5-Amino-4-sulfanylphthalhydrazide as a chemiluminescence derivatization reagent for aromatic aldehydes in liquid chromatography

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Abstract

5-Amino-4-sulfanylphthalhydrazide (ASPH) was synthesized as a chemiluminescence derivatization reagent for aromatic aldehydes in liquid chromatography (LC). Benzaldehyde, 4-tolualdehyde, 4-chlorobenzaldehyde, 4-formylbenzoic acid, 4-hydroxybenzaldehyde and vanillin were used as model compounds to optimize the derivatization conditions. This reagent, ASPH, reacts selectively with aromatic aldehydes in the presence of sodium sulfite and disodium hydrogenphoshite in acidic medium at 100°C to give the corresponding highly chemiluminescent 2-arylbenzothiazole derivatives. The resulting derivatives generated intense chemiluminescence by reaction with hydrogen peroxide and potassium hexacyanoferrate(III) in alkaline solution. The ASPH derivatives of aromatic aldehydes were separated by reversed-phase liquid chromatography with isocratic elution, and detected chemiluminometrically after mixing with oxidizing agents. The detection limits (signal-to-noise ratio=3) for aromatic aldehydes are in the range 0.2–4.0 fmol for a 20-μl injection volume. Currently, the method is not effective for aliphatic aldehydes because of interfering LC peaks. © 2000 Elsevier Science B.V. All rights reserved.

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1. Introduction

Several aromatic 1-amino-2-sulfanyl compounds, i.e. 2-aminobenzenethiol [1], 2-amino-4,5-methyl-enedioxybenzenethiol [2], 2-amino-4,5-dimethoxy-benzenethiol [2] and 2,2′-dithiobis(1-amino-naphthalene) which is derived to 1-amino-2-sulf-anylnaphthalene when required for use [3], have been developed as fluorogenic reagents for liquid chromatographic (LC) determination of aromatic aldehydes. These reagents themselves are not fluorescent or weakly fluorescent, but their derivatives of aromatic aldehydes fluoresce intensely; this is based on the cyclization reaction between the 1-amino-2-sulfanyl moiety in the reagents and the formyl moiety in aromatic aldehydes to form the highly fluorescent 2-arylbenzothiazole or 2-arylnaphthothizole derivatives [1–3].

Recently, luminol-type chemiluminescence (CL) detection systems have been successfully introduced

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in LC analysis owing to their high sensitivity and wide dynamic range. We have developed some luminol-type CL derivatization reagents, i.e. 4,5-diaminophthalhydrazide (4,5-DPH) for α-keto acids [4], α-dicarbonyl compounds [5] and aromatic aldehydes [6], and 4-aminomethylphthalhydrazide for 5-hydroxyindoles [7]. These luminol-type reagents composed of a cyclic phthalhydrazide as a CL emitter and a reactive group against the target compounds. The CL intensities generated from luminol-type compounds are known to be partially dependent on the fluorescent intensities of the emitters. Thus, one of our strategies to develop a novel CL reagent is to introduce a reactive group, which itself is not fluorescent or weakly fluorescent but is derived with target compounds to yield the highly fluorescent products, to the emitter. As described above, aromatic 1-amino-2-sulfanyl compounds, which themselves are weakly fluorescent, react selectively with aromatic aldehydes to form the highly fluorescent benzothiazole derivatives.

The aim of this study is to develop a highly sensitive and selective luminol-type derivatization reagent for aromatic aldehydes. Based on the above strategy, we designed and synthesized a CL derivatization reagent, 5-amino-4-sulfanylphthalhydrazide (ASPH) (Fig. 1). ASPH has a 2-aminobenzenethiol moiety as a reaction site with aldehydes and a cyclic phthalhydrazide structure as a CL emission site. ASPH reacts selectively with aromatic aldehydes in the presence of disodium hydro-genphoshite and sodium sulfite in acidic medium, and the resulting derivatives produce intense CL by reaction with hydrogen peroxide in the presence of potassium hexacyanoferrate(III) in alkaline medium. In this work, we examined the optimum derivatization, CL reaction and LC conditions, and developed a method for the determination of aromatic aldehydes using ASPH, based on LC with CL detection. Furthermore, the structures of CL products, the ASPH derivatives of aromatic aldehydes, were examined by the use of electrospray ionization (ESI) mass spectrometry (MS) coupled with LC.

