2-[*p*-(5,6-Methylenedioxy-2*H*-benzotriazol-2-yl)]phenethylamine as Fluorescence Derivatization Reagent for Carboxylic Acids in High Performance Liquid Chromatography

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2-[*p*-(5,6-Methylenedioxy-2*H*-benzotriazol-2-yl)]phenethylamine (MBPA; **1**) and two related compounds (**2** and **3**) were synthesized as highly sensitive fluorescence derivatization reagents for carboxylic acids. We investigated the fluorescence characteristics and the chromatographic behavior of their benzamide derivatives produced by the reaction with benzoic acid. Among them, MBPA was found to be the best reagent for use in high performance liquid chromatography (HPLC). Thus it was applied to the determination of ibuprofen in human serum. MBPA reacted with ibuprofen in the presence of 2-bromo-1-ethylpyridinium tetrafluoroborate to give the corresponding fluorescent amide at room temperature. Linear relationships were found between the peak areas and the concentrations of ibuprofen in the range of 5–100 ng/ml and 0.5–5 ng/ml with relative standard deviations of 7.49% and 15.02%, respectively. The detection limit for ibuprofen in serum was 1.5 pg per injection.

Keywords Fluorescence derivatization, 2-[*p*-(5,6-methylenedioxy-2*H*-benzotriazol-2-yl)]phenethylamine, high performance liquid chromatography, carboxylic acid, ibuprofen, labeling reagent

In a previous paper¹, a new fluorescence derivatization reagent 2-(*p*-aminomethylphenyl)-*N,N*-dimethyl-2*H*-benzotriazolyl-5-amine (AMDBA), for the determination of carboxylic acids by high performance liquid chromatography (HPLC) was reported. AMDBA is a highly sensitive reagent and reacts with several carboxylic acids in the presence of 2-bromo-1-ethylpyridinium tetrafluoroborate (BEPT) at room temperature.

In the present work, we synthesized some triazole derivatives, 2-[*p*-(5,6-methylenedioxy-2*H*-benzotriazol-2-yl)]phenethylamine (MBPA; **1**), *p*-(5,6-methylenedioxy-2*H*-benzotriazol-2-yl)benzylamine (MBBA; **2**) and *p*-(5,6-dimethoxy-2*H*-benzotriazol-2-yl)benzylamine (DBBA; **3**) as even more sensitive fluorescence derivatization reagents for carboxylic acids in HPLC (Chart 1). The fluorescence characteristics and the chromatographic

behavior of the benzamide derivatives of these reagents were investigated. MBPA was selected and applied to the determination of carboxylic acid in the biological sample. The highly sensitive HPLC method for ibuprofen in human serum was established.

Experimental

Apparatus

A Hitachi 650-60 fluorescence spectrophotometer was used for fluorescence measurements. All melting points were measured with a Yanagimoto micro melting point apparatus and were uncorrected.

HPLC apparatus: A Shimadzu LC-4A pump equipped with a Shimadzu RF-535 fluorescence HPLC mon-

MBPA (**1**; *n* = 2)

MBBA (**2**; *n* = 1)

DBBA (**3**)

Chart 1

itor and a Rheodyne model 7125 injection valve with a 200 μ l sample loop were used. A NOVA PAK C₁₈ (150 $\times$ 3.9 mm i.d.; Waters Assoc.) was used as an analytical column, in combination with a Nucleosil 5C₁₈ (30 $\times$ 4.0 mm i.d.; Macherey-Nagel) as a guard column. A Shimadzu Chromatopac C-R2AX data processor was used for the data treatment.

HPLC conditions: CH₃CN-H₂O mixture (13:10) was used as a mobile phase at a flow rate of 1.0 ml/min.

Reagents and materials

Ibuprofen and human serum type AB were purchased from Sigma Chemical Co. and Flow Laboratories Inc., respectively. Bond Elut columns (Si and C₁₈) were purchased from Analytichem International. The other chemicals were of reagent grade.

Synthesis of fluorescence derivatization reagents

Reagent compounds were synthesized by the same method described previously.¹

2-[*p*-(5,6-Methylenedioxy-2*H*-benzotriazol-2-yl)]phenethylamine (MBPA; 1): *N*-(*p*-Aminophenethyl)-2,2,2-trifluoroacetamide hydrochloride (2.7 g; 10 mmol) was diazotized and coupled with 3,4-methylenedioxyaniline (1.4 g; 10 mmol) by the usual manner. The resulting azo compound was recrystallized from DMF-MeOH as red needles; mp 165–166°C. The azo compound (2.5 g; 6.6 mmol) was dissolved in pyridine (40 ml) and an ammoniacal cupric sulfate solution (10 g of CuSO₄·5H₂O and 5 g of NH₄Cl were dissolved in 80 ml of H₂O and neutralized with NaHCO₃) was added to the stirred pyridine solution. The reaction mixture was refluxed for 1 h. After addition of H₂O (200 ml), the precipitates were collected, washed with 1% NH₄OH and dissolved in pyridine (30 ml). 1 M NaOH (30 ml) was added to the pyridine solution. The solution was refluxed for 1 h and then poured into H₂O (200 ml). The precipitates were collected, washed with H₂O and recrystallized from MeOH to give MBPA as colorless needles (Table 1).

