THE UTILITY OF 4-(2-THIENYL)PYRIDINES AS A DERIVATIZATION REAGENT FOR HPLC AND CE

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Abstract To emphasize the capability of a fluorescent thienylpyridine skeleton, the derivatization of carboxylic acid, aldehyde, ketone, and glycine were examined and applied to the following determinations: glycine with 5-(4-pyridyl)-2-thiophene-carbaldehyde using CE, carboxylic acids with 5-(4-pyridyl)-2-thiophenemethanol or 4-(5-diazomethyl-2-thienyl)pyridine using HPLC, carboxylic acids with 1-(2-ammonioethyl)-4-(2-thienyl)pyridinium dibromide using CE, and aldehydes and ketones with 4-(2-thienyl)pyridinium acetohydrazide chloride.

Key words thienylpyridine, quaternary pyridinium salt, fluorescent Girard reagent

Recently, we have reported that a thienylpyridine skeleton is a new fluorophore, which exhibits two different spectra in acidic and basic solutions, respectively[1]. The compounds including this skeleton are classified into two groups i.e. non-ionic compounds and quaternary pyridinium salts. The above-mentioned spectra were observed in the former compounds and the latter compounds in both acidic and basic solutions exhibited the same spectra as in the former compounds in acidic solution. To extend the capabilities of this skeleton, the following compounds were prepared and used for the determination of carboxylic acid, aldehyde, etc. using HPLC or CE (capillary zone electrophoresis): 5-(4-pyridyl)-2-thiophenecarbaldehyde(1), 5-(4-pyridyl)-2-thiophenemethanol(2), 4-(5-diazomethyl-2-thienyl)pyridine(3), 1-(2-ammonioethyl)-4-(2-thienyl)pyridinium dibromide(4), and 4-(2-thienyl)pyridinium acetohydrazide chloride(5). Detection limits and linear ranges for most of analytes were superior to those reported previously[2-3].

Experimental

The instruments were same as those previously used[4,5]. Reagents 1[6], 2[4], 3[7], and 4[5] were prepared by the methods described previously as outlined in Scheme 1. 1-Hexyl-4-(2-thienyl)pyridinium bromide(10) was prepared by a method similar to that for preparation of 4.

Preparation of 5. 1-(Ethoxycarbonylmethyl)-4-(2-thienyl)pyridinium chloride(9): A mixture of 4-(2-thienyl)pyridine(7)[1](0.77 g, 4.8 mmol) and ethyl chloroacetate (0.61 g, 5.0 mmol) was refluxed in ethanol(1.2 ml) for 2 h. After cooling a precipitate was separated and recrystallized from ethanol-concd hydrochloric acid(60:1 v/v) to give light yellow needles 9 in a 52% yield(0.71 g). Mp 225.5-226.5°C(decomp). Found: C, 54.98; H, 4.94; N 4.97%; (M-Cl)⁺, 248(SIMS, in a glycerol matrix). Calcd for C₁₃H₁₄ClNO₂S: C, 55.02; H, 4.97; N, 4.94%; M, 283. ¹H NMR(400MHz, D₂O) δ=1.39(3H, t, J=7.1Hz, CH₃), 4.42(2H, q, J=7.1Hz, CH₂), 5.49(2H, s, CH₂), 7.28(1H, dd, H-4 in thiophene ring), 7.91-7.92(2H, m, H-3 and H-5 in thiophene ring), 8.07(2H, d, J=7.1Hz, H-3 and H-5 in pyridine ring), and 8.60(2H, d, J=7.1Hz, H-2 and H-6 in pyridine ring). ¹³C NMR(100MHz) δ=15.95(q), 62.39(t), 66.69(t), 124.65(d, 2C), 132.93(d), 135.07(d), 137.72(d), 139.27(s), 147.79(d, 2C), 152.69(s), and 170.20(s, C=O).

