Thienylpyridines as a New Fluorescent Reagent. I. Determination of Primary Alkylamines with 5-(4-Pyridyl)-2-thiophenecarbaldehyde Using HPLC

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5-(4-Pyridyl)-2-thiophenecarbaldehyde has been prepared as a new fluorescent derivatization reagent and utilized for the determination of primary alkylamines. A definite detection limit was achieved for the amines by using a method based on the combination of precolumn derivatization and postcolumn hydrolysis–detection of the original fluorescent reagent regenerated from the derivatives. Approximately a 0.1 pmol (10 μl injection) (S/N=2) of detection limit was obtained. A dynamic linear range was over 5 orders of magnitude.

Since thienylpyridine was found to be a new skeleton for various fluorophores in our laboratory,[1] we wish to report on the preparation of a new fluorescent compound, 5-(4-pyridyl)-2-thiophenecarbaldehyde (**1**), and its application to the determination of primary alkylamines.

The Schiff bases prepared by the reaction of **1** with primary amines were non-fluorescent in aqueous methanol and decomposed with an acid to produce the fluorescent aldehyde **1**. The following system was, therefore, devised for the determination of primary amines: formation of Schiff bases, separation on a HPLC column, hydrolysis of the eluting Schiff bases, and fluorescence detection of the original aldehyde. The primary alkylamines were consequently determined with nearly equal sensitivity. The detection limit was superior to those of known reagents, except for the SINC derivative[2] and NBI–SO_2Cl,[3] and the dynamic linear range was extremely wide as far as we know.

Experimental

The ^1H and ^{13}C NMR spectra were recorded on a JEOL JNM-GX400 FT spectrometer in $CDCl_3$ using TMS as the internal standard. Mass spectra were recorded on a Hitachi M-80B mass spectrometer. UV-VIS spectra and fluorescence spectra were obtained on a Shimadzu UV-265 spectrophotometer or a Shimadzu RF-5000 spectrofluorophotometer, respectively, and recorded with a Fujitsu FM 16β computer connected with these photometers. Melting points were not corrected.

Isocratic liquid chromatography experiments were conducted on a HPLC system consisting of a Shimadzu LC-6A high-pressure pump, a Rheodyne Model 7125 fixed loop injector, a Shim-pack CLC-ODS column (150 mm×6.0 mm i.d.) placed in a Shimadzu CTO-6A column oven (40 °C), a mixing coil (30 cm×0.3 mm i.d.) merged with a stream of acid solution delivered by a Mitsumi peristaltic pump, and a RF-5000 spectrofluorometer equipped with a 12 μl flow cell or a Shimadzu RF-535 fluorescence monitor. Gradient elution experiments utilized two Tosoh excellent CCPE pumps equipped with an MX-8010 mixer. Chromatograms were recorded with a Shimadzu C-R3A chromatopac or with the computer connected with the chromatopac.

Reagent. All chemicals were of reagent grade and were used as received from commercial sources, except those noted below. Solvents were distilled and water was purified through a Milli-QII system.

Preparation of 1. 2-Iodo-5-methylthiophene (2): A mixture of iodine (22.2 g, 87.4 mmol), periodic acid dihydrate (6.66 g, 29.2 mmol), and 2-methylthiophene (20.0 g, 204 mmol) was stirred in 80% acetic acid (200 ml) at 65 °C for 8 h. After cooling the precipitated heavy oil was separated, the acidic solution was made alkaline, the residual product was extracted with chloroform, the combined oil and extract was steam-distilled from a sodium hydroxide and sodium thiosulfate solution, and the crude product was then distilled in vacuo to give **2**, yellowish oil in an 80% yield; bp 90 °C/4.1 kPa (lit,[4] bp 88.2—89.3 °C/1.9 kPa).