2. Experimental

2.1. Apparatus

Proton nuclear magnetic resonance (^{1}H-NMR) spectra were measured on a Jeol (Tokyo, Japan) JNM-GX-400 spectrometer at 400 MHz in [^{2}H$_6$]dimethylsulfoxide with tetramethylsilane as an internal standard. Splitting patterns were designated as follows: s, singlet; br, broad. Fast-atom bombardment (FAB) mass spectra were taken with a Jeol DX-300 spectrometer. A high-resolution FAB–MS measurement was made on a Jeol JMS-HX110 instrument. Uncorrected melting points were determined on a Gallenkamp (Loughborough, UK) melting-point apparatus.
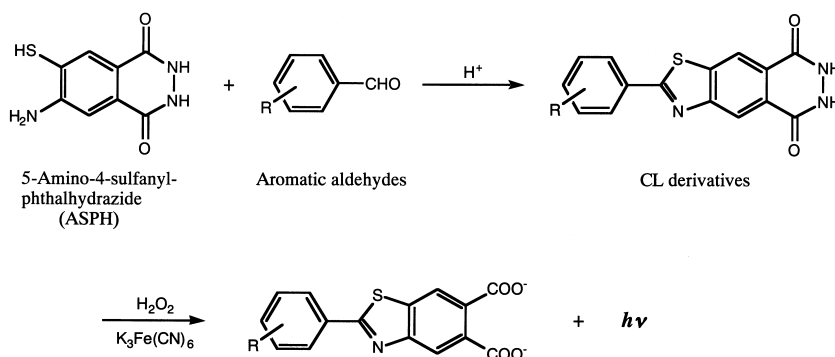

Fig. 1. Derivatization of aromatic aldehydes with ASPH and CL reaction of the derivatives.

2.2. Chemicals and solutions

All chemicals and solvents were of analytical-reagent grade, unless stated otherwise. Distilled water, purified with a Milli-QII system (Millipore, Milford, MA, USA), was used for all aqueous solutions. Hydrogen peroxide (31%, w/v) was purchased from Mitsubishi Gas Kagaku (Tokyo, Japan). Aromatic aldehydes and 4-aminophthalimide were obtained from Tokyo Chemical Industry (Tokyo, Japan), and used without further purification.

Stock solutions of aromatic aldehydes (10 mM) were prepared in N,N-dimethylformamide (DMF), stored at $-20°C$, and diluted further with water to the desired concentrations before use. ASPH (2.5 mM), hydrogen peroxide (15 mM) and potassium hexacyanoferrate(III) (10 mM) solutions were prepared in DMF, water and 1.5 M sodium hydroxide, respectively, and were used within 24 h.

2.3. Synthesis of ASPH

ASPH was synthesized via compound I from 4-aminophthalimide by the following method (as illustrated in Fig. 2).

2.3.1. Compound I

4-Aminophthalimide (6.5 g) and ammonium thiocyanate (6.1 g) were suspended in methanol (100 ml). To the suspension, a methanolic solution of bromine (15 g/50 ml) was added dropwise at 0°C. The solution was kept stirring for 2 h at 0°C and for 8 h at room temperature. The resulting precipitates were collected, dried under reduced pressure and recrystallized from DMF–benzene (1:2, v/v) to give compound I as a yellow crystalline powder (2.7 g, 30.8%). FAB–MS, m/z 220 [M+H]$^+$.

2.3.2. ASPH

Compound I (2.2 g) was dissolved in ethanol (100 ml) at 60°C. To the solution, hydrazine monohydrate (100 ml) was added and the mixture was refluxed for 72 h. The solvent was evaporated under reduced pressure and the residue was recrystallized from ethanol to afford ASPH as a pale yellow crystalline material (1.4 g, 66.9%), m.p. 119–121°C. ^{1}H-NMR, δ 3.31 (br, 2H+1H, hydrazino moiety and SH), 6.19 (br, 2H, NH$_2$), 7.03 (s, 1H, aromatic proton, H$_B$ in Fig. 2), 7.67 (s, 1H, aromatic proton, H$_A$ in Fig. 2). FAB–MS, m/z 210 ([M+H]$^+$, base peak). High-resolution FAB–MS, m/z 210.1257 (as calculated for $C_8H_8N_3SO_2$).

The total yield of the ASPH synthesis was ca. 20%. ASPH was stable in the crystalline state at room temperature in the dark for at least 3 months in a desiccator containing silica gel. ASPH was stable for at least 24 h even in the solution prepared in DMF. The ASPH solution, however, was used within 24 h after the preparation, because ASPH might be oxidized to the disulfide compound in the solution during the long storage.

