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Determination of psilocybin in *Psilocybe semilanceata* by capillary zone electrophoresis

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Abstract

A capillary zone electrophoretic (CZE) method was developed for the rapid determination of psilocybin in *Psilocybe semilanceata*. Following a simple two step extraction with 3.0+2.0 ml methanol, the hallucinogenic compound was effectively separated from matrix components by CZE utilizing a 10 mM borate–phosphate running buffer adjusted to pH 11.5. The identity of psilocybin was confirmed by migration time information and by UV spectra, while quantitation was accomplished utilizing barbital as internal standard. The calibration curve for psilocybin was linear within 0.01–1 mg/ml, while intra-day and inter-day variations of quantitative data were 0.5 and 2.5% R.S.D., respectively. In addition to psilocybin, the method was also suitable for the determination of the structurally related compound baeocystin. © 1997 Elsevier Science B.V.

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1. Introduction

Psilocybe semilanceata is the most abused hallucinogenic mushroom in the Scandinavian countries. Dried mushrooms have been shown to contain up to 2% of psilocybin (Fig. 1A) which is the major active constituent. The dephosphorylated compound, psilocin (Fig. 1B), is only found in trace amounts. However, up to 0.3% of the demethylated derivative of psilocybin, baeocystin (Fig. 1C), has been detected [1]. In a study of mushrooms from several European countries, *Psilocybe semilanceata* and *Panaeolus subbalteatus* proved to be the only psilocybin-containing fungi that can be gathered in Middle and Northern Europe in sufficient quantities

to permit abuse [2]. However, psilocybin has been detected worldwide in a variety of mushrooms belonging to the genera of *Psilocybe*, *Panaeolina*, *Panaeolus*, *Copelandia*, *Conocybe*, *Gymnopilus*, *Stropharia* and *Pluteus* [3,4]. Some recent reports include the detection of psilocybin in mushrooms from Thailand [5], the Venezuelan Andes [6], the Hawaiian islands [7] and Thailand [8].

Since psilocybin is a controlled compound and considered a narcotic drug, it must be reliably identified and quantified in mushroom samples. Identification of psilocybin, psilocin and baeocystin has been carried out by thin-layer chromatography [2,9]. Due to their high polarity and amphoteric nature, high-performance liquid chromatography (HPLC) either in the normal-phase mode [10] or in the reversed-phase mode [11] has been a preferred

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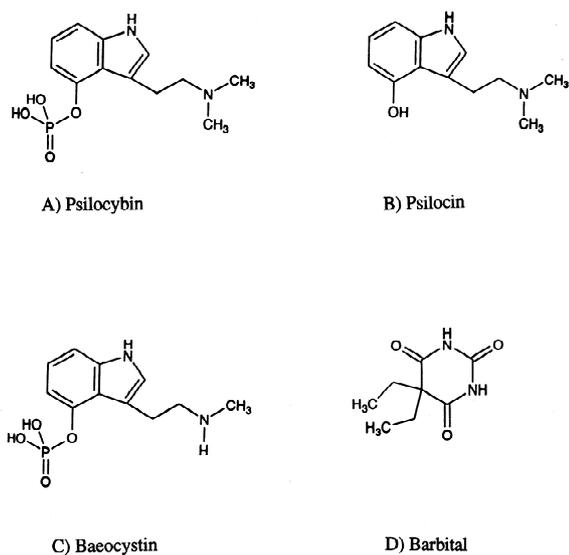


Fig. 1. Structures of (A) psilocybin, (B) psilocin, (C) baeocystin and (D) barbital.

method for their quantification, utilizing either UV, fluorescence or electrochemical detection [12]. Gas chromatography has been carried out after derivatization [9], and GC–MS analysis has been performed for recording of mass spectra for court evidence [13].

Capillary electrophoresis (CE) has been used increasingly to analyse seized drugs, and a variety of methods has been worked out for narcotic drug analysis [14]. CE can analyse drugs which are nonvolatile, polar and thermally degradable, and would in principle be an excellent technique for the analysis of psilocybin and baeocystin in mushroom samples. Psilocybin has previously been separated from other narcotic drugs in a MEKC forensic drug screen using SDS as surfactant [15]. No CE method has, however, been reported for the separation of the closely related compounds psilocybin and baeocystin in hallucinogenic mushrooms. Therefore, the aim of the present study was to develop a CE method for the rapid separation, identification and quantification of psilocybin in mushroom samples. In order to maintain analytical simplicity, the work was carried out with capillary zone electrophoresis. Because of high importance within the European countries, the assay was focused on the mushroom *Psilocybe semilanceata*.

