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Journal of Chromatography A, 767 (1997) 277–284

JOURNAL OF CHROMATOGRAPHY A

Separation of aporphine alkaloids by micellar electrokinetic chromatography

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Received 7 August 1996; revised 28 November 1996; accepted 2 December 1996

Abstract

The separation of nine aporphine alkaloids, lindcarpine, laurolitsine, *N*-methyllindcarpine, boldine, norpredicentrine, isocorydine, laurotetanine, *N*-methyllaurotetanine and isoboldine, was investigated by micellar electrokinetic chromatography. Optimization of separation was realized with the univariate approach by studying the effects of five factors relevant to run buffer on migration times. Quantitative analysis on a Lauraceous plant showed a similar result to that obtained with HPLC previously performed.

Keywords: Alkaloids; Aporphine alkaloids; Lindcarpine; Laurolitsine; *N*-Methyllindcarpine; Boldine; Norpredicentrine; Isocorydine; Laurotetanine; *N*-Methyllaurotetanine; Isoboldine

1. Introduction

Aporphine alkaloids, present extensively in Lauraceous plants [1], exhibit many interesting pharmacological and biological activities, e.g., choleretic and smooth-muscle relaxing actions of boldine [2,3], and hypotensive and hyperlipidemia-reducing actions of dicentrine in tested animals [4,5].

In view of the large number of species of the Lauraceous plants growing in Taiwan [6], an efficient high-performance liquid chromatographic method [7] has been recently developed in this laboratory to facilitate the study of the alkaloidal constituents in these plants.

Capillary electrophoresis (CE) has gained acceptance as an alternative technique to the common liquid chromatography in natural product research because of its merits such as high resolution, high mass sensitivity, small sample volumes, extraordinary small mobile phase consumption and rapid separation [8]. Micellar electrokinetic chromatography (MEKC), an important mode of CE, can be used to separate not only charged but also unionized compounds by means of its capacity to partition molecules between aqueous mobile phase and pseudostationary micellar phase [9].

Among the factors which influence the separation of MEKC, those pertaining to the run buffer compositions are always of critical importance. Vindevogel and Sandra had optimized the resolution of testosterone esters through the investigation of five parameters of the buffer media [10].

The aim of present work was to develop an MEKC method for the rapid separation of nine aporphine alkaloids isolated from the Lauraceous plants, i.e., lindcarpine, laurolitsine, *N*-methyllindcarpine, boldine, norpredicentrine, isocorydine, laurotetanine, *N*-methyllaurotetanine and isoboldine

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PII S0021-9673(96)01093-X

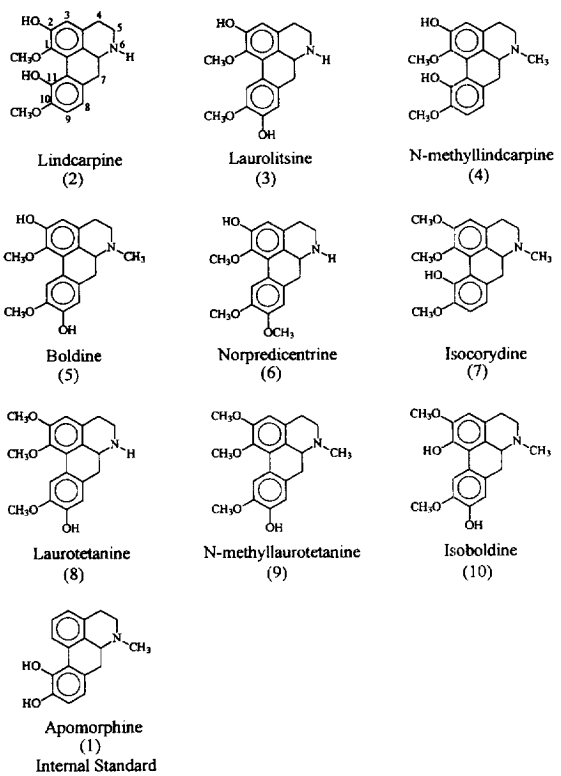

Fig. 1. Aporphine alkaloids used for the analysis of Lauraceous plants.

(Fig. 1). In this work, the factors relevant to the run buffer media: background electrolyte (sodium tetraborate) concentration, surfactant (sodium dodecylsulfate) concentration, organic modifier (acetonitrile) concentration, ion-pairing agent (sodium heptanesulfonate) concentration and pH were considered and their effects on the separation were studied.