Reagent 5: A mixture of 9(501.4 mg, 1.77 mmol) and 100%hydrazine hydrate(347.6 mg, 7.0 mmol) was stirred in ethanol(12.2 g) at 60°C for 1 h to give a precipitate, a portion of the precipitate(509.9 mg) was dissolved in methanol(50.4 ml), and then concd hydrochloric acid(9.6 ml) was added to afford a precipitate, i.e. white needles 5 as hydrous hydrochloride, in a 22% yield. Mp 284.5-285.4°C. Found: C, 40.63; H, 4.43; N, 12.93%; (M-2HCl-H₂O)⁺, 233(SIMS, in a glycerol matrix). Calcd for C₁₁H₁₂ClN₃OS·HCl·H₂O: C, 40.75; H, 4.66; N, 12.96%; M, 323. ¹H NMR(D₂O) δ=5.56(2H, s, CH₂), 7.29(1H, dd, J=3.9 and 5.1Hz, H-4 in thiophene ring), 7.93(1H, dd, J=1.1 and 5.1Hz, H-5 in thiophene ring), 7.95(1H, dd, J=1.1 and 3.9Hz, H-3 in thiophene ring), 8.11(2H, d, J=7.1Hz, H-3 and H-5 in pyridine ring), and 8.63(2H, d, J=7.1Hz, H-2 and H-6 in pyridine ring). ¹³C NMR δ=61.51(t, CH₂), 124.74(d, 2C), 132.96(d), 135.18(d), 137.84(d), 139.30(s), 147.93(d, 2C), 152.93(s), and 167.69(s, C=O).

Results and Discussion

Derivatization reagents 1-5 were prepared from 4-(5-methyl-2-thienyl)pyridine(6) or 7, as outlined in Scheme 1. The structure of 1, 2, and 4-9 was proved by their ^1H and ^{13}C NMR spectra, MS, and elemental analysis. Diazomethane 3 was not isolated in pure state and its content was estimated to be about 60% from the esterification with stearic acid.

Scheme 1. i) SeO_2 , $(\text{CH}_3)_2\text{C}_6\text{H}_4$; ii) NaBH_3CN , $\text{HCl-MeOH}(\text{pH}3)$; iii) $\text{N}_2\text{H}_4\cdot\text{H}_2\text{O}$, MeOH ; iv) MnO_2 , CHCl_3 , KOH-EtOH ; v) $\text{Br}(\text{CH}_2)_2\text{NH}_2\cdot\text{HBr}$, CH_3CN ; vi) $\text{CH}_2\text{ClCOOEt}$, EtOH ; vii) $\text{N}_2\text{H}_4\cdot\text{H}_2\text{O}$, EtOH .

Determination of glycine with aldehyde 1 using CE. Our initial studies on the determination of primary alkylamines with 1 using HPLC were based on the combination of precolumn derivatization and postcolumn hydrolysis-detection of the original fluorescent reagent regenerated from the derivatives[6] as outlined in Scheme 2. Recently, we found that amino acids were determined by this method if a base catalyst was used in the precolumn reaction and that fluorescent

Previous work:

Present work:

Scheme 2. Reaction procedures and flow chart of separation and detection systems.

β -[5-(4-pyridyl)-2-thienyl]serine(11) was synthesized from 1 and glycine(12)[8]. The present method, therefore, was designed to detect only 12 in various amino acids, in which the above-mentioned combination of pre- and postcolumn reactions is used as a precolumn reaction and this new fluorescent amino acid separated from 1 by CE was determined with fluorescence detector (Scheme 2); 0.25 ml of ethanol containing both 5.0 μmol of 1 and 1.0 μmol of 10, and 0.50 ml of 0.2 M ($\text{M}=\text{mol dm}^{-3}$) potassium hydroxide aqueous solution were added to 0.25 ml of water or aqueous ethanol containing amino acids, the mixture was allowed to stand for 24 h at room temperature, and then 0.05 ml of 0.5 M hydrochloric acid was added to a 0.05-ml portion of the mixture, followed by the addition of 0.01 ml of 2.0 M potassium hydroxide aqueous solution. Each 6 nl of the solution was subjected to CE and determined by monitoring the fluorescence intensities. Figure 1 shows a typical electropherogram of 12 derivatized with 1. The linear range for 12 was 4.0×10^{-5} – 4.0×10^{-3} M; the linear-correlation coefficient was 0.9984 and the detection

limits($S/N=4$) 4.0×10^{-5} M. The coefficient of variation for 1.2 mM **12** solution ($n = 5$) was 5.7%. The following L-amino acids had no influence on the determination of **12** in the presence of a 20-fold excess: Ala, Asp, Cys, His, Met, Phe, Tyr, and Val.