2. Experimental

2.1. Chemicals

Psilocybin was supplied by Sandoz (Basel, Switzerland). Methanol of HPLC grade and analytical grades of sodium tetraborate and sodium dihydrogenphosphate were from Merck (Darmstadt, Germany). The internal standard barbital was purchased from Norsk Medisinaldepot (Oslo, Norway). 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. CE system

The CE system was a P/ACE 5000 Series from Beckman (Fullerton, CA, USA) equipped with automatic sampling and execution of electrophoretic runs. Unless otherwise stated, the column used was a 57 cm × 50 μm fused-silica capillary with the detector window 7 cm from the outlet. For electrophoresis in the normal direction (50 cm effective capillary), sample introduction was accomplished by hydrodynamic injection with pressure (0.5 p.s.i. (1 p.s.i. = 6894.76 Pa), 5 s). Reversal of the voltage was also tested with the 57 cm column performing separation in the 7 cm capillary from the detector outlet to the detector window. In this case, sample introduction was accomplished by electrokinetic injection (10 kV, 3 s). The compounds were detected on-column at 220 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. Water and methanol were used as markers for the electroosmotic flow.

2.3. Preparation of running buffers

The running buffers consisted of 10 mM borate and 10 mM phosphate. The pH was adjusted either with 1 M NaOH or 1 M HCl 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. Preparation of samples

Standard solutions of psilocybin (0.005–1.0 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 [10]. For quantitative analysis, 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 of barbital (internal standard). After 10 min of centrifugation at 3200 rpm and transfer of the supernatant, the extraction was repeated with 2.0 ml methanol containing barbital. Following combination, the extracts were directly subjected to pressure injection. In cases of electrokinetic injection, sample extracts were diluted 1:1 (v/v) with the running buffer.

2.5. Validation

The quantitative analyses of psilocybin were based on peak area measurements relative to the internal standard. A calibration graph was constructed for the concentration range 0.01–1.0 mg/ml of psilocybin. Methanolic extracts of *Psilocybe semilanceata* and methanolic solutions of psilocybin (0.02, 0.1 and 0.05 mg/ml) were analysed for intra-day ($n=6$) and inter-day variations ($n=6$). The limit of detection was determined at a signal-to-noise ratio of 2 ($S/N=2$), while the limit of quantification was defined at a signal-to-noise ratio of 10 ($S/N=10$).

3. Results and discussion

3.1. Separation considerations

As discussed in Section 1, the zwitterionic compound psilocybin (Fig. 1A) is the major constituent contributing to the hallucinogenic effect of the mushroom *Psilocybe semilanceata*. Therefore, the method developed in the present work was primarily focused on the rapid and unequivocal determination of this particular compound. However, *Psilocybe semilanceata* also contains considerable amounts of baecocystin (Fig. 1C), which is a demethylated de-

rivative of psilocybin. Although required for a reliable assay, separation of psilocybin and baecocystin may be difficult in general owing to their structural similarity. Therefore, resolution of these two closely related tryptamine derivatives by capillary zone electrophoresis was extensively investigated in the first part of the present study.

Owing to the zwitterionic nature of psilocybin and baecocystin, their separation was expected to be highly dependent on pH of the 10 mM borate-phosphate running buffer. Within the pH range 4–10, the net negative charge of both psilocybin and baecocystin were expected to be almost identical; the phosphate group of both compounds was negatively charged to the same extent (up to -2), while the amino groups were totally protonated ($+1$). In this pH range therefore, both compounds migrated towards the anode by electrophoretic mobilities principally determined by their size. Unfortunately, the structures of the two compounds differed only by the presence of a single methyl group, and the experiments revealed that separation based on this minor difference in size was impossible (Fig. 2).