4-(5-Methyl-2-thienyl)pyridine (3): A mixture of palladium amalgam (0.46 g, 1.5 mmol), sodium hydroxide (14.7 g, 368 mmol), 4-iodopyridine[5] (7.50 g, 36.6 mmol), and **2** (16.4 g, 73.2 mmol) was refluxed in 47 ml of water containing hydrazine hydrate (2.34 g, 46.8 mmol) for 6 h with stirring. The resulting mixture was filtered, and the residual metal was washed with hot chloroform and hot water. The separated organic layer was reduced to 50 ml, treated with 15 ml of 25% hydrochloric acid, and the crude product was back-extracted into chloroform after the separated acid solution was made alkaline. After evaporation of the chloroform, the residue was distilled in vacuo using a ball tube distillation apparatus. The distillate (150 °C/0.4 kPa) was chromatographed on silica gel using acetone–chloroform (1:1) as an eluent and **3** was obtained as the first fraction in a 30% yield: white solid, mp 136.0—136.6 °C. Found: C, 68.53; H, 5.06; N 8.05%; *m/z*, 175.0427. Calcd for $C_{10}H_9NS$: C, 68.54; H, 5.18; N, 7.99%; M, 175.0454. ^1H NMR (400 MHz, $CDCl_3$) δ=2.527 (3H, d, $^4J_{H-Me}$=1.1 Hz, Me), 6.782 (1H, dd, *J*=3.6 Hz and $^4J_{H-Me}$=1.1 Hz, H-4 in thiophene ring), 7.311 (1H, dd, *J*=3.6 Hz and $^5J_{H-Me}$=0.5 Hz, H-3 in thiophene ring), 7.401 (2H, dd, *J*=4.4 and 1.6 Hz, H-3 and H-5 in pyridine ring), and 8.541 (2H, dd, *J*=4.4 and 1.6 Hz, H-2 and H-6 in pyridine ring). ^{13}C NMR (100 MHz) δ=15.62 (q), 125.44 (d, 2C), 126.75 (d), 126.78 (d), 138.57 (s), 141.68 (s), 142.43 (s), and 151.15 (d, 2C).

Aldehyde 1: A mixture of **3** (1.00 g, 5.7 mmol) and selenium (IV) oxide (957 mg, 8.6 mmol) in 35 ml of *m*-xylene was refluxed with stirring for 8 h. After evaporation of the solvent, the residue was distilled in vacuo using a ball tube

distillation apparatus. The crude product (bp 200 °C/0.8 kPa) was chromatographed on silica gel using ethyl acetate as an eluent and the aldehyde (0.71 g, 66%) obtained as white plate crystals, mp 135.0—136.0 °C. Found: C, 63.46; H, 3.78; N, 7.17%; *m/z*, 189.0246. Calcd for $C_{10}H_7NOS$: C, 63.47; H, 3.73; N, 7.40%; M, 189.0247. ^{1}H NMR δ=7.546 (2H, dd, J=4.4 and 1.7 Hz, H-3 and H-5 in pyridine ring), 7.589 (1H, d, J=4.0 Hz, H-4 in thiophene ring), 7.803 (1H, d, J=4.0 Hz, H-3 in thiophene ring), 8.691 (2H, dd, J=4.4 and 1.7 Hz, H-2 and H-6 in pyridine ring), and 9.949 (1H, s, CHO). ^{13}C NMR δ=120.29 (d, 2C), 126.11 (d), 136.97 (d), 140.12 (s), 144.26 (s), 150.11 (s), 150.69 (d, 2C), and 182.82 (d).

