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Separation and determination of *n*-alkylamines and histamine by capillary zone electrophoresis using salicylaldehyde-5-sulfonate as a derivatizing reagent

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Abstract

Sodium salicylaldehyde-5-sulfonate (SAS) was investigated as a derivatizing reagent for the separation and determination of primary amines by capillary zone electrophoresis (CZE). The amines were derivatized with SAS to the corresponding Schiff bases before their determination. Optimal conditions for the formation reactions of the Schiff bases and the CZE analysis were investigated in details. The Schiff bases were formed almost completely within 9 min in 40%(v/v) ethanol solution at 40°C. A migrating solution containing 40%(v/v) ethanol and 20 m*M* phosphate buffer (pH 7.8) was found to be preferable for the stability of the Schiff bases. Eight kinds of *n*-alkylamines were derivatized with SAS under the optimal conditions and the derivatives were successfully separated by a CZE analysis. The proposed method allows simultaneous, sensitive and sufficiently precise determination of the *n*-alkylamines with the alkyl chain length from 3 to 12 of methylene groups. The derivatization process with SAS was successfully applied to the detection of histamine at a very low level. The detection limit was $2.5 \cdot 10^{-6}$ *M*, and it was improved in the order of 8 times compared with the CZE analysis without derivatization.

Keywords: Derivatization, electrophoresis; Alkylamines; Amines; Histamine; Salicylaldehydesulfonate; Sulfonates

1. Introduction

Separation and determination of amines are very important in pharmaceutical science. However, simultaneous analytical methods for mixed samples containing various kinds of amines have been reported since alkylamines show neither natural UV–visible absorption nor fluorescence. In precolumn methods, *n*-alkylamines were derivatized before injection and were separated and determined by high-performance liquid chromatography [1–3]. In the precolumn and the post column derivatization of amino-groups, such derivatizing reagents as 1-fluoro-2,4-dinitrobenzene [1], 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole [2], and salicylaldehyde diphenylboron chelate [3] have been widely used to improve the chromatographic determination of primary amines. The derivatized products provide large molar absorptivity in UV–visible spectral regions and/or sensitive fluorescence. Micellar electrokinetic chromatography and capillary gas chromatography have been applied to the analysis of biogenic amines after the derivatization of the amines by 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate [4] and trifluoroacetylacetone [5]. Indirect detection methods based

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on capillary elecrophoresis separation were also used for the determination of the biogenic amines in wine [6] and polyamines in tumor cells [7,8].

Capillary zone electrophoresis (CZE) is a promising method for the separation and determination of charged species. However, the analysis of amines by CZE has been less investigated, because of the lack of suitable derivatizing reagents.

In several studies dealing with water-soluble Schiff bases and metal complexes, salicylaldehyde-5-sulfonate (SAS) exhibited good reactivity with amines [9,10], where the synthesis of the metal complexes was carried out with diamines in the presence of metal ions. The main advantages in this synthesis were: the reaction simplicity and the formation of charged chelate complexes as a result of the reaction between the charged Shiff bases and metal ions.

In the present work, SAS has been examined as a derivatizing agent first for *n*-alkylamines, and second for histamine, which is a biogenic amine possessing primary and secondary amine groups in its structure. It has been well known as an endogenous substance [19] that is widely disturbed in body tissues and can, on release from its storage sites, exert a variety of pharmacological effects of varying intensity. In addition to its toxicological risk, it is found in a great variety of protein-rich foods, fermented foods and beverages, it can be an indicator of their food quality.

Derivatizing conditions and CZE conditions for the analysis of eight kinds of *n*-alkylamines (alkyl chain length: 3–12 of carbons) were investigated for a simultaneous and sensitive determination of these products. Derivatization of histamine was also developed and a sensitive CZE analysis was proposed.

