Journal of Food and Drug Analysis

Volume 19 | Issue 4 Article 13

2011

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Journal of Food and Drug Analysis, Vol. 19, No. 4, 2011, Pages 452-456 藥物食品分析 第十九卷 第四期

Simultaneous Analysis of Blasticidin S and Kasugamycin with Micellar Liquid Chromatography

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(Received: January 31, 2011; Accepted: July 22, 2011)

ABSTRACT

A simple and fast micellar liquid chromatographic (MLC) method for simultaneous analysis of the antibiotic fungicides of blasticidin S and kasugamycin was developed. Chromatographic separation was achieved on a Phenomenex® Aqua 5μ C_{18} 125 A column. The mobile phase consisted of an aqueous mixture of 69.3 mM sodium dodecyl sulfate (SDS) with deionized water (50 : 1, v/w). The flow rate was set at 1.0 mL/min, and the absorbance was monitored at 210 nm with a photodiode array detector. The total run time was less than10 min, and the retention times (t_r) were 2.47 min for blasticidin S ion, and 7.15 min for kasugamycin ion. Good linear relationships were obtained with correlation of coefficients (r) of 0.997 for kasugamycin, and 0.999 for blasticidin S. The limits of detection (LODs) of blasticidin S and kasugamycin were 0.5 μg/mL and 0.75 μg/mL, respectively, and the limits of quantitation (LOQs) for blasticidin S and kasugamycin were 1.5 μg/mL and 2.2 μg/mL, respectively. The recoveries of blasticidin S and kasugamycin in blank irrigation water ranged from 94.9 - 97.3% and 87.8 - 99.7%, respectively. By using MLC method blasticidin S and kasugamycin could be analyzed simultaneously.

Key words: antibiotic fungicide, blasticidin S, kasugamycin, micellar LC (MLC), sodium dodecyl sulfate (SDS)

INTRODUCTION

Blasticidin S, 1-(4-amino-1,2-dihydro-2-oxopyrimidin-1-yl)-4-[(*S*)-3-amino-5- (1-methylauanidinp) valeramido]-1,2,3,4-tetradeoxy-*β*-D-*erythro*-hex-2- enopyranuronic acid (IUPAC) (Figure 1)[(1)], is an antifungal antibiotic produced by *Streptomyces griseochromogene*s[(2)], and it is used for the control of the rice blast, caused by the phytopathogenic fungus *Piricularia oryzae* (or *Pyricularia oryzae*)[(3)].

Kasugamycin, [5-amino-2-methyl-6-(2, 3, 4, 5, 6-pentahydroxycyclohexyloxy) tetrahydropyran-3-yl]-amino-*α*-iminoacetic acid (IUPAC) (Figure 1)[(1)], is produced by *Streptomyces kasugaens*is[(4)], and it has a strong preventive effect against rice blast caused by *Piricularia oryza*e[(4)]. Both blasticidin S and kasugamycin can be applied for the control of rice blast at the same time; therefore it is necessary to develop a method for simultaneous determination of blasticidin S and Kasugamycin in tank-mixture, or in commercial products. Several HPLC and HPCE methods have been developed for the quantification of blasticidin S[(5-7)] and kasugamycin[(8,9)], but these methods could analyze only one fungicide each time. A simultaneous method for analyzing these two fungicides has not been reported, and our previous RP-HPLC tests using phosphate buffers and organic solvents as mobile phases indicated that these two fungicides were neither retained with capacity factor (k´) close to zero, nor separated with selective factor (α) close to 1.

Micellar liquid chromatography (MLC) was first reported in 1980[(10)], the technique has received much attention due to its numerous capability and advantages, such as simultaneous separation of charged and uncharged solutes[(11)], separation of a set of 11 phenol derivatives with different hydrophobicity in isocratic mode[(12)], simultaneous separation of quercetin, hesperetin and chrysin in honey[(13)], and simultaneous analysis of five quinolones of quinolone antibiotics in fish[(14)].

