Journal of Chromatography, 502 (1990) 178–183
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 22 062

Note

Phthalimidylbenzenesulphonyl chlorides as fluorescence labelling reagents for amino acids in high-performance liquid chromatography

YASUTO TSURUTA, YUUKO DATE and KAZUYA KOHASHI*

Faculty of Pharmacy and Pharmaceutical Sciences, Fukuyama University, 985 Higashimura-cho, Fukuyama, Hiroshima 729-02 (Japan)

(First received August 8th, 1989; revised manuscript received October 3rd, 1989)

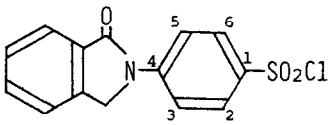
Benzenesulphonyl chloride, which reacts with amines to give the corresponding sulphonamides, is a useful reagent for separating primary and secondary amines[1,2]. N,N-Dimethylamino-*p*-aminobenzeneazosulphonyl chloride[3,4] and dimethylaminonaphthalenesulphonyl chloride (DNS-Cl)[5] have been used as labelling reagents for amino acids in high-performance liquid chromatography (HPLC) with visible and fluorescence detection, respectively. Previously, we developed some fluorescence derivatization reagents having phenylphthalimidine as a fluorophore for hydroxyl and amino compounds[6] and for thiol compounds[7].

This paper deals with the preparations of 4-(N-phthalimidyl)benzenesulphonyl chloride (Phisyl-Cl) and 2-methoxy-5-(N-phthalimidyl)benzenesulphonyl chloride (M-Phisyl-Cl) (Fig. 1) as fluorescent derivatization reagents for amines and amino acids and their reactivities towards amino compounds using thin-layer chromatography (TLC) and HPLC.

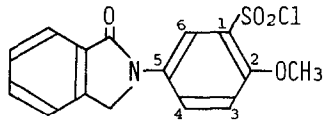
EXPERIMENTAL

Chemicals and solvents

All chemicals were of analytical-reagent grade, unless stated otherwise. *n*-Propylamine, piperidine and aniline were purchased from Wako (Osaka, Japan) and amino acids from Kyowa Hakko (Tokyo, Japan). Amines were dissolved in acetone, and amino acids (alanine, asparagine, aspartic acid, cysteine, glutamic acid, glycine, histidine, hydroxyproline, isoleucine, leucine, lysine, methionine, ornithine, phenyl-



Phisyl-Cl



M-Phisyl-Cl

Fig. 1. Structures of Phisyl-Cl and M-Phisyl-Cl.

0021-9673/90/$03.50

alanine, proline, serine, threonine and valine) in 0.01 *M* hydrochloric acid, except for cystine and tyrosine (in 0.05 *M* sodium hydroxide solution). Organic solvents were purified by distillation prior to use.

Synthesis of Phisyl-Cl. *o*-Phthalaldehyde (2.68 g, 20 mmol) in diethyl ether (100 ml) and aniline (1.86 g, 20 mmol) in diethyl ether (20 ml) were mixed. After overnight stirring at room temperature, the precipitate of phenylphthalimidine was filtered off, washed with diethyl ether and recrystallized from methanol (yield 3.30 g). Chlorosulphonic acid (6.6 g, 50 mmol) was dropped onto crystals of N-phenylphthalimidine (2.09 g, 10 mmol) in an ice-bath over 20 min with vigorous stirring, followed by heating at 60°C for 2 h. On adding crushed ice (*ca.* 30 g) to the resulting mixture, a white precipitate of Phisyl-Cl was obtained (yield 1.21 g). Recrystallization from benzene gave fine colourless needles, m.p. 186–187°C. Calculated for $C_{14}H_{10}NO_3SCl$, C 54.63, H 3.28, N 4.55; found, C 55.22, H 3.30, N 4.71%. Mass spectrum: *m/z*, 307 (M^+). ^1H NMR (C^2HCl_3–[$^2H_6$] DMSO): δ, 4.94 (2H, singlet, CH_2), 7.44 (4H, multiplet, phthalimidyl ArH), 8.10 (2H, doublet, J = 10 Hz, 3- and 5-H), 8.18 ppm (2H, doublet, J = 10 Hz, 2- and 6-H).

