# High-Performance Capillary Electrophoretic Method for the Determination of Antibiotic Fungicide Kasugamycin in Formulated Products

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A high-performance capillary electrophoretic (HPCE) method with a UV−vis detector was used to determine kasugamycin content in commercial products. The results indicated that this method was capable of analyzing the kasugamycin content in formulated products with an instrument detection limit of 0.44 $\mu$g/mL and a method detection limit of 0.51 $\mu$g/mL. Recoveries of kasugamycin in formulated products were in the region of 99.9−103.2%. Relative standard deviation (RSD) values of HPCE determination of kasugamycin in formulated products ranged from 0.57 to 2.22%. All products collected from markets contained kasugamycin and were real products. High recovery, low detection limit, and low RSD values confirmed that the HPCE method is a sensitive and selective method. Thus the present official bioassay method could be replaced by the HPCE method.

**Keywords:** *HPCE; antibiotic fungicide; kasugamycin; detection limit; sensitivity; selectivity*

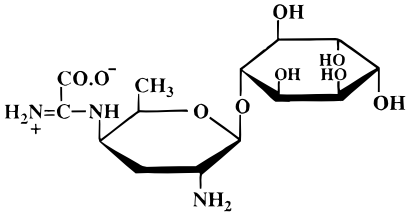
## INTRODUCTION

The content of kasugamycin in commercial formulated products is traditionally determined by bioassay which cannot distinguish the actual antibiotic from false products, because the bioassay method is based on the growth inhibition of specific bacteria, and this inhibition may be caused by many inorganic or organic bactericides. For example, blasticidin S is an antibiotic fungicide, and the bioassay method indicated that all seven commercial products collected from markets were active, but the HPCE method proved that one of them contained no blasticidin S (Lo et al., 1995), and indicated that HPCE is a promising method in distinguishing between the real antibiotic compounds and possible imitation antibiotic fungicides. Furthermore, the procedures for bioassay are complex, and time-consuming. Thus the possibility of using the HPCE method to analyze kasugamycin in formulated products is conducted.

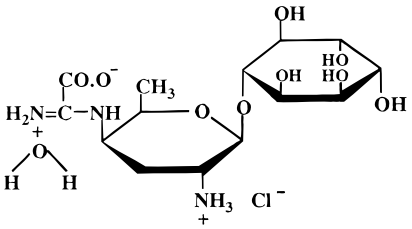
Kasugamycin is an effective fungicide for prevention of rice plant disease by *Piricularia oryzae* (Umezawa et al., 1965). Its chemical structure is identified as [5-amino-2-methyl-6-(2,3,4,5,6-pentahydroxycyclohexyloxy)tetrahydropyran-3-yl]-amino-α-iminoacetic acid (Suhara et al., 1965) (Figure 1, top). Kasugamycin contains an amino group and imino acetic acid group; thus capillary zone electrophoresis was used for kasugamycin separation (Lo et al., 1995). Kasugamycin absorbed UV light with maximum absorption at 201 nm in pH 2.5 phosphate buffer (Figure 2). Therefore, 210 nm was selected for reliable kasugamycin detection. We describe here the development of an HPCE method for the determination of kasugamycin in commercial formulated products purchased from markets.

## MATERIALS AND METHODS

**Solvents and Chemicals.** Standard of kasugamycin monohydrochloride hydrate (MW = 433.8) was supplied by Great Victory Chemical Company (Taiwan). The purity was >99.9% based on HPCE analysis (area percentage). Commercial formulated samples were purchased from markets during the year of 1995. Samples A, B, C, and D contained 2% kasugamycin, samples E and F were mixed fungicides containing 4% carbendazim (methyl-2-benzimidazole carbamate) and 3% kasugamycin hydrochloride (MW = 415.9) which was equivant to 2.7% kasugamycin (MW = 379.4), and samples G and H were mixed fungicides containing 75.6% copper oxychloride and 5.7% kasugamycin hydrochloride (equivant to 5.2% kasugamycin). Solvents and chemicals were analytical grade. Phosphate buffer (0.1 M, pH 2.5) and wash solution used in HPCE analysis were purchased from Bio-Rad.




