

Determination of Kanamycin Using Flow Injection Analysis Coupled with Resonance Rayleigh Scattering Detection

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A flow injection analysis (FIA) method coupled with resonance Rayleigh scattering (RRS) detection for the determination of kanamycin sulfate (KANA) was developed. The method is based on the ion-association reaction of KANA with acid triphenylmethane dyes such as aniline blue W. S (AB) and methyl blue (MB) by virtue of electrostatic and hydrophobic interaction forces, which results in a significant enhancement of RRS intensity; their maximum scattering peaks are all at 346 nm. The optimum conditions of the reactions and the flow-through parameters including the length of reaction tubing, the flow rate of the carrier stream and the sample injection volume are discussed in this paper. Under the optimum experimental conditions, the linear ranges for both systems are 0.02–12.0 $\mu g\,mL^{-1}$. The detection limits (3σ) are 4.2 $ng\,mL^{-1}$ for the AB system and 5.0 $ng\,mL^{-1}$ for the MB system and the relative standard deviation for 9 replicate measurements of 2.0 $\mu g\,mL^{-1}$ KANA solution was 1.1%. The proposed method was successfully applied to the determination of KANA in commercial KANA injection, urine and serum samples, and the sample throughput was 30 h^{-1}.

One of the aminoglycoside antibiotics, kanamycin sulfate (KANA), has an extensive antimicrobial spectrum and is available at a low price. As an important bactericidal agent of treating Gram-negative bacteria infection, KANA is widely used at present in clinical therapy. The antibiotic effect and clinical efficacy of KANA depends upon its concentration in serum. Too high a serum concentration is harmful to patients' ears and kidneys. Therefore, it is very important to develop an accurate and quick determination technique for KANA in serum. Nowadays, the primary methods for the determination of KANA include microbiological assay,[1] high-performance liquid chromatography,[2,3] electrochemistry,[4,5] fluorescence assay,[6,7] and spectrophotometry.[8,9]

In recent years, resonance Rayleigh scattering (RRS) has become popular as a new analytical technology for its remarkable characteristics of high sensitivity, rapid assay, and simple instrumentation. It has been used for the determination of nucleic acids,[10] proteins,[11,12] heparin,[13,14] metal ions,[15] nonmetal ions,[16] and pharmaceuticals such as alkaloids,[17,18] vitamins,[19] and antibiotics,[20–23] etc. Most pharmaceutical analyses using RRS methods are based on the ion-association reaction between drugs and reagent molecules by virtue of weak binding forces such as electrostatic and hydrophobic interaction. Some of the ion-associates are not stable in aqueous solution; consequently, water is not a suitable solvent for some compounds. The intensity of RRS is affected by experimental factors such as the medium and temperature of the reaction, reaction times, and so on. As a result, the reproducibility and accuracy of the method are sometimes poor. Flow injection analysis (FIA) is a technology for dealing with a solution in nonuniformity and non-equilibrium conditions and has distinct advantages in reproducibility, sample throughput, and cost performance. FIA has been frequently combined with chemiluminescence,[24] ultraviolet–visible spectroscopy,[25] fluorescence spectroscopy,[26] and electrochemistry.[27] However, FIA coupled with RRS detection has rarely been reported. Up to now, only one study on FIA incorporating the Rayleigh light scattering technique for total protein determination has been reported.[28]

This work is concerned with the use of aniline blue W. S (AB) or methyl blue (MB) as a binding reagent for the determination of KANA by a flow injection analysis manifold with resonance Rayleigh scattering detection. The effects of acidity, ionic strength, dye concentrations, length of reaction tubing, flow rate of the carrier stream, and sample injection volume on the RRS intensity are discussed. Under optimized experimental conditions, the calibration range and equations, the sensitivity, and the repeatability of the method were investigated. A possible mechanism of RRS enhancement has been discussed.

Experimental

Reagents. The stock concentration of kanamycin sulfate (KANA, Sigma Co.) was 40.0 $\mu g\,mL^{-1}$ and a series of working concentrations were prepared by the appropriate dilution of the standard solution with water before use.

