ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
16 (1998) 717–722

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

Determination of the main tropane alkaloids from transformed *Hyoscyamus muticus* plants by capillary zone electrophoresis

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Received 2 January 1997

Abstract

A capillary zone electrophoretic method (CZE) was developed using an uncoated fused silica capillary for the separation and determination of the main tropane alkaloids. The applicability of the developed method for analysis of plant samples was examined by analyzing samples of transgenic Egyptian henbane *Hyoscyamus muticus* (L.) plants. A simple 40 mM phosphate buffer at pH 7.8 using a voltage of 20 kV was found the best for this purpose. The main tropane alkaloids, atropine and scopolamine as well as nor-(−)-scopolamine, and tropic acid, the precursor of tropane alkaloids, could be separated in less than 13 min. The linear concentration range for atropine was 5.00–140 μg ml^{-1}, for scopolamine 7.50–210 μg ml^{-1} and for tropic acid 2.50–70.0 μg ml^{-1}. © 1998 Elsevier Science B.V.

Keywords: Capillary zone electrophoresis; Tropane alkaloids; Transgenic plants

1. Introduction

The tropane alkaloids atropine and scopolamine are widely used as parasympatolytic, anticholinergic and anti-emetic drugs that are produced by Solanaceous plants. Since the production of these compounds with plant cell cultures has several beneficial features when compared to conventional methods, such as plantation growth and synthetic production, there have been numerous attempts to produce them with conventional or genetically transformed plant cell cultures or alternatively with transgenic plants [1]. Thus rapid and simple analytical methods are often needed to enable the analysis of numerous samples in a short period of time.

Tropane alkaloids are currently analyzed by several methods including gas chromatography (GC) [2,3], high performance liquid chromatography (HPLC) [4,5], and immunological methods [6,7]. Even though HPLC can successfully be used in tropane alkaloid analysis, complex sample purification is often needed [4,5]. The GC methods ordinarily provide the means to analyze many compounds simultaneously but they often involve

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PII S0731-7085(97)00121-0

the use of analytical derivatization prior to the analysis [2] or the use of a specific detector system [3].

Due to their high selectivity, immunological methods can be utilized in the analysis of unpurified plant samples [6,7]. The major problems with immunological methods are the material costs and the fact that simultaneous analysis of several compounds is impossible. When attempting to improve tropane alkaloid biosynthesis in plant cell cultures to develop an economically feasible production method for the end products of the biosynthetic pathway, atropine and scopolamine are the most interesting. Therefore, a simple and fast method which enables the simultaneous analysis of both these compounds would be ideal. Because of the high efficiency, resolving power and low sample consumption of capillary zone electrophoresis (CZE), it can be considered as a perfect complement to traditional methods. The major problem in using CZE in bioanalysis is the relatively high detection limits originating from the very small injection volumes [8]. This often requires concentration of samples, which can lead to other difficulties such as matrix effects caused by changes in sample viscosity.

The aim of this study was to develop a CZE method for the separation and determination of the main tropane alkaloids directly from unpurified plant samples. The influence of the pH and ionic strength of the buffer on separation is discussed.

2. Experimental

2.1. Materials and methods

Atropine (I) as the sulphate, scopolamine (II) as the hydrobromide and tropic acid (IV) were obtained from Sigma (St. Louis, MO) and nor-(−)-scopolamine (III) was synthesized according to the literature [9] (Fig. 1). All other reagents were of analytical grade. Deionized water was obtained in the laboratory from a Milli-Q water purification system (Millipore Corporation, Bedford, MA). All the solutions were filtered through 0.45 μm nylon filters (Gelman Nylon Bulk Acrodisk 13) and degassed in a sonication bath for 10 min before use.

Phosphate buffers (10–60 mM) at pH 5.4–8.2 were used as running electrolytes in method development. The buffers were made by titrating the Na_2HPO_4 solution of appropriate concentration to the desired pH value with an equimolar KH_2PO_4 solution.