p-(5,6-Methylenedioxy-2*H*-benzotriazol-2-yl)benzylamine (MBBA; 2): MBBA was synthesized in the way described in the synthesis of MBPA. *N*-(*p*-Aminobenzyl)-2,2,2-trifluoroacetamide was used instead of *N*-(*p*-aminophenethyl)-2,2,2-trifluoroacetamide hydrochloride. Recrystallization from MeOH gave MBBA as colorless needles (Table 1).

p-(5,6-Dimethoxy-2*H*-benzotriazol-2-yl)benzylamine (DBBA; 3): DBBA was synthesized in the same way as described for the synthesis of MBPA. 3,4-Dimethoxyaniline and *N*-(*p*-aminobenzyl)-2,2,2-trifluoroacetamide were used instead of 3,4-methylenedioxyaniline and *N*-(*p*-aminophenethyl)-2,2,2-trifluoroacetamide hydrochloride, respectively. Recrystallization from EtOH gave DBBA as colorless needles (Table 1).

Preparation of the fluorescent amide derivatives (1a–3a)

Benzoic acid (0.5 mmol) was dissolved in CH₂Cl₂ (10 ml). After addition of BEPT (150 mg), 9-methyl-3,4-dihydro-2*H*-pyrido[1,2-*a*]pyrimidin-2-one (MDPP; 200 mg) and the fluorescence derivatization reagent (MBPA, MBBA or DBBA; 0.5 mmol), the reaction mixture was stirred for 30 min at room temperature. After filtration, the filtrate was concentrated *in vacuo*. The residue was dissolved in CHCl₃ (2 ml) and chromatographed on a silica gel column with CHCl₃. The eluate was concentrated *in vacuo* and the residue was recrystallized from CHCl₃-MeOH to give the corresponding amide (Table 1).

Derivatization procedure

A serum sample (1 ml) was acidified with 1 M HCl (0.1 ml) and then passed through a Bond Elut (C₁₈) column. After successive washings with H₂O (10 ml) and 50% MeOH (6 ml), the desired fraction was eluted with 85% MeOH (2 ml). After the eluate was dried under a stream of nitrogen, the residue was dissolved in CH₃CN (0.5 ml). To this solution, 50 μ l each of BEPT (3.7 mM), MDPP (2.7 mM) and MBPA (3.7 mM) solutions in CH₃CN were added. After mixing for 10 s, the mixture was allowed to stand for 90 min at room

Table 1 Physicochemical and analytical data

Compd. No.	Appearance	Yield, %	mp/°C	Formula	Analysis, % : Calcd/Found		
					C	H	N
1	colorless needles	55.6	145–147	C ₁₅ H ₁₄ N ₄ O ₂ ·1/4H ₂ O	62.82 62.66	5.10 5.21	19.54 19.55
2	colorless needles	53.7	202–203	C ₁₄ H ₁₂ N ₄ O ₂ ·1/4H ₂ O	61.64 61.86	4.62 4.64	20.54 20.53
3	colorless needles	45.7	155–156	C ₁₅ H ₁₆ N ₄ O ₂ ·1/2H ₂ O	61.42 61.54	5.84 5.83	19.10 19.05
1a	colorless needles	65.9	262–264	C ₂₂ H ₁₈ N ₄ O ₃ ·1/4H ₂ O	67.60 67.72	4.77 4.82	14.33 14.31
2a	colorless needles	56.3	261–262	C ₂₁ H ₁₆ N ₄ O ₃	67.73 67.67	4.33 4.35	15.05 14.92
3a	colorless needles	56.3	225–226	C ₂₂ H ₂₀ N ₄ O ₃	68.03 67.95	5.19 5.30	14.42 14.48

temperature. Then, 0.5 ml of the reaction mixture was passed through a Bond Elut (Si) column and eluted with 1.5 ml of CH₃CN. The eluate was diluted with CH₃CN to make 2.5 ml. The resulting solution (20 μ l) was injected into the chromatograph.

Results and Discussion

Evaluation of fluorescence derivatization reagents

In order to select the fluorescence derivatization reagent for carboxylic acids, the fluorescence characteristics of the benzamide derivatives of the reagents, **1a**–**3a**, were measured in CH₃CN solution. As shown in Table 2, the fluorescence properties of the derivatives were almost the same. The excitation and emission maxima of these derivatives were much shorter than that of AMDBA (λ_{ex} ; 390 nm, λ_{em} ; 488 nm). Figure 1 shows the fluorescence spectra of **1a** in CH₃CN.

