

AMINOGLYCOSIDE ANTIBIOTICS



Aminoglycoside broad-spectrum antibiotics, which include mainly streptomycin, gentamycin, apramycin and neomycin, are used in both human and veterinary medicine.

In addition, streptomycin is used as a plant protection agent (trade name: Plantomycin) for the treatment of the bacterium *Erwinia amylovora* on pomaceous fruit. It is applied during the flowering period and can pass via the pollen into honey. Residues of aminoglycoside antibiotics in food could potentially be very detrimental to health.

For the analysis of amino glycoside antibiotics, PICKERING provides the systems PINNACLE PCX for post-column derivatization. Their configuration (number of reagent pumps, reactor volume, etc.) differs according to the respective derivatization method.

For the aminoglycoside antibiotics gentamycin, apramycin and neomycin, Pickering also supplies complete application kits. The kits for aminoglycoside antibiotics contain, in addition to the columns, reagents, diluents and eluents.

A kit for the analysis of Streptomycin is not available. Therefore, the user has to supply column, eluents, reagents, etc..

Description of the Method

Gentamycin, Apramycin and Neomycin react spontaneously with *o*-phthalaldehyde (OPA) and Thiofluor[®] (2-mercaptoethanol derivative) to fluorescing isoindole derivatives. Separation can be performed on either the special Pickering ion-exchange column or on a reversed-phase-column (depending on literature).

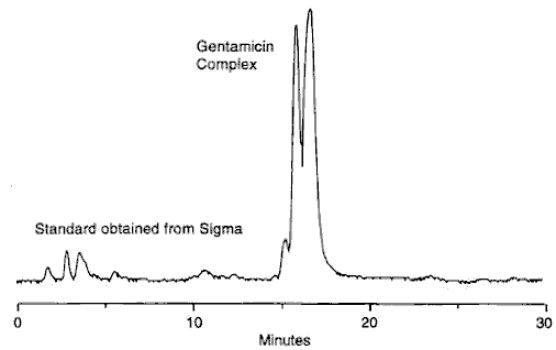
Derivatization of Streptomycin is performed in a one-step reaction by conversion with 1,2-naphthoquinone-4-sulfonic acid (NQS) to a fluorescing derivative in a highly alkaline environment. NQS, the actual derivatization reagent, is a component of the mobile solvent. Post column, the pH-value is adjusted by addition of sodium hydroxide solution and the reaction is completed in the reactor by heating.



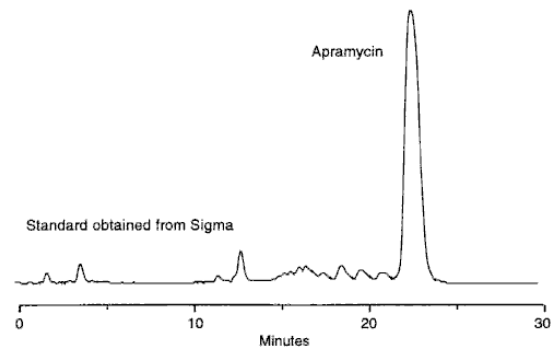
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Chromatograms

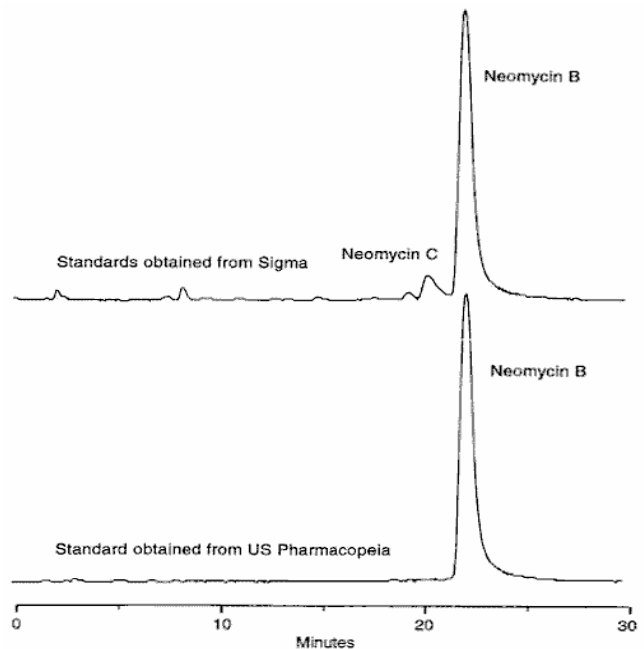
Chromatogram of Gentamicin Standard
5 µg Gentamicin-Complex abs.



Chromatogram of an Apramycin Standard
11,1 nmol Apramycin abs.



