Stig Pedersen-Bjergaard
Knut Einar Rasmussen
Ellen Sannes

School of Pharmacy,
University of Oslo, Oslo, Norway

Strategies for the capillary electrophoretic separation of indole alkaloids in *Psilocybe semilanceata*

While the hallucinogenic mushrooms *Psilocybe semilanceata* have previously been analyzed for the indole alkaloids psilocybin and baeocystin by capillary zone electrophoresis (CZE) at pH 11.5, the present work focused on the development of an alternative and complementary capillary electrophoretic method for their identification. Owing to their structural similarity and zwitterionic nature, the compounds were difficult to resolve based on different interactions with cationic or anionic micelles. However, while the attempts with micellar electrokinetic chromatography (MEKC) were unsuccessful, rapid derivatization with propyl chloroformate and reanalysis by CZE at pH 11.5 was effective to support identification of the two indole alkaloids. Psilocin was difficult to analyze by CZE at pH 11.5 owing to comigration with the electroosmotic flow. For this compound, the pH of the running buffer was reduced to 7.2 to effectively enhance the electrophoretic mobility.

1 Introduction

The hallucinogenic compound psilocybin has been detected worldwide in a variety of mushrooms belonging to the genera of *Psilocybe, Panaeolina, Panaeolus, Copelandia, Conocybe, Gymnopilus, Stropharia and Pluteus* [1, 2]. Within the Scandinavian countries, *Psilocybe semilanceata* is the most abused hallucinogenic mushroom. This mushroom has been shown to contain up to 2% of psilocybin (Fig. 1A) as the major active constituent. The dephosphorylated compound psilocin (Fig. 1B) is only found in trace amounts, while up to 0.3% of baeocystin has been detected as a demethylated derivative of psilocybin (Fig. 1C) [3]. Since psilocybin is a controlled compound and considered a narcotic drug, it must be reliably identified and quantified in mushroom samples. Psilocybin, baeocystin, and psilocin have been identified by thin-layer chromatography [2, 4]. Due to the high polarity and amphoteric nature of these compounds, high-performance liquid chromatography (HPLC), either in the normal phase mode [5] or in the reversed phase mode [6], has been a preferred method for their separation and quantification, utilizing either UV, fluorescence, or electrochemical detection [7]. In addition, gas chromatography has been carried out after derivatization [4], and GC/MS analysis has been performed for court evidence [8].

Recently, a new method for the analysis of psilocybin in *Psilocybe semilanceata* was published based on capillary zone electrophoresis (CZE) [9]. Compared with existing methods, this CZE assay was found to be fast, reliable, and simple. Owing to these attractive features and because identification of controlled drugs should be based on at least two independent analytical methods, a new and complementary capillary electrophoretic method was developed in the present work to support the identification of psilocybin. In addition, some attention was focused on the identification of the dephosphorylated compound psilocin bacause this closely comigrated with the electroosmotic flow in the original CZE system [9].

Correspondence: Dr. Stig Pedersen-Bjergaard, School of Pharmacy, University of Oslo, P.O. Box 1068 Blindern, 0316 Oslo, Norway (Tel: +47-2285-6576; Fax: +47-2285-4402; E-mail: stig.pedersen-bjergaard@farmasi.uio.no)

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2 Materials and methods

2.1 Reagents

Psilocybin and psilocin were supplied by Sandoz (Basel, Switzerland). Propyl chloroformate (98%) was purchased from Aldrich (Steinheim, Germany). Methanol of HPLC grade and analytical grades of sodium tetraborate, sodium dihydrogenphosphate, sodium hydroxide, potassium hydrogencarbonate, and potassium carbonate were from Merck (Darmstadt, Germany). Hexadecyltrimethylammonium bromide (CTAB) of 98% was purchased from Fluka AG (Buchs, Switzerland), while sodium dodecyl sulfate (SDS) of 99% was obtained from Sigma (St. Louis, MO, USA). Deionized water was obtained from a Milli-Q system (Millipore, MA, USA), and samples of *Psilocybe semilanceata* were supplied by The Bureau of Crime Investigation (Oslo, Norway).

