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Resonance Rayleigh scattering spectra for studying the interaction of aminoglycoside antibiotics with pontamine sky blue and their analytical applications

Abstract In a weakly acid medium, some aminoglycoside antibiotics, such as kanamycin (KANA), gentamicin (GEN), tobramycin (TOB), and neomycin (NEO), or acid bisazo dye pontamine sky blue (PSB) can only produce very weak resonance Rayleigh scattering (RRS) signals. However, when the two agents react with each other to form the ion association complexes, the RRS intensity can be enhanced greatly and a new RRS spectrum and a significant enhancement of the RRS intensity in the wavelength range 350–600 nm can be observed. The maximum scattering peak is at 580 nm. There is a linear relationship between the RRS intensity and the antibiotic concentration in the range 0.01–6.0 μg mL^{-1} at 580 nm. This RRS method has therefore been developed for the determination of trace levels of aminoglycoside antibiotics. The detection limits (3σ) of the four antibiotics, whose order of sensitivity is KANA>NEO>TOB>GEN, are 5.8–6.9 ng mL^{-1}. This method has a good selectivity and has been successfully applied to the quick determination of antibiotics not only for injections and ear drops, but clinic serum samples as well. In addition, quantum chemistry-based analysis of the reaction mechanism, the factors influencing the RRS spectra, and the reasons for the enhancement of RRS are discussed.

Keywords Resonance Rayleigh scattering · Aminoglycoside antibiotics · Pontamine sky blue · Determination

Introduction

Aminoglycoside antibiotics with similar chemical and antibiotic properties are a type of indican antibiotic formed by the condensation of aminocyclitols with amino sugars. Owing to their good solubility in water, stable quality, wide antimicrobial spectrum, powerful sterilization, ability to be absorbed and drained without difficulty, cooperative effect with β-lactam medicines, and at lower price, aminoglycoside antibiotics have been popularly used in clinic since their invention [1], especially against tuberculosis and Gram-negative bacteria. Kanamycin, gentamicin, tobramycin, neomycin, and streptomycin are very common aminoglycoside antibiotics used in the clinic. They are usually sulfate derivatives, and consequently their antibiotic effects depend upon their concentrations, and their clinic curative effects and toxicities are closely related to their serum concentrations. In the interest of using this kind of antibiotic scientifically and securely, finding a safe range, an optimum dosage and reasonable intervals, and establishing an individualized therapy, it is very important to develop an accurate and quick examination technique for antibiotic serum concentration. Nowadays, the primary methods for the determination of aminoglycoside antibiotics include microbiological assay (MA) [2, 3], high-performance liquid chromatography (HPLC) [4, 5], ultraviolet visual spectrophotometry (UV-VIS) [6, 7], and immune assay (IA) [8, 9]. However, MA is too trivial and time consuming to satisfy the need for a quick determination with severely injured and emergency patients, so it is being gradually phased out. Though it is accurate, HPLC is expensive and the pretreatment of sample is inconvenient. UV-VIS is easy to manipulate, but has a lower sensitivity. IA has a wide application using little sample and is highly specialized, but it relies on expensive special kit. RRS is a new analytical method developed in recent years and has a series of predominant qualities. Intensive RRS is used for researching biological macromolecules [10, 11, 12, 13, 14] and determining trace amounts of levels of inorganic ions [15, 16, 17, 18] and is based on aggregation of conjugated structure in biological macromolecules [19] and ion association complexes by virtue of electrostatic and hydrophobic interaction between small molecules [20]. Nevertheless, there is no report about the determination of aminoglycoside antibiotics with the RRS method thus far.