The concentrations of the stock solutions for methyl blue (MB, Shanghai Sample Pattern Factory) and water soluble aniline blue (AB, Shanghai Sample Pattern Factory) were 1.0×10^{-3} mol L^{-1}, and the working solutions (1.0×10^{-4} mol L^{-1}) were prepared by diluting the stock solutions with water.

Britton–Robinson buffer solutions of different pH were prepared by mixing the mixed acid (composed of 0.04 mol L^{-1} H_3PO_4, H_3BO_3, and HAc) with 0.2 mol L^{-1} NaOH.

The KANA standard consisted of biochemical reagents, and the other reagents were of analytical-reagent grade. Doubly distilled water was used throughout the experiment.

Apparatus. A Hitachi F-2500 spectrofluoremeter with a 90 μL quartz flow cell (Tokyo, Japan) was used as the detector for recording RRS spectra by scanning synchronously with the same excitation and emission wavelengths and measuring RRS intensity by time scan pattern. The determination parameters were the slit (EX/EM) of 5.0 nm/5.0 nm and PMT voltage 400 V. A UV–vis

Published on the web February 10, 2006; DOI 10.1246/bcsj.79.247

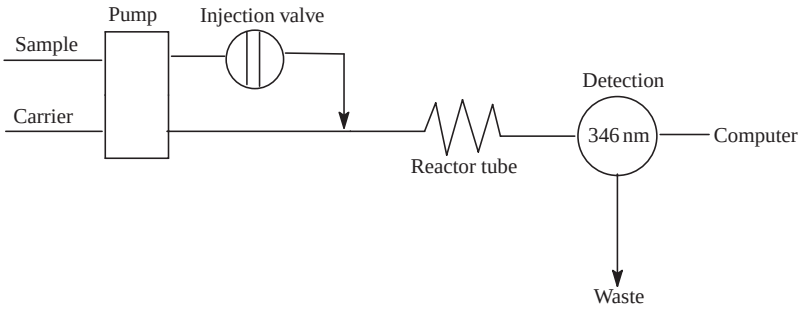

Fig. 1. Schematic diagram of FIA system for KANA determination with RRS detector. Reactor length: 80 cm, carrier flow rate: 3.5 mL min^{-1}, injection volume: 60 μL.

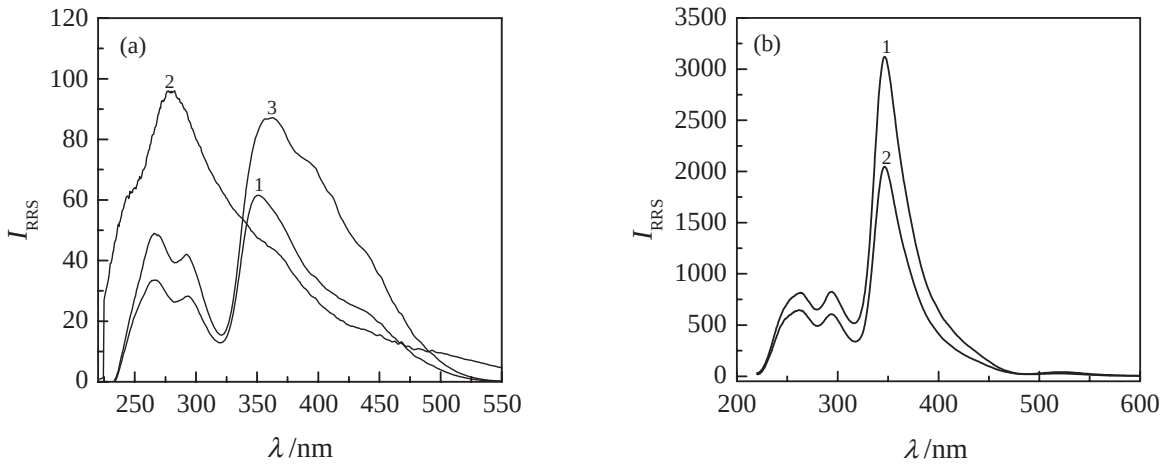

Fig. 2. RRS spectra. (a) 1, AB; 2, KANA; 3, MB; (b) 1, AB-KANA; 2, MB-KANA.

