Analysis of Aminoglycosides with a Zwitterionic HILIC Stationary Phase and Mass Spectrometry Detection

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A method was developed to analyse three model aminoglycosides (neomycin, apramycin, and kanamycin) using a zwitterionic HILIC (hydrophilic interaction liquid chromatography) stationary phase and mass spectrometry (MS) detection. High separation efficiency and good peak shape were obtained with an optimal combination of mobile phase composition and gradient profile. Gradient elution proved to be reproducible and a good way to combine both HILIC and electrostatic interactions to obtain unique selectivity. The possible lower limit of quantification (LLOQ) was under 0.25 µg/mL using HILIC coupled with a single quadrupole ESI-MS instrument.

Introduction

Aminoglycosides are bactericidal antibiotics that have amino-modified sugar in their structures [1-3]. This particular group of antibiotics are widely used as clinical and veterinary medicines to treat infections caused by gram-negative or some gram-positive organisms, and are classified as bactericidal agents because of their interference with bacterial replication. However, theses antibiotics can also cause varying degree of ototoxicity and nephrotoxicity. McGlinchey et al. described in a recent review that the overuse of antibiotics and exposure to the food animals are the two major routes attributed to the antibiotic resistance [3]. Therefore, it is very important to develop sensitive and reliable analytical methods for determining and monitoring aminoglycosides residue in different sample matrices.

Aminoglycosides are normally very hydrophilic and carry several amino groups, which mean they are positively charged at neutral pH condition. It is very difficult to separate them by reversed-phase liquid chromatography (RPLC) unless an ion-pair reagent or pre-column derivatization is applied. Furthermore, aminoglycosides usually have no chromophores in the molecular backbone, which limits the use of light absorption detection. Despite these technical barriers, scientists have developed several LC methods [2,3], e.g. RPLC with pre-column derivatization and UV or fluorescent detection [4-7], ion-pair RPLC with evaporative light scatter detection (ELSD) or mass spectrometry (MS) [8,9], and ion chromatography with electrochemical or fluorescent detection [11,12]. HILIC-MS has also emerged in recent years as an alternative technique for the separation and detection low levels of aminoglycosides [12-15].

HILIC is a straightforward chromatographic separation mode, which is suitable for separation of polar and hydrophilic compounds without any sample derivatization or ion-pair reagent in the mobile phase. It is ideal to combine the HILIC with MS because HILIC uses large proportion of organic solventthat enhances the ionization and desolvation in ESI-MS detection. Through literature searching, it was found that most published HILIC-MS methods for aminoglycoside separation are based on a zwitterionic HILIC stationary phases with ESI-MS in positive mode [12-14]. As yet the separation mechanism on such HILIC phases has not been clarified. In this work, we aim to understand what are the most critical factors affecting separation efficiency, selectivity and detection sensitivity under HILIC conditions.

Experimental

Chemicals and Reagents:

Neomycin, apramycin, kanamycin and ammonium acetate were all purchased from Sigma-Aldrich (Steinheim, Germany). Acetonitrile (HPLC grade) was obtained from Merck (Darmstadt, Germany). Formic acid was from J.T. Bakers (Deventer, The Netherlands). Water was purified by a bench-top Milli-Q water purification system from Merck Millpore (Billerica, MA, USA).

LC-MS Instrumentation and Conditions

HPLC Pump:	Shimadzu LC-20AD HPLC Pump
Autosampler:	Shimadzu SIL-20AC
Column:	SeQuant ZIC®-HILIC, 100 x 2.1 mm, 3.5 µm/100 Å (Merck, P/N 1504410001)
Mobile Phase:	A: acetonitrile with 1 w% formic acid B: 150 mM ammonium acetate with 1.5 w% formic acid
Flow Rate:	0.4 mL/min
Injection:	20 µL
Temperature:	50 °C
UV Detection:	Shimadzu SPD-20A UV/Vis Detector
MS Detection:	Shimadzu LCMS-2010EV; ESI in positive mode; neomycin: m/z 615; Apramycin: m/z 540; Kanamycin: m/z 484; capillary voltage: 4 kV; Block temperature 250 °C; spray gas: nitrogen at 1.5 L/min; detector voltage: 2 kV.
Data Acquisition:	Shimadzu LCMSsolution Chromatography Software; Version 3.30

Gradient program:

Time (min)	Flow rate (mL/min)	A (%)	B (%)
0	0.4	50	50
4	0.4	5	95
8.99	0.4	5	95
9	0.4	50	50
17	0.4	50	50

Sample Preparation

The stock solutions containing 20 mg/mL each of Neomycin, Apramycin and Kanamycin were prepared by dissolving the appropriate amount of solid in 30/70 (v/v) acetonitrile/water solution. The standard solutions were prepared by mixing the stock solution with the same acetonitrile/water solution as described above.