In this work, a micellar liquid chromatography method using aqueous SDS as mobile phase was developed, and the results showed that blasticidin S and kasugamycin could be separated with selectivity factor (α) close to 4.2.

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Journal of Food and Drug Analysis, Vol. 19, No. 4, 2011

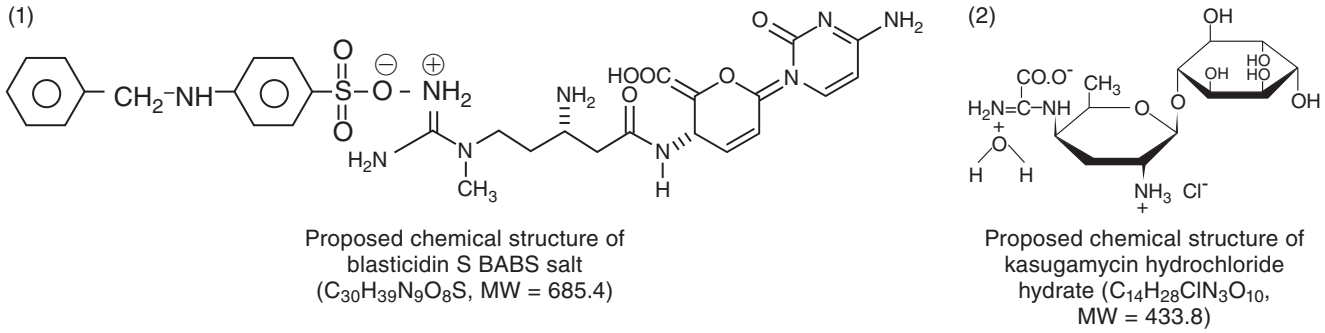

Figure 1. Proposed chemical structures of blasticidin S BABS salt (1), kasugamycin hydrochloride hydrate (2).

MATERIALS AND METHODS

I. *Chemicals and Reagents*

Blasticidin S BABS salt (4-(*N*-benzylamino) benzensulphorate salt of blasticidin S) (94.0% purity) and kasugamycin hydrochloride hydrate (99.9% purity) were kindly provided by Kaken Pharmaceutical Co., Ltd. (Tokyo, Japan), and Hokko Chemical Industry Co., Ltd. (Tokyo, Japan) (Figure 1), respectively. Sodium dodecyl sulfate (SDS) was purchased from Sigma (L4390-500G, 99% purity, St. Louis, MO, USA).

II. *Apparatus and Chromatographic Conditions*

The HPLC analysis was performed on a Varian Prostar HPLC system equipped with a Prostar 220 solvent delivery module, and a Prostar 335 photodiode array detector (PDA). Separations were carried on a Phenomenex® Aqua 5μ C_{18} 125 A (150 mm × 4.6 mm, 5 μm particle size) column with a guard column of similar packing (50 mm × 4.6 mm i.d.) using isocratic elution. The mobile phase was a mixture of deionized water and 69.3 mM SDS in the ratio of 50 : 1.0 (v/w) with flow rate of 1.0 mL/min. Photodiode array detector was set at 210 nm for detection of fungicides. All solvents were filtered through a 0.45 μm nylon filter and degassed before use.

III. *Preparation of Stock and Working Solution*

The standard stock solution of blasticidin S BABS salt and kasugamycin hydrochloride hydrate (1000 μg/mL) were prepared by dissolving proper amounts of standard with deionized water in a 100-mL volumetric flask. Six different working solutions were prepared by appropriate dilution of stock solution. The concentration range of working solutions was 0.5 - 10 μg/mL. A molecular weight factors (F_{wt}) of 0.586[6] and 0.875[9] was applied to calculate the concentration of blasticidin S ion and kasugamycin ion in water, because the mass spectral data confirmed that blasticidin S BABS salt and kasugamycin HCl salt dissolved in water were dissociated into ion forms[6,9].