Chromatogram of a Neomycin Standard
2,75 nmol Neomycin abs.



Chromatogram of a Streptomycin Standard
Not available at the time!

HPLC Conditions and Derivatization Parameters

HPLC	
Operation Mode	Ternary gradient
Eluent	A: 0,1 M potassium phosphate buffer, pH 6.0 B: 0,1 M potassium hydroxide solution, pH 13.0 C: 1,0 M sodium chloride solution
Degassing	Helium- or vacuum degassed
HPLC Column	ALKION® Cation-exchange column, K ⁺ -Form
Flow Rate	0.8 mL/min
Post-Column Derivatization	
Pinnacle PCX	Single-pump; 150 µL reactor
Column Oven	40 °C
Reactor Volume	150 µL
Reactor Temperature	45 °C
Reagent	Solution of o-phthalaldehyde (OPA) and Thiofluor® (2-mercaptoethanol derivate) in OD104 diluent (sodium borate buffer, pH 10,4. Concentration: 300 mg OPA and 2 g Thiofluor in 1L diluent
Reagent Flow	0.3 mL/min
Detektion	
Detection Type	Fluorescence detection
Excitation Wavelength	330 nm
Emission Wavelength	465 nm
Flowcell	Analytic; pressure stable up to 7 bar

APPLICATION NOTE

Caution: Extreme pH-range!

The following advice is only applicable to Pickering-Kits! As a consequence of the alkaline regenerant (pH 13), components made from Vespel may not be present in the HPLC system, instead these must be exchanged for components made from pH-inert materials (Tefzel or PEEK). For advice, please contact your LC-representative or consult your technical manual. An inert version (Titanium, PEEK) of the HPLC system is not necessary, a piston seal wash for the pump heads, however, is recommended.

To avoid corrosion of the system and contamination of the ion-exchange column with metal ions, passivating is recommended; this is especially valid for older systems. For advice, please contact your LC-representative or consult your technical manual.

Gradient Programs

METHOD "ALKION® Ion-exchange column for Gentamycin"

Flow rate: 0.8 mL/min; column temperature: 40°C

Step	Time	Interval	K01	K02	K03	
1	0	-	43	31	26	Injection
2	20	20	9	13	78	Linear gradient
3	30	10	9	13	78	Isocratic
4	30.1	0.1	0	22	78	Step change
5	31	0.9	0	22	78	Isocratic
6	31.1	5 - 9	43	31	26	Re-equilibration

METHOD "ALKION® Ion-exchange column for Neomycin"

Flow rate: 0.8 mL/min; column temperature: 40°C

Step	Time	Interval	K01	K02	K03	
1	0	-	60	40	0	Injection
2	15	15	13.2	8.8	78	Linear gradient
3	25	10	11.0	11.0	78	Linear gradient
4	25.1	0.1	0	22	78	Step change
5	26	0.9	0	22	78	Isocratic
6	26.1	5 - 9	60	40	0	Re-equilibration

METHOD "ALKION® Ion-exchange column for Apramycin"

Flow rate: 0.8 mL/min; column temperature: 40°C

Step	Time	Interval	K01	K02	K03	
1	0	-	67	33	0	Injection
2	5	5	67	33	0	Isocratic
3	15	10	14.7	7.3	78	Linear gradient
4	20	5	14.7	7.3	78	Isocratic
5	20.1	0.1	0	22	78	Step change
6	21	0.9	0	22	78	Isocratic
7	21.1	5 - 9	67	33	0	Re-equilibration

APPLICATION NOTE

Streptomycin

HPLC Conditions and Derivatization Parameters

HPLC	
Operation Mode	Isocratic
Eluent	1-Heptane sulfonic acid (10 mmol/L) and 1,2-naphthoquinone-4-sulfonic acid (0,4 mmol/L) in water with 20 % acetonitrile; adjust pH to 3.3 with acetic acid
Degassing	Helium- or Vacuum degassed
HPLC Column	RP C18 with guard column
Flow Rate	0,8 mL/min
Injection Volume	100 µL
Post-Column Derivatization	
Pinnacle PCX	Single-pump; 1.0 mL reactor
Column Oven	40 °C
Reactor Volume	1,0 mL
Reactor Temperature	50 °C
Reagent	Sodium hydroxide 0,2 mol/L
Reagent Flow	0,4 mL/min
Detection	
Detection Type	Fluorescence detection
Excitation Wavelength	263 nm
Emission Wavelength	435 nm
Flowcell	Analytic; pressure stable up to 7 bar

Chemicals and Columns

Post-Column Derivatization Units

Order Number	Description
1153-1012	PINNACLE PCX; single-pump, 150 µL reactor
On request	PINNACLE PCX; single-pump, 1.0 mL reactor

