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Pulsed amperometric detection of calystegines separated by capillary electrophoresis

Hans-Hermann Rüttinger[a], Birgit Dräger[b,*]

[a]*Martin-Luther-Universität Halle-Wittenberg, Institut für Pharmazeutische Chemie, Wolfgang-Langenbeck-Straße 4, D-06120 Halle, Germany*

[b]*Martin-Luther-Universität Halle-Wittenberg, Institut für Pharmazeutische Biologie, Hoher Weg 8, D-06120 Halle, Germany*

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Abstract

Calystegines are polyhydroxyalkaloids with a nortropane skeleton. They are oxidized by pulsed amperometry at a gold electrode due to their vicinal hydroxyl groups similar to monosaccharides, but at a slightly higher potential. Compared to carbohydrates, calystegines exhibit lower acidity, thus the effective electrophoretic mobility as anions in 0.1 *M* NaOH is lower, independent of their molecular mass. The acidity and mobility of calystegines increase with the number of hydroxyl groups. The influence of temperature and power dissipation in the capillary and changes of the inner surface on the migration times was eliminated by cooling and subtraction of the electroosmotic flow velocity. The high resolving power of capillary zone electrophoresis allows the separation of calystegines with the same number of hydroxyl groups. Detection is linear from 2 to 200 mg L^{-1} with a 1 nL injection volume. Calystegines were determined in crude plant sap after filtration without further sample purification.

Keywords: Plant materials; Calystegines; Alkaloids; Hydroxyalkaloids; Nortropanes

1. Introduction

Calystegines are a new group of polyhydroxyl alkaloids with a nortropane skeleton (Fig. 1). The substances were initially found in roots of *Calystegia sepium*, Convolvulaceae, and of *Atropa belladonna*, Solanaceae, but it appears that other Solanaceae contain higher concentrations. Since these substances are interesting monomers in the tropane alkaloid pathway, and also strong inhibitors of glycosidases, the demand for their determination in various matrices is growing rapidly. A versatile analytical procedure for screening calystegines in different plants and plant parts could be very useful. Calystegines in plant tissues usually occur as mixtures of three to six different compounds, the concentration ranging from traces of minor calystegines, <5 μg g^{-1} plant tissue, up to maximal concentrations of the major calystegines A_3 and B_2 of 500–2500 μg g^{-1} plant tissue [1,2]. Liquid chromatography of the original compounds as simple TLC [3] and HPLC with pulse amperometric detection can resolve the different groups of calystegines corresponding to the number of hydroxyl groups, but not of isomers containing the same number of hydroxyl functions. Resolution of the isomers requires GC

*Corresponding author. Tel.: +49-345-552-5765; fax: +49-345-552-7021.

E-mail address: draeger@pharmazie.uni-halle.de (B. Dräger).

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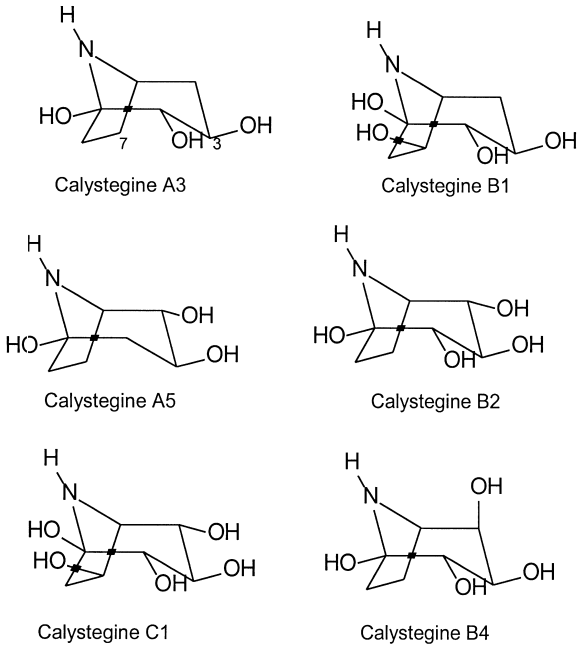

Fig. 1. Calystegine structures.

analysis after silylation of the alkaloids [3]. Before GC, many cumbersome extraction and derivatization steps are necessary which prevent effective screening of plant tissues. Capillary electrophoresis with its high inherent resolving power and low substance demand may be an alternative method for the rapid screening and analysis of minute volumes of plant extracts.