2. Experimental

2.1. Instruments

Electrophoretic separations were carried out using an Applied Biosystems 270 A-HT (Foster City, USA) capillary electrophoresis system, equipped with a UV detector. A fused-silica capillary (GL Sciences, Tokyo) of 50 μm I.D. and (50+22 cm) length was used. In the aim of quick separation, a shorter capillary (30+22 cm) was also used. All the electropherograms were recorded and analysed by a Hitachi D-2500 Chromato-Integrator.

For the determination of histamine, a Hewlett-Packard ^{3D}CE capillary electrophoresis system with a diode–array detector was used. A fused-silica capillary of 75 μm I.D. (Hewlett-Packard) was attached to the system. The total length of the capillary was 50.5 cm with a 42-cm effective length from the injection point to the detector window.

2.2. Chemicals and solutions

Several *n*-alkylamines and histamine were purchased from Tokyo Kasei Kogyo (Tokyo, Japan) and used without further purification. For the SAS synthesis, a reagent-grade salicylaldehyde (Wako, Osaka, Japan) was used. Aniline, sulfuric acid and acetic acid were used as received from Wako. Copper chloride was also obtained from Wako, and was dissolved in water to give a 10^{-2} *M* solution. An aliquot of 0.5 ml of salicylaldehyde was mixed with 15 ml of triethanolamine and diluted to 100 ml with water or with 10^{-2} *M* surfactant solution tested. The salicylaldehyde solution thus prepared was stocked for the further derivatizations. Phosphate buffer solution composed of Na_2HPO_4–KH_2PO_4 (0.1 *M*) was used in the pH range of 7.0–8.0; borate buffers (0.1 *M*) of $Na_2B_4O_7 \cdot 10H_2O$–HCl or –NaOH were used in the pH ranges of 8.0–10.5. All of these buffer components were purchased from Wako. Organic solvents, ethanol and acetone, of a reagent grade were used as received, except for the CZE separation; analytical grade were added in the migrating solution. Deionized and distilled water was used throughout the experiments.

2.3. Synthesis of sodium salicylaldehyde-5-sulfonate monohydrate

Prior to the SAS synthesis, *n*-phenylsalideneimine-5-sulfonic acid was prepared with some modifications of the literature [11]: 35 g of *n*-phenylsalicylaldimine (prepared as previously described [12]) were added to 95 ml of concentrated sulfuric acid, and the mixture was heated at 100°C for 2.5 h under vigorous stirring. After heating, the solution was stepwisely poured into an equal volume

of ice-water while continuously stirring. Yellow precipitate was obtained; it was recrystallized in diluted sulfuric acid. A yellow solid thus obtained was then filtered off, washed with small quantity of ice-water; and then washed with ethanol and acetone. The product was dried under air at ambient temperature (yield is 71%).

A 25-g amount of *n*-phenylsalideneimine-5-sulfonic acid was dissolved in an aqueous solution of Na_2CO_3 (13.8 g/125 ml) and boiled vigorously in an open flask for 2.5 h with periodic replenishment of the evaporated water. Acetic acid was added slowly to 100 ml of the cooled solution until the pH reached 5. An equal volume of ethanol was added and the mixture was cooled to 0°C. The precipitate, sodium salicylaldehyde-5-sulfonate, was obtained; it was filtered off, washed with ethanol, and dried at ambient temperature. The yield of the synthesis is equal to 40.5%.

After every step of the synthesis, the obtained products were identified and confirmed by NMR spectrometry.

2.4. Derivatization procedure of the n-alkylamines for fundamental investigation

The synthesized SAS (0.002 mol) was dissolved in 80 ml alcoholic water (ethanol: 20 ml) in a 100 ml volumetric flask. Then, 0.0005 mol of *n*-alkylamine in 20 ml ethanol was added in the flask, and the mixture was kept at 40°C with continuous stirring for 9 min. Each stock solution thus prepared contained $5 \cdot 10^{-3}$ *M* of sodium *n*-alkylsalicylaldemine-5-sulfonate.