We are still seeking more suitable derivatization and separation conditions because peak 3 in Fig. 1 occupied only 3% of the area estimated from standard **11**, and because the peak included that of a by-product derived from **12** and **1**.

Determination of carboxylic acids with 2 or 3 using HPLC. Recently, we reported that both reagents **2** and **3** reacted with fatty acids to produce the corresponding esters (IFS (Intrinsic fluorescence sensitivity = $\epsilon \cdot \phi_f / W_{1/2}$) = 0.81–2.96) which were separated with ODS column[4, 7] and that the latter reagent also reacted with bile acids without heating and a condensing reagent to produce fluorescent esters which were separated with ODS column [7]. Unsaturated fatty acids(**13**) and glycine-conjugated bile acids(**14**) were derivatized with **3** and the produced esters were separated with ODS column as shown in Fig. 2; 0.2 ml of 4 mM **3** methanol solution was added to 0.2 ml of methanol containing carboxylic acids, allow-

Fig. 1 Electropherogram of a sample prepared by the derivatization of 0.75 μ mol of **12** with 5.0 μ mol of **1**: (1)**10**, (2)**1**, and (3)**11**. The area under peak 3 corresponds to 2.0 pmol of **12**. Capillary inside diameter, 50 μ m, length 100cm(75 cm to the detector); applied voltage, 20kV(20 μ A); electrolyte, 10 mM Na_2HPO_4 : 40 mM NaH_2PO_4 = 1 : 1 v/v; gravity injection from 30 cm for 10 s(6 nl); detector, Shimadzu RF-535 fluorescence HPLC monitor with a homemade capillary holder, λ_{ex} =299 nm, λ_{em} = 366 nm.

Fig. 2 Chromatograms of 5-(4-pyridyl)-2-thiophenemethyl esters of (A)**13** and (B)**14**: (1)**3**, (2)**10**-undecenoic(**13a**), (3)dodecanoic, (4)*cis*-9-hexadecenoic(**13b**), (5)*cis*-9-octadecenoic(**13c**), (6)*cis,cis*-9,12-octadecadienoic(**13d**), (7)*cis,cis,cis*-6,9,12-octadecatrienoic(**13e**), (8)glycocholic(**14a**), (9)glycodeoxycholic(**14b**), (10)cholic, and (11)hyodeoxycholic acids. Column, 150x6 mm i.d. Shim-pack CLC-ODS; eluent, (A)95% methanol, (B)83–100% methanol, 1.0 ml min⁻¹; sample size, 1 μ l(100 pmol); detector, Shimadzu RF-535, λ_{ex} =300 nm, λ_{em} = 360 nm.

ed to stand for 3 h, and each 1 μ l of the solution was subjected to HPLC. The linear ranges for **13** and **14** were from 0.5 pmol to 100 pmol and from 0.15–1.50 pmol to 150 pmol, respectively; the linear-correlation coefficients were 0.9929–0.9983 and 0.9934–0.9964 and the detection limits($S/N=2$) were 0.5 pmol and 30–150 fmol. The coefficients of variation for 1.0×10^{-5} M **13** and **14** solutions($n=6$) were 1.7–3.8% and 2.3–4.4%. Alcohol **2** reacted with **13** under the conditions described previously[4], as well as **3**, to form the corresponding esters which were separated with ODS column. Consequently, both reagents **2** and **3** were available for the determination of **13**. The methods presented here were more excellent in detection limits and linear ranges than those reported previously[2].

Determination of carboxylic acids with 4 using CE. Since reagent **4** reacted with saturated fatty acids in the presence of dicyclohexylcarbodiimide(**15**) at 40°C to produce the corresponding esters which are separated using CE with a lower detection limit of 1.0×10^{-6} M(6 fmol) and plate number of about $1 \times 10^5/75$ cm[5,9], some unsaturated fatty acids(**13a**–**13f**) were examined; 0.1 ml of DMF containing 0.5–500 nmol of carboxylic acids and 0.10 ml of 2.0 mM **3** DMF solution were added to a mixture of **4**(3.5 mg, 9.0 μ mol), **15**(31 mg, 0.15 μ mol) and 4-dimethylaminopyridine(3.6 mg, 30 μ mol), diluted to 0.5 ml with DMF, heated with stirring at 40°C for 2

