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Improved separation of six harmane alkaloids by high-performance capillary electrophoresis

Jing Cheng[a], Keith R. Mitchelson[b,*]

[a]*Department of Pathology and Laboratory Medicine, School of Medicine, University of Pennsylvania, 3400 Spruce Street, Philadelphia, PA 19104-4283, USA*

[b]*ForBio Research Pty. Ltd., 50 Meiers Road, Indooroopilly, Qld. 4068, Australia*

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Abstract

The β-carboline alkaloids of the harmane group (harmine, harmol, harmaline and harmol) are found at high levels in some plants and occur in many natural foodstuffs and beverages. Both harmane and norharman also occur naturally in mammalian neural tissues. The harmane alkaloids are often difficult to fully fractionate using conventional high-performance liquid chromatography. We report here the successful separation of a mixture of six harmane alkaloids (harmane, norharman, harmine, harmaline, harmol and harmalol) to baseline resolution using micellar electrokinetic chromatography. The alkaloids were detected by UV absorption using diode-array spectrophotometry, which allowed characterization of individual peaks. The fractionation was rapid and was highly reproducible, with complete resolution of all six compounds within 14 min. Harmalol could also be detected directly using laser-induced fluorescence.

Keywords: Buffer composition; Alkaloids; Harmane alkaloids

1. Introduction

Harmane alkaloids occur naturally at high concentrations in plants such as *Peganum harmala* [1–3], *Banisteria caapi* [4] and in *Tribulus terrestris* [5], and can be divided into two groups, harmine and harmol, harmaline and harmalol, according to their oxidation stage (see Fig. 1). The alkaloids have long been known to stimulate central nervous system of mammals [4] and have been used in hallucinogenic snuffs of South American peoples [6]. High concentrations of the alkaloids or long periods of exposure are known to be detrimental in herbivores, and after long periods of ingestion of the plant *T. terrestris*, staggers can occur in sheep, indicative of a neurological effect of the drug [5]. The neurological and cytochemical effects of the alkaloids are complex: the β-carbolines, harmane and norharman, occur naturally in the cerebral cortex and other brain tissues, as well as in the liver and adrenal tissues [7]. Harmane and norharman bind to cell membranes at several different binding sites specific for the alkaloids [7–9]. These naturally occurring alkaloids are believed normally to have a natural sedative function in neural tissue, but have also been found at higher levels in tissues of alcoholics and heroin addicts.

β-Carboline alkaloids also occur in natural plant-derived foods and are often associated with fermentation processes such as in the production of wines

*Corresponding author.

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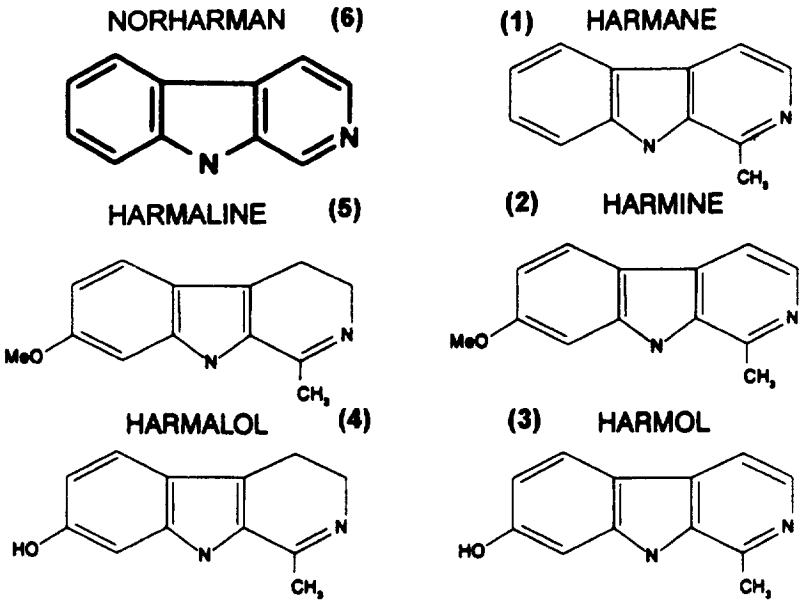

Fig. 1. Structures of the harmane alkaloids (β-carbolines).

and beer, soya bean products and vinegar [10]. Although β-carbolines such as harmane and norharman at endogenous concentrations are not mutagenic per se, at high concentration they are also reported to having potential cytotoxic and carcinogenic activities, causing renal toxicity in male F344 rats at the dietary level of 1000 ppm [11,12]. The development of efficient and sensitive analytical procedures for β-carbolines are therefore important to researchers involved in studies as diverse as (neuro-) pharmacology, toxicology and analytical chemistry.