2. Experimental

2.1. Apparatus

A PRINCE programmable injector for capillary electrophoresis (Model 4-Tray) including a 30 kV high-voltage supplier was obtained from Lauer Labs (Emmen, Netherlands). This was connected with a Dynamax UV-C Absorbance Detector (Rainin, Emeryville, CA, USA) for UV detection. The electropherograms were recorded and analyzed on a 486 DX2 66 PC with an appropriate ADC card and interface, using EZChrom chromatographic data system ver. 6 (Scientific Software, San Ramon, CA, USA) for electrophoresis data processing. Measurements were carried out on a fused-silica capillary of 50 μm I.D. and 375 μm O.D. (Polymicro Technologies, Phoenix, AZ, USA) with total length of 66 cm and detection length of 50 cm, or total length of 59 cm and detection length of 43 cm. A Mettler delta 320 pH meter with an InLab 410 combination electrode (Essex, UK) was used for measurement of pH.

2.2. Chemicals and reagents

Boldine, the internal standard apomorphine, and the surfactant sodium dodecyl sulfate (SDS) were purchased from Sigma (St. Louis, MO, USA). The other eight alkaloids, lindcarpine, laurolitsine, *N*-methyllindcarpine, norpredicentrine, isocorydine, laurotetanine, *N*-methyllaurotetanine and isoboldine, were obtained by isolation from the Lauraceous plants *Litsea cubeba* [11] and *Dehaasia triandra* Merr. [12]. The identities of the substances were verified by UV, IR, ^{1}H NMR and mass spectrometry. Sodium tetraborate was purchased from Merck (Darmstadt, Germany). Sodium heptanesulfonate was purchased from Fluka (Buchs, Switzerland). pH of the buffer electrolyte was adjusted with 0.1 *M* hydrochloric acid (Carlo Erba, Milan, Italy) and 0.1 *M* sodium hydroxide (Fluka), both of analytical grade. Acetonitrile and methanol used as additives to buffer electrolyte in MEKC were of chromatographic grade and purchased from Mallinckrodt (Paris, KY, USA). Ethanol (95%) and chloroform used in the extraction process of sample preparation were obtained locally and distilled before use. Ammonia solution (25%) was purchased from Merck. Water was purified in a Barnstead water purification system (Dubuque, IA, USA).

2.3. Preparation of standard solution

Stock solutions of the nine Lauraceous aporphines, lindcarpine, laurolitsine, *N*-methyllindcarpine, boldine, norpredicentrine, isocorydine, laurotetanine, *N*-methyllaurotetanine and isoboldine, as well as the internal standard apomorphine, were prepared at 1

mg/ml in methanol. A working solution containing each of the above ten compounds at 100 μg/ml was prepared by diluting the stock solutions with methanol–water (40:60, v/v).

2.4. Preparation of sample [1]

The dried leaves of the plant *Neolitsea sericea* var. *aurata* (collected in August 1992 in Lanyu, Taitung County, Taiwan) were extracted with 95% ethanol. Concentration of the ethanolic extract afforded a residue from which the alkaloids were extracted with 0.1 *M* hydrochloric acid. The neutral compounds in the acidic aqueous layer were removed by partitioning with chloroform. The pH of the aqueous layer was adjusted to 9.9 with ammonia solution and the free bases were extracted with chloroform. The chloroform extract after drying was taken as the sample for the determination of the aporphine alkaloids. A stock solution was prepared by dissolving the extract in methanol in an appropriate concentration. An aliquot diluted with methanol–water (40:60, v/v) was used as the preparation for injections.

2.5. Electrophoretic procedure

When a new capillary was used, it was flushed with 1.0 *M* sodium hydroxide for 10 min, followed by 0.2 *M* sodium hydroxide for 10 min.

Prior to use, the capillary was flushed with deionized water for 3 min, 0.2 *M* sodium hydroxide for 3 min, water for 3 min, and then the run buffer for 4 min, successively.

The run buffer solutions were prepared by mixing stock solutions of sodium tetraborate, SDS and sodium heptanesulfonate. After the addition of acetonitrile, water was added to complete the volume. Following the adjustment of pH the buffer solutions were filtered through a 0.45 μm filter (Millipore, Bedford, MA, USA) before use.