2.5. Procedure for the electrophoretic separation of the n-alkylamines for fundamental investigation

The separation of eight kinds of *n*-alkylamines Schiff bases was performed with a migrating solution containing 20 m*M* phosphate buffer (pH 7.8) and 40%(v/v) ethanol. Optimum electrophoretic conditions for favorable separation and sensitive determination were found to be 30 kV applied voltage, 35°C capillary temperature, 250 nm detection wavelength and 3 s injection period by applying a pressure of 5 mmHg (1 mmHg=133.322 Pa). The standard sample solution contained the sodium *n*-alkylsalicylaldemine-5-sulfonates at a concentration of $5 \cdot 10^{-4}$ *M*, and the other composition was the same as the migrating solution.

2.6. Derivatization procedure and electrophoretic determination of the histamine

A sample solution was prepared by mixing in a 10 ml flask, 0.5 ml of histamine solution ($5 \cdot 10^{-3}$ *M*) and 1 ml of SAS solution ($5 \cdot 10^{-2}$ *M*), 2 ml ethanol and 1 ml borate buffer (pH 10.5) were added, then the mixture was diluted to 10 ml with water, put in an ultrasonic bath for 2 min and left for few minutes allowing the reaction completes.

The migrating solution used was composed of 10 m*M* borate buffer (pH 10.5) and 20% ethanol. The prepared sample solution was injected from an anodic end by applying pressure (50 mbar) for 5 s. A voltage of 30 kV was then applied, and the analytes were separated and detected at 220 nm. The temperature of both the capillary and the vials was maintained at 25°C throughout the experiments.

3. Results and discussion

3.1. Investigation of the formation of Schiff bases as derivatives of amines with salicylaldehyde

Salicylaldehydes can react with primary and secondary amines to form corresponding Schiff bases through an easy procedure [13], which needs stirring and heating (40–70°C). Moreover, Schiff bases derived from formylsalicylic acid and amines form stable metal complexes [14,15]. In this study, salicylaldehyde was examined as a derivatizing reagent for amines. When copper chloride was added to the reaction of *n*-hexylamine with salicylaldehyde in the presence of triethanolamine at pH 7.5, a green product precipitated within 5 min, indicating the formation of Schiff base–copper complex.

A micellar system can promote chemical reactions by concentrating the reactants in a small volume of micelle [16], and the changes in the dielectric constant of the matrix and in the viscosity of the micelle also result in a higher reaction probability [17,18]. Another interesting effect of the micelles is their wide variety of solubilization sites/functional

groups, which make solutes more soluble than homogeneous aqueous media. In this study, several surfactants with different surface activities have been investigated in order to solubilize the Schiff base of salicylaldehyde and to increase the formation rate of the Schiff base. Brij 58 as a nonionic surfactant and sodium dodecyl sulfate and sodium octyl sulfate as anionic surfactants, dissolved the Schiff base. The Schiff bases formed between salicylaldehyde and the primary amines, such as *n*-propylamine and *n*-hexylamine, were characterized by micellar electrokinetic chromatography. Although the results allow the detection of the corresponding signals of the Schiff bases, the resolution was not sufficient because of the short elution window and strong hydrophobic character of the Schiff bases; they migrated almost along with the micelle. Such behavior was more serious with the amines having longer alkyl chains. Aiming at getting wider elution windows, mixed micelles (10 m*M* sodium dodecylsulfate+Brij 58 at different concentrations) were investigated. However, the great partition of the Schiff base to the micelle allowed neither good selectivity nor fast reaction rate.

