Determination of the Antibiotic Fungicide Validamycin A in Formulated Products by Micellar Electrokinetic Chromatography

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A micellar electrokinetic capillary chromatographic method (MEKC) was used to determine validamycin A content in commercial products. The results indicated that this method was capable of analyzing the validamycin A content in formulated products with an instrument detection limit of 0.94 μg/mL and a method detection limit of 1.70 μg/mL. Relative standard deviation (RSD) values of MEKC determination of validamycin A in formulated products ranged from 0.61 to 2.09%. Recoveries of validamycin A in formulated products were in the region of 99.5−105.1%. All commercial products collected from markets contained validamycin A. The high percentage of recovery, the low detection limit, and the low RSD values confirmed that the MEKC technique is a senstivie and selective method.

Keywords: *MEKC; antibiotic fungicide; validamycin A; detection limit; recovery*

INTRODUCTION

Capillary zone electrophoresis (CZE) is an efficient separation technique in which charged solutes are differentially transported through open capillaries under the influence of an applied field (Jorgenson and Lukacs, 1981). In our previous work, the CZE technique has successfully separated the antibiotic fungicides blasticidin S (Lo et al., 1995) and kasugamycin (Lo and Hsiao, 1996). However, there is no peak detected when we apply the CZE technique to the detection of imidazolidine-2-thione (ethylenethiourea, ETU) and validamycin A. An alternative approach to detect these compounds is by the addition of surfactant ions to the mobile phase at concentrations above their critical micelle concentration (cmc) (Terabe et al., 1984). This technique was designated micellar electrokinetic capillary chromatography (Burton et al., 1986). By using this technique, we have developed a method for the routine analysis of the carcinogenic compound ETU in commercial ethylenebis(dithiocarbamate) fungicides (EBDC) (Lo and Hsiao, 1997).

The fungicide validamycin A has been isolated from the metabolites of the fungus *Streptomyces hygroscopicus* var. *Limoneous* (Iwasa et al., 1970). Its chemical structure is identified as 1L-(1,3,4/2,6)-2,3-dihydroxy-6-(hydroxymethyl)-4-[(1*S*,4*R*,5*S*,6*S*)-4,5,6-trihydroxymethylcyclohex-2-enylamino]cyclohexyl β-D-glucopyranoside (Figure 1). The official method that has been used for the analysis of validamycin A in formulations is the bioassay method called the "reversed layer method" (Iwasa et al., 1971). This bioassay method usually has its limitation in both selectivity and efficiency. It cannot distinguish the actual antibiotic from false products and is time-consuming (Lo et al., 1995; Lo and Hsiao, 1996). A second method is a gas chromatographic (GC) procedure. The sample is extracted with water, evaporated under reduced pressure, and analyzed by GC using a flame ionization detector after the preparation of validamycin A trimethylsilyl derivative (Nishi and Konishi, 1976). The GC method is also complex and time-consuming. Thus, a fast, efficient method should be developed for routine analysis. Validamycin A is readily soluble in water and is stable in neutral media, indicating that an MEKC method could be used to detect this compound. Therefore, experiments using the MEKC technique were conducted, and we describe here the development of an MEKC method for the determination of validamycin A in commercial formulated products.

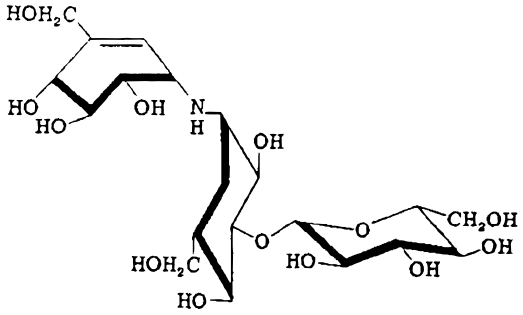

Figure 1. Chemical structure of validamycin A.

MATERIALS AND METHODS

Standard and Samples. Standard of validamycin A (MW = 497.5, purity = 93.9%) was kindly supplied by TAKEDA. Commercial formulated samples were purchased from markets in different areas of Taiwan during 1996−1997. Samples A and B contained 3% validamycin A, and samples C−E contained 5% validamycin A. All of these samples are in soluble liquid (SL) formulation.