**I.** Possible actual structure of kasugamycin (MW=379.4)




**II.** Structure of kasugamycin monohydrochloride hydrate (MW=433.8)

**Figure 1.** Structure of kasugamycin (top) and its monohydrochloride hydrate (below).

**Capillary Electrophoresis and Analytical Column.** High-performance capillary electrophoresis was performed using a Biofocus 3000 automated capillary electrophoretic apparatus. A Biofocus cartridge capillary column coated with hydrophilic polymer (24 cm × 25 $\mu$m) was used. This capillary column was operated at the recommended acidic condition (below pH 3). The column used in these studies was placed in circulating water jackets for ambient temperature control.

A regulated dc power supply delivering 15 kV was used to provide high voltage between the ends of the column filled with

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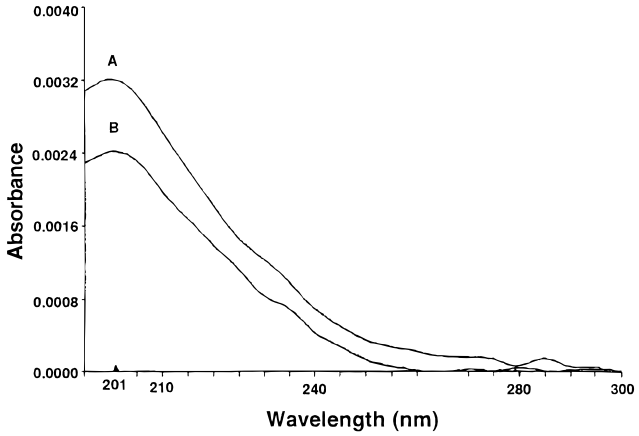


**Figure 2.** UV spectra of kasugamycin (curve A) and 2% solution (curve B). Maximum absorption occurred at 201 nm, and 210 nm was selected for kasugamycin detection.

pH 2.5 buffer solution. The elution of a solute was monitored by an on-column UV−vis detector (210 nm) at the negative pole (Figure 2).

**Sample Extraction for Capillary Electrophoresis (CE) Analysis.** A proper amount of sample was weighed into a 15 mL centrifuge tube. Exactly 6 mL of distilled water was added. The mixture was mixed with a mixer (Thermolyne 37600 Mixer, USA) for 1 min. The extract was centrifuged at 1006*g* for 5 min (Sigma 320, USA). The supernatant was transferred to a 25 mL volume flask. The extraction was repeated twice, the supernatants were combined and made up with distilled water to 25 mL. The contents of the 25 mL volume flask were mixed and diluted with distilled water to a proper concentration in a 10 mL volume flask. Of this solution, a proper aliquot was injected into an autosampler vial through a 0.45 $\mu$m nylon syringe filter (Lida Manufacturing Corp. USA) for CE analysis.

Sample extract was introduced into the capillary vessel using the pressure injection mode at 20 psi × s, and the volume of sample introduced into the capillary vessel during pressure injection was calculated to be 3.7 nL (Bio-Rad Bulletin 1818; Lo et al., 1995).

Column efficiency is expressed in terms of theoretical plates. The theoretical plates ($N$) were determined by the standard molecular diffusion term in chromatography (Jorgenson and Lukacs, 1981; Lo et al., 1995), and the column efficiency was measured by the equation

$$N = 16\left(\frac{t_r}{W}\right)^2 = 5.5\left(\frac{t_r}{W_{1/2}}\right)^2$$

where $t_r$ is the retention time of the peak, $W$ is the peak width at a given peak height (the tangents to the side of the peaks are extrapolated to the base line for $W$), and $W_{1/2}$ is the width at 1/2 peak height. Since the peak was sharp for HPCE, a peak width ($W$) at a given peak height was used for theoretical plate calculation.

The reproducibility of retention time ($t_r$), peak area, linearity, and detection limit was used to evaluate the selectivity, sensitivity, and the reliability of the HPCE method.

**HPCE Calibration Curve.** Kasugamycin hydrochloride hydrate (MW = 433.8) standard (0.1g, purity > 99.9%) was weighed into a 10 mL volumetric flask, diluted with distilled water to obtain kasugamycin (MW = 379.4) stock standard solution of 8.74 mg/mL (0.1 g × 0.999 × 379.4/433.8 = 87.4 mg). The stock standard solution was diluted with distilled water in sequence to obtain the final working standard solution of concentrations 8.75, 17.49, 26.24, 34.98, and 43.73 $\mu$g/mL. The different working standard solutions were used for the calibration curve. Three replications were conducted, and linear regression was used to determine the suitability of the HPCE method.