3.2. Use of sodium salicylaldehyde-5-sulfonate as a derivatizing reagent

On the investigation of Schiff bases, the authors noticed water-soluble Schiff base, an anionic one, and utilized it by CZE analysis. A copper complex of the water-soluble Schiff base was synthesized at first on heating at 60°C in a thermostated bath with continuously stirring a mixture of 0.0011 mol of SAS, 0.0021 mol of $CuCl_2$ and 0.0011 mol of *n*-hexylamine in a 80% ethanol solution, and was synthesized using a mixture of four times the amount of the stoichiometric quantity of the alkylamine (0.0042 mol) at the second time. The solution of the copper complex with *n*-hexylamine Schiff base was operated by CZE after appropriate dilution in different pH buffers containing 3% ethanol. Variations in pH buffer used for diluting the sample solution and the migrating solution were investigated. With an acidic buffer, no peak corresponding to the Schiff base was observed. It should be attributed that the protonation of the amines caused less possibility of reaction with SAS at acidic pH conditions. At higher alkaline pH conditions, the hydrolysis of SAS is prominent, together with the fact that the derivatization is incomplete. From these results, it is preferable to derivatize the amines to the Schiff bases at weakly alkaline pH conditions.

3.3. Selection of reaction solutions and migrating solutions

The pH of the migrating solution was varied with the value between 7.0 and 9.8. Maximum peak area for *n*-hexylamine with better reproducibility was observed in the pH range between 7.0 and 8.6. Although the Schiff base presents better stability at the pH range, it was dramatically degradated after 5 h even when running with a migrating solution containing 1 m*M* $CuCl_2$. Copper ions could contribute to the stability of the Schiff base. However, it can precipitate in the migrating solution and is not adequate for the present aim. Based on these results, the copper ion was found to be less suitable for the CZE separation and it was not used in further experiments. No usage of heavy metal ions requested more strict conditions.

Previous papers [9–12] described that some complexes of SAS Schiff base were obtained by condensation in ethanol medium. Increasing ethanol percentage in the sample solution would improve the stability of the complex, while the addition of ethanol causes extension of the migration time upon increasing the amount. The Schiff bases derived with *n*-propylamine (PROP-SAS), *n*-heptylamine (HEP-SAS) and *n*-dodecylamine (DOD-SAS) were prepared as cited in the previous section. The stability of the Schiff bases was monitored by CZE using 20 m*M* phosphate buffer (pH 7.0) mixed with 20, 30, or 40%(v/v) ethanol as sample solution and migrating solution. Fig. 1 shows the effect of the ethanol percentage on the stability of the Schiff bases. Ethanol added at the amount of 40% (v/v) provided good reproducibility of the signals. Signals corresponding to excess SAS did not emerge within 60 min, which indicates that the solutes did not reach the detector. Suppressed speed of the electroosmotic flow by the addition of ethanol would be attributed to the reason.