Table 1. The RRS Spectral Characteristics

	KANA	AB	AB-KANA	MB	MB-KANA
The maximum RRS peak/nm	280	351	346	362	346
Other RRS peaks/nm	—	266, 294	263, 294	266, 293	262, 294
I_{RRS}	95	61	3122	97	2047

8500 spectrophotometer (Tianmei, Shanghai) was used for recording the absorption spectra. A pHS-3C acidimeter (Shanghai Dazhong Analytical Instrumental Plant) was used to measure pH.

The flow system used consisted of a peristaltic pump (Shanghai Electrical Machine Factory, Shanghai) and an eight-way rotary valve with exchangeable sample loop. Polytetrafluoroe thylene (PTFE) with 1.0 mm internal diameter tubing was used to connect all components in the flow system. A schematic diagram of the flow system used is shown in Fig. 1.

Procedure. The carrier solution, consisting of the dye (MB or MA) solution and a pH 2.5 BR buffer solution was continuously pumped through the system until a stable baseline was obtained. Then, kanamycin standards or sample solutions were injected into the flow system by an eight-way injection valve. The RRS intensity (I) was detected at $\lambda_{ex} = \lambda_{em}$. $\Delta I = I - I_0$, I and I_0 are the RRS intensities of the carrier in presence and absence of KANA, respectively. Typically, three repeated injections of standards and samples were made and the results are reported as the mean value. In the present work, the major flow-through parameters were 80 cm, 3.5 mL min^{-1}, and 60 μL for the length of mixing tubing, flow rate of carrier stream, and injection loop volume, respectively.

Results and Discussion

RRS Spectra. The RRS spectra of AB, MB, and KANA alone, and the ion-associates for AB-KANA and MB-KANA are shown in Fig. 2. The RRS spectral characteristics of the two systems are listed in Table 1. From Fig. 2 and Table 1, it can be seen that (1) the RRS of AB, MB, or KANA alone is very weak, their maximum scattering peaks are at 280 nm for KANA, 351 nm for AB, and 362 nm for MB, respectively; (2) when KANA reacts with AB or MB to form an ion-associate, it can lead to a remarkable enhancement in the intensity of RRS and a new RRS spectrum appearing. The spectral characteristics of both are similar, their maximum RRS peaks are all at 346 nm; (3) the spectral characteristics of the ion-associate are mainly influenced by the RRS of the dye. The maximum scattering peaks (λ_{max}) at 346 nm for the complex is very close to 351 nm for AB and 362 nm for MB and the λ_{max} for the complexes shift to wavelengths 5 and 16 nm shorter than those of the corresponding dyes, respectively. And two other RRS peaks, 263 and 294 nm for AB-KANA and 262 and 294 nm for

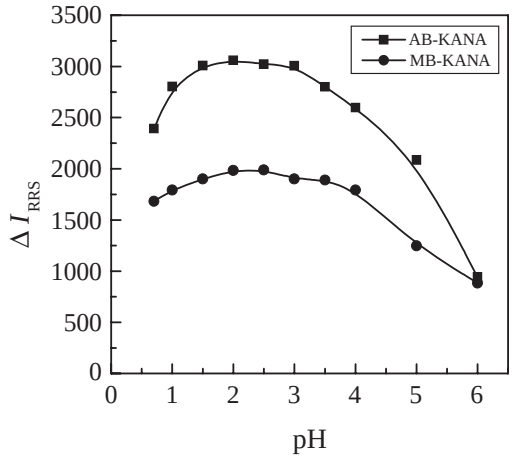

Fig. 3. Effect of acidity on ΔI_{RRS}. KANA concentration: 4.0 μg mL^{-1}; Dyes: 1.0×10^{-4} mol L^{-1}.

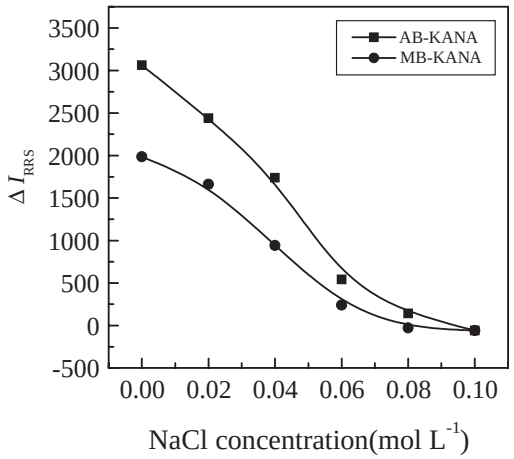

Fig. 4. Effect of ionic strength on ΔI_{RRS}. KANA concentration: 4.0 μg mL^{-1}; Dyes: 1.0×10^{-4} mol L^{-1}.