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In a weakly acidic or near-neutral medium, pontamine sky blue (PSB), an acid bisazo dye, exists as an anion. Under these conditions, aminoglycoside antibiotics exist as cations because of the dissociation of protons from "multisulfo" groups. The experimental results show that separate PSB or aminoglycoside antibiotics produce very weak RRS signals. However, when the two agents react with each other to form the ion association complexes, the RRS intensity can be enhanced greatly and a new RRS spectrum is observed. In this paper, RRS spectral characteristics, optimum reaction conditions, and analytical properties have been studied. The method has high sensitivity and good selectivity and has been applied to the determination of aminoglycoside antibiotics in injections and ear drop samples. Moreover, the composition of the ion association complexes has been determined. By using quantum chemistry methods, not only the structures of the ion association complexes but the different charge distribution before and after reaction have been discussed. The results show that the formation of the ion association complexes of PSB with aminoglycoside antibiotics is not only by virtue of electrostatic and hydrophobic interaction, but also the charge transfer effect that is very important for the stabilization of ion association complexes. In addition, the factors influencing the RRS spectra and reasons for the enhancement of RRS have been discussed.

Experimental

Apparatus

A Shimadzu RF-540 spectrofluorophotometer (Tyoto, Japan) was used for recording and measuring the RRS spectra and the intensities at a given wavelength using a 1-cm path length. The determination parameters were of low sensitivity, ordinate scale ×6, and the slit (EX/EM) of 10.0 nm/10.0 nm. A UV-VIS 8500 spectrophotometer (Tianmei, HongKong) was used for recording the absorption spectra. A pHS-3C acidimeter (Shanghai Dazhong Analytical Instrumental Plant) was used to measure pH.

Reagents

The stock concentrations of kanamycin sulfate (KANA, Sigma Co.), neomycin sulfate (NEO, Sigma co.), gentamicin sulfate (GEN, Sigma Co.), and tobramycin sulfate (TOB, Chongqing Daxin Medicine Plant) were $400.0\ \mu g\ mL^{-1}$, and the working concentrations were $20.0\ \mu g\ mL^{-1}$ or $10.0\ \mu g\ mL^{-1}$.

The stock concentration of the bisazo dye pontamine sky blue (PSB, Chroma Co., Switzerland) was $2.0\times10^{-3}\ mol\ L^{-1}$, and the working concentrations was $2.0\times10^{-4}\ mol\ L^{-1}$ by diluting the stock concentration with water.

Britton–Robinson buffer solutions with 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 in proportion, and adjusting pH values with an acidimeter.

Four antibiotic standards were biochemical reagents, and the other reagents were of analytical reagent grade. Doubly distilled water was used throughout the experiment.

General procedure

Into a 10-mL volumetric flask were added 1.0 mL of PSB solution, 1.0 mL of Britton–Robinson buffer with different pH according to different system, and 1.0 mL antibiotic solution. Finally, the mixture was diluted to the mark with water and mixed thoroughly. RRS spectra of the system were recorded with synchronous scanning at $\lambda_{ex}=\lambda_{em}$ (i.e., $\Delta\lambda=0$ nm) and the RRS intensity I_{RRS} was measured for the interacting system and I_0 for the reagent blank at their own maximum RRS wavelength, $\Delta I_{RRS}=I_{RRS}-I_0$.