Solvents and Chemicals. Anionic surfactant, sodium dodecyl sulfate (SDS), was purchased from Sigma (St. Louis, MO) and sodium tetraborate ($Na_2B_4O_7$) from Osaka (Osaka, Japan). The aqueous running buffer solution (pH 9.0) for MEKC analysis was composed of 100 mM SDS and 50 mM $Na_2B_4O_7$. The dilution buffer was composed of 100 mM SDS and 5 mM $Na_2B_4O_7$. All buffer solutions were filtered through a 0.45 μm nylon filter.

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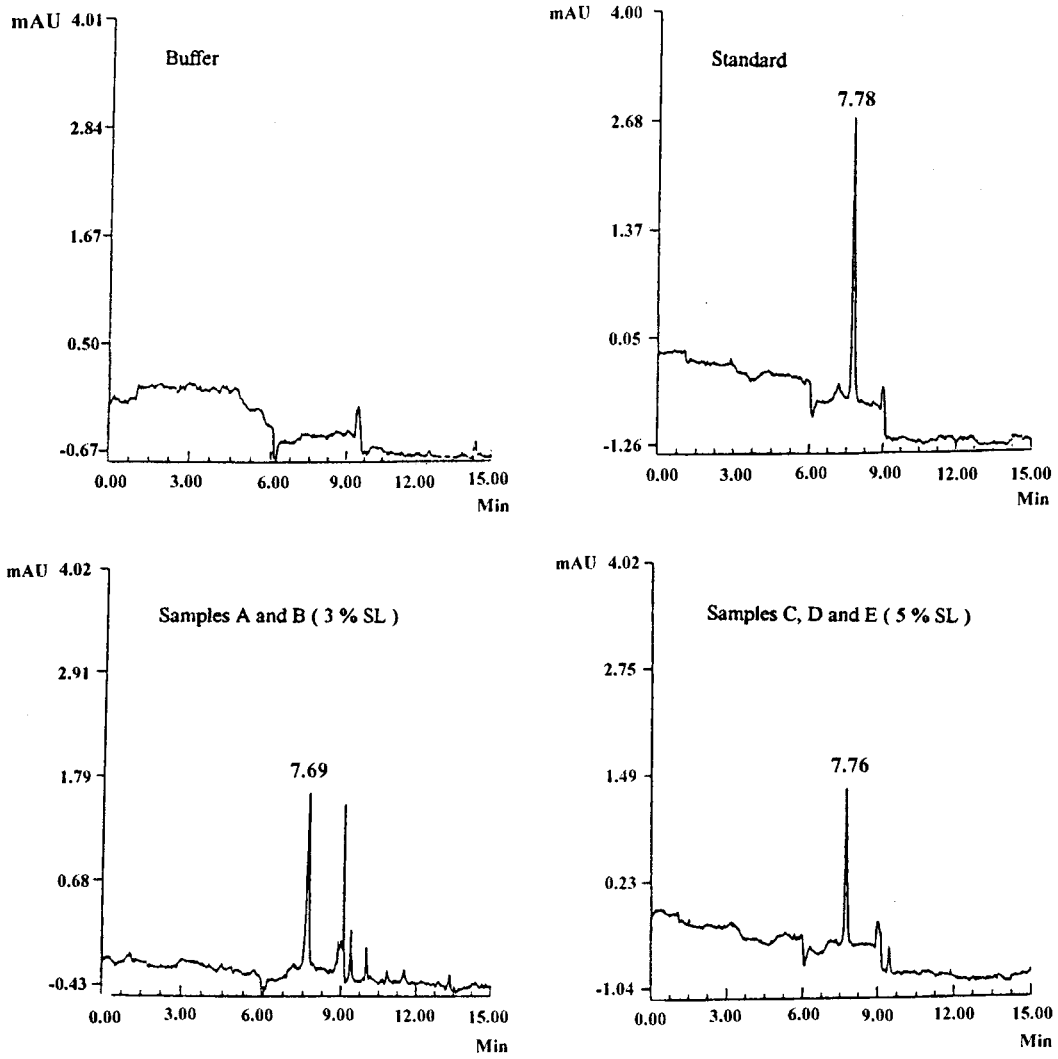

Figure 2. Typical electropherograms of dilution buffer, validamycin A standard, and commercial products. Samples A and B were 3% solution (SL), and samples C−E were 5% solution (SL).