2.4. Derivatization procedure

To a 100-μl aliquot of a test solution of aromatic aldehydes (10^{-5}–10^{-9} M) placed in a screw-capped tube (100×15 mm I.D.), 100 μl each of 3.0 M sulfuric acid, an aqueous solution containing 50 mM sodium sulfite and 0.5 M disodium hydrogenphoshite, and 2.5 mM ASPH solution were added
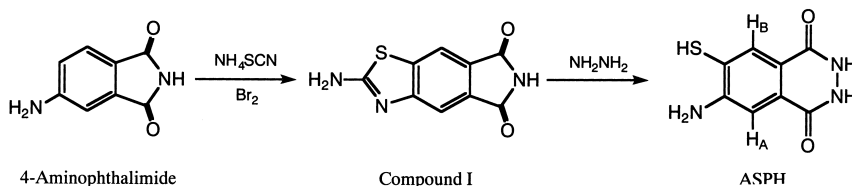

Fig. 2. Synthesis of ASPH.

successively. The tube was tightly closed and heated at 100°C for 20 min.

After the tube was cooled in ice–water, 200 µl of 3 M sodium hydroxide was added to neutralize the reaction mixture, and a 20-µl portion of the final solution was injected into the chromatograph. To prepare the reagent blank, 100 µl of water in place of the test solution was subjected to the same procedure.

2.5. LC system and its operation conditions

Two LC systems, LC–CL system for the determination of aromatic aldehydes and LC–MS system for the structural analysis of the ASPH derivatives, were operated. Fig. 3 shows schematic diagrams of the LC systems.

2.5.1. LC–CL system (Fig. 3A)

Chromatography was performed with a Jasco (Tokyo, Japan) PU-980 liquid chromatograph equipped with a Rheodyne (Cotati, CA, USA) Model 7125 syringe-loading sample injector valve (20-µl loop). The ASPH derivatives of aromatic aldehydes were separated on a reversed-phase column, COS-MOSIL 5C$_{18}$–MS (250×4.6 mm I.D., particle size 5 µm) (Nacalai Tesque, Kyoto, Japan) by isocratic elution. Two mobile phases were used: mobile phase A for the analysis of hydrophobic aldehydes was a mixture of 50 mM sodium phosphate buffer (pH 7.0) — acetonitrile (75:25, v/v) and mobile phase B for hydrophilic aldehydes was that of 50 mM sodium acetate buffer (pH 3.8) — acetonitrile (78:22, v/v). Both the mobile phases were delivered at a flow-rate of 1.0 ml/min. The column temperature was ambient (22±4°C).

The eluate from the LC column was first mixed with a 15 mM hydrogen peroxide solution by the first T-type mixing device, and then with 10 mM potassium hexacyanoferrate(III) solution in 1.5 M sodium hydroxide by the second T-type mixing device, delivered by two Jasco 880-PU LC pumps at flow-rates of 1.0 and 2.0 ml/min, respectively. A
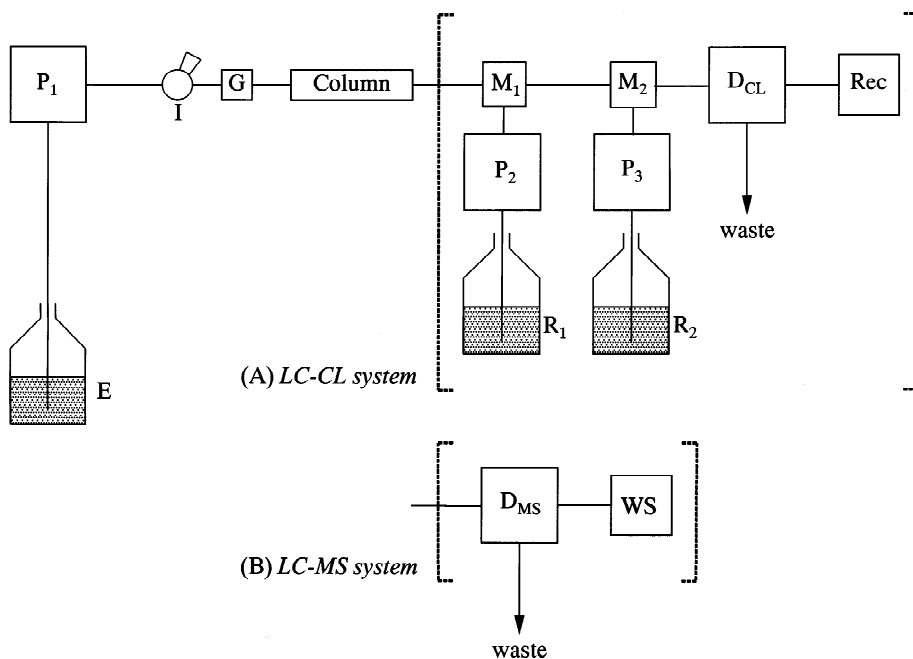

Fig. 3. Schematic flow diagram of (A) the LC–CL and (B) the LC–MS systems. P_1–P_3, LC pumps; I, injector valve; G, guard column; Column, analysis column; M_1 and M_2, mixing devices; D$_{CL}$, CL detector; Rec, integrator; D$_{MS}$, MS detector; WS, work station; E, mobile phase (1.0 ml/min); R_1, hydrogen peroxide solution (1.0 ml/min); R_2, potassium hexacyanoferrate(III) solution (2.0 ml/min).

