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Determination of the Fungicide Validamycin A by Capillary Zone Electrophoresis with Indirect UV Detection

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Validamycin A, a main component of the antibiotic validamycin complex, which is widely used to control sheath blight disease of rice plants, can be determined by capillary zone electrophoresis with indirect UV detection. The influence of various separation conditions including background electrolyte and modifier concentration, pH, and voltage was investigated. By using 10 mM aminopyrine−2 mM ethylenediaminetetraacetic acid at pH 5.2 as the carrier electrolyte, high efficiency separation of validamycin A was achieved with the number of theoretical plates up to 350 000 plates/m. The linear range was across 3 orders of magnitude. The relative standard deviations for migration times and peak areas were less than 0.5 and 3.0%, respectively. The limit of detection for validamycin A was 1.0 μg/mL. The average recoveries ranged from 103.0 to 104.8%. This method has many advantages as compared with high-performance liquid chromatography and micellar electrokinetic capillary chromatography in the determination of commercial formulations.

KEYWORDS: Validamycin A; capillary zone electrophoresis (CZE); indirect UV detection

INTRODUCTION

Validamycin A [1L-(1,3,4/2,6)-2,3-dihydroxy-6-hydroxymethyl-4-[(1*S*,4*R*,5*S*,6*S*)-4,5,6-trihydroxy-3-hydroxymethylcyclohex-2-enylamino]cyclohexyl β-D-glucopyranoside (**Figure 1**)] is a main component of the antibiotic validamycin complex produced by *Streptomyces hygroscopicus* var. *limoneus* (*1*) or *S. hygroscopicus* var. *jinggangensis* (*2*). Validamycin A is a nonsystemic antibiotic with fungicide action, because it inhibits specifically the hyphal extension of *Rhizoctonia solani* without growth inhibition (*3*, *4*). It is most effective against soil borne diseases and is widely used for the control of *Rhizoctonia solani* in rice (sheath blight of rice), potatoes, vegetables, and others as well as damping off diseases in vegetable seedlings, cotton, sugar beets, rice, and other plants (*5*−*9*).

There are mainly three methods used for the analysis of validamycin A (*10*−*12*). Bioassay is the first method used to determine validamycin A (*10*). However, this method is complex and time consuming and usually has its limitation in both selectivity and efficiency. Although gas chromatography (GC), which involves the preparation of validamycin trimethylsilyl derivatives (*11*), is sensitive and precise, it is also laborious. High-performance liquid chromatography (HPLC), which acts as the Chinese official method for the determination of validamycin A in commercial formulations (*12*), is more sensitive than the GC method. It has been used for the routine analysis. However, the complex procedures of sample purification (*12*) and the column regeneration after phase collapse are its drawbacks.

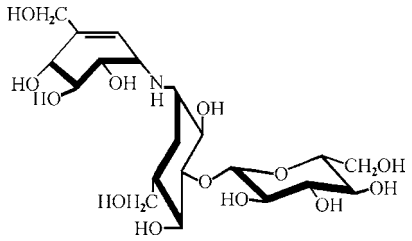

Figure 1. Chemical structure of validamycin A.

In recent decades, capillary electrophoresis (CE) has become a popular analytical method. Among the different CE procedures, capillary zone electrophoresis (CZE) is the most universal and efficient method. Recently, Hsiao and Lo (*13*) reported a micellar electrokinetic capillary chromatography (MEKC) for the determination of validamycin A, which was based upon the fact that there was no peak detected when the CZE technique was applied to analyze validamycin A. The MEKC method has satisfactory reproducibility and high sensitivity. Nevertheless, analyses were somehow interfered by at least one kind of impurity in commercial formulations. Therefore, a simple and reliable method is still required.

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In practice, the determination of validamycin A by CZE with indirect UV detection can be performed by selecting an appropriate carrier electrolyte and optimizing separation voltage. The CZE method described here requires the minimal sample preparation and the lowest operational costs. It is rapid, sensitive, and free from interference.