Results and Discussions

Separation with Zwitterionic HILIC

Stationary Phase

The zwitterionic stationary phase used in this study is a silica base stationary phase bonded with sulfobetaine zwitterionic functionality, which has both a positively charged quaternary ammonium and a negatively charged sulfonic acid group in one single functional moiety. The unique zwitterionic structure and highly hydrophilic character makes it ideal for HILIC separation. The balance between the strong acidic and strong basic group makes this phase independent of pH, and the electrostatic interaction is significantly weaker compared to a conventional ion exchanger [16,17]. Figure 2 shows the proposed retention mechanism for separating polar/hydrophilic compounds on such a zwitterionic HILIC phase. A crucial part in the separation mechanism is that a stagnant water layer is built up when equilibrating the stationary phase with the mobile phase containing 3-50% aqueous buffer solution. A polar/hydrophilic analyte experiences hydrophilic partitioning between the water layer and the less polar mobile phase. In addition, a charged analyte may have weak electrostatic interactions (both attraction and repulsion) by the zwitterionic groups, which greatly contribute to the separation selectivity.

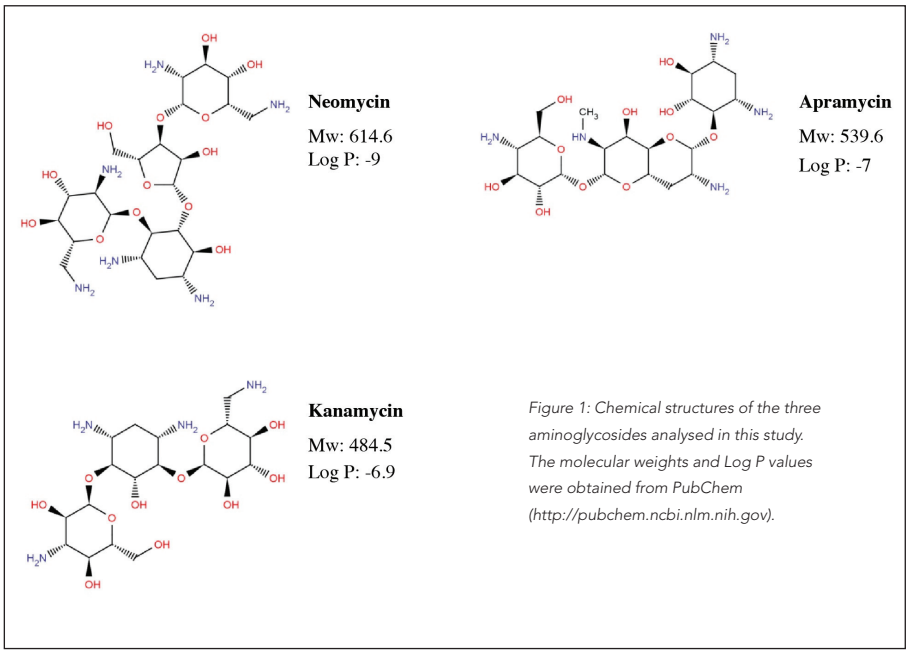

Figure 1: Chemical structures of the three aminoglycosides analysed in this study. The molecular weights and Log P values were obtained from PubChem (http://pubchem.ncbi.nlm.nih.gov).

