A Fluorogenic Reagent, 3-(4,6-Difluorotriazinyl)amino-7-methoxycoumarin, for the Determination of Amantadine by High-Performance Liquid Chromatography

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A sensitive method for determination of amantadine by high-performance liquid chromatography (HPLC) has been developed. Amantadine was derivatized quantitatively into a fluorescent compound through the amino group by treatment with 3-(4,6-difluorotriazinyl)amino-7-methoxycoumarin (FAMC) in benzene at 140 °C for 15 min. The derivative was subjected to HPLC on TSK gel ODS-120T (250 × 4.6 mm i.d.) with acetonitrile-water (5 : 1) as the mobile phase and monitored by fluorescence detection (Ex. 345 nm; Em. 410 nm). The limit of detection was 250 fmol in 100 μl of urine. This method was satisfactory with respect to recovery and precision to quantify amantadine spiked in urine.

Keywords amantadine; precolumn fluorescence labeling; 3-(4,6-difluorotriazinyl)amino-7-methoxycoumarin; high-performance liquid chromatography

Amantadine hydrochloride (Symmetrel) is an antiviral drug which has been increasingly used as an antiparkinsonism agent in recent years. Several methods have been described for the assay of amantadine in biological fluids and tissues by gas chromatography (GC).[1–4] However, the sensitivity was insufficient for the determination of amantadine in biological fluids. We have been developing 4,6-difluorotriazinyl compounds as fluorogenic reagents, and we have found that 3-(4,6-difluorotriazinyl)amino-7-methoxycoumarin (FAMC) reacts with alkyl amines. Based on this finding, we have devised an high-performance liquid chromatography (HPLC) method involving precolumn fluorescence labeling with FAMC and applied it to control urine spiked with amantadine hydrochloride. This method was found to give satisfactory results.

Experimental

Materials Amantadine hydrochloride was supplied by Nihon Ciba-Geigy (Hyogo, Japan) and *n*-decylamine by Nacalai Tesque Inc. (Kyoto, Japan). All reagents and solvents were of analytical grade. Organic solvents were purified by distillation prior to use.

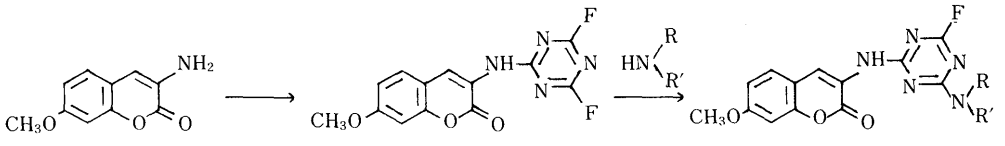
Apparatus Proton-1 and fluorine-19 nuclear magnetic resonance (^{1}H- and ^{19}F-NMR) spectra were recorded on a JNM-GX400 spectrometer (JEOL Ltd., Tokyo, Japan) at 400 MHz for ^{1}H and at 376 MHz for ^{19}F. Chemical shifts were expressed in parts per million relative to tetramethylsilane (δ 0.00) for ^{1}H-NMR and to benzotrifluoride (ϕ 67.75) for ^{19}F-NMR as internal standards. Mass spectra (MS) were obtained with a JMS-DX 303HF spectrometer (JEOL Ltd.). Infrared (IR) spectra were obtained with a Hitachi 270-30 infrared spectrophotometer. For fluorescence measurements, a Hitachi 650-60 spectrofluorometer with a 1 cm quartz cell was used. A Hitachi L-6000 pump coupled with a Hitachi F1000 fluorescence spectrophotometer (Ex. 345 nm; Em. 410 nm) and a Rheodyne model 7125 injection valve with a 20 μl sample loop were used. A stainless steel column (250 × 4.6 mm i.d.) packed with TSK gel ODS-120T (particle size 5 μm, Tosoh, Tokyo, Japan) was used. Acetonitrile–water (5 : 1) was used as a mobile phase at a flow rate of 1.0 ml/min at room temperature. The temperature of derivatization was controlled by using a TAH-1 dry thermo bath (Taiyo, Tokyo, Japan).

Synthesis of FAMC Cyanuric fluoride (1 ml) was added *via* a syringe to a stirred suspension of 3-amino-7-methoxycoumarin[5] (300 mg) in anhydrous ether (100 ml). The mixture was further stirred for 1 h at room temperature, and then the solvent was removed under reduced pressure. The resulting residue was recrystallized from *n*-hexane to give FAMC (186 mg) as colorless prisms (mp 212—215 °C). *Anal.* Calcd for $C_{13}H_8F_2N_4O_3$: C, 50.98; H, 2.63; N, 18.29. Found: C, 50.96; H, 2.85; N, 18.65. MS *m/z*: 306 (M^+, base peak). IR ν_{max}^{KBr} cm^{-1}; 3372 (NH), 1714 (C=O). ^{1}H-NMR (DMSO-d_6) δ: 3.87 (1H, s, CH_3O), 7.00—7.10 (2H, m, 6-, 8-H), 7.70 (1H, d, 5-H), 8.18 (1H, s, 4-H), 10.81 (1H, s, NH). ^{19}F-NMR (DMSO-d_6) ϕ: 89.78 (ABq). FAMC reacts with primary and secondary alkyl amines to give the corresponding derivatives, but does no react with aromatic amines or alcohols.

