Colorimetric Microtiter Plate Assay of Polycationic Aminoglycoside Antibiotics in Culture Broth Using Amaranth

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We present a colorimetric method for the detection of aminoglycoside antibiotics such as neomycin (NEO) using a reddish anionic dye, amaranth (AR⁻). Under acidic conditions, at which NEO exists in fully protonated form (NEOH⁶⁺), the AR⁻ anion associates with the NEOH⁶⁺ cation to form a precipitate, NEOH(AR)₂. The precipitate was soluble in a buffer solution of pH 8.5, yielding a reddish solution with an absorption maximum at around 520 nm. Tobramycin and gentamycin, which exist as pentavalent cations under acidic conditions, gave almost the same results. On the other hand, kanamycin, amikacin, and streptomycin, which would exist as tri- and tetravalent cations, were not precipitated. Thus, the AR⁻ anion could be considered to be an analytical reagent for specific aminoglycosides with polycationic functionality. However, since the precipitation reaction was considerably affected by other anions, a separation method using the tetraphenylborate anion was employed as a pretreatment. The separation method involves precipitating the polycationic aminoglycosides with the tetraphenylborate anion, washing the precipitate with acetonitrile, and re-precipitating the aminoglycosides as hydrochloride salts. Thus, the present method was applied to a microtiter plate assay of the products in an NEO-producing culture broth.

Keywords
Colorimetry, aminoglycoside antibiotics, neomycin, tobramycin, gentamycin, amaranth

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analytical reagent for the colorimetric determination of a specific aminoglycoside. However, it was found that the precipitation reaction was considerably affected by electrolyte anions. Therefore, a separation pretreatment is needed for the colorimetric determination of NEO in culture broth. In a previous paper, we presented a method for the separation of polyamines from culture broth using the tetraphenylborate (TPB–) anion. This method was based on precipitating the polyelectrolyte salts of the analyte in polycationic form with the TPB– anion, washing the precipitate with organic solvent to purify the polyelectrolytes, and re-precipitating the hydrochloride salts. In this study, the method was optimized for the separation of NEO from culture broth. The introduction of the pretreatment made it possible to detect NEO as well as an intermediate in the NEO-producing culture broth.

**Experimental**

**Chemicals**

Neomycin trisulfate was obtained from Aldrich. Tobramycin sulfate (2.5H2SO4) and amikacin disulfate were obtained from Nacalai Tesque. Gentamycin sulfate (2.5H2SO4) was obtained from Panreac. Amaranth (Na3AR) was obtained from Aldrich. Tetraphenylborate sodium salt (NaTPB), 2-morpholino-ethanesulfonic acid (MES), and N-tris-(hydroxymethyl)methyl-3-aminopropanesulfonic acid (TAPS) were obtained from Dojindo. These were used without further purification. Other chemicals were of reagent grade, and were used as received.

The production of NEO by *Streptomyces fradiae* NBRC12773 was performed using a slight modification of the previously described method. S. fradiae NBRC12773 was cultivated in culture medium containing 2% (w/w) starch, 0.5% glucose, 0.5% yeast extract, 0.3% peptone, 0.3% meat extract, 0.02% KH2PO4, 0.06% MgSO4·7H2O, and 0.1% CaCO3 for 5 days at 28°C with rotary shaking. The culture broth was centrifuged, and proteins and non-polar compounds were removed from the resulting supernatant using chloroform extraction. The resulting sample was then subjected to further experiments.

**Measurements**

The absorption spectra were recorded by a JASCO V-630 spectrophotometer with a path length of 1 cm. In the microplate assay, the absorbance was recorded with a Bio-Rad iMark microplate reader.

The NEO-producing culture broths and the pretreated samples were analyzed by HPLC/ESI-MS (Esquire 4000; Bruker) using a reverse-phase column (Sunniest RP-AQUA, 3 μm, 100 × 2.1 mm; ChromaNik Technologies) at 30°C at a flow rate of 0.3 mL min⁻¹ with an initial gradient of 5% acetonitrile in water to 15% acetonitrile over 5 min, which was then ramped to 50% acetonitrile over 10 min. Both the acetonitrile and water contained 0.05% n-heptafluorobutyric acid and 0.05% formic acid.

**Microtiter plate assay**

The microtiter plate assay of NEO was performed as follows.