High-performance liquid chromatography (HPLC) has been generally the means of separation of harmane alkaloids with subsequent fluorescence detection, characterization and estimation of individual alkaloid concentrations [9,13]. High-performance capillary electrophoresis (HPCE) has been established as an important analytical tool due to its high resolving power, speed, automation and reproducibility [14]. Micellar electrokinetic chromatography (MEKC) is an electrophoresis technique by which neutral and ionic compounds, such as steroids [15], alkaloids [16] and barbiturates [17] can be separated by partitioning between the hydrophobic phase (e.g., micelles) and the hydrophillic buffer phase. MEKC has also been successfully used to separate complex mixtures of different pharmacologically active alkaloids (anthraquinones, flavones and glucuronides) from plant material [18]. Here, we report the separation of a mixture of six harmane alkaloids to baseline resolution using MEKC within 14 min. The MEKC separation was achieved using a run buffer of 20 m*M* borate, pH 9.0, 50 m*M* SDS, 0.75 *M* urea and 15% acetonitrile at 25 kV using a capillary of 47 cm×75 μm I.D. at 25°C. The alkaloids were detected using a diode-array detection system which provides for spectral identification of the compound [17]. The resolution of the six harmane alkaloids achieved by MEKC was superior to reported methods using HPLC. Despite the alkaline MEKC buffer conditions, harmalol could also be detected by laser-induced fluorescence (LIF) using an 488 nm Ar laser.

2. Materials and methods

2.1. Reagents

The alkaloids were each purchased from Aldrich (Gillingham, UK): harmine (28 604-4), harmol (11 655-6), harmane (10 327-6), harmalol (H12-5), harmaline (28 603-6) and norharman (N3,310-1).

The alkaloid powders were dissolved in ethanol (analytical-reagent grade) at a concentration of 1 m*M* and stored at 4°C under light-protected conditions. Sodium dodecyl sulphate (Cat. 30175) was from BDH. All other chemicals were high purity grades obtained from Sigma (UK) or from BDH (UK).

2.2. Capillary electrophoresis

2.2.1. MEKC buffer system

The MEKC buffer was freshly prepared and typically consisted of 20 m*M* boric acid (pH 9.0), 50 m*M* SDS, containing 15% (w/v) acetonitrile and 0.75 *M* urea. Other buffer formulations with different pH values, or different concentrations of urea or acetonitrile were tested as described in the text. During formulation of the buffers, 0.4 *M* boric acid solutions, brought to different pH by addition of NaOH, were mixed in different ratios before addition of the other ingredients from stock solutions (500 m*M* SDS, pH 9.0; 5 *M* urea, acetonitrile analytical-reagent grade). The final pH was determined following the complete buffer formulation as the solution pH increased by about 0.2 pH units due to decreased activity of water after the addition of acetonitrile. The buffers were then filtered using a 0.4-μm filter and kept at 4°C until use.

2.2.2. Capillary electrophoresis

MEKC was performed on the P/ACE 5050 system (Beckman) in the normal-polarity mode (positive potential at the injection end of the capillary). The temperature was set at 25°C and UV absorbance was monitored at 254 nm using diode-array detection (DAD) (Beckman Instruments, CA, USA). Peak identity was confirmed by post-run diode-array analysis of the absorption maxima of the individual peaks in a mixture [17]. Post-run analysis of data was performed using the Gold Chromatography Data System (version 8.13). An unmodified fused-silica capillary (J&W Scientific) was used for all analysis. The capillary [47 cm (effective length of 40 cm)×75 μm I.D.] was conditioned daily with five volumes of acetonitrile, 10 volumes of water and by 20 volumes of MEKC buffer and was then subjected to voltage equilibration for 10 min until a stable baseline was achieved. For some experiments using a mixture of four β-carbolines an unmodified fused-silica capillary of 37 cm (effective length 30 cm)×75 μm I.D. was used. Samples were introduced into the capillary by pressurised injection for 1 s. Separations were performed under constant voltage at 530 V/cm for 20 min. The capillary was rinsed for 1 min with water, 15 s with acetonitrile and then 2 min with the MEKC buffer after each run, prior to the next injection. Laser-induced fluorescence was detected using the Beckman LIF detector with a fixed excitation and emission wavelength set at 488 nm and 520 nm, respectively.