EXPERIMENTAL PROCEDURES

Standard and Samples. Crude standard of validamycin A (purity = 87%) was kindly supplied by Professor Yinchu Shen. It was purified by tandem coupling of two reported methods (*11*, *14*). In brief, the crude standard was dissolved in water and fractionated on Dowex 1 × 8 ion exchange column (OH form, 100−200 mesh, 20 cm × 1.1 mm i.d.) using water at a flow rate of 1 mL/min; the fraction only containing validamycin A was concentrated and then precipitated with acetone to give pure crystalline validamycin A (purity > 97%). Samples I−III were purchased from markets in different areas of China. All of these commercial formulations are soluble powder containing about 20% validamycin A.

Reagents. Sodium dodecyl sulfate (SDS) and a Dowex 1X8 ion exchanger were purchased from Sigma (St. Louis, MO). Aminopyrine, ethylenediaminetetraacetic acid disodium salt (EDTA disodium), phosphoric acid, disodium hydrogen phosphate anhydrous, sodium tetraborate ($Na_2B_4O_7$), acetic acid glacial, and methanol were obtained from Shanghai Chemical Reagents (Shanghai, China). The chemicals used were of analytical reagent grade. Water was purified with a Barnstead EASYpure system (Sybron Barnstead, Boston, MA).

Preparation of Standard and Sample Solutions. A validamycin A stock solution (1250 μg/mL) was prepared in water and stored in 4 °C. The standard stock solution was diluted with carrier electrolyte as required. About 500 mg of a sample was weighed into a 50 mL volumetric flask and brought to the mark with water. It was then mixed up evenly and filtered with a quantitative filter paper (Hangzhou Xinhua Paper Industry, Hangzhou, China). A 2 mL amount of the filtrate was then diluted to 50 mL with carrier electrolyte. A proper aliquot of the diluent was centrifuged at 10000*g* for 10 min in a 5415D centrifuge (Eppendorf, Hamburg, Germany), and the supernatants were analyzed automatically by CZE.

CZE. Electrophoresis was performed on a P/ACE MDQ CE system (Beckman Coulter, Fullerton, CA), which was equipped with a P/ACE UV detector module, an autosampler, and a temperature-controlled fluid-cooled capillary cartridge. A computer and MDQ software (2.3 version) were used for instrument control and for data collection and processing.

An uncoated fused-silica capillary (total 60 cm, effective length 50 cm × 75 μm i.d.) from Yongnian Chromatography (Yongnian, Hebei, China) was employed. UV detection was set at 200 nm. Samples were introduced into the capillary by pressure mode (0.5 psi for 10 s). The temperature of the capillary was controlled at 25 °C.

Before each injection, a 3 min purge of capillary with carrier electrolyte was programmed. The pH of carrier electrolyte was adjusted by acetic acid solution. All carrier electrolyte solutions were filtered through a 0.22 μm syringe membrane filter (Shanghai Yadong Hitech, Shanghai, China), and all samples were centrifuged (10000*g* for 10 min) before they were introduced into the system.

Reference Methods. MEKC analysis was carried out on the same capillary and CE system; the UV detector was set at 200 nm. The analysis procedure and other conditions were as described previously (*13*).

A Waters (Milford, MA) HPLC system equipped with a 515 HPLC pump, an injector with a 5 μL sample loop, a 4.6 mm × 75 mm Symmetry C_{18} 3.5 μm column (WAT066224), and a 2487 dual λ absorbance detector, coupled with an analytical work station (Millennium^{32} software 3.2 version), were used. The analyses were done by the method of the national standard of People's Republic of China (*12*).

Separation efficiency is expressed in terms of the number of theoretical plates (*N*)