Synthesis of M-Phisyl-Cl. *p*-Anisidine was used instead of aniline as above in the synthesis of Phisyl-Cl. The reaction with *o*-phthalaldehyde was carried out for 3 days with stirring. The precipitate of N-(4-methoxyphenyl)phthalimidine was collected, washed with diethyl ether and recrystallized from methanol. Chlorosulphonic acid (*ca.* 0.8 ml) was dropped onto crystals of N-(4-methoxyphenyl)phthalimidine (0.3 g, 1.3 mmol) in an ice-bath. After standing at 60°C for 5h, crushed ice was added to the reaction mixture. The resulting precipitate was collected and extracted with chloroform. After evaporation of the solvent, crude M-Phisyl-Cl (yield 0.11 g) was recrystallized from chloroform–carbon tetrachloride as colourless plates, m.p. 215–217°C (decomp.). Calculated for $C_{15}H_{12}NO_4SCl$, C 53.33, H 3.58, N 4.15; found, C 52.64, H 3.65, N 3.94%. Mass spectrum: *m/z*, 337 (M^+). ^1H NMR (C^2HCl_3–[$^2H_6$]DMSO): δ, 4.08 (3 H, singlet, OCH_3), 4.88 (2H, singlet, CH_2), 7.20 (1H, doublet, J = 8 Hz, 3-H), 7.58–7.91 (4H, multiplet, phthalimidyl ArH), 8.05 (1 H, doublet, J = 3 Hz, 6-H), 8.82 ppm (1H, quadruplet, J = 8 and 3 Hz, 4-H).

Derivatization procedure

To a test solution of amine or amino acid (0.05–10 m*M*, 5 μl), sodium hydroxide (0.1 *M*, 20 μl) and reagent solution (5 m*M* in acetone, 150 μl) were successively added and mixed well. The mixture was heated at 50°C for 15 min.

Thin-layer chromatography

An aliquot of the resulting mixture was applied to a silica gel plate (Analtech, Newark, DE, U.S.A.) and developed at *ca.* 25°C with the solvent systems S_1 (benzene–acetone, 8:2, v/v) for amines and S_2 (*n*-propanol–ammonia, 8:2, v/v) for amino acids. Visual detection of the fluorescent spots on the silica gel plate was achieved with a UV cabinet II (Camag, Muttenz, Switzerland).

High-performance liquid chromatography

The apparatus used was a Bip-1 HPLC system equipped with a Model 880-51 two-line degasser (Jasco, Tokyo, Japan), a Rheodyne 7161 injector (20-μl loop) and an F-1000 fluorescence detector (Hitachi, Tokyo, Japan). An ERC-ODS-1161 (3 μm)

column (100 mm × 6 mm I.D.) (Erma, Tokyo, Japan) was used with a gradient system of 2.5 m*M* phosphate buffer (pH 7.3)–methanol in order to examine the reaction conditions of Phisyl-Cl and M-Phisyl-Cl with amino acids. The separation was carried out with a linear gradient from 25 to 75% methanol over 20 min, followed by 75% methanol for 5 min, at a flow-rate of 1.0 ml/min. The separation of a reaction mixture of twenty amino acids with Phisyl-Cl was carried out by using a YMC AM-303 packed column of 5-μm ODS (C_{18}) (250 mm × 4.6 mm I.D.) (Yamamura, Kyoto, Japan). The flow-rate was 0.6 ml/min stepwise elution using solvent systems A [acetonitrile–30 m*M* Tris buffer (pH 6.5) (10:90)] and B [acetonitrile–30 m*M* Tris buffer (pH 6.5). (75:25)]. The elution programme was as follows (%B): 0% (8 min)–10% (12 min–20% (18 min)–30% (10 min)–45% (5 min)–60% (7 min)–100% (5 min)–0% (15 min). A C-KGC-324C-S guard column of 5-μm ODS (C_{18}) (25 mm × 4 mm I.D.) (Yamamura) was used together with each column. A 20-μl volume of the sample prepared from the reaction mixture by ten-fold dilution with eluent was injected. Fluorescence was detected with excitation at 295 nm and emission at 425 nm for Phisyl derivatives and excitation at 298 nm and emission at 445 nm for M-Phisyl derivatives.