2.2 Apparatus and separations

The CE-system was a P/ACE™ 5510 Series from Beckman (Fullerton, CA, USA) equipped with automatic sampling and execution of electrophoretic runs. Separations were performed inside a 57 cm × 50 μm uncoated eCAP™ fused-silica capillary (Beckman) with the detector window placed 7 cm from the capillary outlet. Sample introduction was accomplished by hydrodynamic injection with pressure (0.5 psi, 5 s). The compounds were detected on-column at 225 nm with an aperture of 100 × 800 μm. Electropherograms were recorded and processed with the capillary electrophoresis software for the P/ACE System 5000 series (Beckman). The system was run at 25°C with an applied voltage of 25 kV. With the different buffers used, this voltage generated a current of typically 40–70 μA. Water and methanol were used as markers for the electroosmotic flow.

2.3 Preparation of running buffers

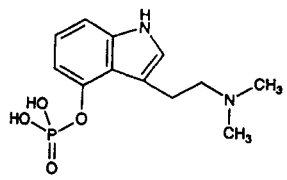
The running buffer for capillary zone electrophoresis of psilocybin consisted of 10 mM borate and 10 mM phosphate. For the experiments with a micelle-modified system, 25 mM of either CTAB or SDS was added to the borate/phosphate buffer. For the capillary zone electrophoresis of psilocin, the running buffer consisted of 10 mM phosphate. In all cases, the pH was adjusted with 1 M NaOH and controlled with a Metrohm 632 pH meter (Metrohm, Herisau, Switzerland). The pH of the system was determined in the final buffer ready for use.

2.4 Standards, sample preparation, and derivatization

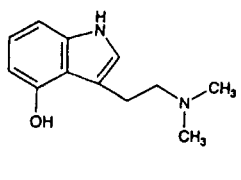
Standard solutions of psilocybin (0.2 mg/mL), psilocin (0.2 mg/mL), and barbital (0.5 mg/mL) were prepared in methanol. The solutions were protected from light and stored at −20°C. Mushroom samples were pulverized and extracted with methanol [5]; four accurately weighed mushrooms (corresponding to approximately 100 mg) were extracted in an ultrasonic bath for 15 min with 3.0 mL of methanol containing 0.5 mg/mL barbital (internal standard). After 10 min of centrifugation at 3200 rpm and transfer of the supernatant, the extraction was repeated for 15 min with fresh 2.0 mL methanol containing barbital. The extracts were combined and directly subjected to pressure injection. Sample derivatization was performed with propyl chloroformate; 2.0 mL of methanolic extract (*Psilocybe semilanceata*) was mixed with 2.0 mL of a 50 mM potassium carbonate/50 mM potassium hydrogencarbonate buffer (pH 9.0). Fifty µL of propyl chloroformate was added, and derivatization was accomplished during 15 min exposure to ultrasonification.

3 Results and discussion

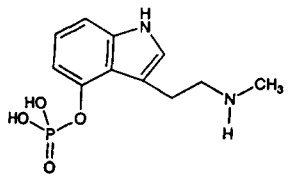
In a recent paper, a new method based on CZE was proposed for the determination of psilocybin in samples of



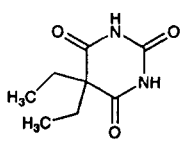
A) Psilocybin



B) Psilocin



C) Baeocystin



D) Barbital

Figure 1. Structures of (A) psilocybin, (B) psilocin, (C) baeocystin, and (D) barbital.

