Multiresidue Analysis of Aminoglycoside Antibiotics using Disposable Pipette Extraction and LC-MS/MS

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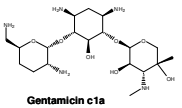
Overview

Aminoglycosides are widely applied antimicrobial drugs in current veterinary practice. In the U.S., USDA Food Safety and Inspection Service (FSIS) uses a semi-quantitative microbial assay to determine violative levels of aminoglycosides (and some other antibiotics) in animal tissues. For positive findings, this assay is followed by a confirmatory LC-MS/MS method based on a laborious sample preparation using cartridge-based solid-phase extraction (SPE). The aim of this study was to develop a streamlined approach for multiresidue LC-MS/MS analysis of aminoglycosides that would provide fast screening and eliminate the microbial assay and the cartridge-based SPE and evaporation steps. The streamlined sample preparation method involves buffer extraction, followed by a disposable pipette extraction (DPX) with a weak cation exchange sorbent. The DPX technique uses disposable pipette tips with a small amount of loosely contained sorbent material that is mixed with the sample solution. DPX speeds the SPE process, reduces material cost and solvent use, and enables easy automation. After elution from the DPX tip, twelve target aminoglycosides are analyzed in ESI positive mode using a fast UPLC-MS/MS method, which employs an ion-pairing agent to retain highly polar aminoglycosides on a reversed-phase column BEH C18 (50 x 2.1 mm, 1.7 µm). During the LC-MS/MS method development, various aqueous normal-phase chromatography conditions were also tested for LC separation of aminoglycosides using different column chemistries (hydride-based silica bonded C18 and hydrophilic interaction LC, HILIC, columns), dimensions, and mobile phase compositions.

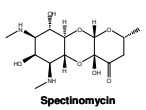
Introduction

Aminoglycoside antibiotics:

◆ Aminoglycosides and closely related aminocyclitols are very polar compounds containing an aminocyclitol group linked to aminosugars. Examples of aminoglycoside structures :



Gentamicin c1a



Spectinomycin

Current USDA-FSIS analytical approach:

◆ Semi-quantitative microbial inhibition assay followed by a confirmation of positive samples using LC-MS/MS.

◆ Confirmatory LC-MS/MS method [1] involves in brief:

- extraction of 2 g sample (bovine, porcine or avian kidney, liver or muscle) with 2x10 mL of an aqueous buffer solution (10 mM KH_2PO_4, 0.4 mM EDTA and 2% (*w/v*) TCA)
- adjustment of extract pH to 7.5-8.0
- SPE clean-up of the entire extract using a weak cation exchange sorbent (500 mg CBX) packed in a cartridge
- elution with 3 mL of 10% acetic acid in methanol
- concentration of the extract in 0.5 mL of 5 mM HFBA (heptafluorobutyric acid)
- LC-MS/MS analysis using 20 mM HFBA as an ion-pairing agent in both water and methanol (run time = 35 min)

Objective of this study:

◆ Develop a streamlined LC-MS/MS-based approach that would replace the microbial inhibition assay and enable fast multiresidue screening and identification of aminoglycosides.

Methods

Sample extraction:

(1) Weight out 5 g of homogenized bovine kidney sample in a 50 mL disposable polypropylene tube.

(2) Add 10 mL of the extraction buffer solution (10 mM KH_2PO_4, 0.4 mM EDTA and 2% (*w/v*) TCA in water).

(3) Add tobramycin as an internal standard. Vortex briefly, shake for 10 min. Centrifuge at 3450 rcf for 5 min.

(4) Decant the supernatant into a clean 50 mL tube. Add 10 mL of the extraction buffer solution to the pellets, vortex briefly, shake for 10 min. Centrifuge at 3450 rcf for 5 min.

(5) Combine supernatants. Adjust pH of the combined extract to 6.5 using 1N NaOH.

Disposable Pipette Extraction (DPX):

(1) Condition a 1 mL DPX tip (DPX Labs, LLC; Columbia, SC, USA) containing 50 mg of a weak cation exchange (WCX) sorbent (30-70 µm particle size) with 1 mL methanol and 1 mL water.