**Limit of Detection.** The instrumental limit of detection (IDL) was determined by injecting a working standard solution

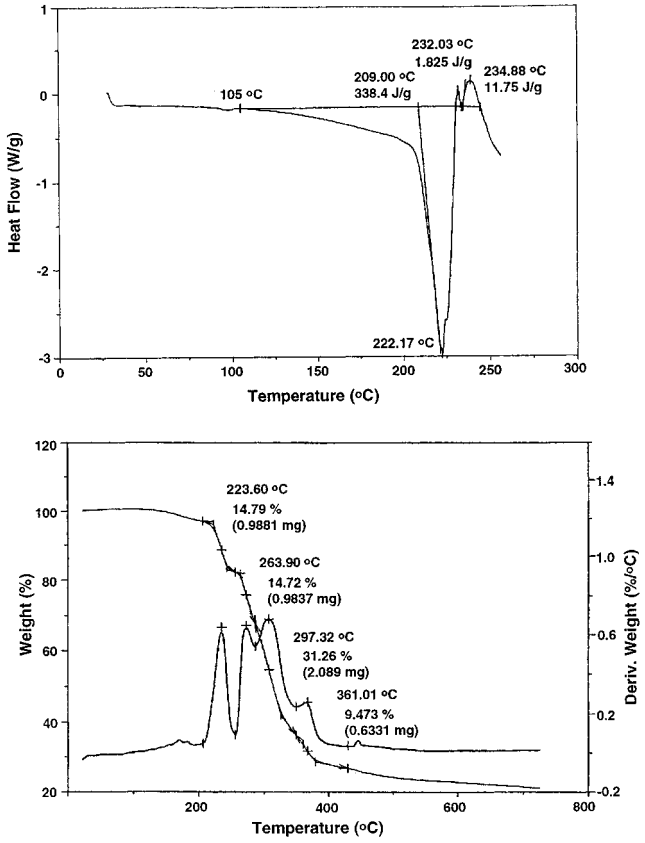


**Figure 3.** Typical DSC (top) and TGA profiles (bottom) of kasugamycin hydrochloride hydrate standard.

to produce a signal that was about three times the signal-to-noise ratio (US EPA, 1984). Low concentrations of working standard solutions of 0.44 and 0.22 $\mu$g/mL were prepared and were used to determine the IDL.

The concentration of working standard solution that corresponds to 5.0 times IDL is used to determine the method detection limit (MDL). Repeated HPCE analyses (seven times) produced data for the standard deviation (SD); 3SD was used as the MDL. Precision expressed by relative standard deviation (RSD) was used in judging the acceptability of the method.

**Recovery.** Because the accurate composition of different commercial formulations of solution (SL) and wettable powder (WP) were unknown, the effect of formulations on HPCE approach were analyzed by recovery. The recoveries of kasugamycin from formulated products were determined by applying with a pipet a 0.5 mL aliquot of kasugamycin standard solution (8.74 mg/mL) to a 80 mg portion of formulated product. To another 80 mg portion of formulated product was added nothing. The spiked and unspiked formulated samples were then mixed separately for 2 min, the samples were analyzed, and percents of recoveries were calculated as the difference between the amounts of kasugamycin found in the spiked and in the nonspiked samples expressed as a percentage of the amount of kasugamycin added.

**Molecular Structure and Melting Point Determination.** The molecular structure, the melting point, and the decomposition temperature of the manufacturer standard were determined with a DSC 10 differential scanning calorimeter (DSC), a 951 thermogravimetic analyzer (TGA), and a 2000 thermal analyzer (DuPont Instruments). Temperatures were set from room temperature to 275 °C for DSC and to 680 °C for TGA. The heating rate was set at 10 °C/min, and the nitrogen flow was set at 80 mL/min. Sample masses used for DSC and TGA were about 6.7 mg.