IV. *Linearity*

Linearity was verified by analysis of working solutions at six different concentrations. Signals (peak area) and concentrations (μg/mL) of the fungicides were subjected to regression analysis to calculate the calibration equation and correlation coefficients. Each concentration was prepared in triplicate, and analyzed three times.

V. *Limits of Detection and Limit of Quantification*

The limits of detection (LOD) and the limit of quantitations (LOQ) were determined at a signal-to-noise ratio (S/N) of 3 and 10, respectively.

VI. *Precision*

The intraday and interday accuracy and precision (RSD) for the proposed method was examined by analysis of fungicides in three different concentrations (1.0, 5.0, 10 μg/mL). Intraday precision was assessed in one day, and interday precision was assessed on three consecutive days.

VII. *Recovery from Irrigation Water*

The recoveries for blasticidin S and kasugamycin in spiked irrigation water (spiked with 0.75, 1.0 and 10.0 μg/mL) were used to determine the accuracy and the reproducibility of HPLC method. Irrigation water (collected from paddy field at Wufong) with no blasticidin S and kasugamycin residue was used as blank irrigation water for recovery test. The recoveries were conducted by adding proper amounts of standard into blank irrigation water, and the final concentration of blasticidin S and kasugamycin spiked in blank irrigation water were, 0.75, 1.0 and 10 μg/mL. Spiked irrigation water of 100 mL was collected and evaporated to dryness with a vacuum rotavapor, and the residue was redissolved in 5 mL distilled water for direct HPLC analysis. The recovery was determined by the percentage of calculated amount versus amount added. Each level was prepared in triplicate, and analyzed three times.

Journal of Food and Drug Analysis, Vol. 19, No. 4, 2011

RESULTS AND DISCUSSION

I. *High Performance Liquid Chromatography*

Chromatograms of the results were shown in Figure 2. Figure 2A was mobile phase (H_2O : SDS = 50 : 1); Figure 2B was blasticidin S-BABS salt (Bs) in deionized water; and Figure 2C was kasugamycin-HCl salt (Ks) in deionized water. Figure 2D was mixture of blasticidin S-BABS salt and kasugamycin-HCl salt standard in deionized water. Peak at 2.47 min was blasticidin S ion, peak at 4.97 min was BABS ion, and peak at 7.15 min was kasugamycin ion. Capacity factors (k´) for blasticidin S and kasugamycin were 1.5 and 6.2, respectively. The k´ values (k'_{Bs} = 1.5, k'_{Ks} = 6.2)