Results and discussion

RRS spectra

The RRS spectra of PSB–aminoglycoside antibiotics are shown in Fig. 1a. It can be seen from Fig. 1a that the RRS of separate PSB or aminoglycoside antibiotics such as kanamycin sulfate, gentamicin sulfate, tobramycin sulfate, and neomycin sulfate is very weak. However, the ion association complexes of PSB with aminoglycoside antibiotics have intensive RRS signals. Their spectral characteristics are similar, having the maximum scattering peak at 580 nm, and two smaller peaks at 368 nm and 470 nm,
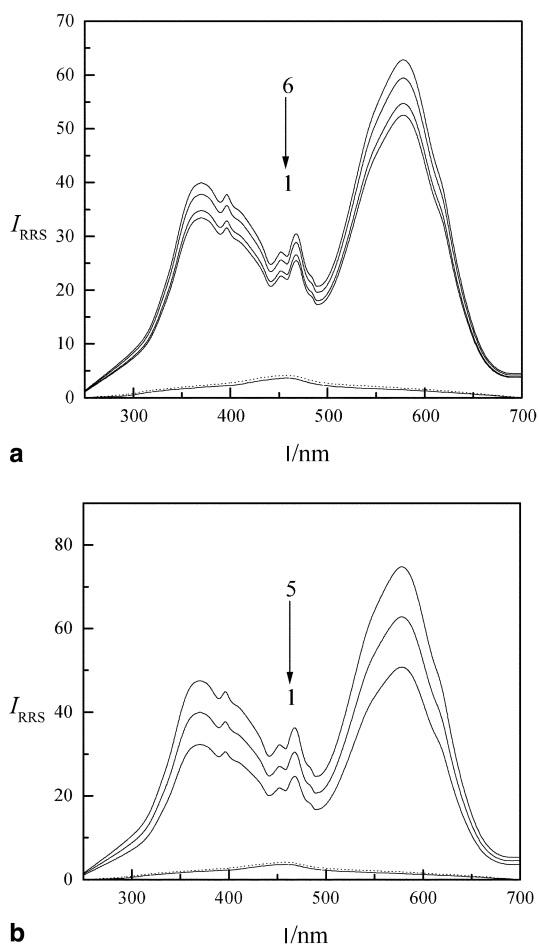
Fig. 1a,b RRS spectra of **a** PSB–aminoglycoside antibiotics. *1* PSB, *2* KANA, *3* PSB–GEN, *4* PSB–TOB, *5* PSB–NEO, *6* PSB–KANA; antibiotics concentration $4.0\ \mu g\ mL^{-1}$, PSB $2.0\times10^{-5}\ mol\ L^{-1}$, pH 4.0; **b** RRS spectra of PSB–KANA. *1* PSB, *2* KANA, *3* PSB–KANA ($3.2\ \mu g\ mL^{-1}$), *4* PSB–KANA ($4.0\ \mu g\ mL^{-1}$), *5* PSB–KANA ($4.8\ \mu g\ mL^{-1}$), PSB $2.0\times10^{-5}\ mol\ L^{-1}$, pH 4.0
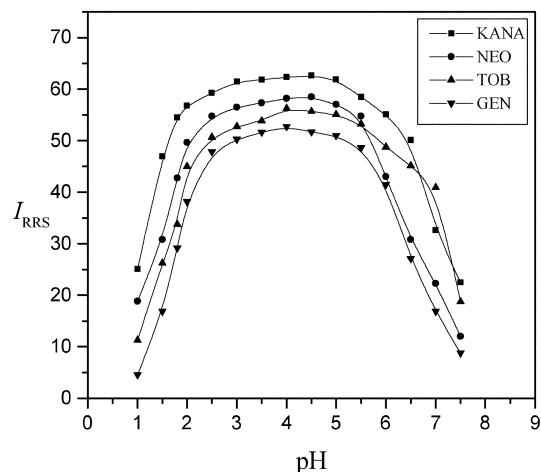
Fig. 2 Effect of acidity on ΔI_{RRS}. PSB concentration 2.0×10^{-5} mol L^{-1}, antibiotics concentration 4.0 µg mL^{-1}

Fig. 3 Effect of ionic intensity on ΔI_{RRS}. PSB concentration 2.0×10^{-5} mol L^{-1}, antibiotics concentration 4.0 µg mL^{-1}, pH 4.0