2. Experimental

2.1. Chemicals

A 0.1 *M* NaOH solution was prepared by dilution of an ampoule solution (Merck) with distilled water and filtered through a 0.25 μm syringe filter. Stock solutions of calystegines were prepared by dissolving the pure compounds in distilled water. Isolated calystegines (pure compounds analysed by CE, GC and TLC) were obtained from Dr. Naoki Asano, Kanasawa, Japan. The stock solutions were maintained at −20°C until use.

2.2. Preparation and clean up of plant extracts

2.2.1. Sample preparation

Plant tissues were obtained as cultured roots of *Atropa belladonna* or as spouts from tubers of *Solanum tuberosum* (potato). For dry mass measurements, aliquots were lyophilized. The alkaloids were extracted from fresh tissue, typically 1 g fresh mass, and homogenized in MeOH–water (1:1), 1 mL per gram fresh mass. Methanol was evaporated under reduced pressure. The aqueous extracts were applied to a cation-exchange column as described [2]. For experiments with plant sap, fresh tissues (1 g) were crushed, and approx. 0.1 mL was filtered through a 0.22 μm filter, mainly for removal of starch granules from potato extracts. This sap was diluted with water and injected directly into the CE column.

2.2.2. Gas chromatography

The quantification of calystegines was performed by gas chromatography, essentially as described previously [1,2]. The method was altered such that azobenzol (0.1 mg mL^{-1}) was used as internal standard for all measurements, and the column was a DB1, 30 m×0.25 mm I.D., 0.25 μm film thickness.

2.3. CE Instrumentation

Capillary electrophoresis (Fig. 2) was performed with a 50 cm fused-silica capillary with an inner diameter of 25 μm (Supelco Deisenhofen, Germany) at a constant voltage for each run ranging from 17 to 21 kV.

High-voltage supplies (F.u.G. Rosenheim, Germany) type HCN 7E-35000 were used for generation of the separation voltage. The end of the capillary was fixed to a micromanipulator (Carl Zeiss, Jena, Germany) and adjusted to a gold microelectrode (90 μm diameter) sealed in a soft glass tube at ca. 20 μm distance. To minimize the potential distortion caused by the IR drop between the capillary end and the detector electrode, the end of the capillary was etched with fluoric acid from the original outer diameter of 350 μm, as provided by Supelco, to an outer diameter of about 100 μm. The capillary was air-cooled by a fan (Conrad Electronic, Hirschau, Germany).

The electrochemical detector (own construction)

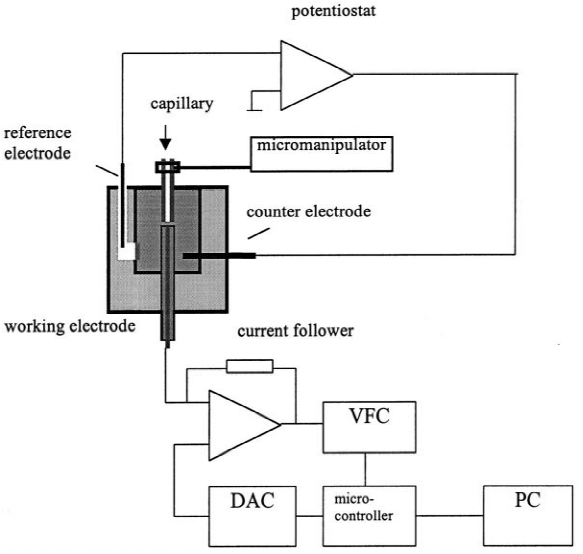

Fig. 2. Scheme of CE with pulsed amperometric detection.