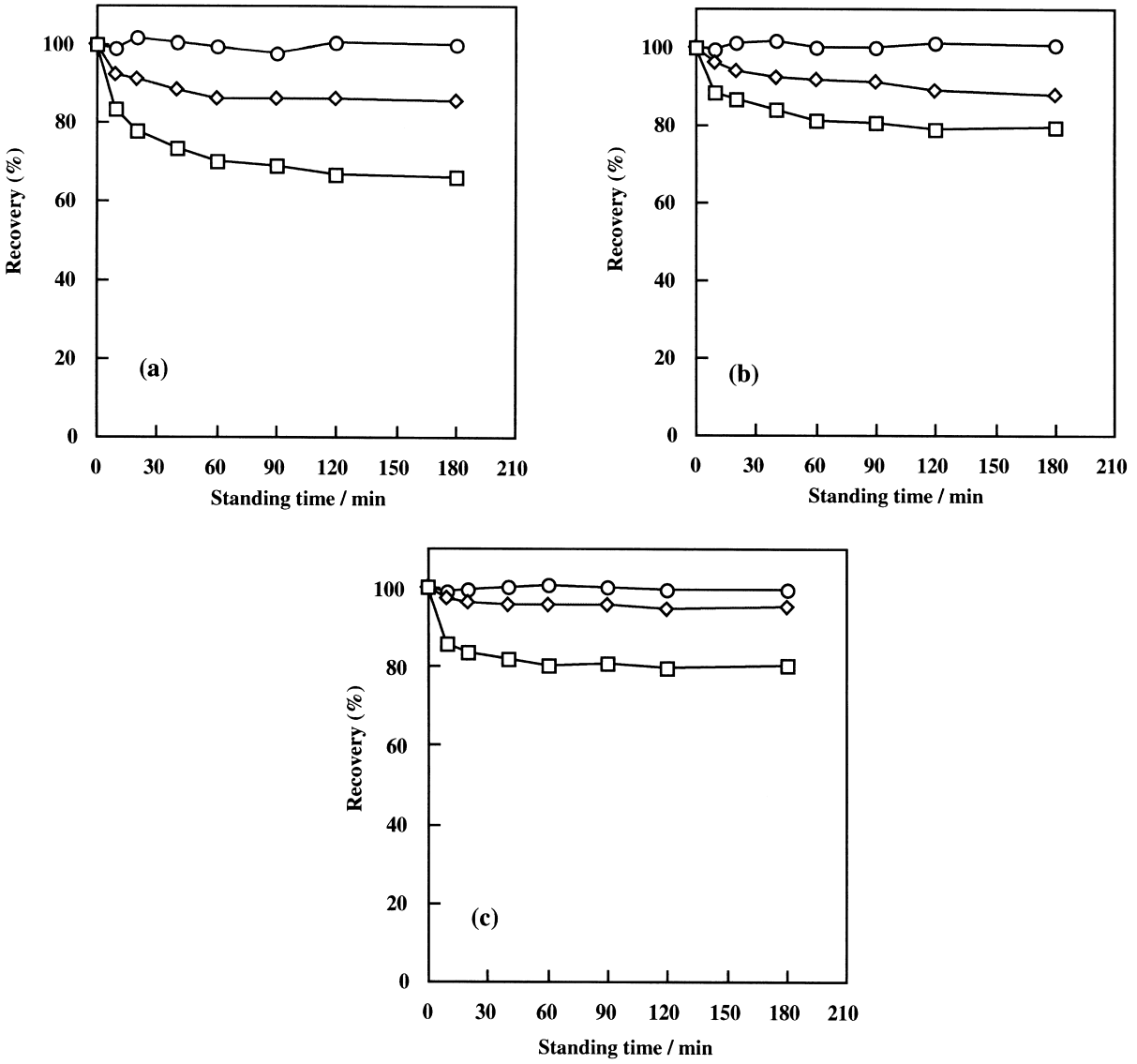

Fig. 1. Influence of ethanol percentage on the stability of the *n*-alkylsalicylaldemine-5-sulfonate after derivatization. Migrating solution: 20 m*M* phosphate buffer (pH 7.0) + ethanol. CE conditions: capillary temperature, 35°C; applied voltage, 30 kV; detection wavelength, 250 nm; injection period, 3s. Amines: (a), *n*-propylamine; (b), *n*-heptylamine; (c), *n*-dodecylamine. Ethanol percentage: □, 20% (v/v); ◇,30% (v/v); ○, 40% (v/v).

3.4. Temperature effects on the derivatization of the n-alkylamines with SAS

Primary amines as analytes of interest were mixed with SAS in 40% ethanol solution, since the Schiff bases show preferable stability at this condition. Derivatization of *n*-propylamine, *n*-heptylamine, and *n*-dodecylamine was examined at 40 and 60°C. A CZE measurement was used to quantify the formed Schiff bases by diluting the reacting solution in the optimized electrolyte: 20 m*M* phosphate buffer (pH 7.8) with 40%(v/v) ethanol. The average of the peak area of three injections of the same solution was considered to determine the amount of the Schiff

base. Fig. 2a and b show the relative peak area for each Schiff base corresponding to standing time at 40 and 60°C, respectively. Yield of the Schiff base reached the maximum within 9 min when derivatized at 40°C, while unexpected partial decomposition of all Schiff bases was observed at 60°C. From the results, derivatization at 40°C would provide more a reliable analytical method.