The effect of H₂O concentration on the fluorescence intensity was examined. As shown in Fig. 2, the fluorescence intensity of **1a** in the aqueous CH₃CN was almost constant at H₂O concentrations of 0–50% (v/v),

but was slightly decreased in proportion to the H₂O concentration over 50% H₂O; at 90% H₂O, the intensity was about 85% of the maximum value. **2a** and **3a** showed the same profiles.

The chromatographic behavior, retention time and detection limit of the amides were also investigated (Table 2). The detection limits of three amides were 0.5 fmol per injection. It was thus found that the intensities of the amides derivatized with reagents synthesized in this work were 30 times that with AMDBA (detection limit; 15 fmol) and that these reagents were one of the most sensitive reagents for carboxylic acids. The retention time of **1a** was larger than those of **2a** and **3a**, indicating that MBPA was more favorable for the separation of the biological samples than MBBA and DBBA. Therefore, MBPA was selected for further investigation to develop an analytical procedure for ibuprofen in human serum.

Conditions for the determination of ibuprofen in human serum

Clean-up of ibuprofen in serum sample. Several clean-up procedures for the determination of ibuprofen

Fig. 1 Excitation and emission spectra of compound **1a** (2×10^{-7} M) in CH₃CN.

Fig. 2 Effect of water concentration on the fluorescence intensity of compound **1a** (2×10^{-7} M) in aqueous CH₃CN.

Table 2 Fluorescence characteristics and chromatographic behavior of triazole derivatives (**1a**–**3a**)

Compd. No.	R	n	Fluorescence characteristics ^a			Chromatographic behavior ^b	
			λ_{ex} /nm	λ_{em} /nm	RFI, %	R_t /min	(fmol/inj.)
1a	5,6-methylenedioxy	2	333	372	100	8.9	0.5
2a	5,6-methylenedioxy	1	333	372	106	7.8	0.5
3a	5,6-dimethoxy	1	332	369	104	6.3	0.5

a. Observed in CH₃CN solution. b. HPLC conditions: column, Nucleosil 5C₁₈ (150×4.6 mm i.d.); guard column, Nucleosil 5C₁₈ (30×4.0 mm i.d.); mobile phase, CH₃CN:H₂O=4:3; flow rate, 1.0 ml/min. c. Detection limit $S/N=5$.

Fig. 3 Effect of reaction time on the peak area. Serum samples spiked with 20 ng of ibuprofen were treated according to the derivatization procedure but for various periods.

in serum have been reported.²⁻⁵ The extraction with the organic solvent is commonly used prior to HPLC. We first attempted the extraction of ibuprofen with isooctane-isopropanol mixture.² But many endogenous substances which could react with MBPA were also extracted, with the result that numerous interfering peaks were found on the chromatogram. Then the solid phase extraction was examined. A Bond Elut C₁₈ column was employed in our work. The serum sample was acidified with 1 M HCl and was passed through the column. Co-existing polar substances were removed with H₂O and 50% MeOH, while ibuprofen was adsorbed on the column and eluted with 85% MeOH. The recovery of this procedure was quantitative. The eluate was evaporated under the stream of nitrogen followed by the derivatization procedure.

Derivatization of ibuprofen with MBPA. Derivatization conditions in this work were the same as those described in our previous paper, except for reaction time. Although the reaction of MBPA with authentic ibuprofen was completed within 30 min to give the corresponding amide, it required a longer time to obtain the sufficient yield when conducted with serum sample. Figure 3 shows the effect of time on the reaction. The maximum and constant peak areas were attained after 1 h.

Excess MBPA was removed from the reaction mixture by a simple pretreatment method, using a Bond Elut Si column, established previously. The typical chromatogram obtained by the assay procedure is shown in Fig. 4. No interfering peaks were detected at the retention time of ibuprofen under the chromatographic conditions.

Evaluation of the assay procedure

The linearity of the peak area with the concentration

Fig. 4 Chromatograms of serum samples obtained after derivatization with MBPA. A, serum blank; B; serum sample spiked with 20 ng of ibuprofen; 1: amide derived from MBPA and ibuprofen.

of ibuprofen was examined. Calibration curves constructed with serum samples spiked with 5–100 and 0.5–5 ng of ibuprofen showed the linearity with relative standard deviations of 7.49% and 15.02%, respectively. The recovery of ibuprofen through the assay was 100.6%. The assay was reproducible and a good precision was obtained by the repeated assay of serum samples spiked with 20 ng of ibuprofen with relative standard deviation, 2.14% ($n=10$). The detection limit for ibuprofen in serum sample was 1.5 pg per injection. The assay procedure established in this work showed a high sensitivity and sufficient reliability.

The new fluorescence derivatization reagent, MBPA, was found to be a highly sensitive reagent and should be useful for the determination of carboxylic acids in the small amounts of biological samples.

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