The detection wavelength was set at 214 nm. Samples were injected hydrodynamically at 40 mbar for 0.2 min. The experiments were performed at room temperature (23±2°C).

In this work with the apparatus PRINCE in its standard configuration only about 20% of the capillary length was in the air-thermostatted chamber. The parts of the capillary exposed to ambient air were shielded with a sponge sleeve.

3. Results and discussion

Before undertaking the MEKC experiments, preliminary studies using the capillary zone electrophoresis (CZE) were attempted. Single and two-component buffers composed of borate, phosphate and citrate (with total concentrations of 40 m*M*), in the pH range from 2.0 to 10.0 were tested. The peaks of the nine aporphines were always crowded together with serious overlapping. On considering the extraordinary resolution power of MEKC compared with the simple CZE technique [9], MEKC using SDS as the micelle-forming surfactant was employed.

At room temperature and an applied voltage of 30 kV, a screening test by adding various modifiers to the buffer media composed of sodium borate and SDS showed that two additives, acetonitrile (as organic modifier) and sodium heptanesulfonate (as ion-pairing agent), could improve the separation selectivities of the aporphine alkaloids. As a matter of fact, the background electrolyte (sodium borate), the surfactant (SDS) and the pH of the media constituted the essential parts of the run buffer media. Consequently these five parameters were studied by the one-variable-at-a-time approach with a view to achieving an acceptable separation within a reasonable analysis time.

3.1. Effect of sodium borate

The nine aporphine alkaloids and the internal standard apomorphine, having one or two phenolic groups and an amine function, are amphoteric, with pK_b and pK_a about 7.0 and 9.0, respectively [13]. Around pH 9.0 a maximum difference of migration times could be envisioned among these compounds when they are subjected to MEKC separation in normal mode, on considering their phenolic acidities [14]. Sodium borate, as a pH buffer with its useful range between 8.0 and 10.0 as well as its complex-forming character, was well suited as the background electrolyte of the buffer for the separation of these aporphines. In the presence of 25 m*M* SDS, 5 m*M* sodium heptanesulfonate, 1% acetonitrile (v/v) and

pH 9.3, a range of 15 m*M* to 25 m*M* tetraborate was tested and an optimum value of 20 m*M* was found. The migration times of the monophenolic aporphines increased to a greater extent than the diphenolic aporphines with the borate concentration in the range tested. This might be due to the stronger binding of diphenolic aporphines to the borates compared to the monophenolics as the borate concentration increased.

3.2. Effect of SDS

On applying MEKC with SDS as micelle-forming agent the separation was dramatically improved (Fig. 2). At low concentrations of SDS in the presence of 20 m*M* sodium tetraborate, 2 m*M* sodium heptanesulfonate, 3% acetonitrile (v/v) and pH 9.3 the peaks remained merged; following the increase of SDS concentration the micelles formed progressively and the aporphine components were separated in the migration order of apomorphine (1)>lindcarpine (2)>laurolitsine (3)>*N*-methyllindcarpine (4)> boldine (5)>norpredicentrine (6)>isocorydine (7)> laurotetanine (8)>*N*-methyllaurotetanine (9)>isoboldine (10). This sequence could be explained by the polarity and geometry of the compounds.

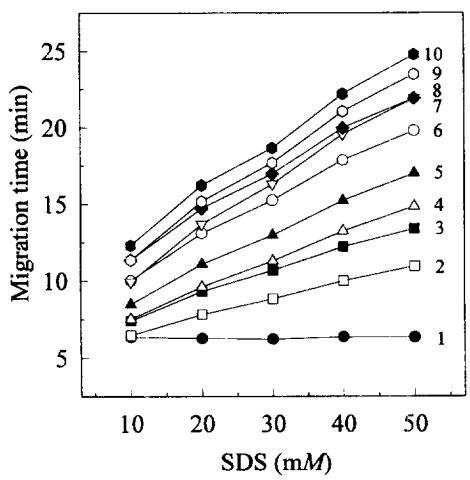

Fig. 2. Migration times versus SDS concentration for the aporphine alkaloids. Electrophoretic conditions: 20 m*M* sodium tetraborate, 2 m*M* sodium heptanesulfonate, 3% acetonitrile (v/v), pH 9.3, with various concentrations of SDS; capillary, fused-silica, 50 μm I.D., detection length 50 cm, total length 66 cm; injection time, 0.2 min; voltage, 30 kV; temperature, 23°C; UV detection, 214 nm. Compound identities shown in Fig. 1.