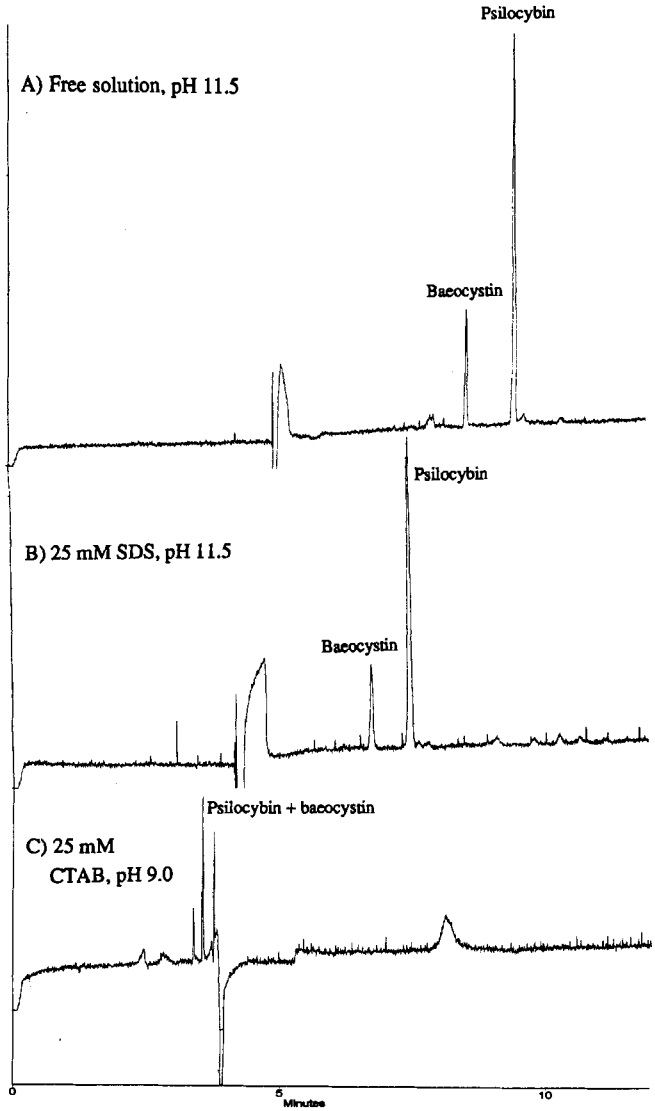


Figure 2. Capillary electrophoresis of *Psilocybe semilanceata* utilizing (A) free solution at pH 11.5, (B) 25 mM SDS at pH 11.5, and (C) 25 mM CTAB at pH 9.0. Common buffer: 10 mM borate/phosphate.

the hallucinogenic mushroom *Psilocybe semilanceata* [9]. Owing to their structural similarity (Fig. 1A, C), CZE separation of psilocybin and the demethylated derivative baeocystin was impossible within the pH range 4–10.5. Above pH 10.5, however, the different deprotonation of the secondary amino group of baeocystin and the tertiary amino group of psilocybin resulted in significant differences in their charge-to-size ratios, and excellent separation of the two zwitterionic compounds was achieved within 10 min at pH 11.5 (Fig. 2A). The identity of psilocybin was confirmed by migration time information and by UV-spectra, while quantitation was accomplished utilizing barbital (Fig. 1D) as internal standard. The consumption of organic solvents was very low with the CZE method, and intra-day and inter-day variations of quantitative data were only 0.5 and 2.5% RSD, respectively. Because identification of controlled drugs should be based on at least two independent analytical methods, the present work was focused on a complementary separation system for psilocybin based on capillary elec-

trophoresis. In addition, some attention was focused on the identification of the dephosphorylated derivative psilocin because the original CZE method suffered for this particular compound owing to a close comigration with the electroosmotic flow.

3.1 Alternative separation strategies for psilocybin and baeocystin

3.1.1 Addition of micelles to the running buffer

In a first experiment, 25 mM SDS was added to the 10 mM borate/phosphate running buffer (pH 11.5) to separate the two compounds by different interaction with anionic micelles. As with CZE, psilocybin and baeocystin were effectively separated (Fig. 2B), but the effective electrophoretic mobility of both compounds was almost unaffected by the addition of SDS. Obviously, both psilocybin and baeocystin were almost excluded from the micelles. This effect arose owing to the zwitterionic nature of the compounds; the negative charges of psilocybin and baeocystin caused an electrostatic repulsion from the micelles. Consequently, separation with SDS in the running buffer was exclusively based on charge-to-size ratios and provided no complementary selectivity as compared with the existing CZE method. As an alternative, 25 mM CTAB was added to the running buffer (pH 11.5) to form cationic micelles. CTAB was adsorbed onto the silica surface of the capillary and formed a bilayer with a net positive charge. Since this positive charge changed the direction of the electroosmotic flow, the applied voltage was reversed. Unfortunately, with CTAB present in this highly alkaline running buffer, psilocybin rapidly decomposed following injection. Thus, even 0.5 mg/mL of psilocybin disappeared in the electropherogram. This problem was generally observed in the pH range from 9.5 to 11.5, while acceptable stability for psilocybin was observed when the pH of the running buffer was adjusted to 9.0. However, at pH 9.0 as well as in more acidic media, psilocybin and baeocystein comigrated as illustrated in Fig. 2C.