Jasco CL-925 intelligent chemiluminescence detector monitored the CL intensity. Stainless-steel tubing (0.5 mm I.D.) was used for all the LC–CL lines. The length of the tubing between the second mixing device (M_2) and the detector cell was set at 5 cm.

2.5.2. LC–MS system (Fig. 3B)

General conditions were the same as described above, except for the mobile phase and the detector. As the mobile phase of LC–MS analysis, a mixture of 50 mM ammonium acetate buffer (pH 3.8) — acetonitrile (78:22, v/v) was used in place of mobile phase B. The effluent from the LC column was directly introduced to the LC–MS interface without adding any oxidizing reagent solutions (Fig. 3B).

A Finnigan (San Jose, CA, USA) LCQ ion trap mass spectrometer with an ESI interface was used. The ion source voltage and the temperature of the heated capillary were set at +4.5 kV and 275°C, respectively. Nitrogen gas was used as both sheath gas (85 p.s.i.) and auxiliary gas (20 p.s.i.; 1 p.s.i.= 68 g 4.76 Pa). The scan range was set at m/z 150–1000.

3. Results and discussion

Benzaldehyde (BA), 4-tolualdehyde (TA), 4-chlorobenzaldehyde (CB), 4-formylbenzoic acid (FB), 4-hydroxybenzaldehyde (HB) and vanillin (VA) were used as model compounds to establish the derivatization, LC separation and CL reaction conditions.

3.1. LC conditions for CL system

Not all the aromatic aldehydes examined could be separated well with an isocratic elution, because the aldehydes were so differed in polarities. Therefore, the six aldehydes were divided into two groups: one is hydrophobic aldehydes (BA, TA and CB; group A) and the other is hydrophilic aldehydes (FB, HB and VA; group B), and their separation conditions were optimized independently.

Good separations of the ASPH derivatives in each group were achieved under the different LC con-
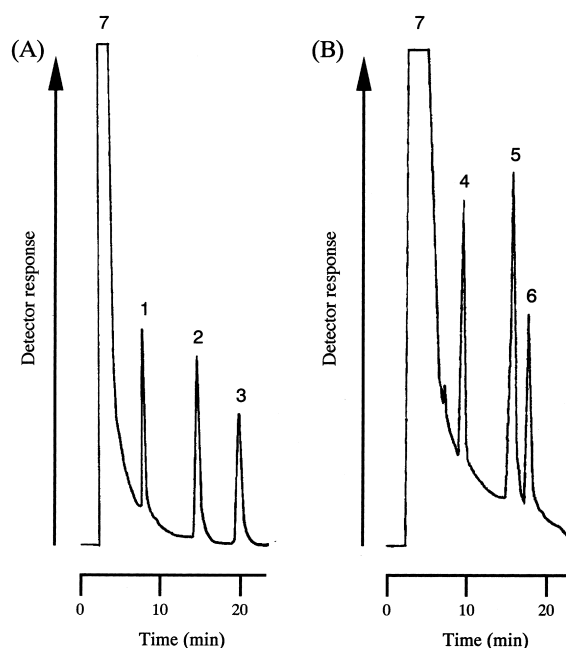

Fig. 4. Chromatograms of the ASPH derivatives of aromatic aldehydes. Chromatogram (A) was obtained from the hydrophobic aldehydes with mobile phase A and (B) was from the hydrophilic aldehydes with mobile phase B. Mobile phases: see Experimental. Peaks and amounts (fmol per injection volume): 1, BA (500); 2, TA (500); 3, CB (500); 4, FB (500); 5, HB (50); 6, VA (250); 7, reagent blank.

ditions as described in Experimental. Typical chromatograms obtained with the standard mixtures of the aromatic aldehydes in groups A and B are shown in Fig. 4A and B, respectively. The ASPH derivatives of the aldehydes in the two groups were all successfully separated within 25 min, and each aldehyde gave a single peak in the chromatograms.

3.2. Derivatization of aromatic aldehydes with ASPH

The derivatization reaction between aromatic aldehydes and ASPH proceeded efficiently in DMF and methanol. On the other hand, acetone, acetonitrile and dimethylsulfoxide gave less intense peaks (about 10–40% of those obtained in DMF). Because ASPH was found to dissolve easily in DMF, it was used for the preparation of the reagent solution. Maximum

and constant peak heights were obtained with 10–50% (v/v) DMF in the reaction mixture. DMF at concentration of 25% (v/v) was selected, which corresponds to 100% (v/v) DMF in the ASPH solution. The concentration of ASPH solution of 2.0–5.0 mM in the reagent solution provided almost maximum and constant peak heights; a 2.5 mM ASPH solution dissolved in DMF was adopted for the preparation of the reagent solution.