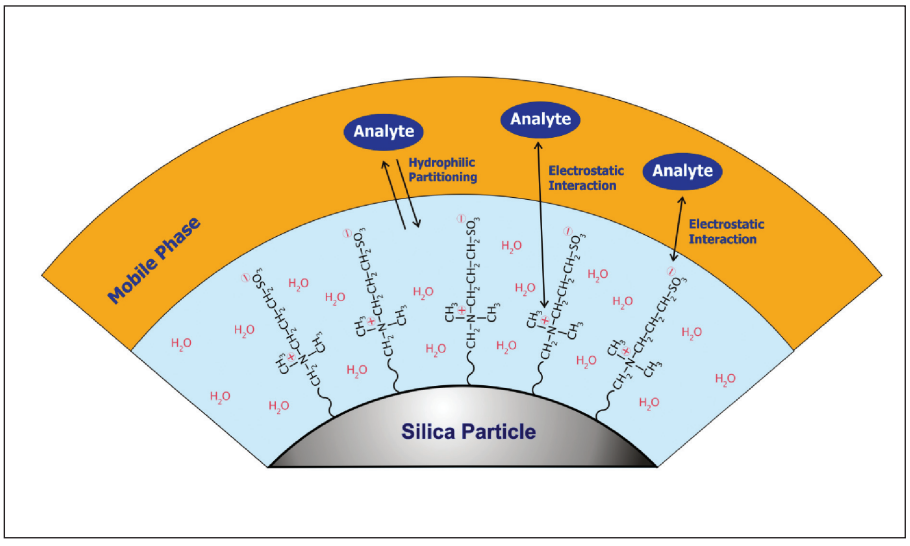

Figure 2: Retention of polar/hydrophilic compounds on a zwitterionic HILIC stationary phase.

At the beginning of this work, we investigated a more neutral zwitterionic stationary phase to separate neomycin and apramycin with a similar gradient elution profile as described in the experimental section. However, the separation resolution between these two aminoglycosides was unfortunately unsatisfactory. It appeared that retention and selectivity based on hydrophilic partition are not sufficient in this case, especially when using a very fast gradient elution. We realized it was actually an advantage to have electrostatic interaction to improve the separation resolution due to the different charge states of neomycin and apramycin. This may be one of the reasons that zwitterionic HILIC stationary phases are the most used phases for the HILIC separation of aminoglycosides.

Separation of Aminoglycosides

Aminoglycosides have multiple positive charges at pH conditions below their pKa values. This indicates that the electrostatic interaction between these analytes and the stationary phase will be much stronger than that for single charged molecules. The electrostatic interaction was examined by isocratic separation of aminoglycoside with a mobile phase containing 20-30% A and 80-70% B, where the separation should be primarily dependent on the electrostatic interaction but not hydrophilic partitioning due to the very low organic solvent content in the mobile phase. It was found that retention factor (k') for neomycin and apramycin decreased from 10.61 and 5.59 to 5.13 and 2.95, respectively, while the mobile phase B was increased from 70% to 80%. It was also found that the eluting peaks became broader. These observations indicate a separation mode based on electrostatic interaction.

Ishii et al., Oertel et al., and Shen et al. [12-14] demonstrated the importance in using a mobile phase with high ionic strength and a fast gradient profile for aminoglycosides separation in HILIC. We used a standard HPLC-MS system to repeat the experiments with the same separation conditions and gradient profiles as those used by Ishii et al. yet neomycin exhibited strong peak tailing,