respectively. The order of the four RRS intensities of PSB–aminoglycoside antibiotics is KANA>NEO>TOB>GEN. Different RRS signals of the PSB–KANA system at different concentrations of KANA are shown in Fig. 1b. Since the RRS intensity enhances with an increase in KANA concentration, the RRS method can be applied to the determination of antibiotics.
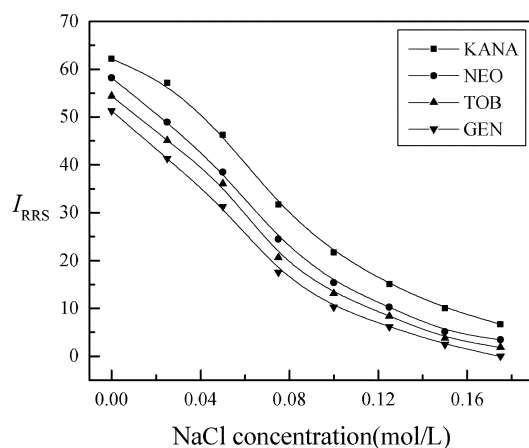
Optimum reactive conditions

Effects of acidity

The effects of acidity on the RRS intensities of the reaction products were tested and the results are shown in Fig. 2. It can be seen from Fig. 2 that this method has a wide acidity range, and the optimum pH range for determining KANA, NEO, TOB, and GEN is 2.5–5.5.

When acidity is too high (pH≤2.5), the dissociation of dye–SO$_3$H will be inhibited, which will go against ion association. When acidity is too low (pH≥5.5), as a result of H$^+$ dissociation, the positive charges of aminoglycoside antibiotics will be decreased, and even aminoglycoside antibiotics could not exist as ions. All these work against the formation of the ion association complexes and make ΔI_{RRS} decline.

Effects of ionic intensity

The effects of ionic intensity on RRS were studied by adding NaCl (as shown in Fig. 3) and the experimental results show that ΔI_{RRS} of the four systems decrease with increasing ionic strength. High Na$^+$ and Cl$^-$ concentrations have competitive effects on the association of PSB–antibiotics. Therefore, ionic intensity should be rigidly controlled in the determination system, and salt should be avoided carefully.

Composition of ion association complexes

In weakly acidic media (pH 4.0), aminoglycoside antibiotic sulfate is dissociated as proton and acid group, while the four protons on the sulfo group of PSB are decompounded completely, being large organic anions. Taking KANA as an example, ion associative reaction of PSB–aminoglycoside antibiotics has been studied as follows.

By using Job's method of RRS to determinate the composition ratio of PSB with KANA in ion association, the result shows that PSB:KANA is 1:1. To define the combinative site of PSB and antibiotic more clearly, the AM1 method of quantum chemistry was applied to calculate the charge distribution of PSB and KANA in the ground state. It can be seen from Fig. 4a that on the whole the distribution of negative charges is uneven in the four sulfo groups of PSB^{4-}. Among them the negative intensity of one SO$_3^-$ of each naphthyl is larger (marked with an asterisk in Fig. 4a). In the case of KANA, two amino groups have more negative intensities, and are protonated easily in acid media to take positive charges (as shown in Fig. 4b). When PSB was mixed with KANA solution, PSB^{4-} reacted with KANA^{2+} to form a 1:1 ion association complex (as shown in Fig. 4c).

RRS spectral characteristics of the ion association complex and reasons for RRS enhancement

Effects of absorption spectrum on RRS

Since PSB^{4-} and KANA^{2+} have opposite charges, electrostatic interaction between them is very strong. In addition, the two naphthyls and one diphenyl group of PSB are hydrophobic groups. Except for the protonizable amino group, the cycloalcohol stem of KANA can be viewed as a hydrophobic group; therefore, it also has an intensive hydrophobic character. When PSB and KANA approach a