In strong basic environments, the net negative charge of both compounds was expected to increase owing to deprotonation of the amino groups. Because baecocystin contained a secondary amino group while the corresponding functionality was tertiary in nature for psilocybin, significant differences in their net negative charge were expected at pH values close to the pK_a for psilocybin. Although no exact pK_a information was available, deprotonation of this tertiary amino group was expected to occur between pH 10.0 and 12.5. Therefore, the experiments with capillary zone electrophoresis were carried out in the pH range 10.0–12.5. At pH 10.5, partial deprotonation occurred and different electrophoretic mobilities resulted in base line separation of psilocybin and baecocystin (Fig. 2). The resolution improved as the pH of the running buffer increased to 12.0. Above this value, however, the separation was gradually deteriorated owing to the strong deprotonation of both compounds; the net negative charge approached two for both compounds in highly alkaline solutions.

Although the deprotonation of the amino groups promoted separation of psilocybin and baecocystin as discussed above, this also caused prolongation of migration times owing to an increase of the net

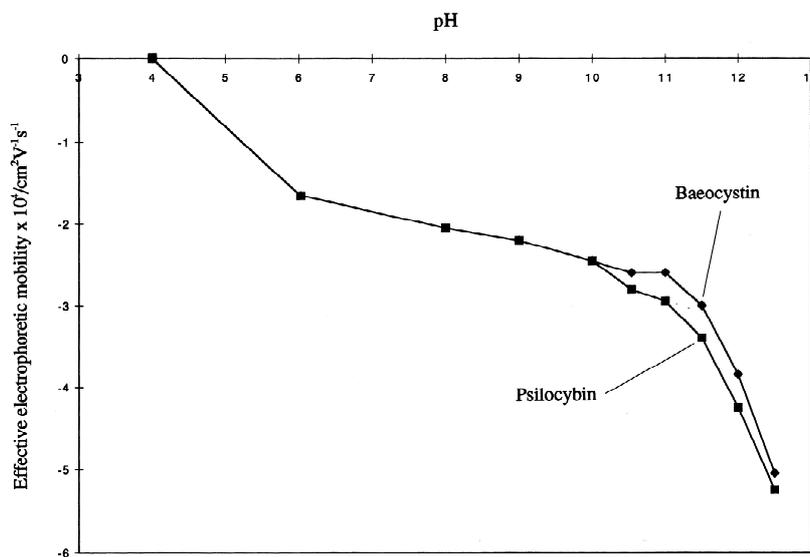


Fig. 2. Effect of running buffer pH on the effective electrophoretic mobility of psilocybin and baeocystin. Buffer: 10 mM borate–phosphate pH adjusted with NaOH or HCl (see Section 2.3).

negative charge for both compounds. Therefore, in order to minimize the analysis time while maintaining acceptable separation, the 10 mM borate–phosphate running buffer was adjusted to pH 11.5 in the final method. As illustrated in Fig. 3, this running buffer provided excellent separation of psilocybin and baeocystin within 7.2 min. In spite of the alkaline conditions, no degradation of psilocybin and baeocystin was detected when present in the running buffer for several days. In contrast, for psilocin, rapid degradation was observed when present in highly alkaline solutions. In addition, psilocin migrated close to the electroosmotic flow in the current system. However, although psilocin was difficult to analyze with the proposed method, this compound is present only at trace levels in *Psilocybe semilanceata* and was therefore of little interest in the present work.

As shown in Fig. 3, the migration times of baeocystin and psilocybin differed by as much as 0.97 min when separation was performed at 25 kV in a 57 cm column (50 cm effective length). Because of this, it was decided to speed up the analysis by utilizing a shorter capillary for the separation. A 25 cm column (18 cm effective length) was tested, and operation at 20 kV provided a 1.7 min time of analysis. However, owing to the higher electric field,

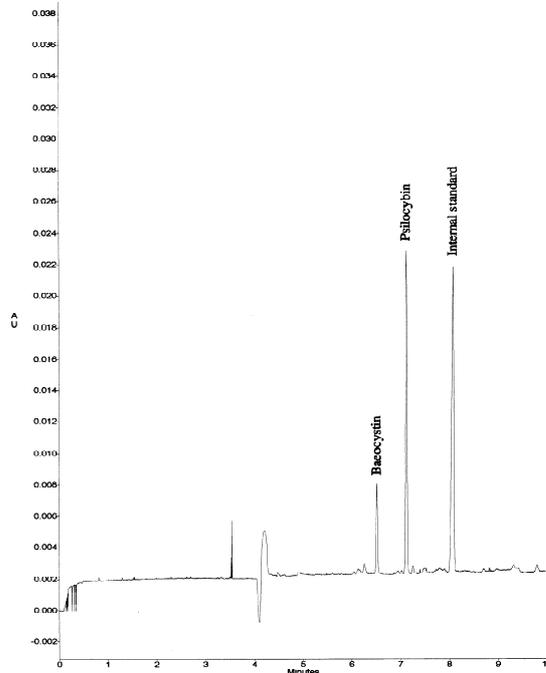


Fig. 3. Analysis of *Psilocybe semilanceata* by capillary zone electrophoresis. Buffer: 10 mM borate–phosphate adjusted to pH 11.5. Detection: 220 nm.