The cyclization reaction between aromatic 1-amino-2-sulfanyl compounds and the formyl group proceeded under strongly acidic conditions (nitric acid, perchloric acid and sulfuric acid) [2,3]. The maximum reaction rate was attained at 3.0–6.0 M sulfuric acid, and other acids could not give the results better than that of sulfuric acid; 3.0 M sulfuric acid was recommended in the procedure. Sodium sulfite and disodium hydrogenphoshite accelerated the derivatization reaction of aldehydes with aromatic 1-amino-2-sulfanyl compounds [2,3]. They also accelerated the ASPH reaction; a mixture of 50 mM sodium sulfite and 0.5 M disodium hydrogenphoshite yielded the highest CL intensity. In the absence of these two salts, the peak heights were less than 20% of those obtained under the recommended conditions.

The derivatization reaction occurred at a temperature above 40°C; higher temperature allowed the CL

to develop more rapidly. However, the temperatures higher than 120°C caused reduction of the CL intensity, probably due to the decomposition of the produced ASPH derivatives. For all the aldehydes examined, the CL intensities reached maxima after heating at 100°C for 15 min, and the resulting CL derivatives were stable at 100°C for at least further 60 min. Heating at 100°C for 20 min was employed for the reproducible results.

The ASPH derivatives of aromatic aldehydes in the final mixture were stable, and still gave constant CL intensities after standing for at least 8 h in daylight and for 48 h at 4°C in the dark.

3.3. CL reaction of the ASPH derivatives

The optimum conditions for the CL reaction of the ASPH derivatives of aromatic aldehydes were examined by setting the flow-rates of the solutions of hydrogen peroxide and potassium hexacyanoferrate(III) at 1.0 and 2.0 ml/min, respectively.

The CL intensities of the ASPH derivatives were greatly influenced by the concentrations of hydrogen peroxide, potassium hexacyanoferrate(III) and sodium hydroxide solutions. The concentrations of these reagent solutions were varied one at a time to establish the maximum obtainable intensity. Based on these experiments (Fig. 5), 15 mM hydrogen
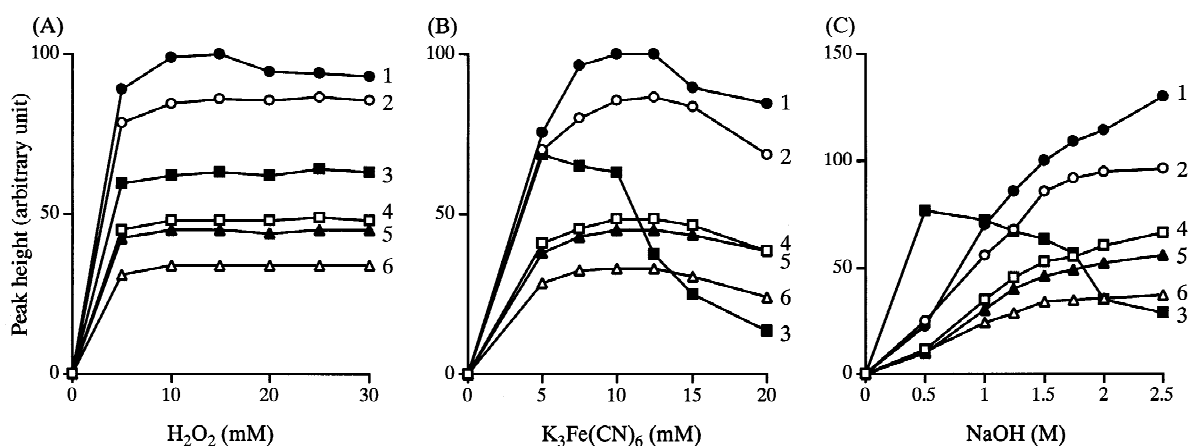

