Alturas Advisor
FALL WINTER 2008-09

Ion-Pair HPLC/MS/MS Bioanalysis: Are There Always Negative Consequences?

Ever since the first reports on the negative consequences from the use of ion-pair reagents in LC/MS/MS, researchers have explored many options to improve the peak shape and retention of basic compounds. Ion-pair reagents are added to the mobile phase in reversed-phase HPLC to increase retention and improve the peak shape for the analysis of polar, basic analytes. Conventional ion-pair reagents are non-volatile and include, octanesulphonic acid and tetrabutylammonium hydrogen sulphate. Volatile ion-pair reagents compatible with LC/MS include trifluoroacetic acid (TFA) and heptafluorobutyric acid (HFBA). The majority of literature references discuss the decrease in ESI/MS signal when using ion-pairing regents with LC/MS/MS (1). Since the strong acid pairs with the basic analyte, the theory holds that the complex is now neutralized. Therefore, the production of positive ions in the ESI source is diminished. Due to the negative influence on ESI/MS signal, researchers have avoided ion-pair reagents for use in LC/MS assays. This is unfortunate since ion-pair HPLC seems to be a perfect match to obtain excellent peak shape and improve retention for the analysis of polar, basic analytes on reversed-phase, silica based columns.

To reduce tailing, vendors have produced much higher purity silica and improved the stationary phase coverage . Other researchers were involved in the development of special stationary phases designed specifically to eliminate the need for ion-pair reagents in LC/MS (2). More recently many methods have been developed using hydrophilic interaction chromatography (HILIC) for the LC/MS analysis of polar analytes (3). The following discussion describes a case study for the LC/MS/MS analysis of the polar and basic aminoglycoside compounds.

The analysis of aminoglycosides is a great example of the need for ion-pair HPLC. The compounds are very hydrophilic and basic. Thus to obtain adequate retention and peak shape, ion-pair reagents have been used in the past with conventional HPLC. Now that newer dose regimens and better analogs have been discovered, the concentrations of aminoglycosides found in biological fluids can be in the low ng/mL range. An accurate and sensitive LC/MS/MS method is an ideal selection for the analysis of these low concentrations.

Method Development: Upon review of the structure of the aminoglycosides and review of the literature, it was expected that the standard reversed-phase LC/MS/MS methods may not be successful for the LC/MS/MS bioanalysis of the compounds. Other alternatives to the addition of ion-pair reagents include HILIC. Several HILIC phases were investigated for the LC/MS/MS analysis of the aminoglycosides. A bare silica column was used in the HILIC mode to attempt to obtain adequate peak shape and retention. Although adequate retention was obtained the peak shape was not optimal (See Figure 1.). A pentafluorophenylpropyl (PFP) column was also used for the LC/MS/MS analysis of the aminoglycosides. However, the compounds were

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OUTREACH 2008-09

The 15th North American ISSX meeting
October 12-16, 2008
Town & Country Resort
San Diego, California.
Poster Presentation: Stabilization of peptides for validation of HPLC/MS/MS methods for the quantitative analysis of these peptides from blood and plasma.
Exhibit booth #25.
(*http://www.issx.org/i4a/pages/index.cfm?pageid=3303*).

CPSA-Short Course: Method Development for LC/MS: Traditional approaches and emerging trends
October 27-30, 2008
Sheraton Bucks County Hotel
Langhorne, Pennsylvania.
(*www.milestonedevelopment.com*).

AAPS 2008
November 16-20, 2008
Georgia World Congress Center
Atlanta, GA. **Exihibit Booth #1656**
(*http://www.aapspharmaceutica.com/meetings*).

60th Pittsburg Conference
March 8-13, 2009
Chicago, IL.
Pittcon Short Course "HPLC Methods Development for LC/MS", oral presentation: achieving ultimate performance for bioanalysis using orthogonal solid phase extraction with reversed phase HPLC/MS/MS. (*http://www.pittcon.org*).

48th Annual Society of Toxicology Meeting and ToxExpo
March 15-19, 2009
Baltimore Convention Center.
Exhibit booth #2309.
Presentations Pending
(*http://www.eshow2000.com/toxexpo/toxwelcome.cfm*).

57th ASMS Conference on Mass Spectrometry
May 31-June 4, 2009
Philadelphia, PA. **Posters pending.**
(*http://www.asms.org/*).

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not adequately retained under several isocratic or gradient conditions. Although more method development may have led to successful HILIC methods, with respect to timelines we decided to move-on and try ion pairing methods. Our first method was to attempt to obtain adequate peak shape and retention using trifluoroacetic acid. Unfortunately, not all of the compounds were well retained with the TFA additive (Fig. 2). The next method was to add HFBA to the mobile phase. With the addition of the HFBA, all of the aminoglycosides were well retained and gave excellent peak shape (See Figure 3.). Notably, the ESI/MS signal was not diminished with the addition of the HFBA. More importantly, the improvement in peak shape actually led to a better overall LC/MS/MS response for the HFBA methods compared to the HILIC methods (with no addition of HFBA).