1. A 50-μL aliquot of the test solution was transferred into the well.
2. The test solution was mixed with 10 μL of the 1 mol/L MES and 0.5 mol/L NaOH buffer (pH 6.2) and 50 μL of the 0.3 mol/L NaTPB aqueous solution; the mixture was centrifuged, and the supernatant was removed to obtain a mixed precipitate containing an NEOHn⁺-TPB⁻ salt.
3. The precipitate was mixed with 100 μL of acetonitrile; the mixture was centrifuged, and the supernatant was removed to purify the NEOHn⁺-TPB⁻ salt.
4. The precipitate was mixed with 5 μL of the 5 mol/L HCl solution and 200 μL of AN; the mixture was centrifuged, and the supernatant was removed to obtain an NEOHCl₆ precipitate.
5. The precipitate was mixed with 200 μL of water; the mixture was centrifuged, and the supernatant was removed to wash the NEOHCl₆ precipitate.
6. The precipitate was mixed with 100 μL of the 0.05 mol/L CH₃COOH and 0.05 mol/L CH₃COONa buffer (pH 8.5) and 25 μL of the 3.3 mmol/L Na3AR solution; the mixture was centrifuged, and the supernatant was removed to obtain an NEOH(AR)₂ precipitate.
7. The precipitate was mixed with 100 μL of water; the mixture was centrifuged, and the supernatant was removed to wash the precipitate.
8. The precipitate was mixed with 100 μL of the 0.1 mol/L TAPS and 0.05 mol/L NaOH buffer (pH 8.5), and the absorbance at 520 nm of the resulting NEOH(AR)₂ solution was measured by the microplate reader. The procedure is illustrated in Fig. 2.
Results and Discussion

Color reaction for NEO using amaranth

In this section, the standard NEO solutions with different concentrations, $c_{\text{NEO}}$, of 0.15x g/L (as trisulfate salt, x = 0 - 2), that is, $c_{\text{NEO}} = x \, \text{mEq/L}$, were used as test solutions. Here, Eq/L denotes the equivalent concentration with reference to NH$_2$-groups, that is, the charge number of the aminoglycoside in fully protonated form. Also, a 1-mL volume of test solution was examined in a centrifugation tube (15 mL) to detect the precipitate consisting of NEO by visual observation.

In the present colorimetric method, first, a 1-mL aliquot of test solution was mixed with 0.1 mL of the 0.5 mol/L CH$_3$COOH and 0.5 mol/L CH$_3$COONa buffer (pH 4.7), and with 0.1 mL of the 10 mEq/L (3.3 mmol/L) Na$_3$AR aqueous solution; the mixture was centrifuged, and the supernatant was removed to obtain the precipitate. It is noted that this operation corresponds to the operation-6 in the microtitre plate assay (Fig. 2). In the case of amaranth, Eq/L denotes the equivalent concentration with reference to the charge number of the AR$^-$ anion.

When $c_{\text{NEO}} = 0.5$ mEq/L, a reddish precipitate was obtained. The pK$_a$-value of the amino groups of NEO have been reported to be 5.7 and 7.6 – 8.8. Thus, at pH 4.7, NEO would exist in the fully protonated form, NEOH$^{n+}$, according to

\[ \text{NEO}^0 + 6\text{H}^+ = \text{NEOH}^{n+}, \]  

(1)

where NEO$^0$ denotes the free base form of NEO. As described below, the precipitate was identified as a 1:2 NEOH$^{n+}$-AR$^-$ electrolyte salt, NEOH(AR)$_2$. Therefore, the precipitation reaction can be written as

\[ \text{NEOH}^{n+} + 2\text{AR}^- = \text{NEOH(AR)}_2, \]  

(2)

Second, the precipitate was mixed with 1 mL of water; the mixture was centrifuged, and the supernatant was removed. Even after this operation, the reddish precipitate remained in the centrifugation tube, suggesting that the NEOH(AR)$_2$ precipitate can be washed with water.

Third, the NEOH(AR)$_2$ precipitate was mixed with 4 mL of the 0.1 mol/L TAPS and 0.05 mol/L NaOH buffer (pH 8.5). After the centrifugation, no precipitate was observed in the mixture, suggesting that the precipitate was completely dissolved in the alkaline solution. This was due to the deprotonation of some NH$_2$-groups of the NEOH$^{n+}$ cation. It must be noted that, in this section, the volume of the TAPS buffer solution was four fold larger than that of the test solution, because of the high absorption coefficient of the AR$^-$ anion.