Preparation of Schiff bases. *N*-[5-(4-Pyridyl)-2-thenylidene]butylamine (4): A mixture of **1** (0.20 g, 1.1 mmol) and butylamine (0.30 g, 4.1 mmol) was refluxed in benzene (20 ml) for 4 h. After evaporation of the solvent, the residue was distilled in vacuo using a ball tube distillation apparatus to give yellow oil **4** in a 54% yield (0.14 g, bp 170 °C/0.5 kPa). Found: C 67.84; H, 6.80%; *m/z* 244.1031. Calcd for $C_{14}H_{16}N_2S$: C, 68.82; H, 6.60%; M, 244.1033. ^{1}H NMR δ=0.952 (3H, t, J=7.3 Hz, CH_3), 1.387 (2H, h, CH_2), 1.691 (2H, q, CH_2), 3.606 (2H, t, J=7.0 Hz, CH_2), 7.278 (1H, d, J=3.7 Hz, thiophene ring proton), 7.456 (1H, d, J=3.7 Hz, thiophene ring proton), 7.482 (2H, dd, J=4.6 and 1.7 Hz, H-3 and H-5 in pyridine ring), 8.337 (1H, s, CH=N), and 8.607 (2H, dd, J=4.6 and 1.7 Hz, H-2 and H-6 in pyridine ring); UV (60% MeOH) 322 (ε 2.37×10^4).

***N*-[5-(4-Pyridyl)-2-thenylidene]octylamine (5):** A mixture of **1** (0.10 g, 0.53 mmol) and octylamine (0.14 g, 1.1 mmol) was refluxed in benzene (20 ml) for 6 h. After evaporaton of the solvent, the residue was distilled twice in vacuo to give yellow-orange solid **5** (240 °C/0.5 kPa) in a 82% yield. Mp 56.0—57.0 °C. Found: C, 71.43; H, 8.21; N, 9.09%; *m/z*, 300.1655. Calcd for $C_{18}H_{24}N_2S$: C, 71.95; H, 8.05; N, 9.32%; M, 300.1659. ^{1}H NMR δ=0.879 (3H, t, J=6.7 Hz, CH_3), 1.274—1.327 (10H, m, $(CH_2)_5$), 1.700(2H, q, =N-CH_2-CH_2), 3.596 (2H, t, J=7.2 Hz, =N-CH_2-CH_2), 7.286 (1H, d, J=4.0 Hz, thiophene ring proton), 7.463 (1H, d, J=4.0 Hz, thiophene ring proton), 7.489 (2H, dd, J=4.6 and 1.5 Hz, H-3 and H-5 in pyridine ring), 8.338 (1H, s, CH=N), and 8.608 (2H, dd, J=4.6 and 1.5 Hz, H-2 and H-6 in pyridine ring); UV (60% MeOH) 323 (ε 2.46×10^4).

***N*-[5-(4-Pyridyl)-2-thenylidene]aniline (6):** A mixture of **1** (0.15 g, 0.79 mmol) and aniline (0.25 g, 2.7 mmol) was refluxed in benzene (20 ml) for 6 h. After evaporation of the solvent, the residue was distilled twice in vacuo to give yellow-orange solid **6** (235 °C/0.4 kPa) in a 67% yield. Mp 148.0—149.0 °C. Found: C, 72.63; H, 4.62; N, 10.34%; *m/z*, 264.0720. Calcd for $C_{16}H_{12}N_2S$ C, 72.70; H, 4.58; N, 10.60%; M 264.0720. ^{1}H NMR δ=7.167—7.203 (3H, m), 7.314—7.353 (2H, m), 7.415 (1H, d, J=3.8 Hz, thiophene ring proton), 7.479—7.455 (3H, m), 8.494 (1H, s, CH=N), and 8.572 (2H, d, H-2 and H-6 in pyridine ring); UV (60% MeOH) 322 (ε 2.06×10^4).

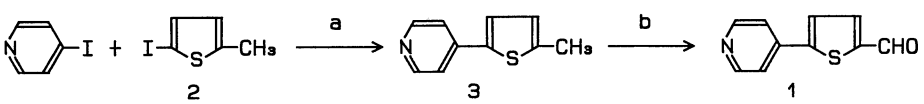
Derivatization Procedure. A mixture of **1** (a 1.1-2.2-fold amount of the total amines) and amines (20—50 μmol for each amine) in methanol(10 ml) was refluxed for 5 h, and then diluted to 1×10^{-3}—2×10^{-8} mol dm^{-3}. Each 5—10 μl of the solution was subjected to an HPLC instrument, and the Schiff bases in eluate were hydrolyzed with 1.0 mol dm^{-3} nitric acid, followed by monitoring the fluorescence intensities of **1** at 395 nm with excitation of 340 nm.