3.1.2 Addition of ion-pair reagents to the running buffer

Because the experiments above with micelles in the running buffer were unsuccesful in the development of alternative separation strategies for psilocybin and baeocystin, the experiments were continued with the addition of ion-pair reagents to the CZE running buffer. The idea was to utilize ion-pair reagents to suppress the positive charges on psilocybin and baeocystin under highly alkaline conditions in order to promote significant shifts in their electrophoretic mobilities. In a first experiment, 50 mM tetramethyl ammonium chloride was added to the 10 mM borate/phosphate running buffer (pH 11.5), but this provided no change in their migration behavior. Obviously, no ion-pair formation occurred during the electrophoretic run. In a subsequent experiment, 50 mM tetrabutyl ammonium bromide was added as a more hydrophobic ion-pair reagent. In this medium, the effective electrophoretic mobility decreased approximately 10%, which indicated a minor tendency for ion-pair formation. Unfortunately, this minor shift in migration time provided no strong complementary information to positively confirm the presence of psilocybin.

3.1.3 Derivatization prior to electrophoretic analysis

As a third attempt to develop an alternative identification method for psilocybin, derivatization of psilocybin (and baeocystin) in aqueous solution with propyl chloroformate was evaluated. The idea was to change the electrophoretic mobility of the two compounds, and subsequently reanalyze the derivatized species with the original CZE system. To accomplish the derivatization, 2.0 mL of the methanolic mushroom extract was diluted with 2.0 mL of 50 mM hydrogencarbonate/50 mM carbonate buffer (pH 9.0). Subsequently, 50 µL propyl chloroformate was added, and derivatization was accomplished in the alkaline medium during 15 min of ultrasonification. As illustrated in Fig. 3A and B with a pure standard solution, psilocybin was almost quantitatively transformed into a single derivative of higher electrophoretic mobility. This shift in migration time by approximately 1.7 min was attractive for the confirmation of psilocybin, and occurred with high efficiency in the real samples (Fig. 3C and D). A quantitative analysis of psilocybin in a mushroom extract was accomplished both before and after derivatization. The quantitative results (1.04 and 1.05% psilocybin in dried mushrooms, respec-

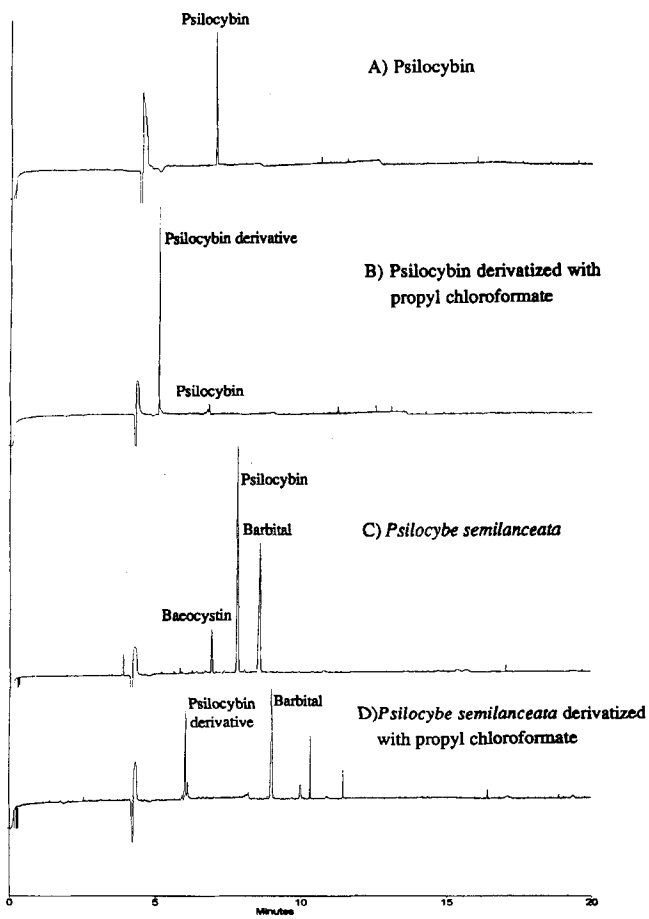


Figure 3. CZE of (A) psilocybin, 0.2 mg/mL, (B) psilocybin, 0.2 mg/mL, derivatized with propyl chloroformate, (C) *Psilocybe semilanceata*, and (D) *Psilocybe semilanceata* derivatized with propyl chloroformate. CZE buffer: 10 mM borate/phosphate adjusted to pH 11.5.

tively) were identical and indicated that no derivatization products of the sample matrix comigrated with the psilocybin derivative. Also, baeocystin was totally derivatized by the propyl chloroformate reagent. The baeocystin derivative was probably the peak which migrated closely behind the psilocybin derivative. However, because no reference standard was available for baeocystin, the location within the electropherogram of the baeocystin derivative was not confirmed.