the baseline was elevated and the noise level increased (Fig. 4A). Therefore, in a subsequent experiment, the 57 cm column was reinstalled, the voltage was reversed, and the sample was introduced at the detector outlet utilizing the 7 cm capillary to the detector window for separation. As illustrated in Fig. 4B, baecocystin and psilocybin were baseline separated even in this short column, and the analysis time was now reduced to 1.3 min. Owing to the long total length of the capillary, the electric field was relatively low and no elevation of the baseline was observed. Unfortunately, only electrokinetic injection was possible with our instrument when sample introduction was performed at the capillary outlet, and this injection technique was found to suffer from a discriminative analyte transfer. Thus, without the

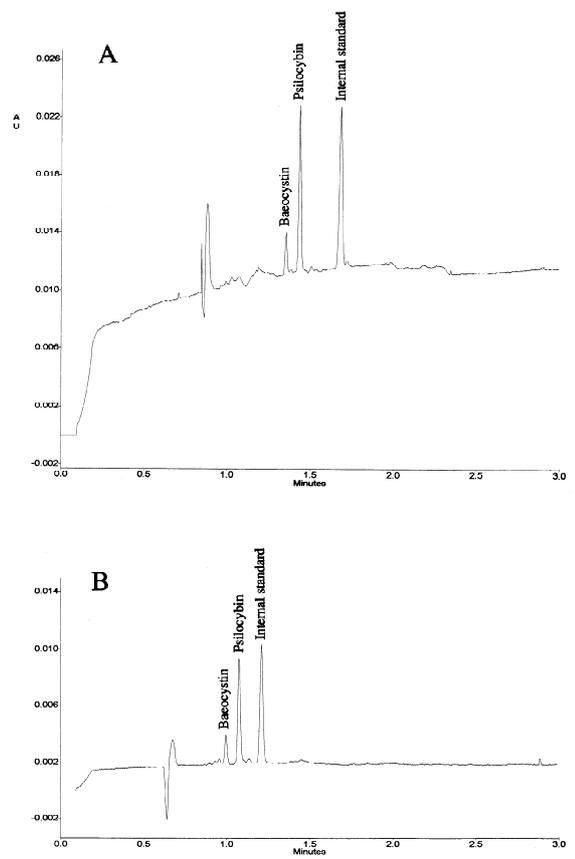


Fig. 4. Analysis of *Psilocybe semilanceata* by capillary zone electrophoresis. (A) 20 kV, 25 cm capillary (18 cm effective length), injection at inlet. (B) 25 kV, 57 cm capillary (7 cm effective length), injection at detector outlet.

facility of pressure injection at the detector outlet, the reversed direction mode was suitable only for screening purposes. Therefore, the validation of the method below was performed in a 57 cm capillary (50 cm effective length) utilizing pressure injection.

3.2. Analysis of mushroom samples

Prior to analysis, *Psilocybe semilanceata* was extracted in two steps with 3.0 and 2.0 ml methanol as described in Section 2.4 to ensure a quantitative recovery (98%) of psilocybin [10]. Owing to the relatively high levels of psilocybin in *Psilocybe semilanceata*, the combined extract was directly subjected to pressure injection without further operations.