MB-KANA, are nearly to identical 266 and 294 nm for AB and 266 and 293 nm for MB.

Optimum Reactive Conditions. Effects of Acidity: The effects of acidity on the RRS intensities of the reaction products were tested and the results are shown in Fig. 3. The results show that the optimal range of pH is 1.0–3.5.

When pH is too low (pH $\leq$ 1.0), the dissociation of dye-SO_3H is inhibited, which goes against ion association. When pH is too high (pH $\geq$ 3.5), as a result of protonated H^+ dissociating, the positive charges of KANA antibiotics are decreased, and even KANA cannot exist as a cation. In these cases, they are unfavorable to the formation of ion-associates, and make ΔI_{RRS} decline. A pH of 2.5 was chosen for the following experiments.

Effects of Ionic Intensity: Sodium chloride was found to be a good ionic strength adjustor. Thus, the effects of ionic intensity on RRS were studied by adding NaCl (as shown in Fig. 4) and the experimental results show that ΔI_{RRS} of the two systems decreases with increasing ionic strength. High Na^+ and Cl^- concentrations have competitive effects on the ion-association of dye-KANA. Therefore, ionic intensity should be rigidly controlled in the determination system, and salts should be carefully avoided.

Effects of Dye Concentrations: The effect of different dye concentrations on relative RRS intensity was investigated in the range of (0.2–2.0) $\times 10^{-4}$ mol L^{-1}. The results show that the relative RRS intensity increased with increasing dye concentration up to 1.0×10^{-4} mol L^{-1}, then it decreased gradually with a further increase of dye concentration. The experimental results indicated that (0.8–1.2) $\times 10^{-4}$ mol L^{-1} give a high I_{RRS} and low blank signal. Thus, a 1.0×10^{-4} mol L^{-1} dye concentration was used in this work.

Optimization of FIA Variables: FIA conditions have been optimized by studying the following factors under the selected reaction conditions described above: (1) Injection volume: The injection volume had a strong influence on the sensitivity and dynamic range of the method. Samples of different volumes (40–100 μL) were injected. In general the higher the sample volume, the higher the peak heights and the longer the residence time of the sample in the detector, requiring a longer time to reach a steady state and greater consumption of sample. Though smaller volumes have the advantage of lower sample consumption and shorter sample residence time (i.e. enhanced sampling frequency), the desired sensitivity and precision must also be considered. In the present study, a volume of 60 μL was selected as a reasonable compromise. (2) Flow rate: The effect of the rate of the carrier stream was examined at different flow rates. The effect of the flow rate on the I_{RRS} was investigated. I_{RRS} increased with increasing flow rate over the range 2.0–5.5 mL min^{-1}. High flow rates led to greater consumption of reagents with little gain in sensitivity. If the flow rate of the carrier stream were too low, it would not be possible to record the maximum intensity when the mixture solution reached the flow cell. Thus, a reasonable compromise of 3.5 mL min^{-1} for the carrier stream was chosen for further studies. (3) Length of reactor: The reaction between the dye and KANA was almost instantaneous, so that the length of the reactor had to only be optimized with regard to sufficient mixing of the carrier stream with the diluted analyte, while at the same time prevent undesirable sample dispersion. A reasonable compromise was a length of 80 cm. The studied range and optimum values are listed in Table 2.