$$N = 16\,(t_r/W)^2$$

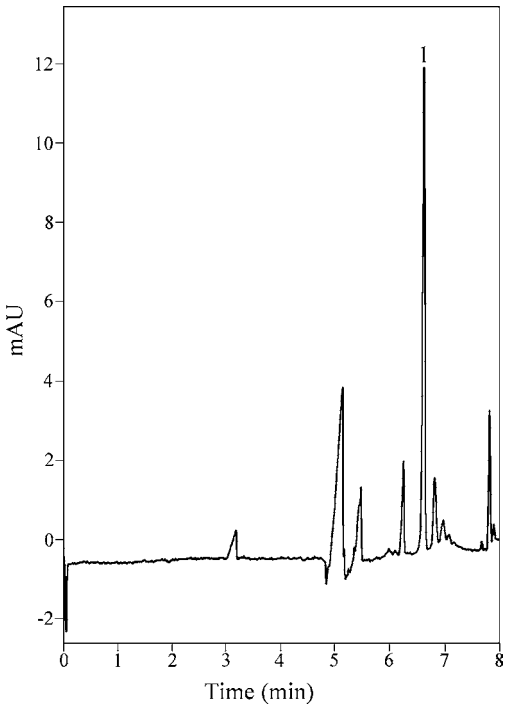

Figure 2. Electropherogram of sample I. Experimental conditions: uncoated fused-silica capillary, 50 cm (effective length) × 75 μm (i.d.); carrier electrolyte, 10 mM aminopyrine−2 mM EDTA (pH 5.2); separation voltage, 15 kV; temperature, 25 °C; indirect UV detection, 254 nm; pressure injection, 0.5 psi for 10 s. The content of validamycin A (peak 1) is 94.6 μg/mL.

where t_r is the migration time (retention time for HPLC) and *W* is the peak width; the values were given automatically by the corresponding software.

RESULTS AND DISCUSSION

CZE Conditions. The method of CZE with indirect UV-absorbing detection was used to analyze validamycin compounds, because of their lack of chromophores. First in the optimization of CZE with indirect UV detection is the selection of a background electrolyte (BGE) with large molar absorptivity and effective mobility similar to that of the analyte canion. Being an aromatic amine compound, animopyrine has strong UV absorption. It is suitable for the analysis of validamycin A as indicated by symmetrical peak (**Figure 2**). So, it was chosen as BGE in the following experiment.

From our previous work (*15*, *16*) and those reported by others (*17*−*23*), it was found that the pH, the concentration of the BGE and modifier, and the separation voltage are factors affecting CE separation. Therefore, the present optimization of separation conditions was based on these factors.

The pH value of a carrier electrolyte is an important parameter that can be manipulated to optimize selectivity in CZE, because it has significant effects on the surface characteristics of the fused silica capillary and also influences the dissociation of the analyte into ions. As a basic compound, validamycin A will be positively charged at acidic pH values. With the voltage at 15 kV and the BGE concentration at 10 mM, the pH values of the carrier electrolyte were tested from 4.4 to 5.6 in 0.2 unit steps. The highest separation efficiency was obtained at pH 5.2. So, it was selected as the optimal pH.

The influence of BGE concentration on the separation efficiency was studied with the concentrations of aminopyine from 5 to 15 mM in 2.5 mM steps at pH 5.2. The high separation

Table 1. Effect of Voltage on Migration Time and Separation Efficiency[a]

separation voltage (kV)	10.0	12.5	15.0	17.5	20.0	22.5	25.0
migration time (min)	9.1	7.5	6.7	5.7	5.0	4.6	4.1
N (theoretical plates/m)	412 000	383 000	358 000	322 000	291 000	262 000	249 000

[a] Experimental conditions: sample is a working standard solution of validmycin A (125 μg/mL). Other experimental conditions as in **Figure 2**.

Table 2. Comparison of Various Methods for the Analysis of Validamycin A[a]

parameter	CZE	MECK	HPLC
RSD for migration time (%, $n = 5$)	0.26	0.71	1.23
RSD for peak area (%, $n = 5$)	1.34	2.45	2.73
N (theoretical plates)	179 000	15 000	6000
linear range (μg/mL)	12.5–1250	5.0–500	1.0–500
regression coefficient	0.9998	0.9994	0.9991
LOD (μg/mL)	1.0	1.0	0.2

[a] Experimental conditions: sample used for the determination of separation efficiency and reproducibility is a working standard solution of validmycin A (125 μg/mL). Other experimental conditions as in **Figure 2**.