MEKC Analysis. The MEKC method was performed using a Biofocus 3000 automated capillary electrophoretic apparatus. A Biofocus cartridge capillary 148-3040 (50 cm × 50 μm i.d., uncoated) was employed with a length of 45.4 cm from the point of sample introduction to the point of detection. The column temperature was 20 °C. A regulated dc power supply delivering 18 kV was used to provide high voltage between the ends of the column filled with running buffer. The sample was introduced into the capillary vessel using pressure injection mode at 5 psi × s. The elution of a solute was monitored by an on-column UV−vis detector (195 nm) at the negative pole (Figure 2).

Column efficiency is expressed in terms of theoretical plates (N) (Jorgenson and Lukacs, 1981; Lo et al., 1995)

$$N = 16(t_r/W)^2 \quad (1)$$

where t_r is the elution time of the peak and W is the peak width at a given height (the tangents to the side of the peaks are extrapolated to the baseline for W).

Capillary conditioning between runs was conducted by rinsing running buffer for 90 s at high-pressure mode.

The reproducibility of elution time (t_r), peak area, linearity, and detection limit were used to evaluate the selectivity, sensitivity, and reliability of the MEKC method.

MEKC Calibration Curve. Validamycin A standard (0.0100 g) was weighed into a 10 mL volumetric flask and diluted with dilution buffer to obtain validamycin A stock standard solution of 1000 μg/mL. The stock standard solution was diluted with dilution buffer in sequence to obtain the final working standard solution of concentrations of 40.0, 80.0, 120.0, 160.0, and 200.0 μg/mL. These final working standard solutions were used to determine the calibration curve. Three replications were conducted, and linear regression was applied to determine the suitability of the MEKC method.

Sample Preparation and Analysis. A proper amount of sample was weighed into a 10 mL volumetric flask and diluted with dilution buffer. The mixture was mixed with a mixer (Thermolyne 37600 mixer) for 1 min, and a proper aliquot was injected into an autosampler vial through a 0.45 μm nylon syringe filter (Lida Manufacturing Corp.) for MEKC analysis.

Recovery. Because the accurate compositions of different commercial formulations were unknown, the effects of formulations on the MEKC approach were analyzed by recovery. The recoveries of validamycin A from formulated products were determined by pipetting a 0.1 or 0.2 mL aliquot of validamycin A stock standard solution (1000 μg/mL) into each of the formulated samples (spiked samples). Other portions of the formulated samples served as blanks (nonspiked samples). The spiked and nonspiked samples were then mixed separately (1 min) and analyzed. Recoveries were calculated as the difference of the amount of validamycin A found in the spiked sample and the nonspiked sample, expressed as a percentage of the amount of validamycin A added.

Limit of Detection. The instrument limit of detection (IDL) was determined by injecting a low concentration of working standard solution to produce a signal that was ~3 times the signal-to-noise ratio (U.S. EPA, 1984). The concen-

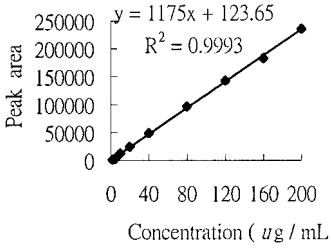

Figure 3. MEKC calibration curve of validamycin A standard.

Table 1. Precision of Elution Time of Validamycin A Standard Analyzed by MEKC Method

concn (μg/mL)	elution time[a]		corrected elution time[b]	
	min	% RSD	min	% RSD
40.0	7.95	1.03	1.75	1.83
80.0	7.83	0.15	1.80	1.60
120.0	7.81	0.27	1.78	0.17
160.0	7.80	0.32	1.78	0.32
200.0	7.76	0.26	1.74	0.66

[a] Mean of three measurements. [b] Corrected elution time = compound elution time − electroosmotic flow time.