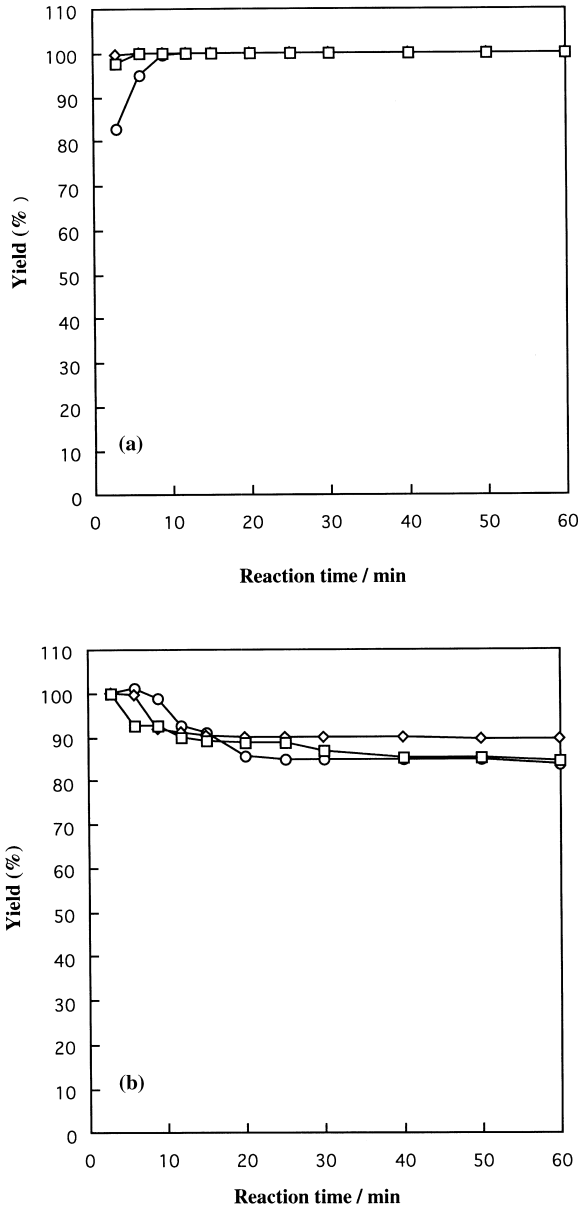

Fig. 2. Yield of the Schiff bases as a function of time. CZE signals were used for the quantification. CZE conditions are the same as in Fig. 1 except for the ethanol percentage; ethanol percentage: 40%(v/v). Reaction temperature: (a), 40°C; (b), 60°C. Amines: □, *n*-propylamine; ◇, *n*-heptylamine; ○, *n*-dodecylamine.

3.5. Separation and determination of the n-alkylamines as Schiff bases by CZE

Optimal conditions for stabilization of Schiff bases and the detection by CZE were obtained by running with 20 m*M* phosphate buffer (pH 7.0–8.6) containing 40%(v/v) ethanol. However, long migrating times for the Schiff bases up to 34 min was observed in the presence of 40%(v/v) ethanol, though good base line resolution for eight kinds of *n*-alkylamines was obtained with a (50+22) cm capillary. Increasing the pH condition from 7.0 to 8.6 has a small effect on the improvement of the shortening of analysis time. Beyond pH 8.6, no significant effect on the shortening of analysis time was obtained, whereas some part of the Schiff bases decomposed. Based on these results, the optimal migrating solution is composed of 20 m*M* phosphate buffer (pH 7.8) with 40%(v/v) ethanol.

The effective length of the capillary was reduced from 50 to 30 cm to shorten the analysis time. More sharp signals without any decrease in the resolution among all the Schiff bases were obtained within 13 min. A representative electropherogram is shown in Fig. 3. Migration order of the derivatives was from a long-alkyl chain to a short-alkyl one, which agrees with the principle of the CZE, and therefore the derivatization and the migration are successfully operated.