Table 3. Contents of Validamycin A in Commercial Formulations ($n = 3$)

sample	CZE	MECK	HPLC
soluble powder I	24.45 ± 0.45	50.72 ± 0.83	24.11 ± 0.51
soluble powder II	21.25 ± 0.30	51.29 ± 2.13	21.78 ± 0.99
soluble powder III	22.79 ± 0.54	43.08 ± 2.19	21.64 ± 0.48

efficiencies ($N > 350\,000$ theoretical plates/m) were obtained when the concentrations were above 10 mM. In general, higher BGE concentrations had higher buffering capacities resulting in better reproducibility. However, the increase of the BGE concentration leads to a decrease in sensitivity (S/N) and an increase in migration time. Because reproducibility is an important issue for commercial product analyses, the concentration of 10 mM was chosen. It is the best compromise between sensitivity and reproducibility.

Because metal ions move faster than validamycins, many metal ion peaks appeared before the peaks of validamycins and therefore interfered with the detection of validamycin A. It can be avoided by chelating metal ions with EDTA (*24*), because EDTA can combine with many metal ions to form stable anionic chelates (metal−EDTA chelates), which migrate slower than validamycin A. The concentrations of EDTA ranging from 1 to 5 mM were investigated. The optimized concentration was found to be 2 mM.

Separation voltage has a great influence on migration time and separation efficiency. The lower voltage increased operating time, but the separation efficiency was better than that of the high voltage, as illustrated in **Table 1**. In this experiment, the separation voltage of 15 kV was applied. It is a compromise between analysis time and separation efficiency.

From the study above, the best separation conditions were found to be the following: 10 mM aminopyrine, 2 mM EDTA at pH 5.2, and an applied voltage of 15 kV.

Validation of the CZE Method. A good linear correlation ($r^2 = 0.9998$) between the peak area (X) and concentration (Y) was found in the concentrations of 12.5−1250 μg/mL. The regression equation was $Y = 2.209 \times 10^{-3} \times X + 3.359$. It was used to calculate the content of validamycin A in the commercial products.

The limit of detection (LOD) of validamycin A was determined by injecting a low concentration of working standard solution to produce a signal-to-noise ratio (S/N) of three. It was estimated to be 1.0 μg/mL.

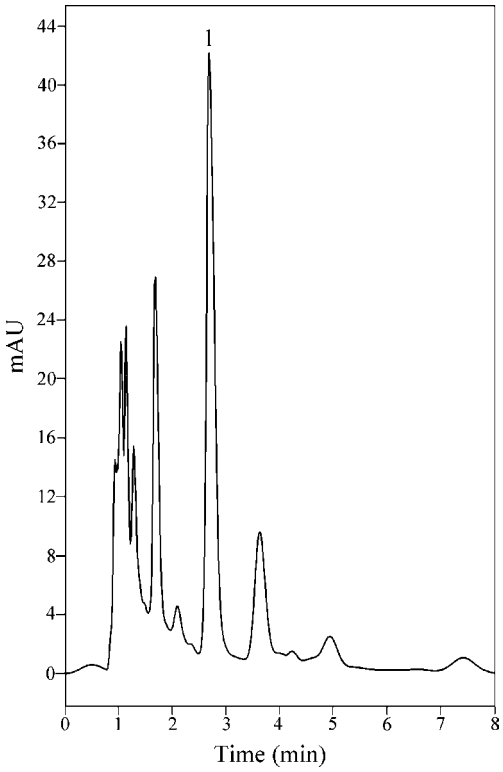

Figure 3. Chromatogram of sample I. Experimental conditions: Waters 4.6 mm × 75 mm symmetry C_{18} 3.5 μm column; mobile phase, 5 mM phosphate (pH 7.0) + 3% methanol; injection volumn, 5 μL; detection wavelength, 210 nm. The content of validamycin A (peak 1) is 93.3 μg/mL.

Repetitive analyses (five consecutive injections) of a standard solution of validamycin A were performed at the concentration of 125 μg/mL. The relative standard deviations (RSDs) for migration time and peak area were 0.26 and 1.34%, respectively.