3. Results and discussion

HPLC has generally been the means of separation of harmane alkaloids with subsequent detection by alkaloid fluorescence from diverse sources: from rich sources such as plant tissues and seeds [2–5], fermented or uncooked foods [19], to low abundance sources such as animal tissues [9,13,20]. In some studies, mass spectroscopy was linked to high-performance liquid chromatography (LC–MS) to provide additional accurate quantification and characterization of the alkaloids [10,21]. Efficient fractionation was also reported using reversed-phase HPLC [10,20,21] with peak resolution similar to the resolution achieved in the present study using MEKC.

Individual harmane alkaloids have reasonable solubility in polar solvents such as ethanol and methanol. Solutions containing approximately 170 μ*M* each of combinations (or alone) of harmane, norharman, harmine, harmaline, harmol and harmalol (Fig. 1) in ethanol were pressure injected for 1 s onto the column and were analysed for 20 min. Fig. 2 shows separate electrophoretic profiles of four harmane alkaloids. The electrophoretic profile of each alkaloid was complex, with a single major (often doublet) peak and a second smaller peak representing a lower amount of a contaminating unknown compound, indicated as C. In addition, several minor peaks eluting at about 3 to 4 min, which may represent the ethanol solvent, were seen in each run. The harmane alkaloids had highly reproducible migration times over six runs, when electrophoresed using identical MEKC conditions (Table 1).

Fig. 3 shows the UV absorption spectra of

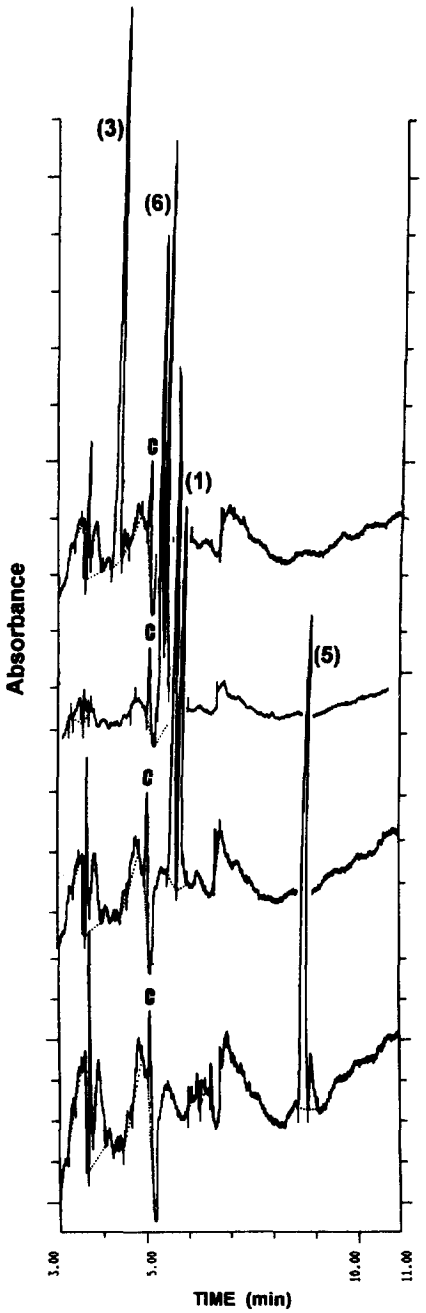

Fig. 2. The MEKC profile of four alkaloids, harmol (3), norharman (6), harmane (1) and harmaline (5), on an uncoated silica capillary of 37 cm×75 μm I.D. Buffer: 20 m*M* borate, pH 9.0, 50 m*M* SDS, 0.6 *M* urea and 15% acetonitrile. Voltage applied, 25 kV; temperature, 25°C. Peaks detected using diode array absorption at 254 nm. An unknown contaminant (C) common to all the four alkaloid preparations can be seen. Peaks are identified as in Fig. 1.