The 0.5 mEq/L NEO standard solution gave a reddish TAPS buffer solution, the absorption spectrum of which is shown by curve (a) in Fig. 3. An absorption maximum was observed at around 520 nm, and the absorbance, $A_{520}$, was determined to be $A_{520} = 0.89 \pm 0.01$. The spectrum was identical to that for the 0.125 mEq/L Na$_3$AR, 0.1 mol/L TAPS, and 0.05 mol/L NaOH solutions, indicating the presence of $-0.125x (x = 0.5/4)$ mEq/L AR$^-$ in the TAPS buffer solution. Therefore, it can be considered that the precipitation reaction of Eq. (2) occurred quantitatively. At pH > 9, the absorption maximum shifted to a shorter wavelength and the absorbance decreased. This was attributed to the deprotonation of the phenolic hydroxyl group of amaranth. Thus, the TAPS buffer (pH 8.5) was used in this operation. In Fig. 4, the $A_{520}$-value is plotted against $c_{\text{NEO}}$. The $A_{520}$ was proportional to $c_{\text{NEO}}$ at $c_{\text{NEO}} < 1$ mEq/L, and remained a constant value at $c_{\text{NEO}} > 1$ mEq/L, indicating again that the precipitation reaction occurred quantitatively.

The 0.5 mEq/L NEO standard solution was examined with different pHs in the first operation of the colorimetric method. In the range of pH < 6, at which NEO would exist in the form of penta- and/or hexavalent cationic species, the $A_{520}$ remained at almost the same value. The $A_{520}$-value decreased sharply at $6 < \text{pH} < 7.5$, and became nearly equal to zero at pH > 7.5. Therefore, the AR$^-$ anion may not precipitate the NEO species with a smaller charge number, i.e., NEOH$^{n+}$ ($n < 5$ or 6).

Color reaction for other aminoglycoside antibiotics

The 0.5 mEq/L TOB standard solution also gave a reddish TAPS buffer solution, the absorption spectrum of which is shown by curve (a) in Fig. 5. The spectrum is identical with curve (a) in Fig. 3, indicating that the fully protonated TOB (TOBH$^{n+}$) was precipitated with the AR$^-$ anion quantitatively to form TOB(AR)$_3$. The 0.5 mEq/L GEN test solution gave almost the same results as the 0.5 mEq/L NEO and TOB solutions. On the other hand, no significant precipitate was formed with the 0.5 mEq/L streptomycin, kanamycin, and amikacin (Fig. S2, Supporting Information) standard solutions. Under acidic conditions, these aminoglycoside antibiotics would
The 0.5 mEq/L spermidine and putrescine solutions gave no precipitate with the AR3– anion. On the other hand, the 0.5 mEq/L spermine solution gave the reddish TAPS buffer solution, the spectrum of which is identical to that given by the 0.5 mEq/L NEO solution. The larger charge number may be an important factor also for the precipitation of the straight-chain polyanimes with the AR3+ anion. However, since spermine was not separated by the pretreatment with TPB– anion, and would not be detected by the present microtitre plate assay. Chitosan oligosaccharides17 and nourseothricin (a mixture of streptothricin12,13 F, E, and D) samples would give polycationic species under the acidic condition. However, the 0.5 mEq/L streptothricin solution gave no precipitate with the AR3+ anion. Thus, the larger charge number may be an important factor for the precipitation of aminoglycosides with the AR3+ anion.

In the acidic condition, putrescine, spermidine, and spermine would exist as di-, tri-, and tetravalent cationic species. Thus, the larger charge number may be an important factor for the precipitation of aminoglycosides with the AR3+ anion.

When $c_{\text{NEO}} = 0.5 \text{ mEq/L}$, a yellow precipitate was obtained. Although the yellow precipitate was dissolved in alkaline solutions, the solutions remained colorless due to the hydrolysis of the molybdosilicate anion. Also, the NEOH6+ cation was not precipitated by the addition of solutions containing 10 mEq/L bis(2-ethylhexyl) phosphate and sulfosuccinate anions17 and dipiryelamine anion.11,13 Thus, the AR3+ anion can be considered to be a superior analytical reagent for NEO, TOB, and GEN, and their analogues.