(2) Condition the tip with 1 mL of the extract. Load 5 mL of the extract onto the tip 5x1 mL portions. Each time let the sorbent interact with the extract for about 30 s.

(3) Wash the tip with 1 mL of water.

(4) Elute the tip with 1 mL of 10% formic acid in water (for manual elution, place the elution solution into a polypropylene autosampler vial, load it onto the tip and make 5 pumps).

LC-MS/MS conditions:

(a) LC-MS/MS instrument:

A Waters Acquity UPLC instrument coupled to a Waters TQD triple quadrupole in electrospray (ESI) positive mode; MassLynx software with QuanOptimize and TargetLynx

(b) LC column: Waters BEH C18 (50 x 2.1 mm, 1.7 µm)

(c) LC column temperature: 40 ℃

(d) Autosampler temperature: 7 ℃

(e) Injection volume: 5 µL (partial loop with needle overfill)

(f) Divert valve program:

0-0.7 min to waste, 0.7-2.5 min to MS, 2.5-3.0 min to waste

(g) Mobile phase:

A: 20 mM HFBA in water-acetonitrile (95:5, *v/v*)

B: 20 mM HFBA in acetonitrile

(h) Flow rate: 500 µL/min

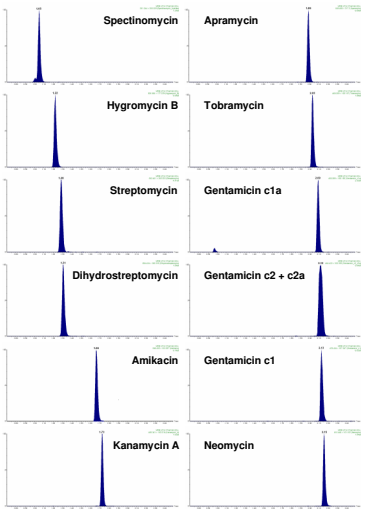
(i) Mobile phase linear gradient:

Time	%A	%B
Initial	100	0
0.50	80	20
1.00	80	20
2.00	60	40
2.05	10	90
2.50	10	90
2.55	100	0
3.00	100	0

(j) MS/MS conditions: ESI+, 5 ms dwell time

Analyte	Precursor *m/z*	Product *m/z*	Cone (V)	Coll (V)
Spectinomycin	351.2	333.3 100.2	40	20 30
Gentamicin c1a	450.4	160.2 322.4	25	25 15
Gentamicin c2+c2a	464.4	322.4 160.2	25	15 25
Tobramycin	468.4	163.2 324.3	30	25 15
Gentamicin c1	478.4	157.2 322.4	30	30 15
Kanamycin A	485.4	163.2 324.3	30	20 15
Hygromycin B	528.4	177.2 352.4	45	30 25
Apramycin	540.4	217.2 378.3	35	25 15
Streptomycin	582.4	263.3 221.3	65	35 40
Dihydrostreptomycin	584.4	263.3 246.3	55	35 40
Amikacin	586.4	163.2 425.3	35	35 20
Neomycin	615.4	161.2 163.2	45	35 35

UPLC-MS/MS chromatograms of 12 aminoglycosides in bovine kidney extract spiked at 2 µg/g:



Results

Optimization of the LC-MS/MS method:

- Aminoglycosides are not retained under typical LC reversed phase (RP) conditions, therefore two strategies were tested:

(1) Aqueous normal phase (ANP) separations using:
- **(a)** a hydride-based silica bonded C18 column [2]
- **(b)** HILIC columns

(2) RP separation with an ion-pairing agent (HFBA)

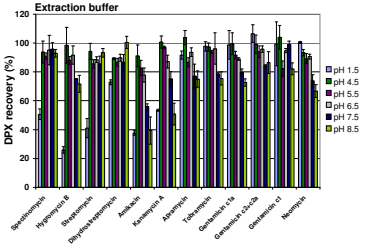
- The ANP LC used acetonitrile and water with formic acid as a mobile phase additive, therefore could be combined with a typical RP analysis of other antibiotics or veterinary drugs using alternate runs in RP and ANP mode [2].
- However, the RP chromatography with HFBA provided more rugged and reliable results.
- The HPLC method was sped up using UPLC with a short, 1.7 µm column, resulting in more than 10-fold reduction of the LC-MS/MS analysis time (35 min *vs.* 3 min).