Table 1
Average migration times of individual harmane alkaloids run on a 47 cm×75 μm I.D. capillary

Alkaloid	Peak (min)	Minor (min)
Harmol	5.40	6.50
Harmalol	6.40	5.30
Norharman	7.50	6.50
Harmane	8.30	6.50
Harmine	9.65	6.50
Harmaline	13.45	6.43

Buffer: 20 m*M* borate, pH 9.0, 50 m*M* SDS, 0.6 *M* urea and 15% acetonitrile. Minor peak refers to two unknown contaminant compounds detected in the alkaloid preparations which had average migration times of 5.30 min and 6.50 min, respectively, over 6 runs. Diode array UV spectra analysis of representitive peaks of the two unknown compounds are shown in Fig. 4 as unknown (4) and unknown (5).

individual harmane alkaloid peaks in the wavelength range of 200–300 nm analysed by the diode array detector and the P/ACE Gold software. The spectra were unique for each alkaloid and were sufficiently different from one another to provide for peak identification of harmaline and harmalol. However, although the UV absorption profiles of some β-carboline were very similar, such as harmol and harmine, or norharman and harmane, the migration times of the alkaloids on the MEKC system were different; with harmol and harmine eluting at 5.4 min and 9.65 min, respectively, and norharman and harmane eluting at 7.5 min and 8.3 min, respectively (Table 1). The individual alkaloids were readily identified by UV absorption profile, or migration time, and by a combination of these parameters.

Each of the six commercially purchased β-carboline preparations were found to contain small amounts of contaminating alkaloid which separated electrophoretically from the major alkaloid peak by the MEKC system. Fig. 4 shows a comparison of the DAD UV absorption profile of the two unknown peaks contaminating the harmalol and harmaline preparations to the UV spectra of authentic harmalol and harmol. One minor contaminant, unknown (5), which was present in each of the norharman, harmine, harmaline, harmol and harmane preparations appears to be harmalol, based on similarity in both migration time (see Table 1) and UV spectrum to authentic harmalol. The second contaminant, unknown (4), which was found in the harmalol prepa-

