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Short communication

Development of liquid chromatographic method for the analysis of kanamycin residues in varicella vaccine using phenylisocyanate as a derivatization reagent

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Abstract

A liquid chromatographic method for the determination of the aminoglycoside kanamycin in varicella vaccine is described. Kanamycin sulfate was derived with phenylisocyanate (PIC) and triethylamine for 10 min at 70°C and chromatographed on a alkylamide-bonded column, Suplex pKb-100. A derivative of kanamycin sulfate was attached to four phenylisocynato groups and that molecular mass was confirmed with liquid chromatography–electrospray ionization mass spectrometry (LC–ESI-MS). The kanamycin-PIC derivative was found to have a retention time of 11.7 min using an eluent composed of 40% acetonitrile in water at 1.2 ml/min column flow-rate. Detection was at a wavelength of 240 nm. Recoveries ranging from 97.5 to 99.8% were found. The correlation coefficient was greater than 0.9998 over the range between 10 and 100 μg/ml. The method precision of within-day assay showed a 0.5 to 4.0% coefficient of variation ($n=5$) ranging from 10 to 70 μg/ml of kanamycin concentration levels. Kanamycin-PIC derivative in reaction solution was stable for 24 h at room temperature. A simple and efficient method for the analysis of the kanamycin in varicella vaccine was developed and validated.

Keywords: Derivatization, LC; Kanamycin; Phenylisocyanate

1. Introduction

Kanamycin sulfate, which is produced during fermentation of *Streptomyces kanamyceticus*, is a water-soluble broad spectrum antibiotic with narrow therapeutic range of gram-positive and gram-negative bacteria. Kanamycin sulfate is composed of the main substance kanamycin A and only small amounts of related substances kanamycin B and C (Fig. 1) [1].

Antibiotics are used in media for cultures. However, their use in media for cultures should often be discouraged because of contamination of residual antibiotics. Kanamycin sulfate is used in combination with the culture media of varicella as an antibiotic. Therefore, an analytical method for the determination of kanamycin residue in varicella vaccine is needed to control quality and assurance.

Several liquid chromatographic methods for the determination of aminoglycosides have been reported [2–6]. Most of these methods use electrochemical detection because of the lack of the chromophores and fluorophores. Gas chromatographic methods

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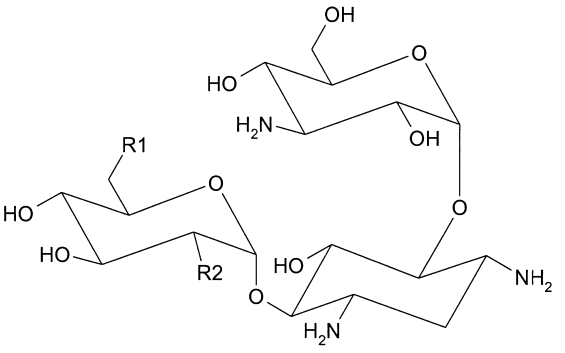

	R1	R2
Kanamycin A	NH_2	OH
Kanamycin B	NH_2	NH_2
Kanamycin C	OH	NH_2

Fig. 1. The structures of kanamycin isomers.

have been published, most of them requiring trimethylsilylation to derivatize hydroxyl as well as amino groups [7–9]. Vogel and co-workers have reported a capillary elecrophoresis–electrochemical detection method for the analysis of aminoglycosides [10]. Yevtushenko described a method for the analysis of kanamycin B and tobramycin using isocyanatobenzene as a derivatization reagent [5].

In this study, we describe a validated, simple and precise high-performance liquid chromatographic (HPLC) method for the analysis of kanamycin residue in varicella vaccine using phenylisocyanate as a derivatization reagent.

2. Experimental

2.1. Chemicals and reagents

Varicella vaccine was commercialized by Life Science R&D Institute in LG Chemical Research Park (Commercial name: LG Varicella Vaccine inj.). All solvents used in this study were HPLC grade and purchased from J.T. Baker company (NJ, USA). Kanamycin sulfate, phenylisocyanate (PIC) and triethylamine (TEA) were purchased from Sigma Chemical (MO, USA). Distilled and deionized water was used in this study (Milli-Q water purification system, Millipore, MA, USA).

2.2. Apparatus

2.2.1. Chromatographic conditions

Chromatography was carried out on a Waters HPLC system consisting of two 510 HPLC pumps, a Rainin AI-1A autosampler (Rainin, CA, USA) and a Waters 486 tunable wavelength detector (Waters, MA, USA). Data integration and process were performed with MILLENNIUM[32] software (Rev. 3.05.01, Waters). The HPLC column used was a Suplex pKb-100 (4.6 mm I.D.×250 mm L, 5-μm, Amide hexadecyl bonded silica, Supelco, PA, USA).

The mobile phase was prepared by mixing 400 ml of acetonitrile with 600 ml of water. This mobile phase was filtered and degassed through a 0.45-μm membrane filter. The flow-rate was performed at 1.2 ml/min. The UV detector was set at a wavelength of 240 nm.

