stability Studies

There was a 15.7 ± 2.5% (n = 3) decrease in the peak height observed when the stock solution of 5-BMF was stored at room temperature for two weeks. No attempts were made to identify degradation products because they did not interfere with quantitation of the palmitic acid conjugate peak. When stored at 4°C, there was no apparent decrease in the peak height for the same amount of 5-BMF injected on-column after three months, indicating that the solution degradation of 5-BMF had been reduced at lower temperature. The reagent was also found to be stable when heated at 76°C for one hour. The peak height of the conjugate as monitored over a period of 24 hours was found to have decreased only by 1.4%. There were no additional peaks observed in the chromatogram after 24 hours of monitoring which indicates the absence of degradation of the conjugate. Photodegradation products were observed when a laser power of 200 mW was employed but the peak height of the derivative was maintained with no apparent degradation at 25 mW. This indicates that photodecomposition of the derivative did not occur over the time period and conditions used for chromatographic separation and LIF detection.

Limit of Detection of Palmitic Acid

For the purpose of determining the detection limit of palmitic acid, the original reaction mixture was successively diluted in the mobile phase. The concentration detection limit was calculated at a signal to noise ratio of 3 and found to be 6×10^{-9}M of palmitic acid using conventional fluorescence. This corresponds to 0.3 picomole of palmitic acid on-column. The noise was determined using the mean peak to peak noise (n = 3) of the chromatogram baseline across the elution window of the conjugate. The concentration detection limit for palmitic acid was determined to be 7.56×10^{-10} M using the laboratory constructed LIF detector. This corresponds to 38 femtomoles of palmitic acid on-column. The LIF response was linear through the range of 1.78×10^{-6} M (89 picomoles on-column) - 2.26×10^{-9} M (113 femtomoles on-column) of palmitic acid with a correlation coefficient of 0.9998. It should be pointed out that the detectability of palmitic acid was calculated based on the chromatographic system which was optimal only for the separation and monitoring of the reaction products. It is possible to lower the detection limit further by increasing the modifier concentration in the mobile phase. This would reduce the retention time of the conjugate presently eluting at 28 min. and increase the corresponding peak height. Thus, the signal to noise ratio will be significantly enhanced for the same on-column amount of the conjugate, resulting in a further lowering in the detection limit at S/N of 3.

Comparison with Other Reported Results

Fluorescence detection following derivatization has been utilized for the determination of fatty acids to obtain high sensitivity. 4-bromomethyl-7-methoxycoumarin (16) has been employed to analyze fatty acids in the range of 9×10^{-7}- 9×10^{-6} M. Using anthryldiazomethane, a concentration detection limit of 7.3×10^{-7} M was achieved for palmitic acid (17). Ishibashi et. al. reported detection limits in the range of 2.5×10^{-8}- 5×10^{-9} M for eight fatty acids, using 4-(aminosulphonyl)-2,1,3-benzoxadiazole derivatives. The derivatization procedure had the limitation of prolonged reaction time and poor stability of the reagent (18). Our concentration based detection limit was, therefore, better than or comparable to detection limits reported with these reagents, using conventional fluorescence. Other workers have utilized anthryldiazomethane (19), 9-aminophenanthrene (20) and 9-chloromethylanthracene (21) to obtain on-column detection limits at the picomole level for fatty acids. The concentration detection limits could not be determined from these reports due to a lack of information. The on-column detection limit (0.3 picomole) achieved with 5-BMF was lower than these reports. Tsuchiya et al. were able to obtain a detection limit of approximately 10 femtomoles for fatty acids using 4-bromomethyl-7-acetoxycoumarin (Br-MAc) (22). This was possible due to post-column alkaline hydrolysis of the fatty acid conjugates, which produced highly fluorescent products.

Recently, a packed capillary LC column with a Helium-Cadmium laser was used to achieve detection limits of approximately 13 attomoles for coumarin derivatives of fatty acids in pure methanol solvent (23). In bioanalysis, samples are not normally limited to nanoliter volumes and concentration detectability becomes more important than ultralow on-column detectability which can be obtained in capillary LC systems. A concentration detection limit of 7.5×10^{-10} M was achieved for palmitic acid with our existing system. This is more than an order of magnitude lower than previously reported (23). With optimization of the chromatographic conditions for detectability and further modification of the LIF instrument, the detectability of palmitic acid can be further lowered. Work is on-going to employ 5-BMF for derivatization of carboxyl containing analytes of pharmaceutical importance requiring ultra-low level quantitation in biological matrices.

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