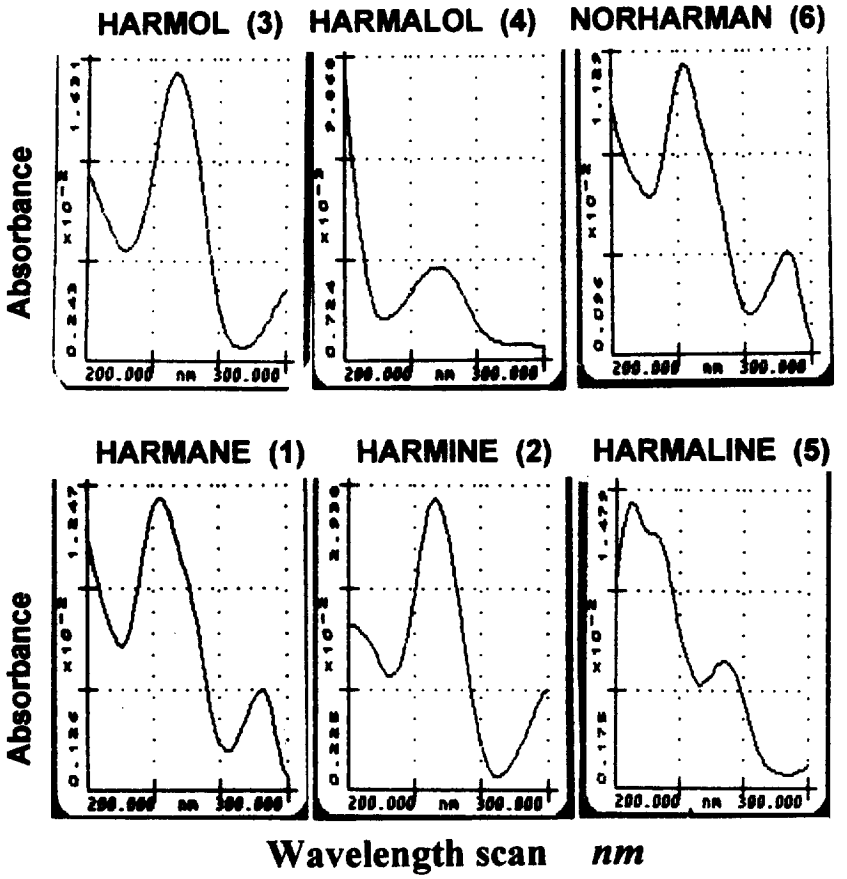

Fig. 3. Diode array UV absorption spectra between 200–300 nm of the individual alkaloid peaks. Peaks are identified as in Fig. 1.

ration appears to be harmol, based on the similarity of migration time (see Table 1) and UV spectrum to the authentic alkaloid.

Fig. 5 shows an electropherogram of an approximately equimolar mixture of the six alkaloid preparations separated in an alkaline MEKC buffer containing 20 m*M* boric acid (pH 9.0), 50 m*M* SDS, 15% (w/v) acetonitrile and 0.75 *M* urea on a 47 cm long capillary. Each of the alkaloids could be resolved to base-line in less than 15 min. The order of migration (first>last) was harmol>harmalol> norharman>harmane>harmine>harmaline. In some runs the peaks were split into a major and minor component, which have identical absorption spectra by diode array analysis and may result from characteristics of the capillary electrophoresis system [22]. The absorption spectra of each peak in the mixture corresponded to the spectral profile of the individual alkaloids, and thus confirmed the identity of each peak in the mixture. Similarly, the migration times for single compounds were in agreement with migration times of each alkaloid in the mixture. During some MEKC runs, the individual harmanes peaks were split into two closely eluting peaks, ranging from almost equal size (e.g., Fig. 5, alkaloids 6 and 1) to major and minor peaks (e.g., Fig. 5, alkaloids 2, 3 and 5). Guttman and Schwartz [22] reported that the shape of the capillary inlet can affect both peak form and peak resolution during MEKC. Although the shape of the capillary inlet may have contributed to the peak resolution, the consistent differences in the splitting of the different harmane alkaloid peaks in the mixtures (see Figs. 2,5 and 6) suggest inlet shape was not the only factor. Although the cause of this peak splitting is not known, one possibility is that partial ionization of norharharman (6) and

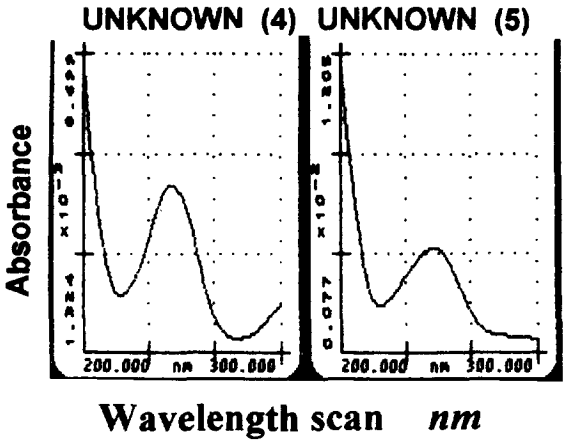

Fig. 4. Comparison of the DAD UV absorption spectra of the contaminant compounds unknown (4) and unknown (5), found in harmalol (4) and harmaline (5) preparations respectively [see Fig. 3 for the respective DAD spectra of harmol (3) and harmalol (4)]. The representitive minor contaminant, unknown (5), had an average migration time of 6.43 min (see Table 1) and displayed an identical DAD UV spectra to the minor contaminant compound found in other alkaloid preparations, each with average migration time of 6.50 min, listed in Table 1.

harmane (1) at pH 9.0 allowed splitting of each compound into two peaks of equal size, while the other harmane alkaloids remained predominantly in unique forms and thus electrophoresed essentially as single peaks, or as major and minor peaks.