Because the synthesized compound III was found impure and it is usually not present in Solanaceous plants [10], it was not used in the quantitative part of this study. Standard solutions were made by dissolving the alkaloids in the running electrolyte and diluting to the desired concentration. Calibration graphs were produced from alkaloid solutions of seven different concentrations (atropine 5.00–140 μg ml^{-1}, scopolamine 7.50–210 μg ml^{-1} and tropic acid 2.50–70.0 μg ml^{-1}).

Two different plant samples obtained from transgenic plant clones T1 and T2 of the Egyptian henbane *Hyoscyamus muticus* (L.) transformed and regenerated as in [6] were prepared by extracting 50 mg of lyophilized plant material with 5.0 ml of 80% (v/v) methanol for 16 h at + 60°C. After centrifugation, 500 μl of the supernatant was lyophilized to dryness and reconstituted with the running electrolyte to the same volume. The standard solutions and samples were analyzed

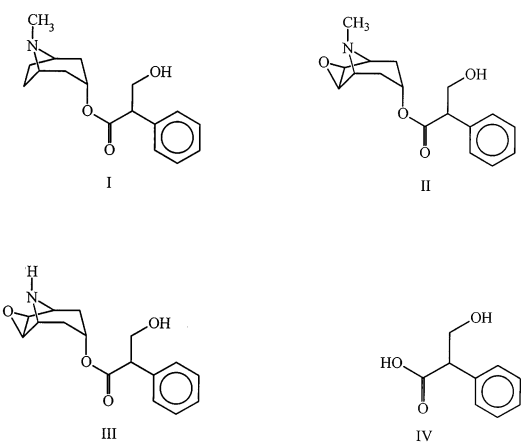

Fig. 1. Structures and symbols of atropine (I, ▲), scopolamine (II, ■), nor-(−)-scopolamine (III, ●) and tropic acid (IV, ◆).

using 40 mM phosphate buffer at pH 7.8 at a constant voltage of 20 kV. All solutions in the quantitative part of this work were injected six times.

At the start of the day, the capillary was purged for 10 min first with 0.1 M potassium hydroxide then with water and finally with the appropriate running buffer. Between runs, the capillary was rinsed with 0.1 M potassium hydroxide solution for 1.5 min followed by deionized water for 1.5 min and equilibration with the running buffer for 2 min. Normal vials were utilized, except for plant samples, for which microvials were used due to the smaller sample volumes. Peak areas were normalized with respect to their migration times during data processing.

2.2. Instrumentation

A Beckman CZE P/ACE System 2200 (Fullerton, CA) equipped with a UV detector, an automatic injector, and an Isco (Lincoln, OR) uncoated fused silica capillary (I.D. 75 μm, effective length 60 cm, total length 67 cm) temperature controlled by liquid cooling was used. The detection wavelength was 214 nm. All the experiments were carried out at 25°C. Hydrostatic sample injection (0.5 p.s.i.) with an injection time of 4 s was employed.

3. Results and discussion

3.1. Method development

Method development was started using 40 mM phosphate buffers at pH range 5.4–8.2. In the pH range 5.4–6.6, baseline separation of the charged basic solutes (I–III) was not possible (Fig. 2). The anionic compound (IV) could always be separated from the basic compounds in the investigated pH range due to its smaller molecular weight and acidic character ($pK_a = 4.1$ [11]). The change from acidic to neutral and basic electrolytes enabled the separation of cationic compounds which migrated in the order of increasing molecular weight. When corrected peak areas were concerned, the most stable pH range proved to be from 7.0 to 7.8 in

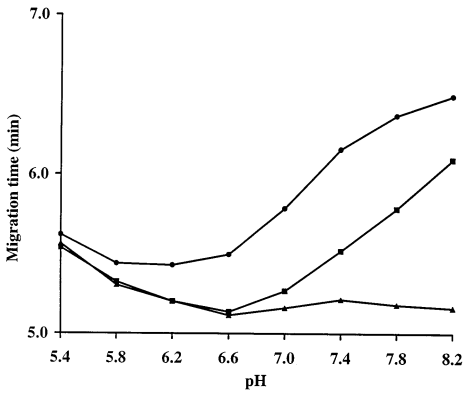

Fig. 2. Effect of pH on migration times of I, II and III. Running electrolytes: 40 mM phosphate buffers, pH 5.4–8.2. Other conditions as in Experimental. Symbols of atropine (I, ▲), scopolamine (II, ■), nor-(−)-scopolamine (III, ●) and tropic acid (IV, ◆).