For trace amounts of amines. To a mixed solution of amines (0.5—1.0 nmol), a 170-fold excess of **1** (6.3×10^{-4} mol dm^{-3} methanol solution) and sufficient methanol were added so as to become 10 ml; after refluxing for 5 h and diluting to 50 ml, 10 μl of the solution was injected into the HPLC instrument.

Results and Discussion

Preparation and Characterization of 1. Reagent **1** was prepared from 4-iodopyridine and 2-iodo-5-methylthiophene, as outlined in Scheme 1, since no formylation of 4-(2-thienyl)pyridine occurred under the conditions in the Vilsmeier-Haack reaction described for the preparation of 2-thiophenecarbaldehyde.[6] The structure of **1** was proved by its ^{1}H and ^{13}C NMR spectra, MS, and elemental analysis. The aldehyde proton of the ^{1}H NMR spectrum appeared at δ=9.949 as a singlet, two thiophene ring protons at δ=7.589 and 7.803 as doublets (J=4.0 Hz), and four pyridine ring protons at δ=7.546 and 8.691 as doublets of doublets (J=4.4 and 1.7 Hz). A cross peak between the high-field thiophene ring proton and the high-field pyridine ring protons was observed in the NOESY spectrum. Their high-field protons were, therefore, assigned to the C4 proton in the thiophene ring and the C3 and C5 protons in the pyridine ring, respectively. The aldehyde, a stable crystalline substance, behaved as a weak base with pK_a 3.74 in 40% aqueous methanol. A protonated form of **1** exhibited excellent fluorescent properties and good stability, while a nonprotonated one decomposed slowly only at concentrations less than 10^{-6} mol dm^{-3} under irradiation of a 150 W Xenon lamp (λ=319 nm, band width=5 nm). The fluorescence spectra and the photometric properties are shown in Fig. 1 and Table 1, respectively.

Properties of Schiff Bases. The new Schiff bases, **4—6**, were prepared in usual way from the aldehyde **1** and the corresponding primary amines, and were characterized by both mass and ^{1}H NMR spectra as well as by elemental analysis. Their physical and fluorescent properties were examined for analytical applications. The behavior of Schiff bases **4—6** was quite different from that of the Schiff bases prepared



Scheme 1. a) $N_2H_4 \cdot H_2O$, PdHg, H_2O, NaOH; b) SeO_2, *m*-$(CH_3)_2C_6H_4$.

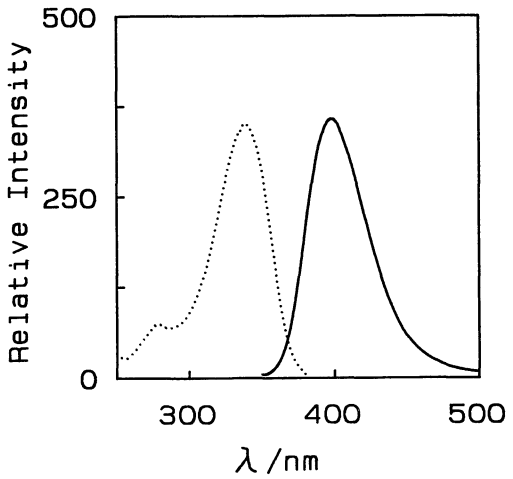


Fig. 1. Excitation (·····) and emission (——) spectra of 7.20×10^{-7} mol dm^{-3} **1** in 60% MeOH at pH 1.53 (λ_{ex}=334 nm, λ_{em}=398 nm, and slit widths=5 nm) (corrected).