**Influence of electrolyte anion in the test solution**

The 0.5 mEq/L NEO and $c_E$ mmol/L NH4Cl solution, where $c_E$ is the electrolyte concentration, also gave a reddish TAPS buffer solution. However, the $A_{520}$-value decreased slightly with $c_E$, as shown by plot (a) in Fig. 6. The $A_{520}$-values did not change when NH4Cl was replaced by NaCl and KCl, indicating that the Cl– anion affects the precipitation of the NEOH6+ cation with the AR3+ anion. The NEOH6+ cation probably associates with the hydrophilic anion to form an ion pair that is stable in aqueous solution. As shown by plot (b) in the figure, the $A_{520}$-value at $c_{\text{NEO}} = 0.5 \text{ mEq/L}$ was also decreased by the addition of KH2PO4 to the test solution.

As shown by plot (c) in Fig. 6, the $A_{520}$-value decreased remarkably with the addition of (NH4)2SO4. When $c_E > 50 \text{ mM}$, no precipitate was formed in the first operation, suggesting a strong interaction between NEOH6+ and SO42– ions and/or the formation of a more stable ion pair. Sometimes, culture media contain high concentrations of (NH4)2SO4 as a nitrogen source. We therefore considered that a separation process was needed for use with the present colorimetric determination of the aminoglycosides in culture broth.

**Separation of NEO using TPB**

In the present separation method, first, a 0.5-mL volume of test solution was mixed with 0.1 mL of the 1 mol/L MES and 0.5 mol/L NaOH buffer (pH 6.2) and with 0.5 mL of the 0.3 mol/L NaTPB solution; the mixture was centrifuged, and the supernatant was removed. This operation corresponds to the operation-2 in the microtitre plate assay (Fig. 2). In this section, we examined a model solution containing $c_{\text{NEO}}$ mEq/L NEO and the components in NEO-producing culture medium, 2%(w/w) starch, 0.5% glucose, 0.5% yeast extract, 0.3% peptone, 0.3% meat extract, 0.02% KH2PO4, and 0.06% MgSO4·7H2O.
The 1 mEq/L NEO model solution gave a large volume of white precipitate, which was likely a mixed precipitate containing the polycationic NEO with the TPB– anion.

It is well known that the TPB– anion forms precipitates with the K+ and NH4+ ions and protonated amines. Therefore, the precipitate would be expected to contain the salts of the monovalent cations with the TPB– anion. However, these 1:1 electrolyte salts were removed in the next operation. Also, it is noted that the 1 mEq/L NEO standard solution did not yield a significant precipitate. However, the precipitation of a polycationic NEO with the TPB– anion would be expected to be facilitated by the precipitation of other cationic species.

Second, the precipitate was mixed with 1 mL of AN; the mixture was centrifuged and the supernatant was removed. The amount of precipitate became much smaller, suggesting that the polycationic NEO salt with the TPB– anion is soluble in AN. On the other hand, NEO was precipitated as the hydrochloride salt, NEOHCl6. Finally, the precipitate was mixed with 0.05 mL of the 5 mol/L HCl solution and with 2 mL of AN; the mixture was centrifuged and the supernatant was removed. By mixing with HCl, the TPB– anion was decomposed to benzene and triphenylborane, which are soluble in AN. On the other hand, NEO was precipitated as the hydrochloride salt, NEOHCl6. At this operation, the precipitate was mixed with 2 mL of AN; the mixture was centrifuged, and the supernatant was removed to wash the NEOHCl6 precipitate.

The separation method was evaluated by chromatography. Figure 7A shows a chromatogram for the 1 mEq/L NEO standard solution. A well-developed elution peak was observed at around 13.3 min. The MS spectrum of the eluted substance showed a signal at m/z = 615 [(M+H)+].

Figure 7B shows a chromatogram for the 1 mEq/L NEO model solution. Although NEO was detected from the model solution, some impurity peaks and significant background were observed in the chromatogram. From a 0.5-mL aliquot of the model solution, an NEOHCl6 precipitate was obtained, and was re-dissolved in 1 mL of water. The aqueous solution gave the chromatogram shown in Fig. 7C. The elution peak for NEO was observed, and the yield was calculated to be 89% from the peak area. Although the solution conditions in the separation process were modified, the yield was not increased. However, no remarkable impurity peak was observed.