Since reference material of psilocybin was commercially available, identification of this compounds in *Psilocybe semilanceata* was principally based on migration time information. To further improve the reliability, the presence of psilocybin was effectively confirmed by UV spectra obtained with the UV photodiode array detector (Fig. 5). In the case of baecocystin, no commercial standards were available. However, UV spectra of this compound have been reported in the literature [16], and are similar to those of psilocybin owing to the common 4-phosphoryloxytryptamine chromophore. This information

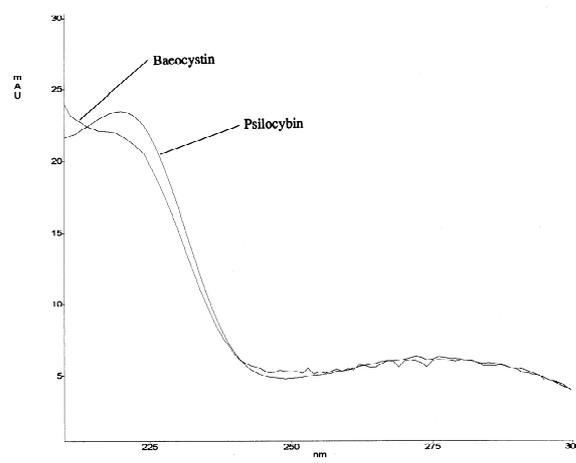


Fig. 5. UV spectra of psilocybin and baecocystin obtained during capillary zone electrophoresis. Buffer: 10 mM borate-phosphate adjusted to pH 11.5.

Table 1
Intra-day and inter-day variations for the quantification of psilocybin by capillary zone electrophoresis

Sample	Intra-day variation ($n=6$) (%)	Inter-day variation ($n=6$) (%)
0.02 mg/ml standard solution	0.87	2.02
0.1 mg/ml standard solution	0.41	2.44
0.5 mg/ml standard solution	0.87	4.30
Mushroom sample	0.49	2.49

was used in the present work for a rapid and reliable identification of baecocystin.

For psilocybin, quantification was accomplished with barbital as internal standard (Fig. 3). At pH 11.5, the internal standard carried a net negative charge (Fig. 1D) and migrated as a perfectly resolved peak at 8.1 min. The calibration curve for psilocybin based on peak areas relative to barbital was linear in the concentration range (0.01–1.0 mg/ml) with a correlation coefficient of 0.9996. Because reference material for baecocystin was not commercially available, this compound was not accurately quantified in the present work.

3.3. Method validation

In order to validate the proposed assay for psilocybin, repeatability and reproducibility of both migration times and quantitative data were investigated. As illustrated in Table 1, the evaluation of quantitative results was carried out for standard solutions at three concentration levels (the lowest corresponding to 4 times the limit of quantification) and for a mushroom extract; the intra-day repeatability for psilocybin was 0.4–0.9% ($n=6$), while the inter-day results ($n=6$) varied within 2.0–4.3%. Similar results were obtained for the migration time of psilocybin (Table 2); for intra-day experi-

ments, the results varied 0.2–0.5% ($n=6$), while 3.1–4.8% values were obtained for inter-day experiments ($n=6$). This excellent migration time stability partially arose from the high pH of the running buffer; small variations in the experimental conditions hardly affected the electroosmotic flow owing to the high ionization of the silanol groups. The detection limit of the proposed CZE method was 0.0009 mg/ml ($S/N=2$), which corresponded to 0.045 mg/g of psilocybin in the mushrooms with the current extraction method. The corresponding values for the limit of quantitation was 0.0045 mg/ml ($S/N=10$) and 0.225 mg/g mushroom. Further improvement of the limits of detection and quantification was not considered in the present paper because the levels of psilocybin in mushroom extracts typically corresponded to 25–50 times the limit of quantification.

4. Conclusions

The present work has demonstrated a new, fast and highly reliable method based on capillary zone electrophoresis for the determination of psilocybin in seized mushrooms. Excellent separation of psilocybin and baecocystin was obtained in free solution with a 10 mM borate–phosphate running

Table 2
Intra-day and inter-day variations for migration times of psilocybin by capillary zone electrophoresis

Sample	Intra-day variation ($n=6$) (%)	Inter-day variation ($n=6$) (%)
0.02 mg/ml standard solution	0.31	4.76
0.1 mg/ml standard solution	0.36	3.93
0.5 mg/ml standard solution	0.49	3.05
Mushroom sample	0.18	3.37

buffer adjusted to pH 11.5. Reliable identification was based on migration times and UV spectra, while repeatable and reproducible quantification was performed utilizing barbital as internal standard. Compared with the recommended HPLC assay [9], the new CZE method was complementary in nature because another principle of separation was utilized. In addition, both the consumption of organic solvents and the analysis time were dramatically reduced with CZE.

Acknowledgments

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