2.2.2. Electrospray mass spectrometry

The HPLC (HP1100, Hewlett–Packard, CA, USA) system was linked to an electrospray interface, which was connected to a Finnigan LCQ Ion-trap mass spectrometer (Finnigan, CA, USA). The mass spectrometer was controlled and data were processed and analyzed using NAVIGATOR software (Rev. 1.2, Finnigan). The following electrospray parameters were used;

Sheath and auxiliary gas; N_2, sheath gas flow-rate; 80 ml/min, auxiliary gas flow-rate; 10 ml/min, capillary temperature; 200°C, capillary voltage; 3.5 kV, scan range; m/z 50~2000.

The mass analyzer was used in the positive-ion mode. The protonated kanamycin-PIC derivative ion, $[M+H]^+$, was detected at m/z 961. Kanamycin-PIC derivative was confirmed for four substituted phenylisocyanato groups on an amino group of kanamycin (Fig. 2).

2.3. Optimization of the derivatization time

The reaction of the kanamycin with phenylisocyanate and triethylamine was optimized using 50 μg/ml of kanamycin in water. The reaction

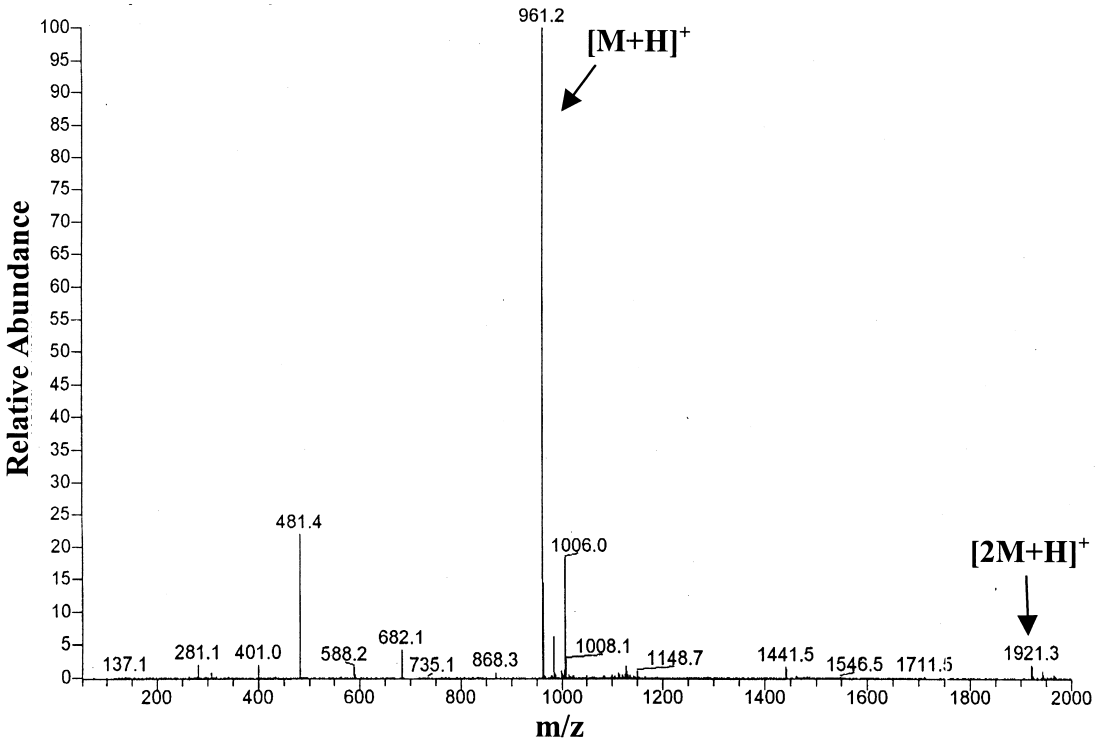

Fig. 2. Mass spectrum of kanamycin-PIC derivative The chromatographic and mass spectrometric conditions were described in the Experimental section.

time was examined varying optimal values from 2 to 20 min at 70°C.

2.4. Sample preparation

2.4.1. Preparation of standard solutions

An aqueous standard solution of 1.0 mg/ml of kanamycin was prepared and diluted with water to obtain the following concentrations; 100, 70, 50, 30, 20, 10 μg/ml kanamycin. A 1-ml volume of each standard solution was added to 500 μl of phenylisocyanate solution (5 mg/ml in acetonitrile) and 500 μl of triethylamine solution (5 mg/ml in acetonitrile). The mixture was reacted in a water bath for 10 min at 70°C and cooled.

2.4.2. Preparation of sample solutions

One vial of varicella vaccine sample was dissolved in 1 ml of water and added to 500 μl of phenylisocyanate solution (5 mg/ml in acetonitrile) and 500 μl of triethylamine solution (5 mg/ml in acetonitrile). This sample solution was heated in a water bath for 10 min at 70°C and cooled rapidly.