Fig. 4 a PSB^{4-} charge distribution in the ground state, **b** KANA charge distribution in the ground state, **c** diagramatic sketch of ion association complex of PSB^{4-}–KANA^{2+}
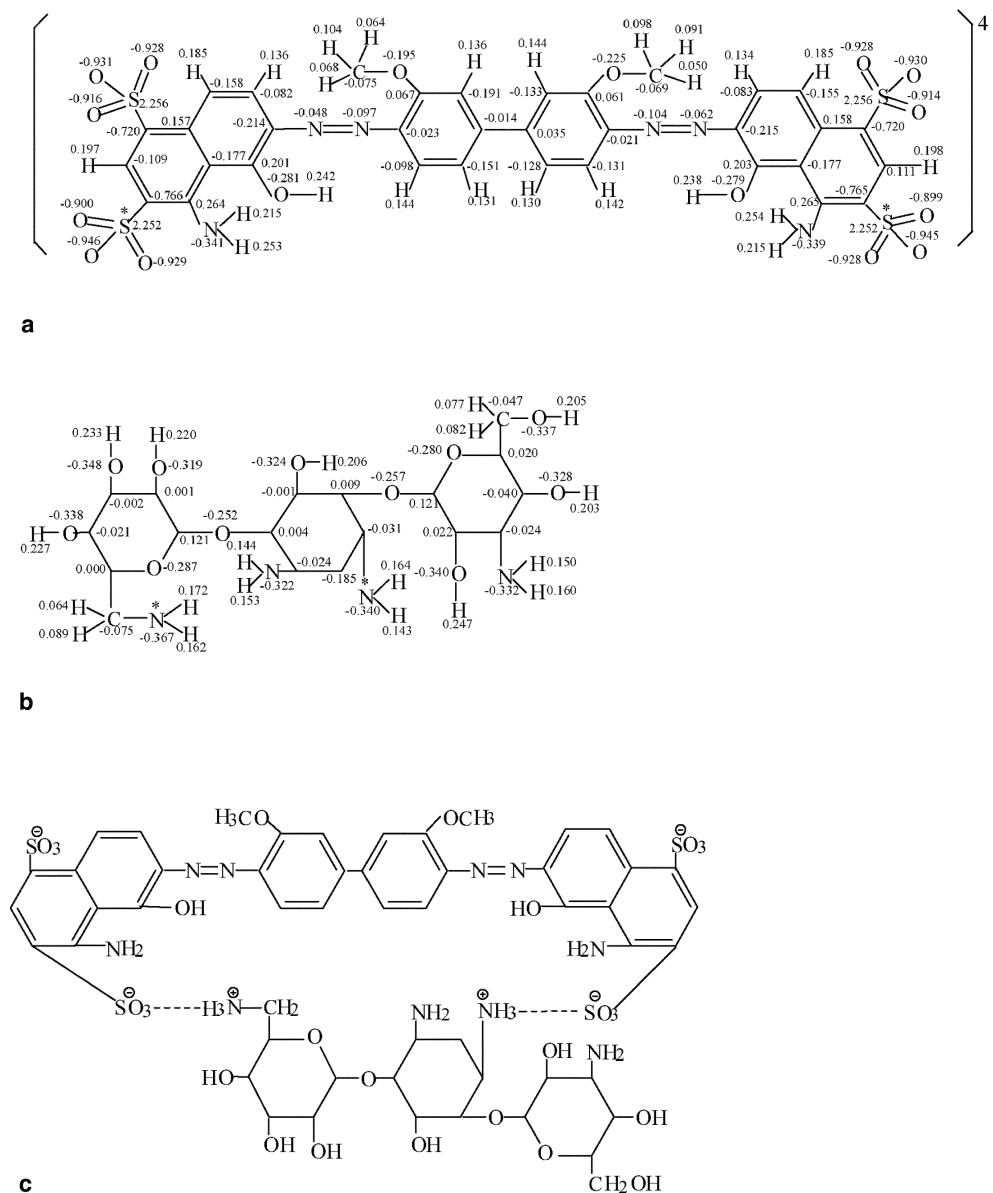