The reproducibility, linearity, and sensitivity of the proposed method were tested and the results are summarized in Table 1. The RSD values for the mobility were less than 2.2% for all analytes and the one for peak area were less than 2.5%, except for *n*-butylamine and *n*-decylamine. Calibration graphs are examined at the concentration ranges from 50 to 500 μ*M*. Correlation coefficients larger than 0.99 were obtained for all analytes. The limit of detection (LOD) based on the peak area were determined at a *S*/*N* ratio of three, where the injection volume was increased to 15 s; they were found to be in the range from 3.5 μ*M* for *n*-propylamine to 10.5 μ*M* for

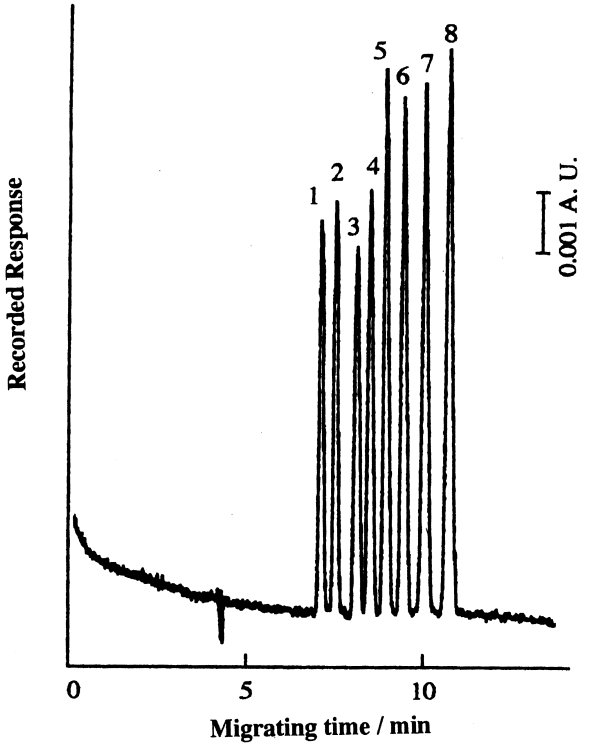

Fig. 3. Electropherogram for *n*-alkylamines after derivatization with SAS. Signals: 1, DOD-SAS; 2, DEC-SAS; 3, OCT-SAS; 4, HEP-SAS; 5, HEX-SAS; 6, AMYL-SAS; 7, BUT-SAS; 8, PROP-SAS. Migrating solution: 20 m*M* phosphate buffer (pH 7.8)+ 40%(v/v) ethanol. Sample solution: $5 \cdot 10^{-4}$ *M* of each derivative, 20 m*M* phosphate buffer (pH 7.8), 40%(v/v) ethanol. CE conditions: applied voltage, 30 kV; capillary temperature, 35°C; detection wavelength, 250 nm; injection period, 3 s.

n-dodecylamine. The resolution among the signals was almost identical when sample injection time was varied from 3 to 15 s.

3.6. Application of the derivatization with SAS to the analysis of histamine

As a diamine, histamine was partially derivatized when operated under the same pH conditions used for *n*-alkylamines, and two peaks corresponding to primary and secondary Schiff bases were observed. For a complete derivatization of both amine groups, SAS reagent was added at a concentration 20 times than that of histamine (0.25 m*M*) with increasing the pH condition at 10.5. As mentioned in the description of the derivatization procedure of histamine, the reaction occurs without heating and no peak corresponding to histamine was detected after a few minutes.

The great advantage on the application of the proposed method to histamine was a low level detection of the corresponding Schiff base compared with that of the histamine itself. Fig. 4 shows the electropherograms of histamine and histamine Schiff base at the concentration of 0.25 m*M* in the same analytical conditions. Moreover, the regression equations for both analytes were examined at 30, 50, 100, 250 and 500 μ*M*. The limit of detection was calculated as the concentration corresponding to three times its relative standard deviation. The injection period was increased to 12 s during this

Table 1
Reproducibility, linearity, and sensitivity for alkylamines by the proposed method