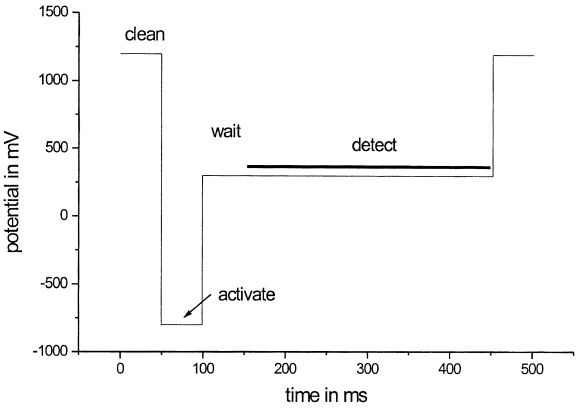

Fig. 3. Potential time function.

was equipped with a Pt counter electrode and a saturated Ag/AgCl reference electrode as shown schematically in Fig. 2. An operational amplifier type CA 3140 (Harris Semiconductors RS Components, Mörfelden, Germany) served as potentiostat, and the detection current was fed to a current follower OPA 110 (Burr-Brown, Filderstadt, Germany) to achieve a gain of 1 V μA^{-1}. The potentiostat kept the reference electrode at ground potential and the electrophoretical current was conducted together with the detection current into the counter electrode.

For data acquisition, a voltage-to-frequency converter VFC 110 (Burr-Brown) was connected to a microcontroller MC68HC11A8 (Motorola MCT Paul & Scherer, Berlin, Germany). The potential/time regime used for the pulsed amperometric measurements was generated by the microcontroller connected to a digital-to-analog converter (MAX 543 Spezial-Elektronik, Bückeburg, Germany). All data were transferred to a personal computer through a RS 232 interface.

2.4. CE procedure

The gold electrode was polished with waterproof abrasive paper and finally with 0.3 μm alumina. Before use it was purified by dipping into concentrated sulfuric acid, containing 20% hydrogen peroxide, rinsed with distilled water, and then mounted in the detection cell. This procedure was repeated at the start of every day. The detection cell was filled with buffer electrolyte and the capillary adjusted with the help of a stereomicroscope and the micromanipulator. After filling the capillary with the electrolyte using a disposable syringe, the source end of the capillary was placed in the source vial. The sample was then injected by application of hydrostatic pressure (gravity injection). The injection volumes were calculated according to the Hagen–Poiseuille law from the time of the applied pressure.

The experimental setup for pulsed amperometric measurements was similar to that described by O'Shea et al. [4] and Lu and Cassidy [5].

The cleaning and activation steps of the potential/time function (Fig. 3) were kept constant, whereas the detection potential was raised every cycle by about 15 mV, starting at −120 mV, to +450 mV. To achieve similar conditions as in the case of CE detection, the capillary was filled with a mixture of analyte–buffer (1:1) and due to the high voltage applied the analyte was pushed against the detecting area.

3. Results and discussion

3.1. Detection potential

Pulsed voltametric measurements were performed

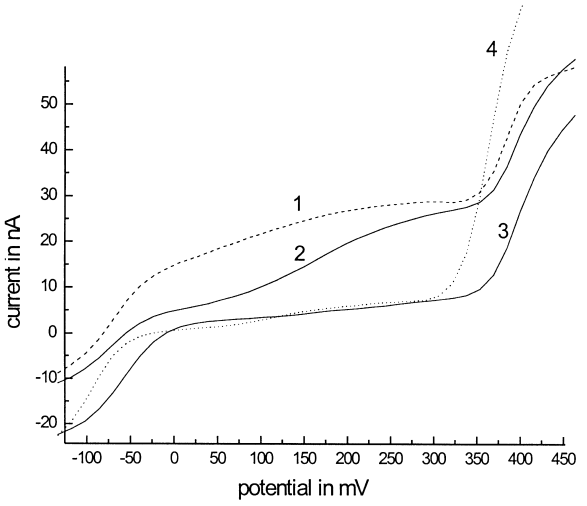

Fig. 4. Pulsed voltametric response of calystegines. 1: Glucose 100 mg L^{-1} in 0.05 *M* NaOH; 2: calystegine B_2 250 mg L^{-1}; 3: blank; 4: blank without electrophoresis current.

to optimize the detection potential. A detection potential of 300 mV gave the highest signal compared to the blank (Fig. 4)