h, and then cooled. Each 6 nl of the solution was subjected to CE, and determined by monitoring the fluorescence intensities. A typical electropherogram is shown in Fig. 3. All the linear ranges for **13a-c** and **13f** were 1.0×10^{-3} – 1.0×10^{-5} M (solutions siphoned); the linear-correlation coefficients were 0.9985–0.9999 and the detection limits ($S/N=2$) were 5.0×10^{-6} – 6.4×10^{-6} M. The coefficients of variation for 5.0×10^{-4} M solutions ($n=7$) were 2.5–6.5%. Although no description on the determination of **13** by CE has been made, the present results were superior in linear ranges and plate number and similar in the lower detection limits to those for short-chain carboxylic acids reported by Zare, et al. [3].

Determination of aldehydes and ketones with fluorescent Girard reagent 5 using CE. Since the lower detection limits of carboxylic acids derivatized with **4** exceeded our expectation, reagent **5** containing the same fluorophore was prepared; its IFS value was 0.27, which was close to that of **4** (0.30). One ml of methanol containing 4.0 μ mol of **5** was added to 1.0 ml of methanol containing each 0.60 μ mol of benz- (**16a**), 4-anis- (**16b**), and 4-isopropylbenzaldehyde (**16c**), heated with stirring at 60°C for 0.5 h, and then cooled. Each 6 nl of the solution was subjected to CE (Fig. 4). Although the optimum derivatization and separation conditions were not sought, the peak height and area of the derivatized aldehydes were close to those of the esters in Fig. 3. Their detection limits and linear ranges may therefore be close to those of the acids. Heptanal, aceto-, propio-, and benzophenone, etc. were also derivatized and determined. Since the sensitivities of the reagents **4** and **5** prepared were better than those we expected, new derivatization reagents containing 5-(1-alkyl-4-pyridinio)-5'-(4-pyridinio)-2,2'-bithienyl in place of 4-(2-thienyl)pyridine is being prepared. Their IFS values were estimated to be 9.4 [1] and the detection limits 0.2 fmol.

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REFERENCES

1. R. Nakajima, H. Iida and T. Hara, *Bull. Chem. Soc. Jpn.*, **63**, 636 (1990).
2. S. Kamada, M. Maeda and A. Tsuji, *J. Chromatogr.*, **272**, 29 (1983).
3. X. Huang, J. A. Luckey, M. J. Gordon and R. N. Zare, *Anal. Chem.*, **61**, 766 (1989).
4. R. Nakajima, K. Shimada, Y. Fujii, A. Yamamoto and T. Hara, *Bull. Chem. Soc. Jpn.*, in press.
5. R. Nakajima, H. Kamiuchi and T. Hara, Proceedings of 52nd Symposium on Analytical Chemistry, Obihiro, May 1991, p. 371.
6. R. Nakajima, A. Yamamoto and T. Hara, *Bull. Chem. Soc. Jpn.*, **63**, 1968 (1990).
7. R. Nakajima, K. Shimada and T. Hara, Proceedings of 52nd Symposium on Analytical Chemistry, Obihiro, May 1991, p. 273.
8. R. Nakajima, E. Nakamoto and T. Hara, unpublished results.
9. The values of the detection limit were obtained under the more suitable separation conditions different from that described in ref. 6.

Fig. 3 Electropherogram of unsaturated fatty acids derivatized with **4**: (1) **4**, (2) **10**, (3) heptanoic acid, (4) **13a**, (5) *cis*-9-tetradecenoic (**13f**), (6) **13b**, and (7) **13c**. Separation conditions were the same as shown in Fig. 1 except for the following: electrolyte, 70 mM NaH_2PO_4 : CH_3CN = 1 : 1 v/v; applied voltage 20kV (8 μ A); concentration of all sample components, 5.0×10^{-4} M.

Fig. 4 Electropherogram of three aldehydes derivatized with **5**: (1) **5**, (2) **5**, (3) **16a**, (4) **16b**, and (5) **16c**. Separation conditions were the same as shown in Fig. 3 except for the following: electrolyte, 50 mM HOAc : 50 mM NOAc : CH_3CN = 1 : 1 : 1.1 v/v/v; applied voltage 20kV (6 μ A).