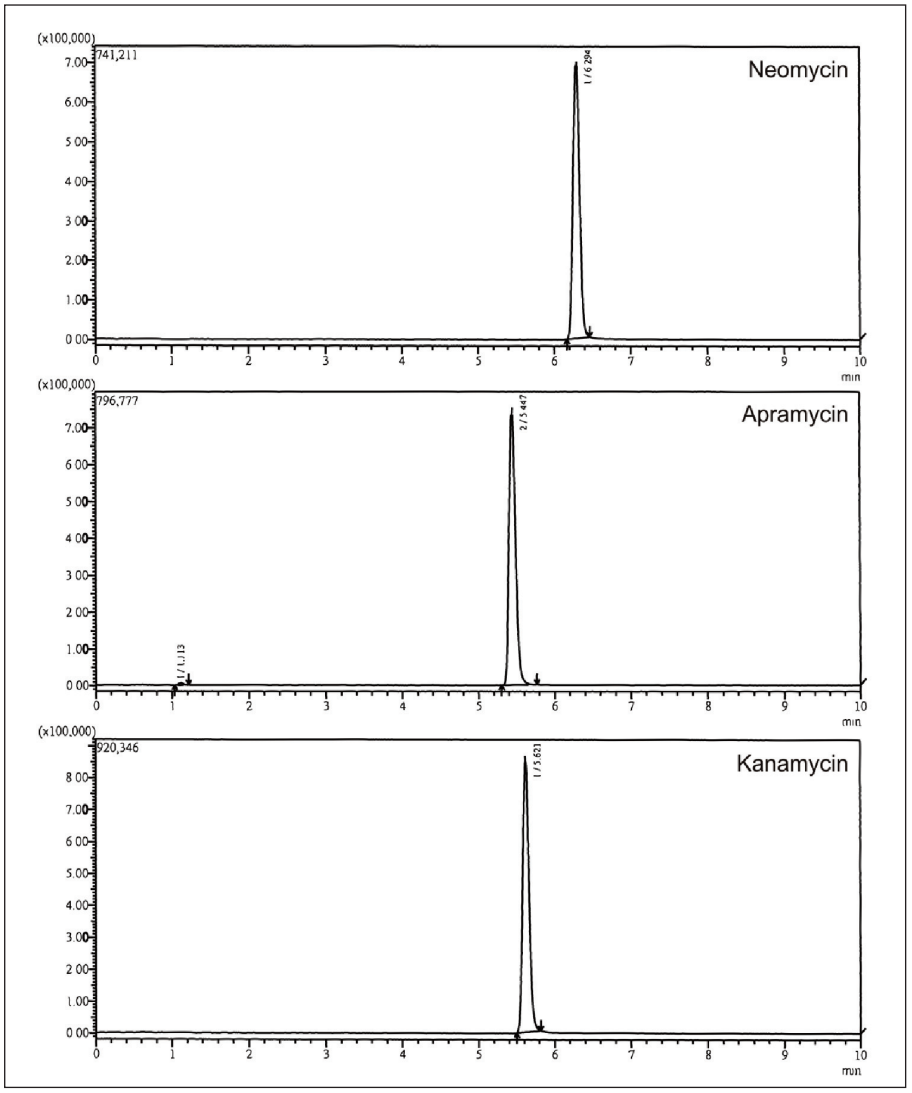

Figure 3: Chromatograms of the three aminoglycosides (5 µg/mL) analysed with a zwitterionic HILIC stationary phase and ESI-MS detection.

especially at sample concentration below 5 µg/mL. The peak shape of all three model aminoglycosides became better when much faster gradient elution, from 5% B to 95% B within one minute, was used. However, the resolution between neomycin and apramycin/kanamycin tended to be poorer. After investigation with a combination of different mobile phase and gradient profiles, best separation was obtained with acetonitrile plus 1 w% formic acid as mobile phase A and 150 mM ammonium acetate plus 1.5 w% formic acid as mobile phase B and a gradient starting from 50/50 A and B. Although many attempts were undertaken in order to reduce the ammonium acetate in mobile phase B and formic acid concentration in both A and B, the conditions described above still gave best results. Figure 4 shows chromatograms of the separation of the three aminoglycosides under optimal conditions as described in the experimental section.

Practical Aspects

Since the aminoglycosides have strong electrostatic interaction with the zwitterionic stationary phases, it is mandatory to use relatively high concentrations of ammonium acetate and formic acid to achieve good separation efficiency and peak shape. Formic acid may act as a weak ion-pair reagent with the amino groups to reduce the electrostatic interaction between analytes and stationary phase. This is especially important for the analysis of aminoglycosides at lower sample concentration.

Another important finding in this work is that the gradient elution must be very fast if the separation starts from around 20-30% of acetonitrile with 1% of formic acid. Otherwise, broad and tailing peaks were normally obtained, which may be due to sample solubility and ionic interaction issues. We found 50% mobile phase A as a starting condition gave best peak shape and chromatographic resolution.

Conclusion

Aminoglycosides exhibited good retention and separation efficiency on a zwitterionic HILIC stationary phase. The separation is based on a mixed mode separation mechanism depending on both hydrophilic partitioning and electrostatic interaction. The addition of formic acid and high buffer concentration in the mobile phases, in combination with fast gradient elution, were key factors influencing the chromatographic performance. Mass spectrometry had excellent compatibility with these HILIC separations and provided good detection sensitivity.

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