Fig. 5. Effects of the concentrations of (A) hydrogen peroxide, (B) potassium hexacyanoferrate(III) and (C) sodium hydroxide on the CL peak heights. Curves: 1, HB; 2, FB; 3, VA; 4, BA; 5, TA; 6, CB.
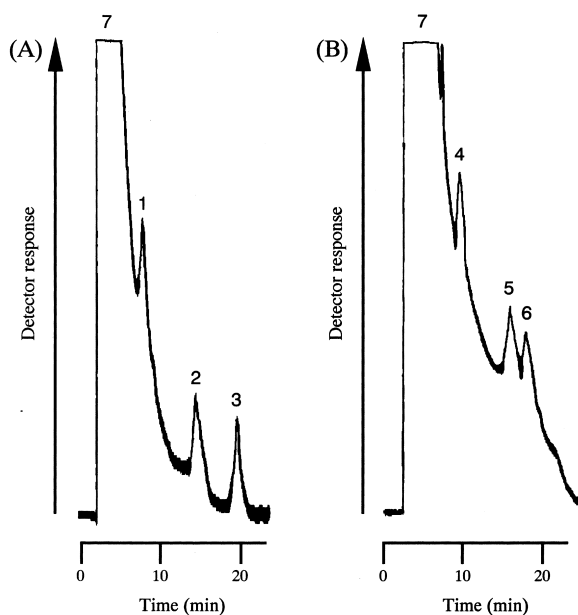
Fig. 6. Chromatograms of the ASPH derivatives of aromatic aldehydes. 100 times diluted sample solutions than that of Fig. 4 were treated according to the described procedure. Peaks and amounts: see Fig. 4.

peroxide, 10 mM potassium hexacyanoferrate(III) and 1.5 M sodium hydroxide were selected. The reason why the optimum conditions of VA were different from other aldehydes has remained unknown.

The CL of ASPH derivatives is generated immediately after mixing the effluent from the column and the oxidizing reagent solutions. Therefore, the length of tubing between the second mixing device (M_2 in Fig. 3A) and the detector has a great influence to the CL responses. The peak heights for all aldehydes and the reagent blank were increased with shortening the length of the tubing. On the other hand, the length did not affect the background levels. Therefore, a 5-cm tubing (0.5 mm I.D.) was utilized as the shortest practicably useful length. This indicates that the CL reaction of the ASPH derivatives proceeds very rapidly (the time lag between the second mixing device and the cell of CL detector was approximately 0.2 s), and is complete within a short period.

On the other hand, pH of buffer in the mobile phases did not affect the optimum CL reaction conditions.

Table 1
Retention times and detection limits for the ASPH derivatives of aromatic aldehydes

Aromatic aldehyde	Mobile phase[a]	Retention time (min)	Detection limit[b] (fmol)
Benzaldehyde	A	8.1	3.6
Piperonal	A	8.3	0.12
4-Methoxybenzaldehyde	A	9.5	0.33
2-Chlorobenzaldehyde	A	12.2	6.5
4-Tolualdehyde	A	14.9	1.9
4-(N,N-Dimethylamino)benzaldehyde	A	15.7	0.02
4-Chlorobenzaldehyde	A	20.1	1.0
4-Bromobenzaldehyde	A	25.0	1.1
3,4-Dihydroxybenzaldehyde	B	9.3	0.14
4-Formylbenzoic acid	B	9.7	4.0
4-Nitrobenzaldehyde	B	11.6	0.56
4-Acetamidobenzaldehyde	B	14.9	0.18
4-Hydroxybenzaldehyde	B	16.2	0.17
Vanillin	B	18.3	1.4
2-Furaldehyde	B	20.3	1.5

[a] Mobile phases: see Experimental.

[b] Defined as the amount in the injection volume (20 μl) giving a signal-to-noise ratio of 3.

3.4. Calibration graph, precision and detection limits

The relationships between the amounts of individual aromatic aldehydes and the peak heights were linear up to at least 6.7 pmol per 20 μl injection volume; the linear correlation coefficients were more than 0.999 ($n = 3$) for all aldehydes. The between-day precision was established by repeated determinations ($n = 8$) using the mixtures of standard aldehydes (33 fmol and 3.3 pmol each per 20 μl injection volume); the relative standard deviations were within 3.8% and 1.6%, respectively, for all the aldehydes examined.

Fig. 6 shows the chromatograms obtained with the diluted sample solution. The detection limits (fmol per 20 μl injection volume, signal-to-noise ratio = 3) for the six aldehydes were as follows: 3.6 (BA), 1.9 (TA), 1.0 (CB), 4.0 (FB), 0.2 (HB) and 1.4 (VA). These sensitivities are at least 20 times higher than other fluorogenic aromatic 1-amino-2-sulfanyl compounds' methods [1–3] and about 2–100 times higher than the CL method with 4,5-DPH [6].