3.1. Buffer conditions

Since the separation mechanism in MEKC involves partitioning between the mobile phase and the micelle phase, any manipulation of the buffer system will have some effect on the distribution coefficient and therefore on the migration of solutes [23]. The mobile phase can be altered with respect to buffer type, pH and organic modifiers. The use of organic solvents such as acetonitrile or methanol reduces the electroosmotic velocity, and thus would expand the migration window [24]. The effect of the buffer composition was examined with a mixture of four alkaloids, harmol, norharman, harmane and harmaline (Table 2). When the amount of acetonitrile was increased from 10% to 20% there was a decrease in the migration time, however the resolution between each of the four alkaloids was only

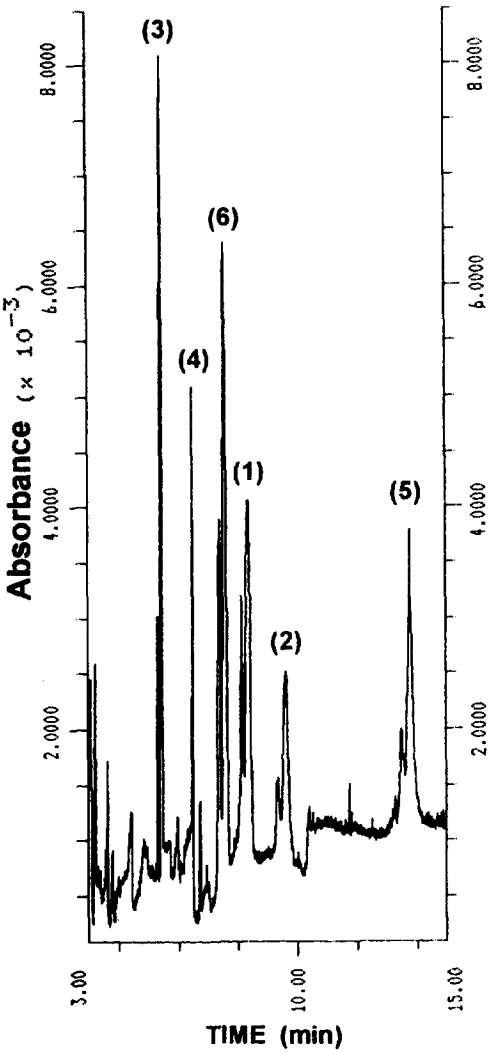

Fig. 5. MEKC separation of the six harmane alkaloids on an uncoated silica capillary of 47 cm×75 μm I.D. Buffer: 20 m*M* borate, pH 9.0, 50 m*M* SDS, 0.75 *M* urea and 15% acetonitrile. Voltage applied, 25 kV; temperature, 25°C. Peak detection using diode array absorption at 254 nm. Peaks are as identified in Fig. 1.

slightly reduced. The effect of adding 1 *M* urea to the buffer containing 15% acetonitrile was to markedly reduce the electrophoretic mobility of each alkaloid, giving a gain in the resolution between each of the four alkaloids in the mixture. The concentration of acetonitrile was set at 15% and the amount of urea varied between 0.5 *M* and 1 *M*. The lower concentration of 0.5 *M* urea did improve the separation between the alkaloids, however, 1 *M* urea