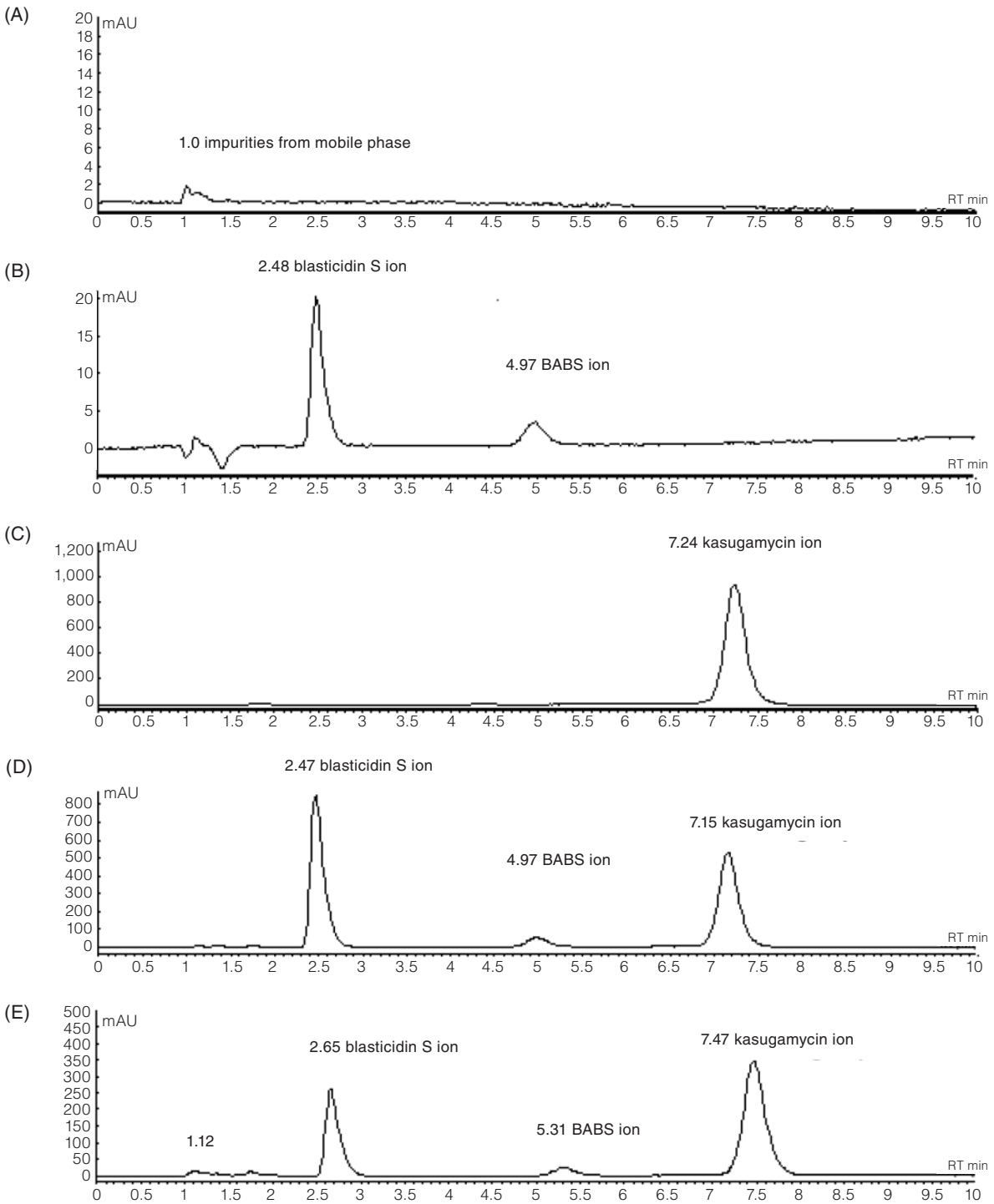

Figure 2. HPLC chromatograms of (A) mobile phases (H_2O : SDS = 50 : 1); (B) blasticidin S-BABS salt (Bs) in deionized water; (C) kasugamycin-HCl salt (Ks) in deionized water; (D) mixture of blasticidin S-BABS salt and kasugamycin-HCl salt standard in deionized water; (E) mixture of blasticidin S-BABS salt and kasugamycin-HCl salt standard in irrigation water.

Table 1. Linear regression analysis, limits of detection (LOD), and limits of quantification (LOQ) of fungicide on MLC method

Fungicides	Linear regression equation	Calibration range (μg/mL)	*r*	LOD (μg/mL)	LOQ (μg/mL)
Blasticidin S	y = 20.842x – 0.0753	0.5 - 10	0.999	0.5	1.5
Kasugamycin	y = 17.285x – 2.5208	0.75 - 10	0.997	0.75	2.2

Table 2. Recovery and precision of the HPLC method for determination of Blasticidin S and Kasugamycin in deionized water

Compound	Concentration (μg/mL)	Intraday precision (%RSD)	Mean recovery (%)	Interday precision (%RSD)	Mean recovery (%)
Blasticidin S	1.0	3.1	102.1	4.7	101.5
	5.0	0.9	99.5	3.3	102.7
	10.0	0.8	99.7	1.6	99.3
Kasugamycin	1.0	0.6	101.7	6.1	98.4
	5.0	4.5	91.1	4.0	90.6
	10.0	2.1	98.2	7.6	95.3

indicated that the separation between mobile phase and target molecules were excellent. The selective factors(α) between blasticidin S and kasugamycin was 4.2 ($\alpha_{Ks/Bs} = 4.2$) indicated that the separation of these two compounds were also excellent. Figure 2E was mixture of blasticidin S-BABS salt and kasugamycin-HCl salt standard in irrigation water. Peak at 2.65 min was blasticidin S ion; peak at 5.31 min was BABS ion. The peak at 7.47 min was kasugamycin ion, and peak at 1.12 min were impurities.