Comparison of CZE with MECK and HPLC. To evaluate the CZE method, the working standard solutions were also analyzed simultaneously by HPLC and MEKC. CZE is prior to MECK and HPLC, regarding with the separation efficiency, reproducibility, and linearity. However, the LOD of CZE is similar with MECK and higher than HPLC (**Table 2**). This is because of the small optical path (50−100 μm) used for UV detection and small injection volumes (2−20 nL) as compared with HPLC (10 mm optical path and 5−100 μL).

Determination of Validamycin A in Commercial Formulations. The contents of validamycin A in soluble powders were listed in **Table 3**. They were all higher than the labeling content. The RSD values for products I, II, and III were 1.85, 1.43, and 2.35%, respectively ($n = 3$). They were all less than 3.00%, indicating that the precision of the CZE method was excellent.

Samples were spiked with validamycin A at a concentration of 125 μg/mL. The average recoveries for samples I, II, and III were 104.1, 104.8, and 103.0%, respectively, with the corre-

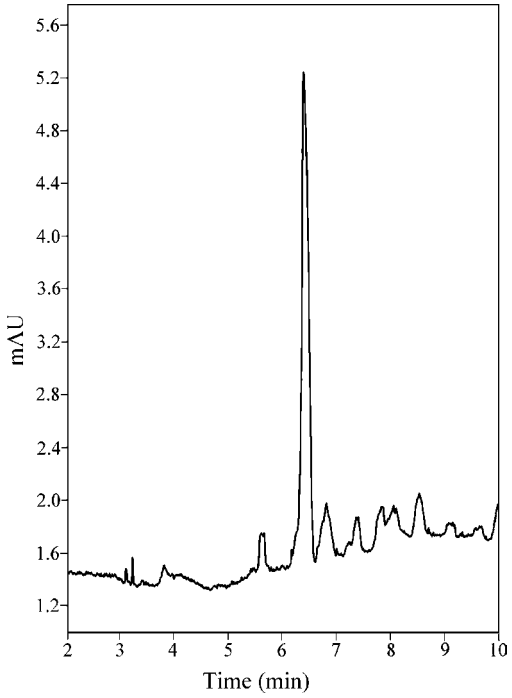

Figure 4. Electropherogram of sample I. Experimental conditions: carrier electrolyte, 100 mM SDS and 50 mM borate (pH 9.0); separation voltage, 20 kV; temperature, 20 °C; UV detection, 200 nm; other experimental conditions as in **Figure 2**.

sponding RSD values of 1.32, 1.46, and 2.86% ($n = 3$). It displayed that the method had a good accuracy. A typical electropherogram (sample I) is shown in **Figure 2**.

The CZE and HPLC yielded similar values for each sample (**Table 3**; **Figure 3**). Nevertheless, the abnormal results obtained from the MEKC method indicated the presence of one or more coeluates in these samples (see the biggest peak in **Figure 4**). It is due to its low separation efficiency (<30 000 plates/m). The results implied that the MEKC method is not suitable for the determination of validamycin A in commercial formulations, at least in these samples.

An important advantage of the CZE method is the possibility of analyzing large quantities of samples (ca. 30 samples with triplicates per day) without poor reproducibility. The MEKC method has good reproducibility, too. However, the reversed phase chromatography with an ordinary C_{18} column does not have satisfactory reproducibility because of the phase collapse of the hydrophobic C_{18} alkyl phases under highly aqueous mobile phase conditions (*25*). As phase collapse progresses, the availability of the alkyl phase to interact with solutes decreases and then the retention time decreases correspondingly. If retention times drop by more than 5%, the column should be purged periodically with a mobile phase containing more than 50% organic modifier. In addition, samples must be pretreated with the ion exchange procedure (*12*). Considering analysis times, CZE offers better values as compared with the HPLC method. Some HPLC columns do not exhibit problems with phase collapse, whereas the expensive prices of the columns increase the run costs.

CONCLUSIONS

A fast and simple CZE method for the determination of validamycin A in commercial products was established. It gives a lower LOD, a wider linear range, good reproducibility, and shorter analysis time. The reduced sample preparation and low run cost are its extra advantages. It can complement or even replace the HPLC method for the determination of validamycin A on a large scale.

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