The derivatization of psilocybin was expected to occur only at the indole nitrogen, which resulted in the formation of a monocarbamate. Because this process increased the size of the compound without affecting the net negative charge, the observed decrease in negative electrophoretic mobility following derivatization was in accordance with the expected reaction scheme. For baeocystin in contrast, derivatization was expected to occur at both nitrogen atoms, resulting in a dicarbamate structure. Thus, the size of the molecule increased significantly, and the positive charge of the secondary amino group was totally eliminated. The former effect was responsible for the assumption that the negative electrophoretic mobility of baeocystin decreased following derivatization, while the latter effect changed the migration order of psilocybin and baeocystin after reaction.

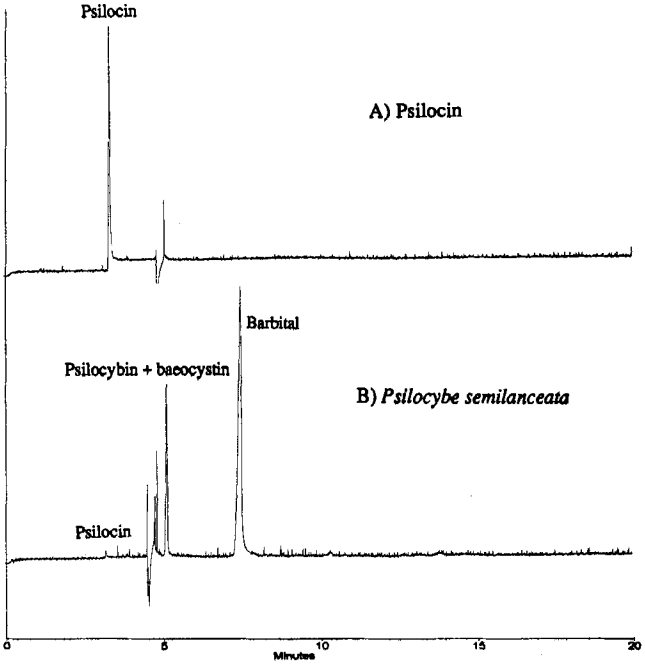


Figure 4. CZE of (A) psilocin, 0.2 mg/mL, and (B) *Psilocybe semilanceata*. CZE buffer: 10 mM phosphate adjusted to pH 7.2.

3.2 Analysis of psilocin

Although psilocin is found only at trace levels in *Psilocybe semilanceata* [3], some attention was focused on this dephosphorylated derivative of psilocybin because it may potentially be present at higher levels in other mushrooms. As mentioned above, psilocin was impossible to analyze by CZE at pH 11.5 because this compound comigrated with the electroosmotic flow. Thus, the psilocin peak was not separated from other neutral components potentially present in the methanolic extracts. In a subsequent experiment, the pH of the CZE system was reduced to 7.2 by utilizing a 10 mM phosphate running buffer. At this pH value, the negative charge of the phenolic group was suppressed, and psilocin carried only a single positive charge. Under these conditions, psilocin migrated as a sharp peak prior to the electroosmotic flow (EOF; Fig. 4A), while psilocybin and baeocystin comigrated after the EOF (Fig. 4B). As shown from the mushroom extract, only traces of psilocin were detected in the extract of *Psilocybe semilanceata*. While solutions of psilocybin were highly stable, psilocin was found to be unstable in methanolic solutions. Consequently, both standard solutions of the latter and mushroom extracts were protected from light and stored at −20°C prior to analysis.

4 Concluding remarks

In the present work, a new and rapid method was developed for identity confirmation of psilocybin in the hallucinogenic mushrooms *Psilocybe semilanceata*. Following quantitative analysis by capillary zone electrophoresis at pH 11.5, mushroom extracts containing psilocybin were treated with propyl chloroformate for 15 min and subsequently reanalyzed under the same electrophoretic conditions. This caused a specific change in the migration behavior of psilocybin and was utilized to support the identification of the hallucinogenic compound. In addition, a capillary zone electrophoretic system for the dephosphorylated derivative psilocin was developed where the pH of the running buffer was reduced to 7.2. At this pH value, psilocin migrated prior to the electroosmotic flow and was effectively analyzed within 3.5 min.

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