3.5. Reaction of other substances with ASPH
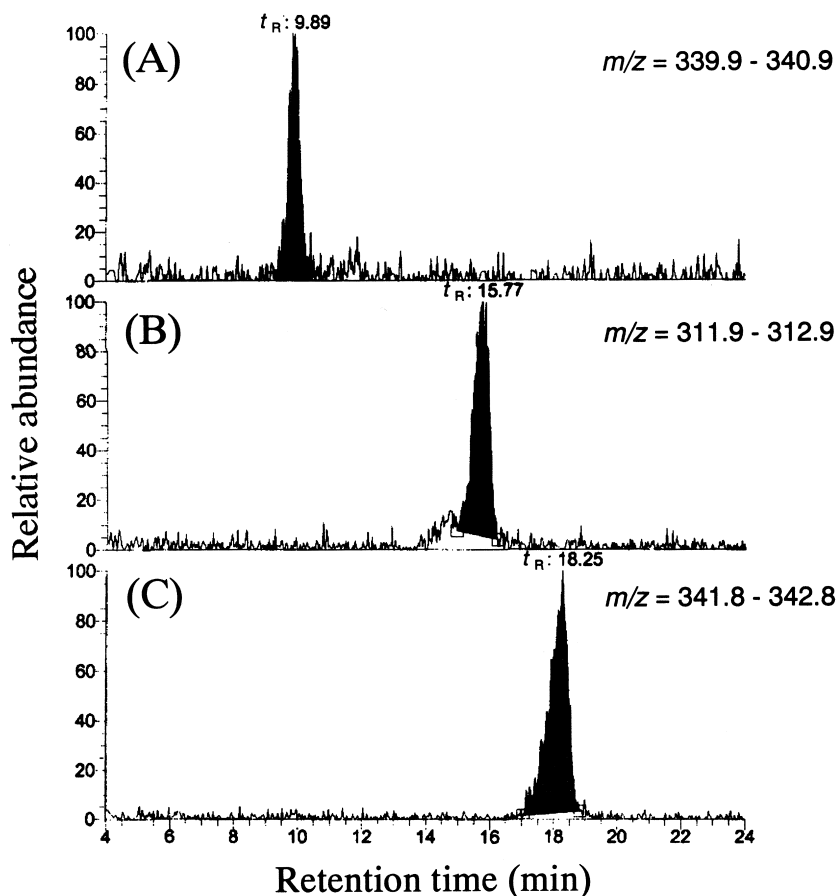
Some other aromatic aldehydes reacted with

Fig. 7. Selected ion chromatograms obtained from the ASPH derivatives of aromatic aldehydes (167 pmol each on column). Chromatograms: (A) FB, m/z 340.4; (B) HB, m/z 312.4; (C) VA, m/z 342.3. t_R means the retention time that gave the highest ion current.
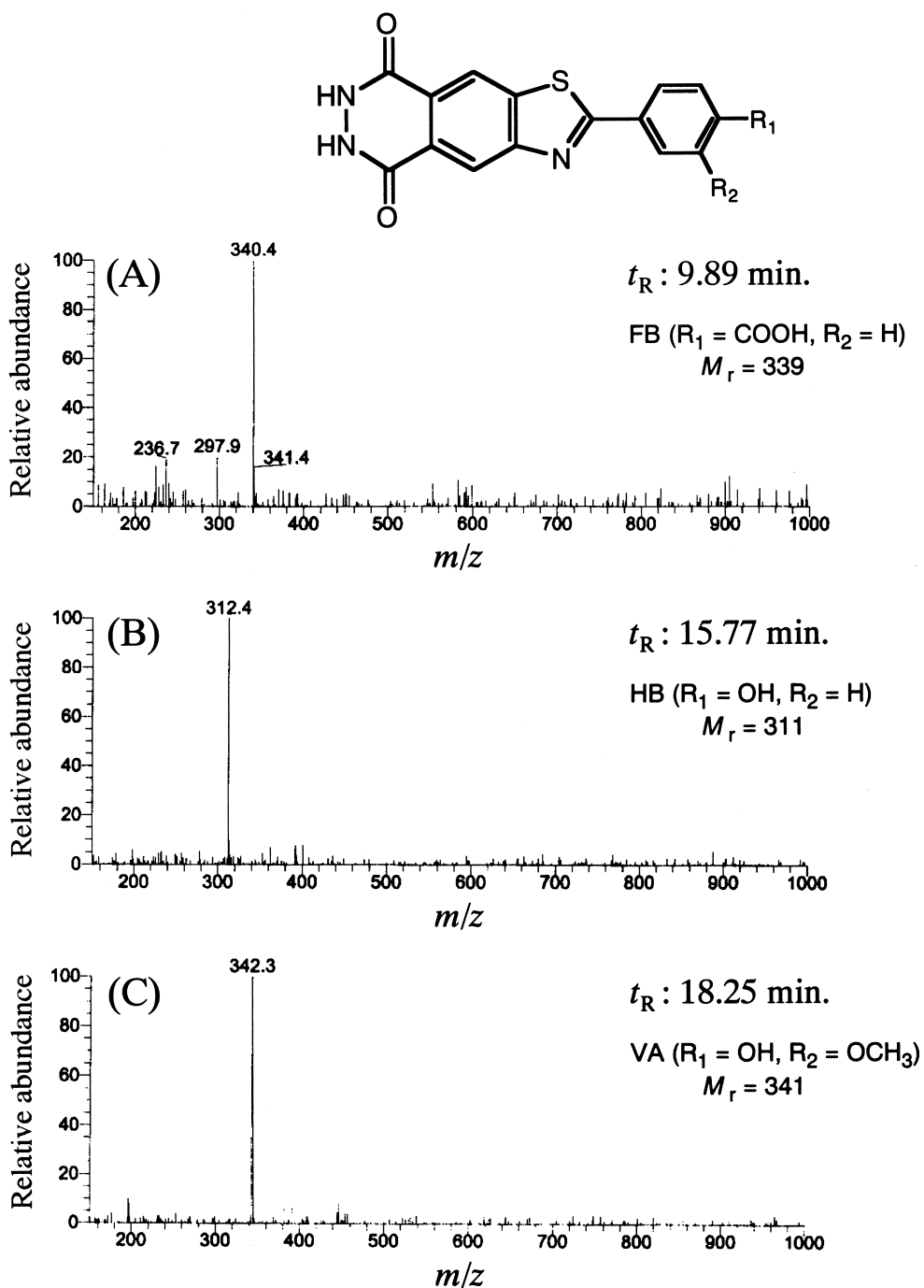
Fig. 8. LC–MS profile spectra of the ASPH derivatives of aromatic aldehydes (167 pmol each on column). Spectra and retention time (min): (A) FB (9.89); (B) HB (15.77); (C) VA (18.25).