Optimization of the DPX procedure:

- The optimized parameters included: sorbent type, particle size and amount; wash and elution solvent composition and volume; and mainly pH adjustment of the extract prior the loading step.
- WCX sorbent (30-70 µm particle size) provided the best overall results for a multiresidue screening method of aminoglycoside residues in bovine kidney samples.

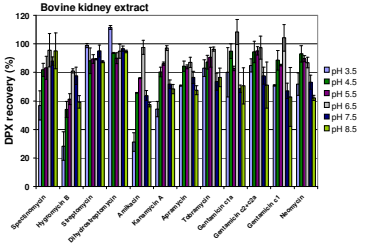
(a) DPX recovery from the extraction buffer at different pH

- 5 mL of spiked extraction buffer without pH adjustment (pH ~1.5) and with pH adjusted to 4.5-8.5, loaded onto a DPX tip with WCX sorbent (50 mg) and eluted with 2x1 mL of 10% formic acid in water.
- Without the pH adjustment, late eluting analytes (apramycin to neomycin) gave very good recoveries but the pH had to be increased for the early eluting aminoglycosides.
- pH 4.5-6.5 provided acceptable recoveries (78-104%) from the extraction buffer for the tested aminoglycosides:



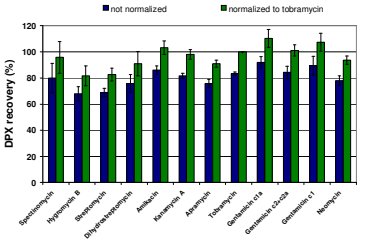
(b) DPX recovery from bovine kidney extract at different pH

- 5 mL of spiked bovine kidney extract (at 2 µg/g) with pH adjusted to 3.5-8.5, loaded onto a DPX tip with WCX sorbent (50 mg) and eluted with 2x1 mL of 10% formic acid in water.
- In addition to pH adjustments to 4.5-8.5, which were tested for the buffer solution, pH 3.5 was also tested in the presence of matrix to evaluate a pH < 4.5.
- pH adjustment to 6.5 provided the best recoveries (81-108%) from the bovine kidney extract for the tested aminoglycosides:



(c) DPX recovery from bovine kidney extract at pH 6.5

- 5 mL of spiked bovine kidney extract (at 2 µg/g) with pH adjusted to 6.5, loaded onto a DPX tip with WCX sorbent (50 mg) and **eluted with only 1 mL** of 10% formic acid in water.
- Aminoglycoside recoveries in 1 mL of the elution solvent were in the range of 82-110% when normalized to the internal standard tobramycin (68-92% without normalization):



Conclusions

The current USDA-FSIS confirmatory method for multiresidue analysis of aminoglycosides can be streamlined by the use of:

◆ UPLC-MS/MS that provides more than 10-fold reduction of the analysis run time.

◆ DPX technique that speeds the SPE process, reduces material cost and solvent use, and enables easy automation.

Future plans:

◆ Semi-automate the developed method using a DPX lever arm extractor for simultaneous processing of 24 DPX 5 mL tips.

◆ Automate the developed method using a DPX system from Gerstel (designed for 1 mL DPX tips).

Acknowledgments

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References

[1] SOP No. CLG-AMG1.02 (2005) US Department of Agriculture, Food Safety and Inspection Service, Office of Public Health Science (www.fsis.usda.gov/PDF/CLG_AMG_1_02.pdf)

[2] Mastovska, K. and Lightfield, A.R. (2008) Reversed phase and aqueous normal phase retention in multiclass LC-MS analysis of antibiotics. *Am. Lab.* (on-line edition) 6-7, 37-40 .