RESULTS AND DISCUSSION

N-Phenylphthalimidine prepared from the reaction of *o*-phthalaldehyde with aniline showed strong fluorescence[8] and was easily converted to Phisyl-Cl by treatment with chlorosulphonic acid. The methoxy derivative, M-Phisyl-Cl, was obtained from *p*-anisidine in a similar manner.

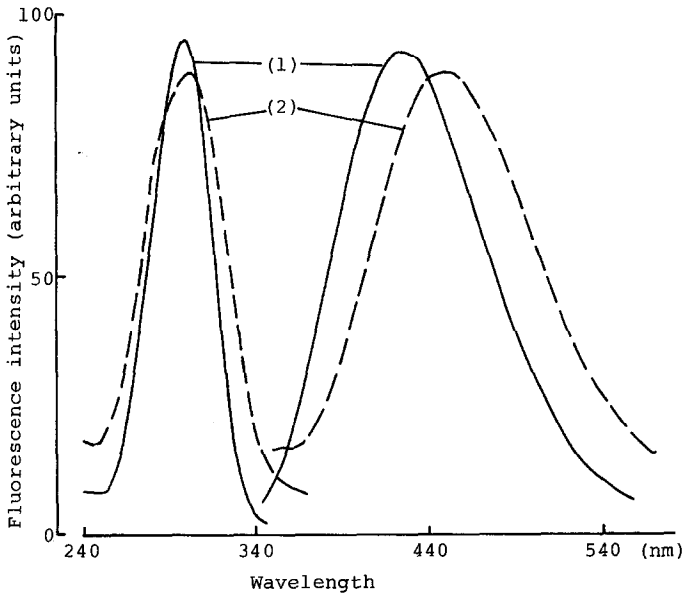


Fig. 2. Fluorescence spectra of the derivatives of alanine with Phisyl-Cl and M-Phisyl-Cl. (1) Phisyl-alanine (λ_{ex} 295 nm, λ_{em} 422 nm); (2) M-Phisyl-alanine (λ_{ex} 298 nm, λ_{em} 445 nm).

TABLE I

FLUORESCENCE WAVELENGTHS OF AMINO COMPOUNDS LABELLED WITH PHISYL-Cl AND M-PHISYL-Cl

Compound	*Phisyl-labelled*		*M-Phisyl-labelled*	
	λ_{ex} *(nm)*	λ_{em} *(nm)*	λ_{ex} *(nm)*	λ_{em} *(nm)*
n-Propylamine	298	407	303	435
Piperidine	297	404	304	431
Aniline	288	404	302	432
Alanine	295	422	298	445

The reactivities of Phisyl-Cl and M-Phisyl-Cl were examined with respect to amines (*n*-propylamine, piperidine and aniline) and an amino acid (alanine) by use of TLC. Reactions of the reagents with amino compounds mostly proceeded in the presence of sodium hydroxide to give the fluorescent derivatives, which were separated on a silica gel plate with solvent system S_1 for amines and S_2 for alanine. Fluorescence spectra of the derivatives of amino compounds with Phisyl-Cl and M-Phisyl-Cl, extracted with methanol from the fluorescent spots on the TLC plate, were measured. Fig. 2 shows the fluorescence spectra of the derivatives of alanine with the reagents. The fluorescence maxima of the extracted derivatives are given in Table I. The emission spectra of the derivatives in methanol–water (1:1) were shifted to a slightly longer wavelength (*ca.* 4 nm) than those in methanol. However, the fluorescence was not affected in the pH range 4–8 in methanol–water (1:1). The wavelengths of the emission maxima of Phisyl derivatives were about 25 nm shorter than those of M-Phisyl derivatives. Lysine and histidine, with more than two amino groups, and tyrosine, with a phenolic hydroxyl group, showed one fluorescent spot on the TLC plate with solvent system S_2 when treated with an excess of the reagents, although two or three fluorescent spots were observed with the reactions of equimolar amounts of reagents and amino acids.