Table 1. Spectroscopic Properties of **1** in 60% MeOH

	Protonated form (pH 1.5)[a]	Non-protonated form (pH 6—12.5)[a]
$\lambda_{abs,max}$/nm	334	319
ε	2.35×10^4	2.08×10^4
$\lambda_{ex,max}$/nm	334	b)
$\lambda_{em,max}$/nm	398	b)
Φ_f	0.06[c]	b)
IFS[d]	0.39	

a) Adjusted with HCl or NaOH. b) Not determined owing to photodecomposition. c) Determined relative to the fluorescence quantum efficiency of 4-(2-thienyl)pyridine (Φ_f=0.07, λ_{ex}=334 nm).[1] d) Calculated from the equation IFS=$\varepsilon\Phi_f/W_{1/2}$, where $W_{1/2}$=3025 cm^{-1}.

from 2-fluorenecarbaldehyde (or 1-pyrenecarbaldehyde) and primary alkylamines[7] regarding the following points: (1) non-fluorescence in aqueous methanol, chloroform, and ethyl acetate, (2) photodecomposition in aqueous methanol, and (3) hydrolysis with acid.[8]

Consequently, the aldehyde **1** could not be used as a precolumn reagent for the usual fluorescent detection of primary alkylamines; however, the aldehyde **1** might be utilized as a derivatization reagent by incorporating the formation and hydrolysis of the Schiff bases into pre- and postcolumn reactions in HPLC.

Amine Analysis. The type and concentration of acids were examined to hydrolyze **5** as a model Schiff base. As can be seen from Fig. 2, nitric acid was better than hydrochloric acid for the hydrolysis of the Schiff base. The results further showed that the fluorescent intensities were constant at a concentration range from 0.5 to 2.0 mol dm^{-3} nitric acid and that the intensities were increased only 1.2 times when the length of the mixing tube was changed from 30 cm to 210 cm.

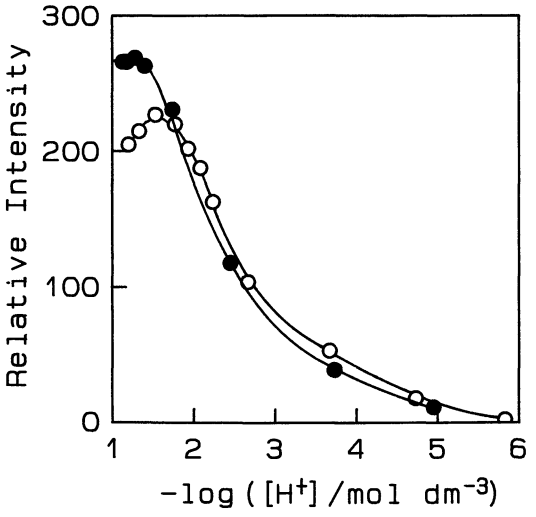


Fig. 2. Relative fluorescent intensity as a function of the concentration of acid used for postcolumn hydrolysis of **5**. Column, 150×6 mm i.d. Shim-pack CLC-ODS; eluent, methanol 1.0 ml min^{-1}; postcolumn reagent, (●) nitric acid or (○) hydrochloric acid at 0.2 ml min^{-1}; sample size, 10 μl (66 pmol); detector, Shimadzu RF-5000, λ_{ex}=334 nm, λ_{em}=398 nm (slit widths 15 nm).