certain distance by virtue of electrostatic and hydrophobic interactions, a new interaction of charge transfer (CT) will appear. The results, attained from calculating the change of charge distribution between PSB^{4-} and KANA^{2+} before and after reaction by AM1 quantum chemistry method, show that the total charge of PSB^{4-} decreased from −4.0 before reaction to −3.31 after reaction, while that of the KANA^{2+} changed from 2.0 before reaction to 1.31 after reaction, respectively. The results suggest the charge transfer interaction has taken place. The changes in absorption spectra also illustrate the existence of CT interaction. KANA alone is colorless and only has an absorption zone near 200 nm in the ultraviolet area, while PSB has a maximum absorption peak at 612 nm. When the ion association complex was formed, the maximum absorption peak (λ_{max}) of the products transferred from 612 nm to 688 nm, $\Delta\lambda$=76 nm, which implies the formation of the CT complex. In the reaction system, the distribution of the π electron cloud was changed, resulting in the decrease of the excitation energy (ΔE) that induces red shift of the absorptive zone. Therefore, besides electrostatic and hydrophobic interaction, charge-transfer interaction also plays a predominant role in the formation of the ion association complex.

Because RRS is a scattering-absorbing-rescattering process brought about by resonance scattering and absorption, RRS should be tied up to the absorption spectrum (as shown in Fig. 5). By comparing RRS with sorption spectrum, it is obvious that RRS is situated in its absorption zone, which is prerequisite for RRS. There must be a corresponding relationship between them.

The RRS peak at 368 nm of the PSB–KANA ion association complexes was very near to its absorption peak at 354 nm. This suggested that the peak at 368 nm was influenced by both absorption spectrum and instrument factors; as a result the scattering signal was strong. Moreover, the RRS spectral characteristic was also affected by
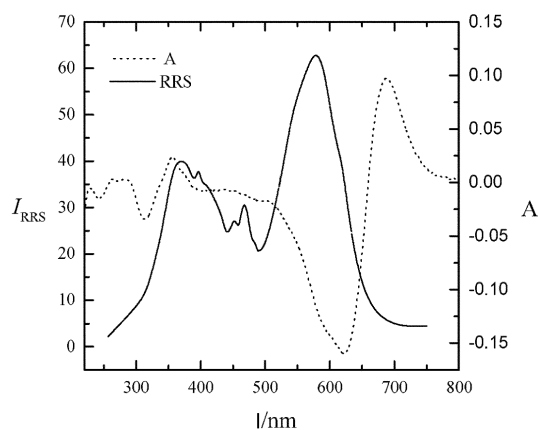
Fig. 5 RRS spectrum and absorption spectrum of the PSB–KANA complex

other factors, such as the emission spectrum of the light source of the apparatus and the response curve of detector on the emission spectrum. The response of the Shimadzu RF-540 spectrofluorophotometer on the scattering signals beyond 600 nm is very weak. Therefore, the peak at 580 nm can be viewed as another characteristic peak of the ion association complexes, which related to the absorption peak at 680 nm.

Enhancement reasons for RRS

1. Rayleigh scattering of the products locates in its absorption band. The process of scattering-absorbing-rescattering brings enhanced resonance effects that make the scattering intensity enhance greatly.
2. Because Rayleigh scattering is the basis for RRS, the enhancement of Rayleigh scattering will favor the enhancement of the RRS signal. According to the simplified Rayleigh formula [21] $I_{RRS} = KCMI_0$
   Where I_{RRS} is RRS intensity, I_0 is the incident intensity, C is the solution concentration, K is a coefficient, and M is the molecular weight. When other factors are constant, the scattering intensity is directly proportional to the molecular weight of the scattering substance. In addition, the increase of the molecular weight from 486.6 to 1,387.4 before and after reaction is conducive to the enhancement of RRS.
3. After the ion association complex was formed, the rotational movement of the aryl moiety in the dye mole-

cule was inhibited because of the binding force between positive and negative charges. As a result, the molecular coplanarity and rigidity increase, which is profitable to the enhancement of RRS.