Calibration Curve A urine sample (100 μl) spiked with various amounts of amantadine hydrochloride (from 5 to 50 pmol) was pipetted into a test tube containing 200 μl of a 50 pmol/ml solution of *n*-decylamine as an internal standard in benzene and 100 μl of 1 M NaOH. The mixture was shaken mechanically for 15 min and centrifuged at 6400 rpm for 6 min. A 20 μl portion of the benzene phase was transferred to a 1.0 ml screw cap vial, to which was added 10 μl of FAMC (2.5 nmol) in benzene. The solution was heated at 140 °C for 15 min. After cooling to room temperature, a 10 μl portion of the resulting mixture was injected into the chromatograph. The peak height ratio of amantadine derivative relative to the internal standard was calculated and a calibration curve was constructed.

Results and Discussion

FAMC was easily prepared by treatment of cyanuric fluoride with 3-amino-7-methoxycoumarin (Chart 1). This reagent was found to be stabel for at least several weeks in benzene solution when protected from light at room temperature. The FAMC derivatives of amantadine and *n*-decylamine were purified by preparative silica gel thin-layer chromatography and subjected to mass spectrometry to confirm their structures. The mass spectra showed the molecular ion peak of the amantadine derivative at *m/z* 438 and that of the *n*-decylamine derivative at *m/z* 443. The excitation and fluorescence spectra of the amantadine



FAMC

Chart 1

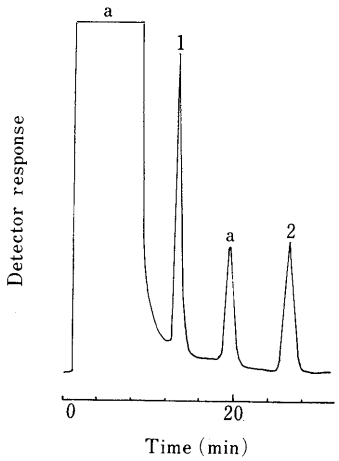


Fig. 1. Chromatogram of Amantadine Derivative Formed with FAMC after Extraction

A urine sample (100 μl) spiked with amantadine hydrochloride (25 pmol) was analyzed according to the procedure described in the text.
Peaks: a, reagent blank; 1, amantadine; 2, internal standard.

derivative in acetonitrile–water (5 : 1) used as the mobile phase showed maxima at 345 and 410 nm, respectively. The quantum yield calculated by the method of Parker and Rees[6] was 0.76 in the same solvent. The reaction of amantadine with FAMC reached a plateau within 15 min. The derivatization yield was *ca.* 94%.

This reaction was applied to the analysis of urine spiked with amantadine. The recoveries of the amantadine from urine at concentrations of 5, 10, 25, and 50 pmol/100 μl were all approximately 96%. Figure 1 shows a chromatogram of the FAMC derivative of amantadine extracted from urine. The standard curve for amantadine spiked in urine showed excellent linearity in the range from 5 to 50 pmol in 100 μl of urine ($y=0.082x+0.132$; $r=0.999$; y axis, peak height ratio; x axis, mass of amantadine (pmol/100 μl urine)). This range encompasses the concentrations of amantadine found in patients' urines.[1] The reproducibility of this procedure was also adequate, the coefficient of variation for 50 pmol of amantadine being 5.5% ($n=4$). The detection limit of amantadine was about 250 fmol in 100 μl of urine (signal-to-noise, 3). The sensitivity for amantadine in urine was about ten times higher than that given by the GC method.[4] This is the first attempt to use HPLC analysis combined with fluorescence labeling for the determination of amantadine hydrochloride.

References

1) M. J. Stumph, M. W. Noall and V. Knight, *Clin. Chem.*, **26**, 295 (1980).
2) A. Sioufi and F. Pommier, *J. Chromatogr.*, **183**, 33 (1980).
3) P. M. Belanger and O. Grech-Belanger, *J. Chromatogr.*, **228**, 327 (1982).
4) K. Kobayashi, K. Uno, C. Nakagawa, S. Minematu, N. Hata and E. Okamoto, *Jpn. Pharmacol. Ther.*, **12**, 195 (1984).
5) D. Chakravarti and R. Das, *J. Indian Chem. Soc.*, **48**, 371 (1971).
6) C. A. Parker and W. T. Rees, *Analyst*, **85**, 587 (1960).