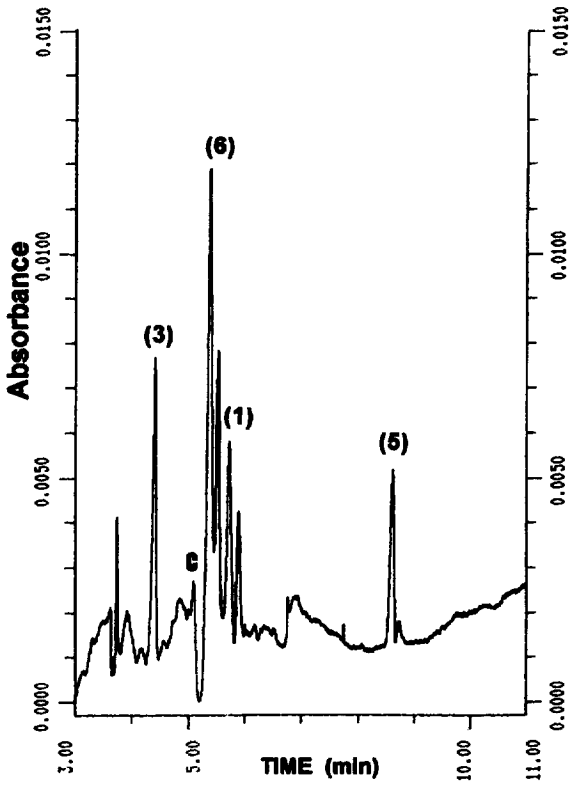

Fig. 6. MEKC separation of the four harmane alkaloids, harmol (3), norharman (6), harmane (1) and harmaline (5), on an uncoated silica capillary of 37 cm×75 μm I.D. Buffer: 20 m*M* borate, pH 9.0, 50 m*M* SDS, 0.75 *M* urea and 15% acetonitrile. Voltage applied, 20 kV; temperature, 23°C. Peak detection using diode array absorption at 254 nm. Peaks are as identified in Fig. 1. The DAD UV spectrum of a contaminant (indicated C) was similar to the sprectrum of unknown (5), shown in Fig. 4.

caused a large reduction in the mobility, with a commensurate increase in migration time (not shown). When a buffer of pH 8.5 was used the alkaloid peaks broadened and good baseline resolution was not achieved (not shown). A buffer of pH 9.5 gave sharp peaks but decreased the resolution between some alkaloids. The run buffer was optimized for maximal resolution of all six alkaloids on a 47 cm capillary when it contained 0.75 *M* urea and 15% acetonitrile, pH 9.0 (Fig. 5), allowed rapid and highly reproducible separation of the six harmane alkaloids by MEKC. Fig. 6 also shows that the base line separation of four alkaloids (harmol> norharman>harmane>harmaline) could be achieved on a capillary of 37 cm (effective length 30 cm) in less than 9 min using the same buffer conditions.

The efficient separation and quantification of six members of the harmane group alkaloids (harmane, norharman, harmine, harmaline, harmol and harmalol) by high-performance MEKC with DAD multispectral analysis presents a new and useful method for the analysis of this pharmacologically and toxicologically important group of compounds. Interestingly, contaminating harmalol was present in commercial preparations of harmane, norharman, harmine, harmaline and harmol which was readily separated from each alkaloid by MEKC. MEKC thus offers a new analysis tool for the determination of alkaloid drug purity. The DAD absorption spectra of individual harmane alkaloid peaks within the mixture were different from one another, and each corresponded to the spectral profile of the individual alkaloids (see Fig. 3), confirming the identity of each peak in the mixture. A similar multiwavelength spectral analysis of barbiturates fractionated by MEKC was presented as a series of 3-dimensional electropherograms [17]. Glucuronide and sulphonate metabolites of the β-carbolines were also present in animal tissues, particularly blood and were generally characterized in other studies using HPLC [13,25]. It would be useful to extend the present work on

Table 2
Effect of acetonitrile concentration on the MEKC migration of the various alkaloids

Alkaloid	10% Acetonitrile	20% Acetonitrile	15% Acetonitrile +0.75 *M* urea
Harmol	5.30	4.42	5.00
Norharman	6.27	5.40	6.35
Harmane	7.00	5.75	6.90
Harmaline	9.10	8.60	10.95

Electrophoresis conditions: 37 cm capillary, 20 kV and temperature 25°C, as in Fig. 6. Buffer: 20 m*M* borate, pH 9.0, 50 m*M* SDS, and 0.6 *M* urea with either 10% or 20% acetonitrile, or 0.75 *M* urea and 15% acetonitrile.

MEKC separation of the harmane alkaloids to a study of the resolution of the glucuronide and sulphonate metabolites of the β-carbolines by MEKC.

3.2. Fluorescence detection

The harmane alkaloids have strong fluorescence at acid pH with absorption in the UV range and emission of blue, or green yellow light. Sasse et al. [2] could detect harmane alkaloid fluorescence in a weak alkaline triethylamine buffer (pH 8.5). In methanolic solutions, harmine and harmol have optimal signals at $\lambda_{ex}/\lambda_{em}$=304/355 nm, while harmaline and harmalol can be detected at $\lambda_{ex}/\lambda_{em}$= 396/475 nm. Thus, under the alkaline pH 9.0 conditions necessary for efficient separation by MECK, fluorescence detection should still be possible. The Beckman LIF fluoro-spectrophotometer has a fixed Ar laser excitation/emission at 488/520 nm respectively, which should not excite the alkaloids. As expected, the laser-induced fluorescence was found to be low for all alkaloids, except for harmalol which displayed a strong emission of 15 relative fluorescence units (RFU) on a 37 cm capillary (Fig. 7). A mixture of all six alkaloids presented a similar profile to that seen with harmalol alone. The fluorescence profiles of the five other individual alkaloids were less than 0.3 RFU for each one (not shown).