Consequently, this potential was chosen for all CE separations for detection. Times for cleaning and activation were kept as short as possible to achieve a high sampling rate, which is mandatory for the registration of narrow peaks. The curves in Fig. 4 show that the calystegines require a slightly more positive potential to be oxidized than glucose. Comparison of curve Nos. 3 and 4 with and without electrophoretic current further shows the influence of the capillary current on the working electrode. Even the use of a very narrow bore capillary and etching a narrow capillary end did not prevent a shift of about 50 mV of curves Nos. 1, 2 and 3 compared to curve 4. Our attempts to introduce a junction prior to the end of the capillary that would keep the electrophoretic current away from the detection point [6] failed, because the diaphragm was not stable enough against 0.1 *M* NaOH for more than two to four runs. Therefore, direct end column detection, placing the detector electrode at a minimal distance from the capillary end with some potential distortion, but almost no peak broadening, appeared to be a reasonable compromise.

3.2. Identification and quantification of calystegines in plant extracts

All of the investigated reference calystegines were separated, if they occurred in the same concentration range, despite the unfavorable conditions for capillary electrophoresis such as low ionization (calystegines are basic compounds due to the nitrogen and are difficult to deprotonize) and minimal differences in structure. The electrophoretic mobilities indicate that ionization depends on the number of hydroxyl groups in the molecule (Table 1).

The migration times varied due to changes in EOF caused by (1) differences in column length, (2) fluctuations in temperature, either ambient temperature or different heating at different voltages, and (3) the capillary surface increasing slowly in acidity in the alkaline electrolyte.

Shorter columns and higher temperatures caused shorter migration times, which could be overcome by an encapsulated column with constant length and temperature. The alkaline electrolyte also led to shorter migration times after 20–40 runs [7]. The effective ion mobility (μ_{eff}), however, remained constant within narrow limits of deviation (Table 1).

The electropherogram of an extract from roots of *Atropa belladonna* containing calystegines A_3, A_5 and B_2 shows sharp baseline-resolved peaks (Fig. 5).

Table 1
Migration times of calystegines and similar compounds. μ_{eff}, effective ion mobility $\times 10^{-6}$. Migration times were influenced by changes in EOF due to capillary surface variations and temperature effects [7]. Standard deviations of the effective ion mobility are given for those calystegines that were measured as reference compounds as well as in plant extracts

Compound	Migration time (s)	μ_{eff} ($cm^2/V\,s$)
2,7-Dihydroxy-nortropane	395	7.80
A_3	415	22.0±0.8
A_5	422	27.1±0.5
B_4	426	29.8±1.3
B_2	432	33.6±9
B_1	446	42.2±3
C_1	480	61.1
Sucrose	514	77.1±1.5
Glucose	684	131.0±0.9

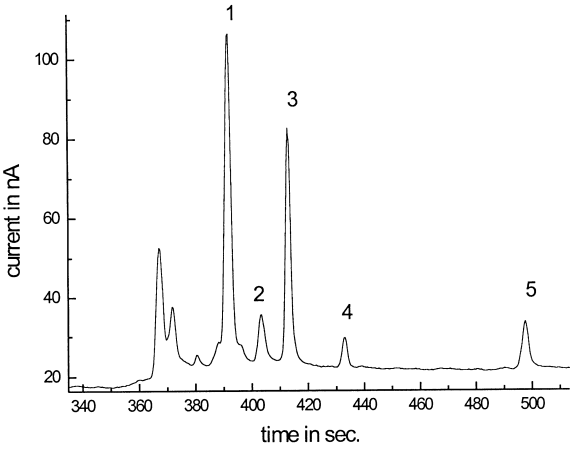

Fig. 5. Root culture extract from *Atropa belladonna*, purified by ion-exchange. Separation voltage 17.5 kV, capillary length 50 cm. 1: Calystegine A_3, 2: calystegine A_5, 3: calystegine B_2, 4: calystegine B_1, 5: sucrose.