## RESULTS AND DISCUSSION

**Determination of the Molecular Weight of the Kasugamycin Standard.** The DSC diagram showed

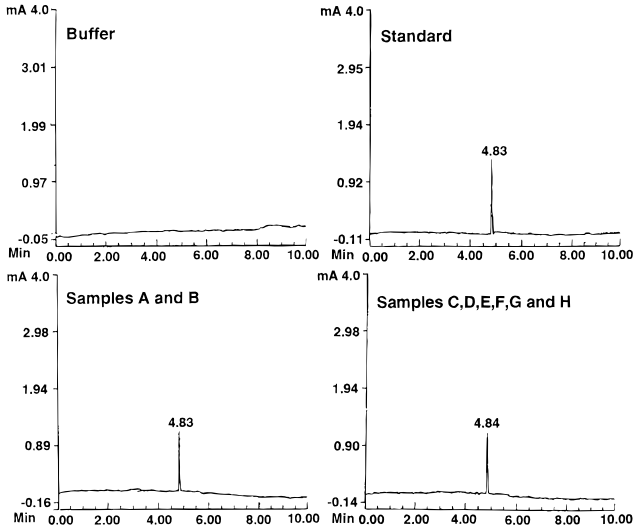


**Figure 4.** Typical electropherograms of phosphate buffer, kasugamycin standard, and commercial products. Samples A and B were soluble products, and samples C−H were wettable powder products.

that the manufacturer standard started to melt at 105 °C, and decomposed after heating up to 222.17 °C. The melting points of the manufacturer standard of three replications were 207.7, 208.8, and 209.9 °C (Figure 3, top), which were a little higher than the reported melting point of kasugamycin hydrochloride (202−204 °C) under decomposition (Umezawa et al., 1965). The TGA diagram showed that there was almost no weight loss below 200 °C, and the weight loss started at near 223 °C (Figure 3, bottom). This was in agreement with the DSC observation of decomposition temperature at 222.17 °C. The weight percent losses at 223 °C were 14.79, 14.88, and 15.38% of three replications; this could result from the loss of HCl and $H_2O$ and some other unknown compound (about 2.2%).

$$\frac{MW_{HCl} + MW_{H_2O}}{MW_{\text{kasugamycin hydrochloride hydrate}}} \times 100\% = \frac{36.5 + 18}{433.9} \times 100\% = 12.6\%$$

The HCl might come from the ammonium group of the tetrahydropyran group, and the chloride ion adjacent to the ammonium. The $H_2O$ might come from the water adjacent to the imino group of acetic acid (Figure 1, bottom). Ikekawa et al. investigated the structure of kasugamycin hydrobromide by X-ray crystallographic analysis, and found that the bromide ion was linked with the ammonium group and water was attached to the imino group (Ikekawa et al., 1966). The behavior of chloride ion is similar to that of bromide ion; thus the structure of manufacturer standard kasugamycin hydrochloride hydrate was proposed as shown in Figure 1 (bottom).

**Chromatogram of Kasugamycin Standard.** A typical electropherogram of the kasugamycin standard is shown in Figure 4. The retention times were very consistent ranging from 4.77 to 4.88 min and RSD from 0 to 0.41%. The retention time was not influenced by the concentration selected from 8.8 to 43.7 μg/mL. A good linear correlation ($r^2$ = 0.9991) between the concentration ($X$) and peak area ($Y$) was found in the concentrations of 0.52−874.60 μg/mL, and the region of 8.75−43.7 μg/mL was used to calculate the kasugamycin concentration in the formulated products.

**Table 1. HPCE Determination of Kasugamycin (KSM) in Formulated Products**

| | formulation (% ai claimed) | HPCE (%, RSD) | tolerance (%) |
|---|---|---|---|
| A | 2% SL[a] (2% KSM) | 1.99, 1.46 | 1.60−2.40 |
| B | 2% SL (2% KSM) | 1.75, 1.06 | 1.60−2.40 |
| C | 2% WP[b] (2% KSM) | 1.95, 0.57 | 1.60−2.40 |
| D | 2% WP (2% KSM) | 2.04, 0.81 | 1.60−2.40 |
| E | 43% WP (3% KSM·HCl[c] or 2.7% KSM) | 2.87, 0.88 | 2.18−3.28 |
| F | 43% WP (3% KSM·HCl or 2.7% KSM) | 2.88, 1.01 | 2.18−3.28 |
| G | 81.3% WP (5.7% KSM·HCl or 5.2% KSM) | 5.42, 0.19 | 4.15−6.22 |
| H | 81.3% WP (5.7% KSM·HCl or 5.2% KSM) | 5.70, 2.21 | 4.15−6.22 |

[a] Solution. [b] Wettable powder. [c] Kasugamycin hydrochloride.