which the corrected peak areas of the solutes were nearly constant. After the most suitable pH range was found, six different phosphate buffers from 10 to 60 mM were used to study the effect of buffer concentration on the migration times and the shape of the peaks. The increase of the buffer concentration increased the migration times and peak areas of the solutes substantially, which is generally the case because of the decreased electro-osmotic flow caused by an increase in the ionic strength of the running buffer. All the cationic compounds (I–III) behaved identically in the investigated concentration range. The corrected peak areas between different buffer concentrations from 30 to 60 mM were found to be close to constant (Fig. 3).

In addition, borate and Tris buffers were tested at the chosen pH. The use of these complex buffers did not have any beneficial effect on the separation of the analytes. Therefore, the 40 mM phosphate buffer was chosen for further studies. Despite the fact, that Joule heating might deteriorate the repeatability of the method, a voltage of 20 kV was used in quantitative studies. The 40 mM phosphate buffer at pH 7.8 was found to be the best for the analysis of tropane alkaloids. Under these conditions, the main alkaloids migrated through the capillary in less than 7 min and the tropic acid in less than 13 min (Fig. 4).

3.2. Determination of tropane alkaloids

There are not many publications written about the applicability of CZE for quantitative alkaloid analysis [12,13]. In spite of that, the calibration graphs for the analytes I, II and IV showed excellent linearity. The analytical parameters are shown in Table 1. The repeatability of the method expressed as relative standard deviations ($n = 6$) was 4.18% (I), 3.44% (II) and 7.06% (IV) for the smallest standard concentration, and 1.00%, 1.45% and 0.960% for the highest concentration, respectively.

The detection limits for the analytes I, II and IV are shown in Table 1. They were determined visually by diluting the standard solution to the concentration which gave the signal for the analytes that was approximately three times the baseline noise. Often the detection limit in CZE is higher than in chromatographic methods [8]. In this case, despite the differences in analytical wavelengths, the same limit was achieved as in reported HPLC methods using UV detection [4,5].

The recovery was examined by using the following procedures: (a) A solution containing 100 μg ml^{-1} of I, 150 μg ml^{-1} of II and 50.0 μg ml^{-1} of IV was prepared in the same way as described in Experimental for plant material. The recoveries were 100.1%, 95.40% and 102.0% for I, II and IV, respectively, with relative standard deviations ($n = 6$) of 4.62%, 5.27% and 4.92%, respectively; and (b) Two samples were prepared from the plant extracts and spiked by dissolving the lyophilized samples instead of the buffer into a standard solution containing 5.00 μg ml^{-1} of both I and II (Fig. 5b). 107.3% of the added I and 94.11% of II were found. In both the recovery tests the scopolamine concentrations in the samples were found to be lower than expected. Additionally, in spiked plant samples (Fig. 5b) a peak with the migration time of tropic acid could be seen. A partial explanation to this phenomenon might be the degradation of scopolamine during the sample processing despite the fact that the hydrolysis of I at the used pH has been found to be approximately 2.5 times faster than that of II, as calculated from the data in [14].

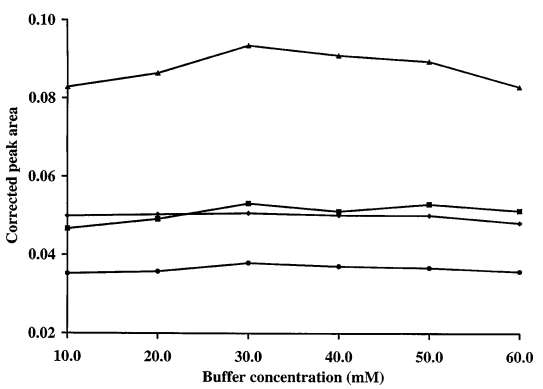

Fig. 3. Effect of buffer concentration on corrected peak areas of I–IV. Running electrolytes: 10–60 mM phosphate buffers pH 7.8. Other conditions as in Experimental. Symbols of atropine (I, ▲), scopolamine (II, ■), nor-(−)-scopolamine (III, ●) and tropic acid (IV, ◆).