Table 2. Determination of Validamycin A Content in Solution Products (SL) by MEKC Method

formulation (% w/w)	MEKC method (% w/w,[a] RSD)	tolerance (%)
A, 3% SL	3.60, 0.86	2.40−3.60
B, 3% SL	2.76, 0.78	2.40−3.60
C, 5% SL	5.05, 0.61	4.00−6.00
D, 5% SL	4.33, 2.09	4.00−6.00
E, 5% SL	5.11, 1.80	4.00−6.00

[a] Mean of three measurements.

tration of working standard solution that corresponds to 5.0 times the IDL is used to determine the method detection limit (MDL). Repeated MEKC analyses (seven times) produced data for the standard deviation (SD); 3 times the SD was used as the MDL. Precision expressed by relative standard deviation (RSD) of elution time was used in judging the acceptability of the method. Three replications were conducted in all analyses.

RESULTS AND DISCUSSION

Chromatogram of Validamycin A Standard. Typical electropherograms of the validamycin A standard and buffer are shown in Figure 2. The elution times and corrected elution times of validamycin A standards ranged from 7.76 to 7.95 min and from 1.74 to 1.80 min, respectively. The RSD values ranged from 0.15 to 1.03% for elution time and from 0.17 to 1.83% for corrected elution time. The data indicated that the elution time was not influenced by the concentration selected from 40.0 to 200.0 μg/mL (Table 1).

A good linear correlation ($r^2 = 0.9993$) between the concentration (X) and peak area (Y) was found in the concentrations of 2.0−200.0 μg/mL and was used to calculate the validamycin A concentration in the formulated products (Figure 3).

Column Efficiency. The column efficiency expressed in terms of theoretical plates (N) was calculated using eq 1 to be 8378 for the MEKC column. The elution time (t_r) and the peak width (W) of validamycin A determined according to the MEKC method were 7.78 and 0.34 min, respectively.

Sensitivity of the MEKC Method. The IDL, defined as 3 times the baseline noise, was estimated at 0.94 μg/mL. The MDL was calculated to be 1.70 μg/mL.

Table 3. Effect of Concentration on the Recovery of Validamycin A in Formulated Products

sample	validamycin A added (g)	no. of determinations	recovery (%)	
			range	av
B, 3% SL	100	3	98.5−101.4	99.5
	200	3	100.6−102.1	101.2
C, 5% SL	100	3	96.0−101.6	99.5
	200	3	97.4−103.0	100.3

Table 4. Recovery of Validamycin A Fortified at 0.5% w/w in Formulated Products by MEKC Method

sample	recovery (%)	mean (%)	RSD (%)
A, 3% SL	103.7, 107.5, 104.0	105.1	2.12
B, 3% SL	98.5, 101.4, 98.7	99.5	1.61
C, 5% SL	96.0, 101.6, 101.0	99.5	3.08
D, 5% SL	102.8, 102.4, 102.3	102.5	0.26
E, 5% SL	99.8, 106.3, 100.3	102.1	3.52

Table 5. Effect of Commercial Formulation on the Analysis of Validamycin A by the MEKC Method

formulation (% w/w)	concn (%, w/w)		
	calcd[a]	corrected[b]	difference[c] (%)
A, 3% SL	3.60	3.43	4.72
B, 3% SL	2.76	2.77	−0.36
C, 5% SL	5.05	5.08	−0.59
D, 5% SL	4.33	4.23	2.31
E, 5% SL	5.11	5.01	1.96

[a] Concentration calculated from calibration curve. [b] Calculated concentration corrected with recovery. [c] Difference = [(calcd concn − corrected concn)/calcd concn] × 100%.

Determination of Validamycin A Content in Commercial Formulated Products. The official tolerance for active ingredients of <10% in commercial formulation ranged from +20% to −20%. The MEKC analyses showed that the contents of active ingredients in all commercial samples were within the official tolerance level (Table 2). Their typical electropherograms are shown in Figure 2.

Precision of the MEKC Method. The precision of the analytical method as measured by RSD values in the determination of validamycin A in commercial formulated products ranged from 0.61 to 2.09% (Table 2). All of the RSD values were <10%, indicating that the precision of the method was excellent (McFarren et al., 1970).