Apomorphine (1), having a catechol structure, could form a complex with borates which was readily soluble in aqueous phase and migrated out the first. The four diphenolic aporphines, lindcarpine (2), laurolitsine (3), *N*-methyllindcarpine (4) and boldine (5) migrated after apomorphine (1) and before the four monophenolic aporphines, norpredicentrine (6), isocorydine (7), laurotetanine (8) and *N*-methyllaurotetanine (9). Isoboldine (10), although it is a diphenolic aporphine, migrated out the last. With the only exception of isoboldine, all the *O*- and *N*-methylated compounds were eluted after their non-methylated counterparts, e.g., norpredicentrine (6) and *N*-methyllindcarpine (4) migrated after laurolitsine (3) and lindcarpine (2), respectively, because the methylated compounds were more lipophilic and thereby more solubilized in micelles. It is interesting to note that in each of the three pairs of positional isomers, i.e., lindcarpine (2)–laurolitsine (3), *N*-methyllindcarpine (4)–boldine (5) and isocorydine (7)–*N*-methyllaurotetanine (9), the compounds with hydroxy group at 11 position (see lindcarpine in Fig. 1 for numbering of positions) were always eluted before their isomers with hydroxy group at 9 position. The steric repulsion between the two benzene rings in 9-OH isomers (compounds 3, 5 and 9) is less than that in 11-OH isomers (compounds 2, 4 and 7), therefore, the 9-OH isomers take a more planar structure compared with the 11-OH isomers. From the above migration orders it was reasonable to suppose that planar compounds were more readily solubilized to the micelles in comparison with their strained isomers. Isoboldine assumes the most planar structure among the nine aporphine alkaloids and thereby it was eluted the last although it possesses two phenolic substitutions.

3.3. Effect of acetonitrile

In MEKC, the addition of organic solvent to the buffer can lengthen the migration time and widen the migration window [15,16]. Its use contributes to the alteration of selectivity and improvement of resolution [17]. Typically <10% (v/v) of organic modifiers were used to avoid breaking down the micellar structure. In this work the aporphine peaks were heavily overlapped in the absence of organic modifier and following the addition of acetonitrile

the separation was greatly improved, with the buffer containing 20 m*M* sodium tetraborate, 30 m*M* SDS and 2 m*M* sodium heptanesulfonate, at pH 9.3 (Fig. 3). In Fig. 3 various migration behaviors of the aporphines in the presence of acetonitrile could be found and these were depicted as follows.

First, unlike all the other nine aporphines, the migration time of apomorphine (1) increased with the concentration of acetonitrile within the experimental range, due to the decrease of electroosmotic flow caused by the addition of acetonitrile. Secondly, contrary to apomorphine, the migration times of the four diphenolic aporphines (compounds 2, 3, 4 and 5) decreased with the percentage of acetonitrile added to the buffer. This might be attributed to the increased partitions of the four diphenolics into the aqueous phase, which led these compounds to faster migrations. Thirdly, for monophenolic aporphines with methyl substitution on nitrogen, i.e., isocorydine (7) and *N*-methyllaurotetanine (9), the migration times increased with the addition of acetonitrile up to a maximum at 7.5% of acetonitrile and then decreased. However, for monophenolic aporphines without methyl substitution on nitrogen, i.e., norpredicentrine (6) and laurotetanine (8), the migration times also increased initially but the maxima were earlier reached at 5% of acetonitrile. Because of this, the reversion of migration order between isocorydine (7) and laurotetanine (8) took place at the point of 7.5% of acetonitrile (see Fig. 4). Lastly, for isoboldine (10), although it belongs to a diphenolic aporphine, its migration behavior looked like isocorydine (7) and *N*-methyllaurotetanine (9). This divergent behavior might be as well caused by the special geometry of isoboldine molecule as described above (Section 3.2).

The optimum of the overall resolution for these ten aporphines was situated at 5% of acetonitrile beyond which the resolution commenced to decay. Methanol had also been tested but a like separation as obtained with acetonitrile could never be attained, with its concentration added up to 20% (v/v).