II. *Method Validation*

(I) *Linearity*

The calibration curves were constructed by plotting the peak areas (Y) versus the concentration (X) of each analytes. The regression equation obtained for blasticidin S was y = 20.842x – 0.0753, the coefficients of correlation (*r*) was 0.999, and the range of linearity was 0.5 - 10 μg/mL (Table 1). The regression equation obtained for kasugamycin was y = 17.285x – 2.5208, the coefficients of correlation (*r*) was 0.997, and the range of linearity was 0.75 - 10 μg/mL (Table 1). The high coefficients of correlation (*r*) indicated that the linearity of the calibration curves of these two compounds were good.

(II) *Limits of Detection and Limit of Quantification*

The limits of detection (LOD) and quantitation (LOQ) were determined at a signal-to-noise ratio (S/N) of 3 and 10, respectively. LOD values for blasticidin S BABS salt and kasugamycin HCl salt were calculated as 0.5 μg/mL and 0.75 μg/mL, respectively. LOQ values for blasticidin S BABS salt and kasugamycin HCl salt were selected as 1.5 and 2.2 μg/mL, respectively (Table 1).

Table 3. Recovery and precision of the HPLC method for determination of spiked blasticidin S and kasugamycin in blank irrigation water

Fungicides	Concentration (μg/mL)	Recovered (μg/mL)	Recovery (%)	RSD (%)
Blasticidin S	0.75	0.73	97.3	1.37
	1.0	0.95	95.3	1.60
	10.0	9.49	94.9	4.97
Kasugamycin	0.75	0.66	87.8	0.51
	1.0	0.94	94.0	1.49
	10.0	9.97	99.7	0.38

Sheu *et al*. (2010) also reported that LOQ of kasugamycin was 2.2 μg/mL when an Agilent HP1050 with UV detector was applied[9], two results were in good agreement.

(III) *Accuracy and Precision*

The results of intraday and interday precision expressed as relative standard deviation (RSD) were shown in Table 2. Recoveries for two fungicides in distilled water were between 91.1 and 102.1% with RSD of less than 4.5% in intraday assays. The recoveries in interday assays were between 90.6 and 102.7% with RSD of less than 7.6%.

(IV) *Recoveries of Fungicides from Blank Irrigation*

The recoveries of blasticidin S in blank irrigation water ranged from 94.9 - 97.3% with RSD ranged from 1.37 - 4.97% (Table 3), and the recoveries of kasugamycin in blank irrigation water ranged from 87.8 - 99.7% with RSD ranged

from 0.38 - 1.49% (Table 3). The high recoveries and low RSD values of the developed HPLC method indicated that the method was reliable (Table 3).

We have tried several aqueous organic mobile phases, and several C_{18} RP columns, but they all failed to separate these two fungicides. Ionic organic compounds were only weakly or not retained in RPLC when pure buffer or water was used as the eluents[15]. SDS is an anionic surfactant, its action on charged modification or micelle formation on these two ionic fungicides might result in the increase of retention capacity. The use of SDS above cmc (8.27 mM) has been proved to be effective separation methods in HPCE[16-18] and HPLC[10,12-14,19]. With the help of SDS, the retention times of two fungicides on RP column increased.

ACKNOWLEDGMENTS

We are grateful to Kaken Pharmaceutical Co., Ltd. (Tokyo, Japan), and Hokko Chemical Industry Co., Ltd. (Tokyo, Japan) for providing analytical standard of blasticidin S-BABS salt and kasugamycin hydrochloride hydrate.

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