Influence of Formulations on the MEKC Performance. The analysis of validamycin A in commercial formulation products was validated by the standard addition method, and the recovery of the added validamycin A standard was calculated. The effect of concentration on the recovery of validamycin A in formulated products was first investigated. Two formulated samples, B and C, with different concentrations were selected, and the average recoveries for sample B were 99.5% of 100 μg added and 101.2% of 200 μg added. The average recoveries for sample C were 99.5% of 100 μg added and 100.3% of 200 μg added (Table 3). The results indicated that the recovery of validamycin A was not influenced by the amount added, and 100 μg was selected for recovery study.

The recoveries of validamycin A in all commercial products were then conducted by adding 100 μg (0.5% w/w in formulation). The result indicated that the recoveries ranged from 99.5 to 105.1%, with RSD values in the range of 0.26−3.52% (Table 4); therefore, there is no matrix interference, and the concentration of each sample calculated from the calibration curve and the corrected recovery are shown in Table 5.

Conclusion. The new MEKC method offers good precision, accuracy, linearity, and sensitivity. No matrix interference was observed. In addition, the simple and quick sample extraction procedure was another advantage of the MEKC method. It clearly demonstrated MEKC's efficiency in saving labor and chemicals. Therefore, the new MEKC method is a better method than the bioassay method and GC method when the selectivity and efficiency are concerned.

ABBREVIATIONS USED

CZE, capillary zone electrophoresis; ETU, ethylenethiourea; GC, gas chromatography; IDL, instrument limit of detection; MDL, method detection limit; MEKC, micellar electrokinetic capillary chromatography; RSD, relative standard deviation; SD, standard deviation; SDS, sodium dodecyl sulfate; SL, soluble liquid; UV−vis, ultraviolet−visible.

LITERATURE CITED

Burton, D. E.; Sepaniak, M. J.; Maskarinec, M. P. Analysis of B6 vitamins by micellar electrokinetic capillary chromatography with laser-excited fluorescence detection. *J. Chromatogr. Sci.* **1986**, *24*, 347−351.

Iwasa, T.; Yamamoto, H.; Shibata, M. Studies on validamycin, new antibiotics. I. *Streptomyces hygroscopicus* var. *limoneus* nov. var., validamycin producing organism. *J. Antibiot.* **1970**, *23*, 595−602.

Iwasa, T.; Higashide, E.; Shibata, M. Studies on validamycin, new antibiotics. III. Bioassay methods for the determination of validamycin. *J. Antibiot.* **1971**, *24*, 114−118.

Jorgenson, J. W.; Lukacs, K. D. Zone electrophoresis in open-tubular glass capillaries. *Anal. Chem.* **1981**, *53*, 1298−1302.

Lo, C. C.; Hsiao, Y. M. High-performance capillary electrophoretic method for the determination of antibiotic fungicide kasugamycin in formulated products. *J. Agric. Food Chem.* **1996**, *44*, 2231−2234.

Lo, C. C.; Hsiao, Y. M. Comparison of micellar electrokinetic capillary chromatographic (MECC) method with high-performance liquid chromatographic (HPLC) method for the determination of imidazolidine-2-thione (ethylenethiourea) in formulated products. *J. Agric. Food Chem.* **1997**, *45*, 3118−3122.

Lo, C. C.; Lee, Y. J.; Chang, C. J. High-performance capillary electrophoretic method for the determination of blasticidin S in formulated products. *J. Agric. Food Chem.* **1995**, *43*, 2892−2895.

McFarren, E. F.; Lishka, R. J.; Parker, J. H. Criterion for judging acceptability of analytical methods. *Anal. Chem.* **1970**, *42*, 358−365.

Nishi, K.; Konishi, K. Validacin. *Anal. Methods Pestic. Plant Growth Regul.* **1976**, *8*, 309−318.

Terabe, S.; Otsuka, K.; Ichikawa, K.; Tsuchiya, A.; Ando, T. Electrokinetic separations with micellar solutions and open-tubular capillaries. *Anal. Chem.* **1984**, *56*, 111−113.

U.S. EPA. *Fed. Regist.* **1984**, *49* (209, Oct 26), Part 136.

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