3.4. *Effect of sodium heptanesulfonate*

An ion-pairing agent, often used in reversed-phase high-performance liquid chromatography, was suggested as an additive to the buffer to improve the resolution [17,18]. Under the optimized condition determined by all the other factors (in the absence of sodium heptanesulfonate), peak pairs of laurolitsine (3)–*N*-methyllindcarpine (4), norpredicentrine (6)–isocorydine (7) and laurotetanine (8)–*N*-methyl-

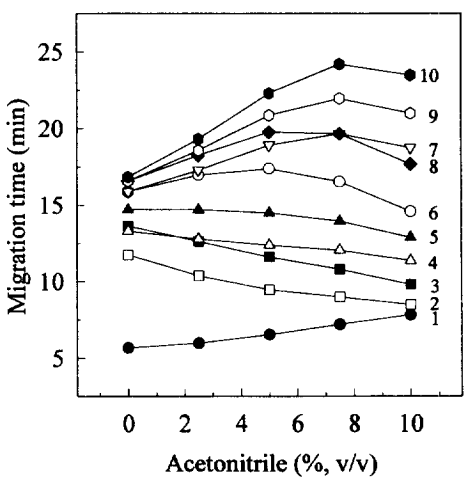

Fig. 3. Migration times versus acetonitrile volume percentage for the aporphine alkaloids. Electrophoretic conditions: 20 m*M* sodium tetraborate, 30 m*M* SDS, 5 m*M* sodium heptanesulfonate, pH 9.3, with various volume percentages of acetonitrile; other conditions as in Fig. 2. Compound identities shown in Fig. 1.

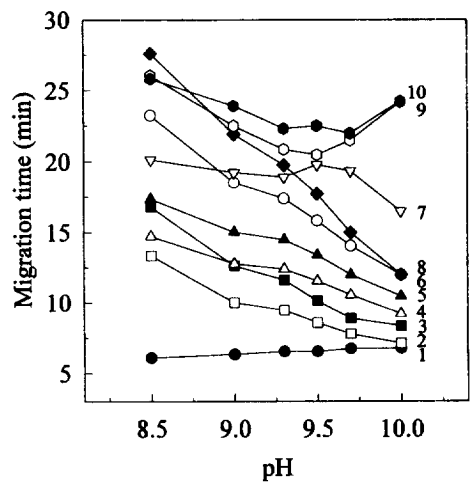

Fig. 4. Migration times versus pH for the aporphine alkaloids. Electrophoretic conditions: 20 m*M* sodium tetraborate, 30 m*M* SDS, 5% acetonitrile (v/v), 5 m*M* sodium heptanesulfonate, with various pHs; other conditions as in Fig. 2. Compound identities shown in Fig. 1.

laurotetanine (9) were not completely separated. With the addition of sodium heptanesulfonate the migration times of the nine aporphines decreased slightly at 5 m*M* and then increased to 20 m*M* of sodium heptanesulfonate. The overall resolutions of the aporphines were always improved by the addition of sodium heptanesulfonate and an optimum was found at 5 m*M*. It seems improper, here at pH 9.3, to attribute this improvement to ion-pair formation between the heptanesulfonate anion and the amine function [18], because at this pH the amine existed entirely as the basic form. Possible explanations for the improvement of separation are as follows: (1) the decrease of the electroosmotic flow and the widening of the elution range caused by the increasing concentration of the ion-pairing agent resulted in the selectivity change and resolution improvement [18]. (2) Sodium heptanesulfonate itself does not form micelles whereas it might incorporate with SDS to form the mixed micelles [10], because the interaction between the aporphines and the mixed micelles differed somewhat from the SDS micelles, the selectivity and the resolution changed.

As the concentration of sodium borate, SDS, or sodium heptanesulfonate increased, the resolution of all the adjacent peaks improved except for the pair isocorydine (7)–laurotetanine (8) which became coalesced at higher concentrations of the above buffer constituents. The elution order of this pair was eventually reversed at increasingly higher concentrations of the buffer constituents (not shown in these figures). The structural relation between isocorydine and laurotetanine was similar to that between *N*-methyllindcarpine (4) and laurolitsine (3); however, the separation tendency was different for these two pairs of compounds within the experimental ranges in this work. It seemed that in isocorydine–laurotetanine pair the effect of molecular planarity [determined by the position of OH group on 9 or 11 position, as described above (Section 3.2)] outweighed that of *N*-methylation on the partition of solutes into micelles, while in laurolitsine–*N*-methyllindcarpine pair the effect of *N*-methylation prevailed.