From the half-width we calculated a theoretical plate number of 160 000 for calystegine B_2. Linear calibration curves for calystegine A_3 and calystegine B_2 were obtained for concentrations from 2 to 200 mg L^{-1}: concentration of calystegine A_3 (mg L^{-1}): intercept (nA s) 18.05 ($\pm$11.5), slope (nA s L mg^{-1}) 9.61 ($\pm$0.15), regression coefficient $R = 0.9990$. Concentration of calystegine B_2 (mg L^{-1}): intercept (nA s) 73.15 ($\pm$32.4), slope (nA s L mg^{-1}) 11.71 ($\pm$0.29), regression coefficient $R = 0.9981$.

One nanoliter was injected routinely. The slope diminishes at concentrations higher than 200 mg L^{-1} from 11.7 to 10.1 nA L mg^{-1} in the case of calystegine B_2, because the electrode is deactivated during the detection step due to the high analyte concentration, i.e. deposition of oxidized product on the electrode surface. The limit of detection was determined (3σ, three times the baseline noise) to be 500 μg L^{-1} calystegine B_2. The relative standard deviation of the peak area was $<5\%$ at a concentration of 100 mg L^{-1}. The results show good reproducibility, but the detector has to be recalibrated when the electrode surface is polished or the distance to the electrode is changed.

The determination of calystegines by CE in semi-purified plant extracts was consistent with GC analysis of a silylated mixture within a standard deviation of 10%. The extract shown in Fig. 5 contained calystegine A_3 240 mg L^{-1}, calystegine A_5 67 mg L^{-1}, calystegine B_1 27 mg L^{-1}, and calystegine B_2 171 mg L^{-1}, determined by CE after 1:4 dilution. The same extract measured by GC contained calystegine A_3 239 mg L^{-1}, calystegine A_5 59 mg L^{-1}, calystegine B_1 25 mg L^{-1}, and calystegine B_2 167 mg L^{-1}.

3.3. Identification and quantification of calystegines in crude plant saps

In contrast to GC, crude plant saps could be subjected directly to CE separation (Fig. 6). Crushed potato sprout sap was diluted and injected directly, and calystegines A_3 (668 mg L^{-1}), B_2 (402 mg L^{-1}) and B_4 (316 mg L^{-1}) were identified. Calystegine determination was confirmed by GC analysis. Other soluble compounds in the plant saps did not interfere with calystegine determination by CE; they either had a different electrophoretic migration time (sugars such as glucose or sucrose) or they were not oxidized under the conditions used. Because of the injection volume of a few nanoliters only, the content of a single cell vacuole (5–100 nL) is sufficient for analysis. The preparation of single vacuole saps by micromanipulators will enable single cell analysis. Electrophoresis of filtered plant saps can be auto-

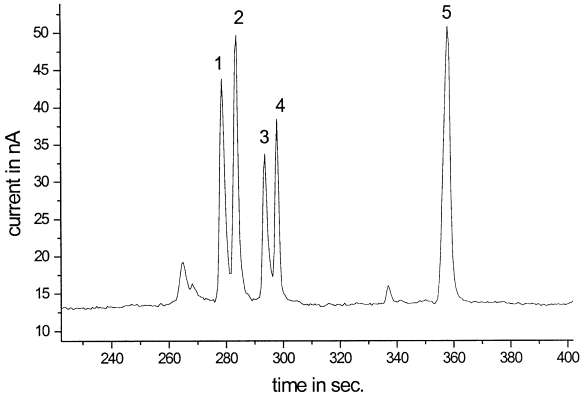

Fig. 6. Crushed potato sprout tissue sap, directly injected, dilution 1:11. Separation voltage 21 kV, capillary length 48 cm. 1: Unknown, 2: calystegine A_3, 3: calystegine B_4, 4: calystegine B_2, 5: sucrose.

mated by using an auto-sampler. In this way, a rapid and simple analysis of plant tissues for calystegine content is possible

4. Conclusions

Capillary zone electrophoresis with pulsed amperometric detection for calystegine determination in plant extracts is rapid, because sample preparation can be reduced to a minimum. Plant saps can be analyzed directly after filtration. The measurement is sensitive and selective for calystegines because of the specific oxidation voltage. The small injection volume should also enable analysis of single cell contents. Quantification of calystegines is possible, but requires frequent recalibration.

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