Interference from other compounds in tissues has frequently hampered the assay of β-carbolines. The quantitative determination of the alkaloids by UV absorption is only possible after separation from other UV absorbing materials, and is not very sensitive [26,27]. It can be used however, as in the present study, when working with pure compounds. The derivatization of the alkaloids has been used to improve the purification from other phenolic compounds and to reduce the fluorescence background of other compounds in the biological samples [19,21]. The harmane alkaloids are also strongly fluorescent compounds, each with characteristic emission spectra. However, Sasse et al. [2,3] showed bathochromic transitions in the excitation and emission spectra of the alkaloids in different methanol: water solutions, and also that emission intensity was greatest under acidic conditions (<pH 6.5). In methanolic solution,

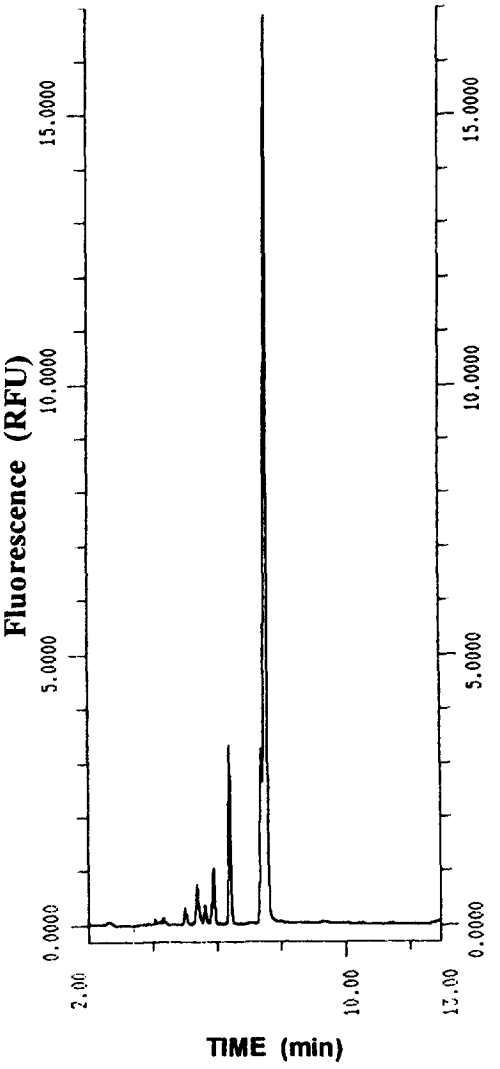

Fig. 7. Laser induced fluorescence (LIF) detection of 170 μ*M* ethanolic solution of harmalol. The electrophoresis conditions were the same as in Fig. 2 and the LIF settings were as in Section 2.2.2.

harmine and harmol had emission maxima at 355 nm with optimal excitation at 304 nm. Harmaline and harmalol displayed no emission at these wavelengths, but were fluorescent at 475 nm (excitation 396 nm). In solutions of water–methanol of 9:1, harmine and harmol fluorescence occurred at 425 nm when excited at 324 nm. The alkaline buffer conditions employed in this study for efficient MEKC separation of the harmane alkaloids were therefore

not optimal for fluorescence detection due to the pH quenching. Although the fixed-wavelength Ar laser (Beckman Instruments) is also not ideal with an excitation wavelength at 488 nm, a strong fluorescence signal was detected for harmalol at 520 nm. Apparatus with a broader range of excitation wavelengths would allow fluorescence detection of additional harmane alkaloids. We hope that the MEKC separation of the harmane alkaloids shown in the present paper will stimulate application of these techniques for pharmacological and medical investigations.

Acknowledgments

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