In addition, NEO was separated with ~90% yield from the model solution containing the components in other culture medium, starch, 0.5% glucose, 1% (NH4)2SO4, 0.5% yeast extract, 0.158% Na2HPO4, 0.136% KH2PO4, 0.05% MgSO4·7H2O, 0.004% ZnSO4·7H2O, and 0.003% FeSO4·7H2O (pH 6.8). Thus, we can expect that NEO would be separated from the culture broths.

The aqueous solution (1 mL) prepared from a 0.5 mL of the 1 mEq/L NEO model solution was assayed by the present colorimetric method. The aqueous solution gave a reddish TAPS buffer solution, the spectrum of which is shown by curve (b) in Fig. 3. The A520 was determined to be 0.78 ± 0.03, and the yield was calculated to be 88%, which is close to that obtained above. The 1 mEq/L TOB model solution also gave a white precipitate, which was assayed by the present colorimetric method. Curve (b) in Fig. 5 shows the spectrum of the resulting TAPS buffer solution. The A520 value was 0.72 ± 0.01. The 1 mEq/L GEN model solution gave A520 = 0.45 ± 0.01. The 1 mEq/L kanamycin model solution gave a white precipitate. However, as described earlier in this paper, kanamycin was not detected by the colorimetric assay with AR+ anion. Thus, the polycationic aminoglycosides, such as NEO, TOB, and GEN, can be detected from the model solutions.

**Microtiter plate assay of NEO**

The microtiter plate assay was performed with the model solutions having different CNEO values and containing 2%(w/w) starch, 0.5% glucose, 0.5% yeast extract, 0.3% peptone, 0.3% meat extract, 0.02% KH2PO4, and 0.06% MgSO4·7H2O. It is noted that NEO concentrations up to 5 mEq/L are capable of being determined under the solution conditions described in Fig. 2. The resulting TAPS buffer solutions are shown in Fig. 8. The model solution of CNEO = 0 gave a colorless TAPS buffer solution, and the redness increased remarkably with CNEO. Thus, although a part of NEO would be lost during the separation process, even the 0.1 mEq/L level of NEO can be detected by visual observation. The absorbance at 520 nm for the TAPS buffer solution in the well, A520, was determined with the plate
The eluted substance showed a signal of the intermediate in the separation process. The separation process should be useful for the biosynthetic analysis of NEO in the range tested. The regression line is given as:

\[ A'_{520} = (0.56 \pm 0.05)(C_{\text{NEO}}/\text{mEq L}^{-1}) + (0.08 \pm 0.04), \]

with a mean square of errors of $3.3 \times 10^{-4}$. The detection limit was calculated to be $3(3.3 \times 10^{-4})^{0.56} = 0.1 \text{ mEq L}^{-1} (\sim 90 \mu\text{g mL}^{-1}, \text{as trisulfate salt}),$ indicating that the 0.1 mEq/L or 100 μg/mL level of NEO can be determined.

**NEO-producing culture broth**

*S. fradiae* was cultivated in the culture medium. As shown in Fig. 9A, the production of NEO was confirmed by the chromatogram shown in Fig. 9B. A well-developed peak was observed earlier than that for NEO (12.8 min). The eluted substance showed a signal of $m/z = 616,$ suggesting that the substance is an intermediate of the NEO biosynthesis, 6‴′-deamino-6‴-hydroxyneomycin C (Fig. S3, Supporting Information). This would exist as a pentavalent cationic species.

The culture broth was assayed by the present colorimetry with the separation process. Using the calibration curve of Fig. 8, the produced amount was calculated to be 1.06 ± 0.05 mEq/L, which is somewhat larger than that determined by chromatography. The positive error might be due to the existence of the intermediate. In fact, the intermediate was detected from the NEOH6⁺-AR3⁻ precipitate. This underscores the importance of detecting not only the final products but also the intermediates in biosynthetic studies.

In conclusion, the present colorimetric method with the separation process should be useful for the biosynthetic analysis of polycationic aminoglycosides.

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**Supporting Information**

Chemical structure of tobramycin, gentamycin, streptomycin, kanamycin, amikacin, and 6‴-deamino-6‴-hydroxyneomycin C. This material is available free of charge on the Web at http://www.jsac.or.jp/analsci.

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