Amines	RSD (%)[a]		Correlation coefficient[b]	LOD (μ*M*)[c]
	Mobility (10^{-4} cm^2 V^{-1} s^{-1})	Peak area		
n-Propyl-	1.1	1.2	0.997	3.5
n-Butyl-	1.1	3.8	0.998	5.0
n-Amyl-	1.5	2.1	0.997	5.0
n-Hexyl-	1.2	2.0	0.998	6.5
n-Heptyl-	2.1	2.5	0.997	5.5
n-Octyl-	2.0	1.4	0.998	7.5
n-Decyl-	1.4	3.2	0.995	10.0
n-Dodecyl-	1.6	2.4	0.991	10.5

[a] Values analyzed with eight measurements of $5 \cdot 10^{-4}$ *M* alkylamines.
[b] Concentration range: 50–500 μ*M*.
[c] Values at $S/N=3$.

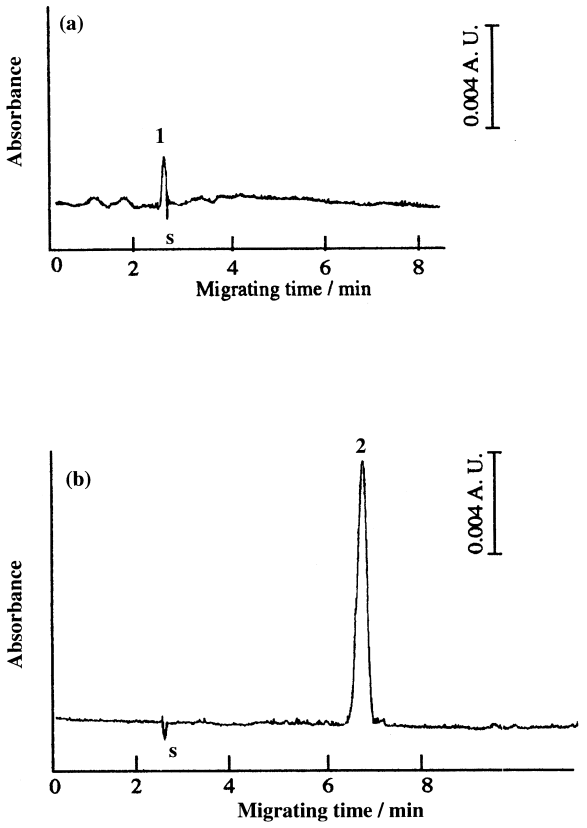

Fig. 4. Electropherograms for histamine before and after the derivatization. Migrating solution: 10 m*M* phosphate buffer (pH 10.5)+20%(v/v) ethanol. Sample solutions: (a), 250 μ*M* histamine; (b) 250 μ*M* derivatized histamine. CE conditions: applied voltage, 30 kV; capillary temperature, 25°C; detection wavelength, 220 nm; injection period, 5 s. Signals: 1, histamine; 2, histamine Schiff base; s, electroosmotic flow.

experiment. Table 2 presents the results obtained. This experiment supports the evaluation of satisfactory reliability of the present method and allows it to be indicated for the determination of histamine in commercial samples and the pharmacological field.

4. Conclusion

Use of salicylaldehyde-5-sulfonate as a derivatizing agent has provided a rapid, simple and sensitive CZE method for the determination of the amines. The conditions for the reaction and the analysis are suitable for such primary amines as having a longer alkyl chain length and other biogenic amines. Compared with other methods reported, this method has an advantage concerning the reaction time, the sample material, and the sensitivity.

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Table 2
Regression, correlation coefficient, and detection limit of the proposed method for the determination of histamine and histamine Schiff base

Analyte	$y = ax + b$[a]	Correlation coefficient	LOD (μ*M*)
Histamine	$a = 0.16 \pm 2.00.\ 10^{-3}$ $b = -1.24 \pm 0.11$	0.987	18.0
Histamine Schiff base	$a = 1.00 \pm 1.4.\ 10^{-2}$ $b = -2.18 \pm 0.24$	0.998	2.5

[a] Values analysed with five injections, *a*: slope, *b*: intercept.

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