Relation between RRS Intensity and KANA Concentrations: Under optimum conditions, KANA with different concentrations reacted with dyes and their RRS intensities were measured. A typical recording of a calibration run for KANA is shown in Fig. 5. It demonstrates the low noise of the baseline, the high reproducibility of the measurements, and the short response and washout times. The calibration graphs of ΔI_{RRS} against the concentration of KANA were then constructed. Correlative parameters are listed in Table 3. It can be seen from Table 3 that correlation coefficients are approximately 0.9990–0.9993, and the linear range is 2 times wider

Table 2. Optimization of Experimental Conditions

Variable	Studied range	Optimum value
Acidity/pH	1.0–4.5	2.5
Dye (MB, AB) concentration /$\times 10^{-4}$ mol L^{-1}	0.2–2.0	1.0
Injection volume/μL	40–100	60
Carrier flow rate/mL min^{-1}	2.0–5.5	3.5
Reactor length/cm	60–160	80

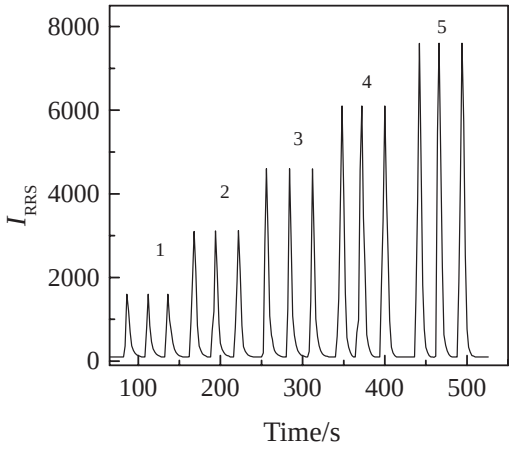

Fig. 5. Calibration peak signals for KANA determination by FIA. Carrier flow rate: 3.5 mL min^{-1}; reactor length: 80 cm; injection volume: 60 μL; KANA concentration 1–5 (μg mL^{-1}): 2.0, 4.0, 6.0, 8.0, and 10.0.

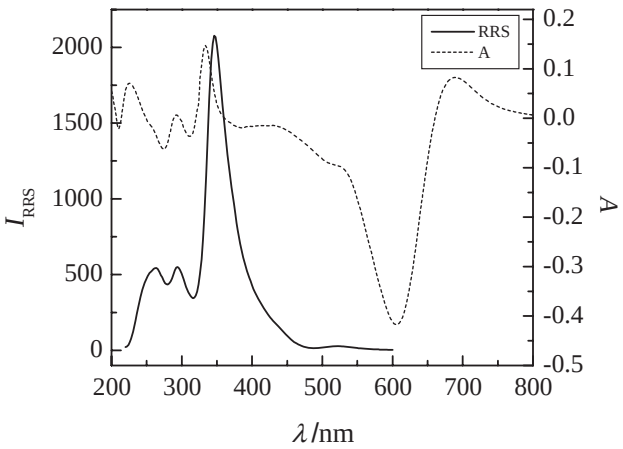

Fig. 6. RRS spectrum and absorption spectrum of the AB-KANA.

Table 3. Correlation Coefficient, Linear Ranges, and Detection Limits for Standard Curves

System	Measurement wavelength/nm	Linear regression equation/μg mL^{-1}	Correlation coefficient (r)	Linear range /μg mL^{-1}	Detection limit (3σ)/ng mL^{-1}
AB-KANA	346	$\Delta I = 35.3 + 750.6C$	0.9993	0.02–12.0	4.2
MB-KANA	346	$\Delta I = 66.3 + 481.7C$	0.9990	0.02–12.0	5.0

than that of the RRS method in the literature.[20–23] The results show that methods for the determination of KANA have very high sensitivity, i.e. 4.2 ng mL^{-1} for the AB system and 5.0 ng mL^{-1} for the MB system, which is much higher than common analytical methods. For example, the sensitivity of the method is 100 times higher than that of spectrophotography,[8] dozens of times higher than that of HPLC,[2,3] electric chemistry,[4] bioluminescence,[29] and slightly higher than that of RRS methods.[20–23] Therefore, the FIA-RRS method is particularly suitable to determine trace levels of KANA. Since the sensitivity of the AB-KANA system is the highest, the AB system was taken as the example for investigating the selectivity of this method.

Composition and Formation Constant of Ion-Association Complexes: By using Job's method of RRS and the equilibrium shift method to determine the composition ratio and the formation constant of KANA with dye in ion association, the results show that the KANA: dye ratio is 1:2 and the apparent formation constant is 5.6×10^9.