The reaction conditions for Phisyl-Cl and M-Phisyl-Cl were studied with five amino acids (alanine, histidine, phenylalanine, proline and tyrosine, 0.25 m*M* each) by HPLC using an ERC-ODS-1161 column. Peaks of Phisyl and M-Phisyl derivatives of amino acids were clearly separated by the gradient system described under Experimental. The fluorescence maxima of each peak eluate were λ_{ex} 294–296 nm and λ_{em} 422–426 nm for Phisyl-amino acids, and λ_{ex} 296–299 nm and λ_{em} 444–445 nm for M-Phisyl-amino acids. The reaction temperature affected the derivatization reactions. At 25°C the reactions were incomplete and decomposition of Phisyl derivatives of histidine, proline, phenylalanine and tyrosine took place at 100°C. As shown in Fig. 3, maximum peak heights of Phisyl-amino acids were obtained at 50°C in 15 min. The Phisyl derivatives were stable for at least 1 week at room temperature. M-Phisyl-Cl gave similar results. However, the peaks of Phisyl derivatives were about five to twenty times higher than those of M-Phisyl derivatives. This suggests that Phisyl-Cl is suitable for the determination of small amounts of amino acids.

The fluorescence intensities (peak heights) of the Phisyl-amino acids (glycine, methionine and proline) were compared with those of DNS-amino acids under the same HPLC conditions (ERC-ODS-1161 column) as described above. The reactions

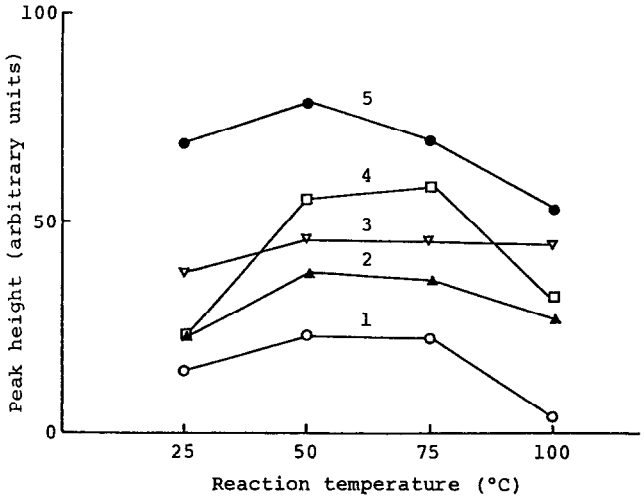


Fig. 3. Effect of reaction temperature on the peak heights of amino acids labelled with Phisyl-Cl. 1, Proline; 2, phenylalanine; 3, alanine; 4, histidine; 5, tyrosine.