ASPH under the proposed derivatization condition. Table 1 shows the mobile phase used for the separation, the retention times for the ASPH derivatives and the detection limits of aromatic aldehydes. Each aldehyde examined provided a single CL peak in the chromatogram. Highly sensitive detection in the range of 0.02–6.5 fmol could be achieved. The reason for the relatively low sensitivities of benzaldehyde, 2-chlorobenzaldehyde and 4-formylbenzoic acid might be due to the low reaction yields and/or fluorescence quantum yields of the CL products.

Aliphatic aldehydes (formaldehyde, acetaldehyde and *n*-decylaldehyde) gave CL peaks, but these peaks were overlapped with those for the reagent blank components under the selected LC conditions. Furthermore, the optimum derivatization conditions were different from those for aromatic aldehydes. Studies on the derivatization reaction and LC separation conditions for aliphatic aldehydes are now in progress. On the other hand, the following compounds of biological importance, at a concentration of 10 nmol/ml, afforded no peaks under the present conditions; the compounds tested were seventeen different L-α-amino acids, biogenic amines (ammonia, dopamine, epinephrine, histamine and serotonin), α-keto acids (α-ketoglutaric acid and phenylpyruvic acid), other acids (acetic acid, palmitic acid, oxalic acid, uric acid and L-ascorbic acid), sugars (D-glucose, D-fructose, D-galactose, D-ribose, *N*-acetyl-D-galactosamine, maltose and sucrose), nucleic acid bases (adenine, guanine, thymine, cytocine and uracil) and other compounds (cholesterol, creatine, creatinine and urea). These observations suggest that the present CL derivatization method is usefully selective for aromatic aldehydes.

3.6. CL products in the determination of aromatic aldehydes

The structures of the reaction products between aldehydes and ASPH were investigated by LC–MS. A standard solution of FB, HB and VA was treated as in the derivatization procedure and the resulting ASPH derivatives were analyzed by LC–MS on the

ion trap, in which volatile salt (ammonium acetate) was used instead of unvolatile salt (sodium acetate) in mobile phase B. The ASPH derivatives of the aldehydes gave CL peaks at almost the same retention times by the use of ammonium acetate buffer, but the changing of buffer caused the decreasing the CL intensities and the increasing the background level.

The ion chromatograms and the mass spectra obtained with the ASPH derivatives are shown in Figs. 7 and 8, respectively. At the selected ion monitoring on the positive ionization mode, each derivative was detected as a protonated molecular ion $[M+H]^+$ at almost the same retention times as those in LC–CL system. Furthermore, each $[M+H]^+$ of the ASPH derivatives were assigned as the corresponded base peaks in the mass spectra of each peak fraction. These results indicate that the ASPH derivatives of aromatic aldehydes by the present method were the corresponding 2-arylbenzothiazole structure as well as the reaction products of other aromatic 1-amino-2-sulfanyl compounds [1–3].

4. Conclusions

ASPH was developed as a novel luminol-type CL derivatization reagent for the determination of aromatic aldehydes in LC. Furthermore, it was confirmed that the reaction products of aromatic aldehydes with ASPH was the corresponding 2-arylbenzothiazole structure and the derivatives generated the very intense CL. For aliphatic aldehydes, the optimum derivatization conditions were different, and the CL peaks were overlapped with peaks from the blank components.

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