Discussion of Reasons for Enhancement of RRS: Because RRS is a scattering–absorbing–rescattering process brought about by resonance scattering and absorption, RRS should be tied to the absorption spectrum (as shown in Fig. 6). By comparing RRS with the absorption spectrum, it is obvious that RRS is situated in its absorption band, which is a prerequisite for RRS. The RRS peak at 346 nm of the AB-KANA ion-association complexes was very near its absorption peak at 334 nm. This showed that the peak at 346 nm is influenced by the absorption band. As a result, the scattering signal was enhanced. Moreover, the RRS spectral characteristic was also affected by instrumental factors, such as the emission spectrum of the light source (Xenon lamp) of the apparatus and the response curve of the signal for the detector (photoelectric multiplier) on the emission spectrum. Therefore, the RRS peak, which related to the absorption peak at 692 nm, was not very apparent.

Selectivity of the Method and Analytical Application. Selectivity of the Method: In order to assess the selectivity of the method, the effects of some common metal ions, nucleotide, proteins, amino acids, and sugars on the determination of KANA were investigated by using solutions containing 4.0 μg mL^{-1} of KANA. A substance that gave a relative error less than ±5% was determined not to interfere in the assay. The results show that no interference was found when including up to a 200-fold NH_4^+, Na^+, K^+, Mg^{2+}, Ca^{2+}, Sr^{2+}, Ba^{2+}, Cl^-, NO_3^-, SO_4^{2-}, PO_4^{3-}, 100-fold TritonX-100, carbowax, PVA, EDTA, D-tryptophan, sugars, L-histidine, fructose, maltose, glucose, starch, citric acid, lactose, vitamin C, nicotinic acid, 50-fold heparin, urea, 10-fold Mn^{2+}, Sn^{2+}, Pb^{2+}, Cu^{2+}, 5-fold ctDNA, Fe^{3+}, Al^{3+}, Pb^{2+}, 2-fold HSA, amylase. Therefore, the method has good selectivity.

Analysis of Commercial KANA Injection: The method was used for the determination of kanamycin sulfate in commercial injections and its determination results are listed in Table 4. It can be seen that this method has high accuracy and good repeatability and the results agree well with those specified.

Determination of KANA Concentration in Serum and Urine: The determination of KANA concentrations in serum and urine were compared to the literature method,[21] and all of the results are listed in Table 5. It can be seen from Table 5 that the results of determination by the FIA-RRS method were in good agreement with the common RRS method, but the present method (FIA-RRS) shows lower detection limits and better reproducibility. Hence, the method could be an interesting alternative technique to monitor KANA concentrations of

Table 4. Results for the Determination of Commercial Kanamycin Sulphate Injection

System	Amount on the label/ $mg\,mL^{-1}$	Found/ ($n = 5$, $mg\,mL^{-1}$)	Recovery/ ($n = 5$, %)	RSD/ ($n = 5$, %)
AB-KANA	250	255	102.0	1.6
MB-KAKA	250	248	99.2	1.0

Table 5. Results for the Determination of Real Samples

Method	Dye	Sample	Found/ ($n = 5$, $\mu g\,mL^{-1}$)	KANA added /$\mu g\,mL^{-1}$	Found/ ($n = 5$, $\mu g\,mL^{-1}$)	Recovery/ ($n = 5$, %)	RSD/ ($n = 5$, %)
FIA-RRS	AB	serum	0	4.0	3.9	97.5	1.3
	AB	urine	0	10.0	10.2	102.0	1.5
	MB	serum	0	4.0	4.1	102.5	1.7
	MB	urine	0	10.0	9.8	98.0	1.1
RRS	AB	serum	0	4.0	10.2	102.0	2.5
	AB	urine	0	10.0	4.1	102.5	2.8
	MB	serum	0	4.0	9.7	97.0	2.6
	MB	urine	0	10.0	4.2	105.0	3.1

patients' serum and urine, and provide further valuable information for clinical doctors to explore safety ranges of antibiotics, find optimal dosage and administration intervals, and establish more individualized treatment.

This project was supported by the National Natural Science Foundation of China (No. 20475045) and the Municipal Science Foundation of Chongqing City (No. 2003-8113).

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