Relation between RRS intensity and the antibiotic concentration

Under optimum conditions, the four antibiotics with different concentrations reacted with PSB and their RRS intensities were measured. The calibration graphs of ΔI_{RRS} against concentration of antibiotics were then constructed. Correlative parameters are listed in Table 1. It can be seen from Table 1 that correlation coefficients are approximately 0.9987–0.9996, and the linear range is wide. The results show that this method for the determination of the four aminoglycoside antibiotics has a very high sensitivity. The sensitivity of KANA is the highest and its detection limit is 5.8 ng mL^{-1}, and the order of the sensitivities is KANA>NEO>TOB>GEN. The sensitivities of the four systems are higher than the reported methods. For example, the sensitivity of the method is 100 times higher than that of spectrophotometry and several tens of times higher than those of noctilucence, electrochemistry, HPLC, kinetic fluorimetry, and fluorescence immunoassay (as shown in Table 2). Therefore, the method is very suitable for the determination of trace amounts of aminoglycoside antibiotics.

Selectivity of the method

Taking KANA as an example, we investigated the effects of some common metal ions, nucleotide, proteins, amino acids, and sugars on the determination of aminoglycoside antibiotics, and the results are shown in Table 3. It can be seen from Table 3 that the permissible interfering amounts of Na$^+$, Mg^{2+}, Ca^{2+}, Sr^{2+}, Mn^{2+}, NH$_4^+$, nonionic surfactant TritonX-100, sugars, urea, and vitamin C are large, but the permissible interfering amounts of Sn^{2+}, Pb^{2+}, and Cu^{2+} are small.

Analytical application

Analysis of commercial antibiotics

The method was used for the determination of antibiotics in commercial kanamycin sulfate, gentamicin, tobramycin, and neomycin injections and neomycin sulfate ear drops

Table 1 Correlation coefficients and linear ranges of the calibration graphs, and the detection limits for antibiotics

System	Determination wavelength (nm)	Linear regression equation (µg mL^{-1})	Correlation coefficient (r)	Linear range (µg mL^{-1})	Detection limit $\sigma=3$ (ng mL^{-1})
PSB–KANA	580	$\Delta I=2.0+15.1C$	0.9987	0.01–6.0	5.8
PSB–NEO	580	$\Delta I=1.1+14.3C$	0.9996	0.01–6.0	6.1
PSB–TOB	580	$\Delta I=1.6+13.2C$	0.9989	0.01–6.0	6.6
PSB–GEN	580	$\Delta I=0.9+12.6C$	0.9995	0.01–6.0	6.9

Table 2 Comparison of the sensitivities of RRS with other methods for the determination of aminoglycoside antibiotics

Method[a]	Reagent	Objective	Condition	Linear range ($\mu g\,mL^{-1}$)	Detection limit ($ng\,mL^{-1}$)	Reference
UV-VIS	Bromothymol blue	KANA	421 nm	8–128	200	[22]
UV-VIS	Cu^{2+}	NEO	640 nm	200–2000	20,000	[23
HPLC-IPD	Niacinamide	TOB	268 nm	0.5–10	20	[24]
Polarography	Methanal	KANA	pH 10.0–11.5	0.1–17.5	115	[25]
BLA	Biaminoacridine	KANA, GEN		1–15	100	[26]
Kinetic fluorimetric method	Phthaldialdehyde	TOB	λ_{ex}=335 nm λ_{em}=450 nm	0.05–30.0	30	[27]
FIA	KANA kit	GEN		0.1–5.5	50	[8]
RRS	PSB	KANA etc.	580 nm	0.01–6.0	5.8	This work

[a]*UV-VIS* ultraviolet visual spectrophotometry, *HPLC-IPD* high-performance liquid chromatography-indirect photomation determination, *BLA* bioluminescence assay, *FIA* fluorescence immunoassay, *RRS* resonance Rayleigh scattering

Table 3 Effects of foreign substances(containing kanamycin sulfate $4.0\,\mu g\,mL^{-1}$)