**Table 2. Recovery of Spiked Kasugamycin in Formulated Products**

| | sample | recovery (%) | mean (%) | RSD (%) |
|---|---|---|---|---|
| A | 2% SL[a] (2% KSM) | 99.2, 100.2, 100.0 | 100.0 | 0.12 |
| B | 2% SL (2% KSM) | 101.0, 99.2, 100.2 | 100.2 | 0.88 |
| C | 2% WP[b] (2% KSM) | 102.3, 100.8, 102.7 | 102.0 | 0.98 |
| D | 2% WP (2% KSM) | 103.4, 101.2, 104.8 | 103.2 | 1.77 |
| E | 43% WP (3% KSM·HCl[c] or 2.7% KSM) | 101.0, 102.2, 102.4 | 101.9 | 0.75 |
| F | 43% WP (3% KSM·HCl or 2.7% KSM) | 98.8, 104.3, 102.8 | 102.0 | 2.81 |
| G | 81.3% WP (5.7% KSM·HCl or 5.2% KSM) | 100.3, 100.8, 99.4 | 100.2 | 0.70 |
| H | 81.3% WP (5.7% KSM·HCl or 5.2% KSM) | 100.3, 99.8, 99.7 | 99.9 | 0.29 |

[a] Solution. [b] Wettable powder. [c] Kasugamycin hydrochloride.

**Table 3. Effect of Commercial Formulation on the Analysis of Kasugamycin (KSM) by the HPCE Method Evaluated by Standard Addition**

| | formulation (% claimed) | concentration (%) calculated[a] | corrected[b] | difference[c] (%) |
|---|---|---|---|---|
| A | 2% SL[d] (2% KSM) | 1.99 | 1.97 | 1 |
| B | 2% SL (2% KSM) | 1.75 | 1.75 | 0 |
| C | 2% WP[e] (2% KSM) | 1.95 | 1.89 | 6 |
| D | 2% WP (2% KSM) | 2.04 | 2.00 | 4 |
| E | 43% WP (3% KSM·HCl)[f] or 2.7% KSM) | 2.87 | 2.82 | 5 |
| F | 43% WP (3% KSM·HCl) or 2.7% KSM) | 2.88 | 2.83 | 5 |
| G | 81.3% WP (5.7% KSM·HCl or 5.2% KSM) | 5.42 | 5.41 | 1 |
| H | 81.3% WP (5.7% KSM·HCl or 5.2% KSM) | 5.70 | 5.71 | −1 |

[a] Concentration calculated from calibration curve. [b] Calculated concentration corrected with recovery. [c] Difference = [(calculated concentration − corrected concentration)/calculated concentration] × 100%. [d] Solution. [e] Wettable powder. [f] Kasugamycin hydrochloride.

The retention time of kasugamycin was 4.83 min ($t_r$), and the peak width was 0.15 min ($W$); then the calculated number of theoretical plates [$N = 16(t_r/W)^2$)] of the column was 16 589, which was higher than the theoretical plates number 8078 of blasticidin S in the HPCE analysis (Lo et al., 1995).

**Sensitivity of HPCE Method.** The instrument limit of detection, defined as 3 times the base-line noise, was estimated at 0.44 μg/mL, or 1.63 pg/injection for 3.7 nL of injection volume. The method detection limit was estimated about 0.51 μg/mL.

**Determination of Kasugamycin Content in Commercial Formulated Products.** The official tolerance

for active ingredients less than 10% in commercial formulation ranged from +20% to −20%. The HPCE analysis of kasugamycin showed that all the active ingredient contents of samples A−H were in the official tolerance range (Table 1), and their electropherograms are shown in Figure 4.

The precision of the HPCE method as measured by RSD values in the determination of kasugamycin in commercial formulated products ranged from 0.19 to 2.21%. The RSD values were less than 10% indicating that the precision of the method was excellent (McFarren et al., 1970).

**Influence of Formulations on HPCE Performance.** The analysis of kasugamycin in commercial formulation products was validated by the standard addition method. Commercial samples were fortified with kasugamycin standard, and the recovery of added kasugamycin was calculated. It was found that the average recoveries ranged from 99.9% of sample H to 103.2% of sample D, with RSD values in the region of 0.12−2.81% (Table 2). Therefore there is no major difference between the concentration calculated from the calibration curve and the concentration corrected with recovery (Table 3).

In summary, all seven products are real kasugamycin products. The RSD of kasugamycin retention time in HPCE column is under 0.32%, and the recoveries of spiked kasugamycin in formulated products are in the region of 99.9−103.2%. Both confirm that the HPCE method is a reliable and efficient method. Furthermore, there is no matrix interference on the CE analysis from kasugamycin commercial products. Therefore, the present official bioassay method could be replaced by this new HPCE method.

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