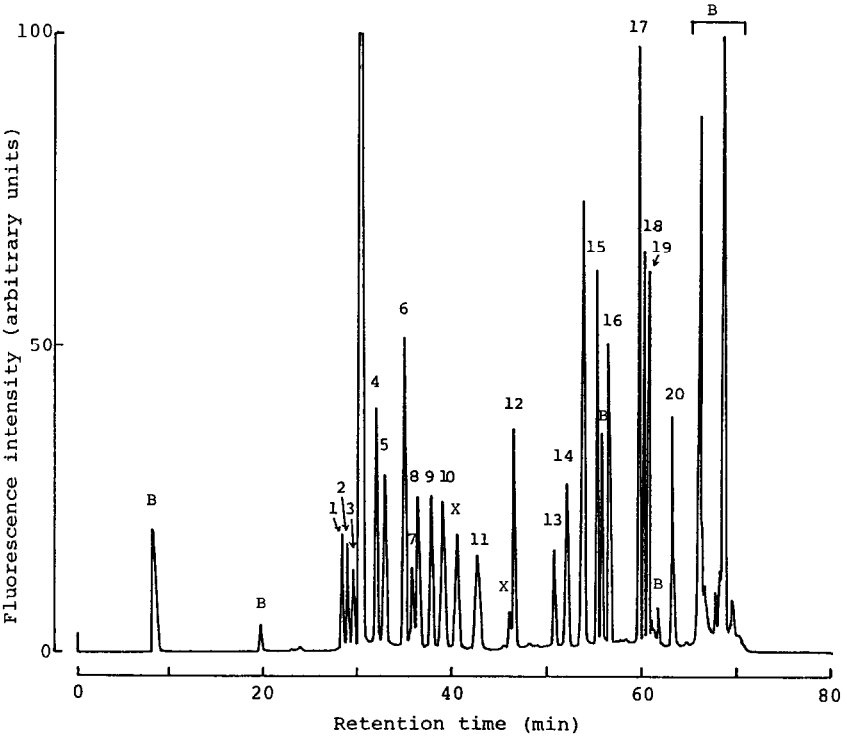


Fig. 4. Separation of amino acids labelled with Phisyl-Cl. A mixture of twenty amino acids (0.25 m*M* each) was treated according to the procedure (each corresponding to 14.2 pmol per injection). Peaks: 1 = cysteine; 2 = aspartic acid; 3 = glutamic acid; 4 = hydroxyproline; 5 = asparagine; 6 = serine; 7 = methionine; 8 = threonine; 9 = glycine; 10 = alanine; 11 = proline; 12 = valine; 13 = isoleucine; 14 = leucine; 15 = phenylalanine; 16 = cystine; 17 = ornithine; 18 = lysine; 19 = histidine; 20 = tyrosine; B = reagent blank; X = unidentified.

of DNS-Cl with amino acids were run according the method of Tapuhi *et al.*[9], and the fluorescence was detected at λ_{ex} 350 nm and λ_{em} 530 nm. The ratios of the peak heights of Phisyl-amino acids to those of DNS-amino acids were >3.

As an example, the separation of a reaction mixture of twenty amino acids with Phisyl-Cl is shown in Fig. 4. The twenty amino acids were successfully separated by using a YMC AM-303 packed column with stepwise elution as described under Experimental. Each peak was assigned by the overlapping method. The sensitivities (signal-to-noise ratio = 2) were less than 0.2 pmol per injection. The peak heights of Phisyl-amino acids were linear at amino acid concentrations up to at least 10 nmol per test-tube. The coefficients of variation (n = 8) for the twenty Phisyl-amino acids were in the range 3.5% (hydroxyproline)–8.2% (histidine) at a concentration of 1.25 nmol per test-tube.

Physil-Cl was stable for more than 1 year at room temperature and the acetone solution was also stable for 1 day in a stoppered vial.

The application of Phisyl-Cl to the simultaneous determination of amino acids in biological fluids is currently under investigation.

REFERENCES

1 O. Hinsberg, *Chem. Ber.*, 23 (1890) 2962.
2 O. Hinsberg, *Chem. Ber.*, 38 (1905) 906.
3 J.-Y. Lin and J.-Y. Chang, *Anal. Chem.*, 47 (1975) 1634.
4 J.-Y. Chang, R. Knecht and D. G. Braun, *Biochem. J.*, 203 (1981) 803.
5 K. Blau and G. S. King (Editors), *Handbook of Derivatives for Chromatography*, Heyden, London, 1977, p. 349.
6 Y. Tsuruta and K. Kohashi, *Anal. Chim. Acta*, 192 (1987) 309.
7 Y. Tsuruta, H. Tomida and K. Kohashi, *Anal. Sci.*, 4 (1988) 531.
8 T. Amano, T. Sakano and S. Mizukami, *Yakugaku Zasshi*, 85 (1965) 1042.
9 Y. Tapuhi, D. E. Schmidt, W. Lindner and B. L. Karger, *Anal. Biochem.*, 115 (1981) 123.