Foreign substance	Concentration ($\times10^{-6}\,mol\,L^{-1}$)	Change in IRRS (%)	Foreign substance	Concentration ($\times10^{-6}\,mol\,L^{-1}$)	Change in IRRS (%)
NH_4^+	450	4.2	NO_3^-	2500	3.8
Na^+	3,000	–2.8	SiO_3^{2-}	125	3.4
K^+	3,500	3.8	$HAsO_4^{2-}$	30	5.8
UO_2^{2+}	6	3.1	SO_4^{2-}	1500	6.3
Cu^{2+}	50	–2.8	EDTA	550	–4.7
Sn^{2+}	2.5	4.3	SLS	55	2.5
Cr^{3+}	55	6.2	TritonX-100	500	5.7
Co^{2+}	40	5.5	Mal	500	3.8
Zn^{2+}	500	–3.2	Lac	500	3.3
Be^{2+}	80	3.4	SDS	40	–4.5
Mg^{2+}	650	4.8	Maltose	750	–5.2
Ca^{2+}	550	2.9	Urea	400	6.1
Sr^{2+}	550	4.6	Fructose	350	3.2
Ba^{2+}	250	3.9	L-Histidine	450	5.7
Mn^{2+}	220	–5.6	Niacinamide	400	5.0
Pb^{2+}	2.0	5.7	α-Amylase	3.5	3.2
Al^{3+}	10	3.6	Vitamin C	800	2.5
Fe^{3+}	50	5.8	Glucose	600	–4.5
PO_4^{3-}	650	3.4	Amylo[a]	10	3.9
$C_2O_4^{2-}$	200	4.3	Hydrochoric thiamine[a]	250	4.1
Cl^-	1,500	5.2	Nicotinic acid[a]	125	3.7
$H_2PO_4^-$	750	–4.2	HSA[a]	2.0	4.6

[a]Unit is $\mu g\,mL^{-1}$

Table 4 Results for the determination of commercial aminoglycoside antibiotics

Sample	Amounts on the label ($mg\,mL^{-1}$)	Antibiotics found (n=5, $mg\,mL^{-1}$)	Recovery (n=5, %)	RSD (%, n=5)
Neomycin sulfate ear drop	2.5	2.47	98.8	1.2
Kanamycin injection	250	256	102.4	1.6
Tobramycin injection	40	41.3	103.3	1.5
Gentamicin injection	40	39.6	99.0	2.6

and their determination results are listed in Table 4. It can be seen that this method has high accuracy and good repeatability.

Determination of medicine concentration in serum

Fifteen serum samples (each 1.00 mL) were taken from 15 healthy people, then 0.25 mL of 2.0 mol L^{-1} trichloro-acetic acid was added to each sample. After mixing thoroughly, each solution was centrifuged at 3,000 rpm for 5 min to let protein deposit completely [28]. Then, 0.5 mL of PSB solution and 0.5 mL of BR buffer (pH 4.0) were added to 0.5 mL of the supernatant fluid. This solution was then diluted to the 5.0-mL mark with water and mixed thoroughly. According to the experimental method, the parallel determination of each concentration was 5 times and the results are listed in Table 5.

Table 5 Results for the determination of serum samples

No.	GEN found	GEN added (μg mL^{-1})	GEN found (n=5, μg mL^{-1})	Recovery (n=5, %)	RSD (n=5,%)
1	Not found	6.0	6.1	101.7	2.1
2	Not found	8.0	8.2	102.5	1.4
3	Not found	10.0	9.7	97.0	1.8

From Table 5, it can be seen that the method has a good repeatability for gentamicin in its safety serum concentration range (4–12 μg mL^{-1}) [29] and the relative standard deviation is 1.4–2.1%; the method also has a good accuracy and its recovery is 97.0–102.5%. Therefore, the method can be applied to the determination of gentamicin in its serum and the study of its pharmacokinetics providing valuable evidence for finding a safe range of serum concentration, an optimum dosage and reasonable intervals, and establishing an individualized therapy.

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