2.5. Validation procedure

2.5.1. Specificity

Specificity of the method was examined from five different batch samples because kanamycin was detected below the limit of detection in each sample. Five different batch samples were derived with phenylisocyanate and sample solutions were determined specifically.

2.5.2. Accuracy

Accuracy was determined by spiking a standard solution of kanamycin into the varicella vaccine sample. For the determination of kanamycin recovery, 1 ml of 10, 20, 30, 50 and 70 μg/ml kanamycin concentrations was added to one vial of varicella vaccine samples, respectively. The recoveries of the kanamycin were determined by

comparison of the concentration found to the calculated concentration.

2.5.3. Precision

Method precision was determined by five replicate within-day assays at five different concentration levels as an accuracy test as described above. These samples were prepared according to the derivatization procedure.

2.5.4. Sensitivity

The limit of detection (LOD) was determined by dilution of kanamycin standard solution until an adequate signal (S/N ratio=3) was obtained. The limit of quantitation (LOQ) was established as five times the limit of detection, mathematically.

2.5.5. Linearity

The linearity of the assay was examined for concentrations ranging from one fifth to twice the regulatory specification of the active substance (product specification: below 50 μg kanamycin per one vial of Varicella Vaccine inj.). A calibration curve of standards of 10, 20, 30, 50, 70 and 100 μg/ml kanamycin in water was used for the determination of the linearity test of kanamycin. The standard solutions were derived according to the derivatization procedure and chromatographed.

2.5.6. Stability of kanamycin-PIC derivative

To estimate the stability of the kanamycin-PIC derivative, the derived kanamycin solution was assayed for an adequate time over a period of 24 h.

3. Results

The peak area counts of kanamycin-PIC derivative for the determination of optimum reaction time were compared from 2 to 20 min. The derivatization of kanamycin showed fast reaction with phenylisocyanate and triethylamine and similar peak area counts in each reaction sample. Then, the optimum time of the derivatization procedure was set at a reaction time of 10 min, adequately.

A representative chromatogram of kanamycin-PIC derivative is shown in Fig. 3a. As shown in Fig. 3b, interference was not found from five different batch samples and kanamycin was not detected (<LOD). The limit of detection (LOD) for kanamycin-PIC derivative was 1 μg/ml of kanamycin. This value was distinguishable from base-line noise.

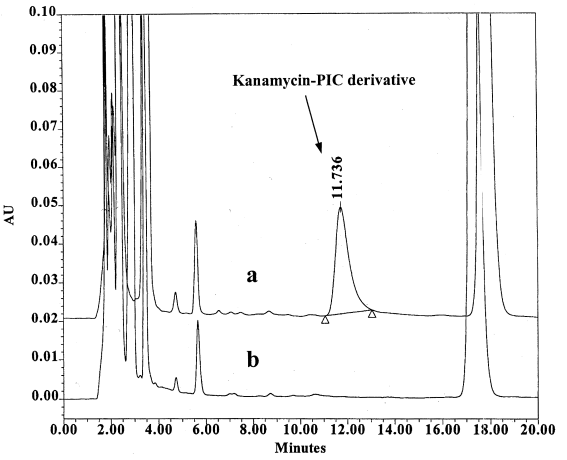

Fig. 3. The representative chromatogram of kanamycin-PIC derivative in varicella vaccine. (a) Spiking 50 μg/ml kanamycin into one vial of varicella vaccine. (b) Blank.

The mean recovery was 98.0% (C.V. 1.1%) over the concentration range between 10 and 70 μg/ml kanamycin. The results of recovery are given in Table 1. Five replicate within-day assays of five different concentration levels show acceptable deviations ranging from 0.5 to 4.0 of C.V. The correlation coefficient in regression line of six-point calibration was 0.9998.

The decomposition of kanamycin-PIC derivative was not found in reaction solutions. The derivatization solutions were stable for 24 h at room temperature.

Table 1
Accuracy and precision in this method for the determination of kanamycin residues in varicella vaccine

Concentration added (μg/ml)	Concentration found (mean value, n=5) (μg/ml)	C.V.[a]	Mean recovery (%)
10.0	10.0	4.0	99.8
20.0	19.4	1.0	97.0
30.0	29.3	1.6	97.7
50.0	48.8	1.2	97.7
70.0	68.3	0.5	97.5

[a] C.V.: Coefficient of variation.

4. Conclusion

We have developed and validated an assay method for the determination of kanamycin residue in varicella vaccine. This method involves a derivatization procedure with phenylisocyanate and triethylamine. The derivatization of kanamycin was simple and fast reaction. In addition, the kanamycin-PIC derivative was stable under reaction solution. This chromatographic method shows good specificity, accuracy, precision and linearity. We have applied this method to the determination of kanamycin residue in commercially available varicella vaccine, LG Varicella Vaccine inj.

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