3.5. Effect of pH

In MEKC, as in CZE, the pH is critical for the separation of weakly ionized compounds. For acidic solutes like substituted phenols the maximum mobility difference among the solutes could be found near the region of their averaged ionization constant [14]. By analogy with apomorphine [13] the pK_as (the phenolic group) of the aporphines in this work was estimated to be about 9.0. A pH range of 8.5 to 10.0 was therefore investigated (Fig. 4). In Fig. 4 great changes in selectivities accompanied with reversions of elution order were observed. Because the electroosmotic flow did not change with pH the decrease of migration times (with exceptions of *N*-methyllaurotetanine (9) and isoboldine (10)) were probably due to the increase of the dissociated forms of solutes which escaped being retained by the micelles. The space available for the selection of an optimum value was very stringent. Following the experiments a satisfactory separation was obtained at pH 9.3 while at pH 9.4 and 9.6 peak pairs of isocorydine (7)–*N*-methyllaurotetanine (9) and *N*-methyllaurotetanine (9)–isoboldine (10) were partially overlapped, respectively.

3.6. Optimization

After the effect of each single parameter on migration time had been studied, the measurements were carried out by combining the optimum values of each parameters, viz., 20 m*M* sodium tetraborate, 30 m*M* SDS, 5% acetonitrile (v/v), 5 m*M* sodium heptanesulfonate and pH 9.3. The resulting electropherogram showed the eluting sequence as apomorphine (1)>lindcarpine (2)>laurolitsine (3)>*N*-methyllindcarpine (4)>boldine (5)>norpredicentrine (6)>isocorydine (7)>laurotetanine (8)>*N*-methyllaurotetanine (9)>isoboldine (10). The ten aporphines were completely separated from each other within 23 min with a minimum resolution between peaks larger than 1.8.

Because this result was obtained from a capillary of total length of 66 cm and detection length of 50 cm, with a view to reducing the analysis time while guarding a basic separation, measurements using a shorter capillary of total length of 59 cm and detection length of 43 cm (which is the limit of the apparatus) were performed, with all the other conditions maintained unchanged. The electropherogram obtained (Fig. 5) showed that except a very minor

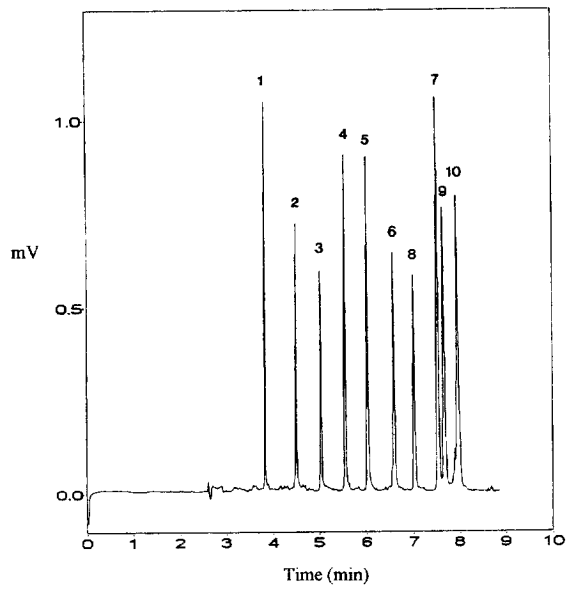

Fig. 5. Electropherogram for the separation of the aporphine alkaloids. Electrophoretic conditions: 20 m*M* sodium tetraborate, 30 m*M* SDS, 5% acetonitrile (v/v), 5 m*M* sodium heptanesulfonate, pH 9.3; capillary, fused-silica, 50 μm I.D., detection length 43 cm, total length 59 cm; injection time, 0.2 min; voltage, 30 kV; temperature, 23°C; UV detection, 214 nm. Compound identities shown in Fig. 1.