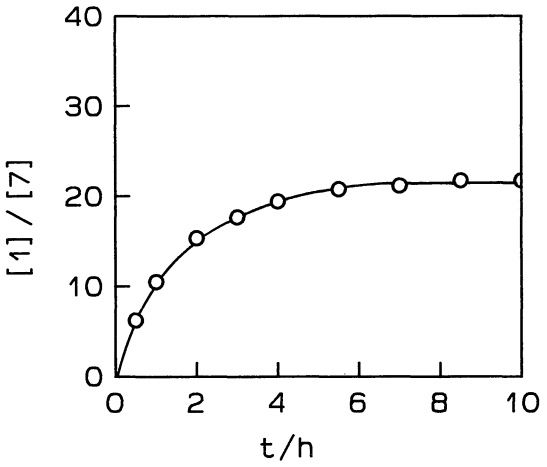


Fig. 3. Influence of precolumn reaction time on the ratio of hydrolysis product **1**, derived from the Schiff base **5**, to octylamine (**7**) used. The ratios were calculated from the amount of **7** used (8.00×10^{-4} mol dm^{-3}) and the peak areas of **1** relative to that of **3** added (3.87×10^{-4} mol dm^{-3}) in the reaction mixture. Conditions are as described under Fig. 2 except that the RF-535 detector (λ_{ex}=340 nm (slit 13 nm), λ_{em}=395 nm (slit 15 nm)) and 1 mol dm^{-3} nitric acid were used.

Therefore, 1.0 mol dm^{-3} nitric acid and a 30 cm-long mixing tube were used in the following experiments.

Fiure 3 shows the influence of the precolumn reaction time on the amounts of **1** derived from the Schiff base. The Schiff base formation was completed within 7 h at the refluxing temperature; therefore, a reaction time of 5 h, which achieved over 90% of the

reaction, was chosen in this study. The amount of the Schiff base was decreased to 1/4—1/7 of that obtained in methanol when chloroform or benzene was used as a solvent; methanol was therefore used in the precolumn reaction. Chromatograms of alkylamines (60—90 pmol injected) are shown in Fig. 4. When ultratrace amounts of amines (6×10^{-7}—9×10^{-7} mol dm^{-3}) were tested under the same conditions as in the above mentioned experiment except that a 170-fold excess of **1** was used to the total amount of the amines, a similar chromatogram was obtained and the amounts of the amines were determined with equal sensitivity (Fig. 4-C). Figure 5 shows the calibration curves for alkyl amines. All alkylamines could be detected with a similar sensitivity. The linear corelation coefficient was more than 0.999 for the following alkylamines in the concentration range of 1×10^{-3} to 2×10^{-8} mol dm^{-3}: butyl-, hexyl-, octyl-, decyl-, dodecyl-, and hexadecylamine. The detection limit (S/N=2) was 0.10—0.15 pmol for the injected sample. This limit was superior to those of the known reagents, except for SINC derivatives[2] and NBI-SO_2Cl.[3] The coefficient of variation for a 1×10^{-5} mol dm^{-3} octylamine solution (n=5) was 1.8%. The precolumn reaction occurred nearly quantitatively, since the calibration curves of octylamine agreed with that of **5** in a methanol solution. Some alkyl and arylamines could be also determined with nearly equal sensitivity as shown in

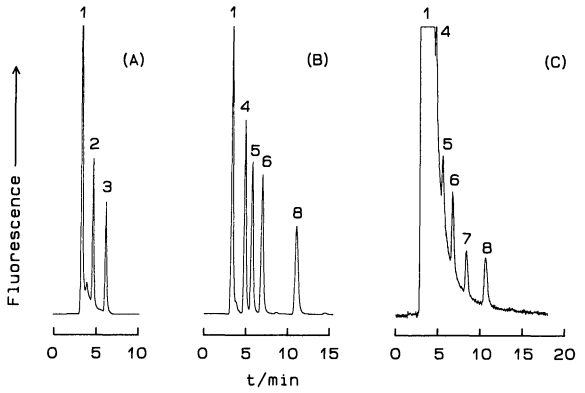


Fig. 4. Separation of the Schiff bases of primary alkylamines: (1) reagent **1**, (2) butyl-, (3) hexyl-, (4) octyl-, (5) decyl-, (6) dodecyl-, (7) tetradecyl- and (8) hexadecylamines. Conditions are as described under Fig. 3 except as follows: eluent, (A) 90% MeOH, (B) and (C) 100% MeOH; sample size, (A) 2μl (65, 86 pmol), (B) 5 μl (60—75 pmol), and (C) 10 μl (1.1—1.9 pmol).