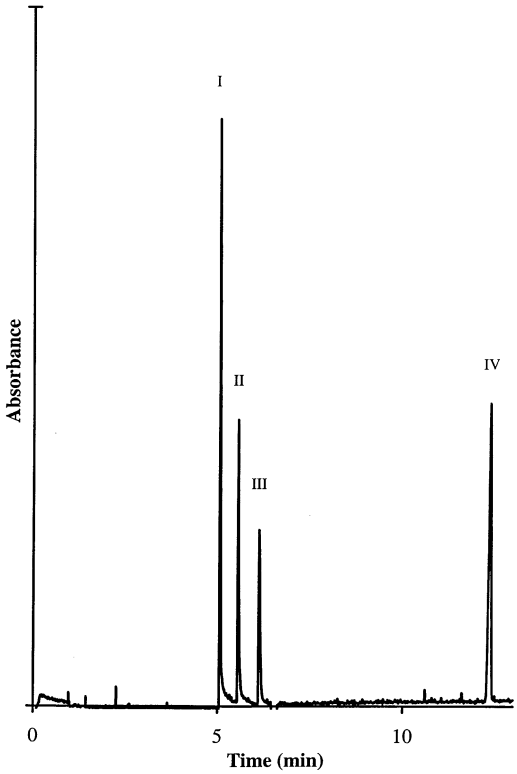

Fig. 4. Electropherogram of a mixture of I–IV. Running conditions as in Experimental.

Table 1
Analytical parameters of the assay of atropine (I), scopolamine (II) and tropic acid (IV)

Parameter		Atropine	Scopolamine	Tropic acid
Regression equation ($y = a \cdot x + b$)	a	1.09E-03 ± 5.30E-06	5.90E-04 ± 9.82E-06	1.32E-03 ± 3.51E-05
	b	(−6.87 ± 43.3)E-04	(−1.34 ± 1.20)E-03	−7.19E-05 ± 1.43E-03
r^2		1.000	0.9986	0.9965
Detection limit (μg ml^{-1})		~1.0	~1.5	~1.0
Measuring range (μg ml^{-1})		5.00–140	7.50–210	2.50–70.0

3.3. Determination of tropane alkaloids from plant material

A typical electropherogram of a plant sample is shown in Fig. 5a. In the analyzed samples the alkaloid concentrations were 86.8 ± 1.01 μg ml^{-1} of I and approximately 1.4 ± 0.24 μg ml^{-1} of II in T1 and 35.3 ± 1.47 μg ml^{-1} of I and approximately 2.5 ± 0.34 μg ml^{-1} of II in T2. Only traces of IV and no III could be found in the analyzed plant samples. The very low scopolamine (II) content of the samples was problematic because the concentrations were beneath the quantitation limit.

4. Conclusions

CZE can be successfully used for the separation and quantitative determination of closely related tropane alkaloids. Minor changes in pH and ionic strength of the buffer did not have any effect on corrected peak areas of the solutes of interest. When analyzing plant samples, very low alkaloid concentrations in the crude plant extracts and matrix effects affected the repeatability substantially. Therefore, further clean-up, the use of a specific extraction method or concentration of the samples would be advantageous.

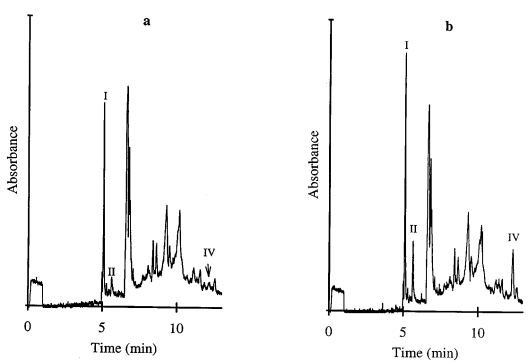

Fig. 5. Typical electropherogram of a plant sample T2 (5a) and the same sample spiked as described in Determination of tropane alkaloids (5b). Running conditions as in Experimental.

Acknowledgements

The authors wish to express their gratitude to Associate Professor Seija Tammilehto for her interest and support during this study.

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