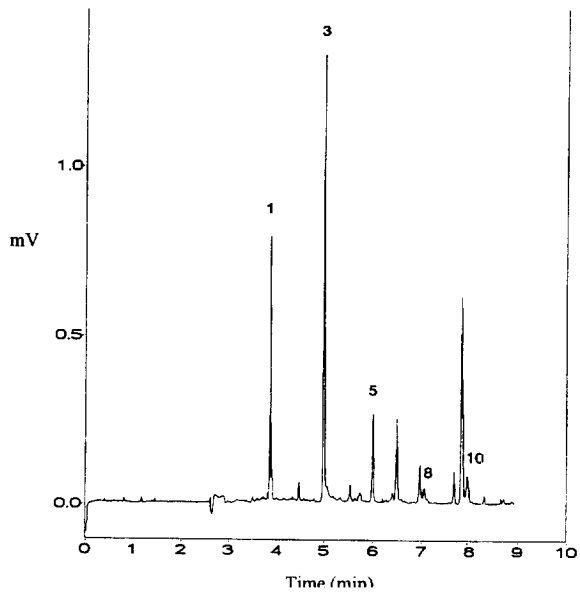

Fig. 6. Electropherogram of the sample preparation from the leaves of *N. sericea* var. *aurata*. Electrophoretic conditions as in Fig. 5. Compound identities as in Fig. 1.

overlapping between isocorydine (7) and *N*-methyllaurotetanine (9) all the other compounds were completely separated from each other within 8 min. The difference in electroosmotic velocities between these two capillaries of different lengths was found to be 4.68 cm/min. However, the elution order between isocorydine (7) and laurotetanine (8) was reversed. This was caused by the stronger field strength yielded on a shorter capillary when the same voltage was applied.

A study to test the repeatability of the separation for the electrophoretic system was performed. The run-to-run relative standard deviations of actual migration times for all the analytes were found within 1.4% ($n=10$), whereas those of relative migration times with respect to apomorphine were within 1.0% ($n=10$).

3.7. Application

In the analysis of the sample prepared from the leaves of *N. sericea* var. *aurata*, four aporphines, i.e., laurolitsine, boldine, laurotetanine and isoboldine, were identified (Fig. 6). Sets of five standard alkaloid solutions covering the range 25–400 μg/ml were run, with apomorphine as internal standard, the detector response for the four alkaloids were linear (peak-height ratios vs. concentration injected) with $r=0.999$, 0.990, 0.998 and 0.993 for laurolitsine, boldine, laurotetanine and isoboldine, respectively.

The quantitation of the four aporphines in leaf parts of *N. sericea* var. *aurata* was carried out by internal standardization. The content of laurolitsine, boldine, laurotetanine and isoboldine was found to be 43, 12, 7.6 and 1.7 ppm on w/w basis, respectively. On comparing this with the result obtained from the method of HPLC [7] (53, 12, 6.8 and 1.1 ppm for laurolitsine, boldine, laurotetanine and isoboldine, respectively), the discrepancies could be attributed to the inefficiency of thermostat control of capillaries in the electrophoretic configuration, especially when used with high voltages and buffers of high ionic strength.

For the comparison of this MEKC with the previous HPLC method [7] for the detection of the

Table 1
Comparison of run time, minimum resolution and limit of detection between the MEKC and the HPLC methods

	Run time (min)	Min. R_s^a	Limit of detection[b] (pg)			
			Laurolitsine	Boldine	Laurotetanine	Isoboldine
MEKC	8.5	1.46	33	30	64	29
HPLC	15	1.32	267	246	593	215

[a] Calculated by the equation $R_s=2(t_2-t_1)/(w_1+w_2)$.
[b] At $S/N=3$.

aporphines, the run time, resolution and the limit of detection for laurolitsine, boldine, laurotetanine and isoboldine are listed in Table 1.

4. Conclusions

MEKC is a viable alternative method for the separation of aporphine alkaloids. In the present work where many aporphine compounds differ from each other merely as positional isomers, the MEKC system proved to be capable of providing the selectivity required for separation. Additives such as sodium heptanesulfonate and acetonitrile were added to the run buffer composed of sodium borate and SDS, otherwise a sound separation could hardly be achieved. As to the quantitative results, although some small differences were present between MEKC and HPLC determinations, if an efficient thermostatting device or even an integrated electrophoresis system was used, MEKC should yield results commensurate with HPLC.

Acknowledgments

The National Science Council of the Republic of China is acknowledged for its financial support (NSC 85-2331-B-002-263).

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