Application-Kits

Order Number	Description
0352-0041	Application kit for gentamycin, neomycin, and apramycin, analysis; consists of:
9410917	ALKION [®] , Ion-exchange column, K ⁺ -Form, 4 x 150 mm
9493020	Guard column, 3 x 20 mm, for column 9410917
O120	o-Phthalaldehyd (OPA), "Chromatographic Grade TM ", 5 g
OD104	OPA diluent, "Chromatographic Grade TM ", 4 x 950 mL
3700-2000	Thiofluor [®] , "Chromatographic Grade TM ", 10 g
1700-1101	Potassium phosphate/borate buffer, 0.1 M, pH 6.0, 4 x 950 mL
1700-1102	Potassium hydroxide solution, 0.1 M, 4 x 950 mL
1700-1103	Potassium chloride solution, 1.0 M, pH 6.0, 4 x 950 mL

Cation-Exchange Columns for Amino glycoside Antibiotics Analysis

Each column for the analysis of amino glycoside antibiotics is tested by applying an calibration standard and running it according to a specific gradient elution protocol. After a column passes this rigorous QC test, it receives a serial number and is packaged with its test chromatogram.

Order Number	Description
9410917	ALKION [®] , Cation-exchange column, K ⁺ -form, 4 x 150 mm
9493020	Guard column; 3 x 20 mm

o-Phthalaldehyde Reagent

Primary amines such as gentamycin, apramycin and neomycin form highly fluorescent compounds when reacted with o-Phthalaldehyde (OPA) and a mercaptan under basic conditions. At a pH >9 and ambient temperature, reaction is generally complete within 1 - 30 seconds. The products of this reaction, 1-alkyl-2-alkylthio-substituted isoindoles, exhibit optimal excitation at 330 nm and maximal emission at 465 nm.

For an oxygen-sensitive reagent like OPA to remain stable for days instead of hours, it is important to start with the purest and most stable ingredients available, and to store and use the reagent under anaerobic conditions.

APPLICATION NOTE

Using the chemicals described below, a long-lasting (up to ten days) OPA reagent for post-column derivatization of primary amines can be prepared. Each chemical is accompanied by clear instructions for formulating the reagent in your laboratory within minutes.

For the preparation of *o*-phthalaldehyde reagent PICKERING offers:

- 1) Ultra-pure, crystalline OPA
- 2) Borate diluent; free of heavy metals, particulates and amines
- 3) Thiofluor; crystalline substitute for 2-Mercaptoethanol

o-Phthalaldehyde



Order Number	Description
O120	<i>o</i> -Phthalaldehyde (OPA), "Chromatographic Grade", 5 g

o-Phthalaldehyde Diluent

For the analysis of gentamycin, apramycin and neomycin Pickering offers a sodium borate buffer with pH 10.4 capable to buffer acidic eluents.

Order Number	Description
OD104	OPA Diluent, "Chromatographic Grade™", 4 x 950 mL

APPLICATION NOTE

Thiofluor[®]

Pickering's Thiofluor, a solid, nearly odorless nucleophile, is a superior substitute for 2-mercaptoethanol in the preparation of OPA reagents. It forms a more stable and longer-lasting fluorophore with OPA than does 2-mercaptoethanol, while possessing the same fluorescence properties.

Unlike the volatile 2-mercaptoethanol, Thiofluor[®] will not migrate through the gas manifold and regulator of the OPA reagent pressurization system.

Order Number	Description
3700-2000	Thiofluor, "Chromatographic Grade TM ", 10 g

Potassium Phosphate Buffer

- 1) Filtered, ready to use
- 2) Free from fluorescent impurities
- 3) Cost-effective: long shelf life; can be used to the last ml
- 4) Consistent elution profiles, bottle to bottle, lot to lot

These buffers are used for the elution of aminoglycoside antibiotics according to the method developed by Pickering.

Order Number	Description
1700-1101	Potassium phosphate/borate buffer, pH 6.0, 4 x 950 mL
1700-1102	Potassium hydroxide solution, 4 x 950 mL
1700-1103	Potassium chloride solution, 4 x 950 mL

Literature

Neomycin

B. Shaikh, E. H. Allen, J. C. Gridley, *J. Assoc. Off. Anal. Chem.* **1985**, 68 (1), 29 – 36.

Gentamycin

D. Guggisberg, H. Koch, *Mitt. Gebiete Lebensm. Hyg.* **1995**, 86, 14 – 28.

Streptomycin

- 1) U. Kocher, *Lebensmittelchemie* **1996**, 50, 112 – 115.
- 2) G. C. Gerhardt, C. D. C. Salisbury, J. D. MacNeil, *J. AOAC International* **1994**, 77 (2), 334 – 336.