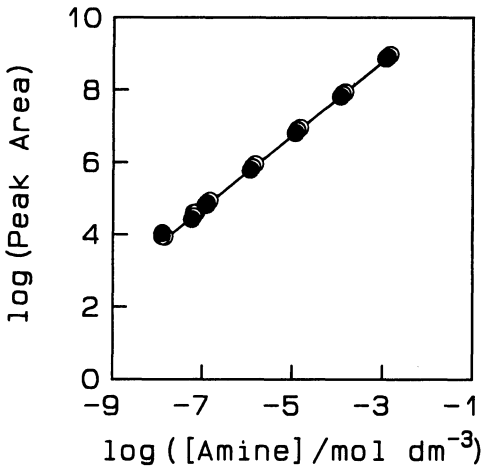


Fig. 5. Calibration curve for primary alkylamines: (●) octyl-, and (○) decyl-, dodecyl-, and hexadecylamines. Conditions are as described under Fig. 4.

Table 2. Retention Time and Relative Response of Schiff Bases[a]

Amine	t_R/min 100% MeOH	t_R/min 90% MeOH	Relative response[b]
Butylamine		4.6	0.79
Hexylamine		6.1	0.72
Octylamine	4.7		1.00
Decylamine	5.5		0.93
Dodecylamine	6.7		0.89
Tetradecylamine	8.3		0.83
Hexadecylamine	10.8		0.84
Octadecylamine	21.3		0.71
Benzylamine		4.0	0.96
Cyclohexylamine		5.0	0.52
1-Phenylethylamine		4.5	0.61
2-Phenylethylamine	3.7	4.7	1.13
1,3-Propanediamine	3.5		2.02
1,8-Octanediamine	4.3		0.61
p-Toluidine		5.1	1.05
p-Chloroaniline	4.0	5.4	0.27[c]
Dopamine hydrochloride		4.1[d]	6.18

a) Conditions as stated in Fig. 4. Retention times of **1** were 3.1 min (100% MeOH) and 3.3 min (90% MeOH). b) Ratio of peak area of amines to that of octylamine at the concentration of 1×10^{-6} mol dm^{-3}. c) If the precolumn reaction was carried out in the presence of KOH, the response of this base could be equal to that of octylamine. d) This retention time was agreement with that of 4-(5-dimethoxymethyl-2-thienyl)pyridine.

Table 2. An increase of retention time or water contents in the eluent decreased the amount of the detectable Schiff bases owing to the hydrolysis of the Schiff bases in the column. The relative response of amines, the ratio of the peak area of amine to that of octylamine, was 0.7 to 1.1, except for that of cyclohexylamine, 1-phenylethylamine, 1,8-octanediamine, and 2-(3,4-dihydroxyphenyl)ethylammonium chloride. In the case of diamine, the response of 1,3-propanediamine was reasonable rather than 1,8-octanediamine. In the case of 2-(3,4-dihydroxyphenyl)-ethylammonium chloride, no precolumn reaction seemed to occur, since its retention time was in agreement with that of 4-(5-dimethoxymethyl-2-thienyl)-pyridine and its response was too large compared that of monoamine. No precolumn reaction occurred with the following amines: 4-aminophenol, 4-aminoacetophenone, 4-aminopyridine, 3-aminoquinoline, L-histidine, L-lysine monohydrochloride, adenine, and hydroxylammonium chloride.

A gradient elution was tried in order to improve the peak broadening which was observed along with an increase in the retention time; the results were unsuccessful: Separation was performed with a linear gradient of 80%MeOH to 100%MeOH or to MeOH–THF (90:10), but the detection limits were increased with an increase of noise level in a base line and with a decrease of the response owing to hydrolysis of the Schiff bases in the column.

It is concluded that the present pre- and postcolumn procedure using isocratic elution is suitable for the determination of primary alkylamines with nearly equal sensitivity.

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8) The decomposition products were identified as **1** and the used amines by GLPC and the formation of **1** was also evidenced by fluorescence spectroscopy.