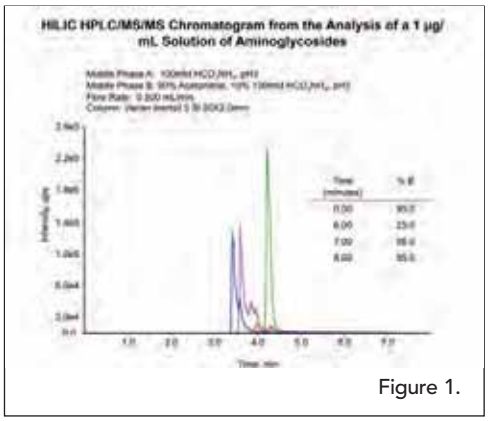


Figure 1.

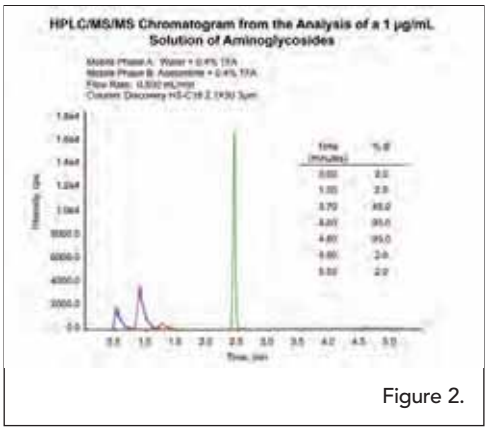


Figure 2.

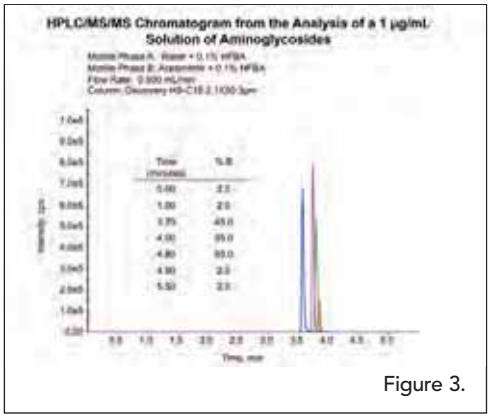


Figure 3.

Although past research suggests that the use of ion-pair reagents should be avoided based on the possibility of a diminished ESI/MS signal, the above data demonstrates that the overall effect to the LC/MS/MS analysis may be a positive influence. This is just the empirical nature of LC/MS/MS, "you can theorize all you want but you never know until you try". Therefore, more recently researchers are reinvestigating the use of ion-pair LC/MS methods for the analysis of polar, basic analytes (4) .

At Alturas, once the LC/MS method with the HFBA addition was refined, bioanalytical methods were developed for the analysis of the aminoglycosides from rat and dog plasma and tissue. The methods were shown to be sensitive, accurate and precise. To date over 1000 samples have been analyzed using the LC/MS/MS method with the addition of HFBA for the analysis of aminoglycosides. For more information regarding the assays developed and validated at Alturas, please visit our website at **www.alturasanalytics.com**.

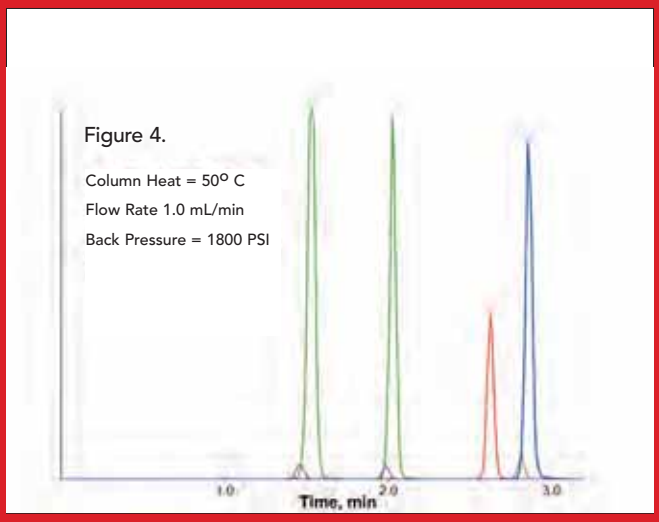
References:
1) F. Kuhlmann et. al. J. Am. Soc. Mass Spectrom. 6 (1995) 1221-1225.
2) S. Needham et. al. J. Chromatogr. B: Biomedical Applications 748 (2000). 77-87.
3) M. Ariffin et. al. J. Chromatogr. A 842 (2006) 91-97.
4) R. Seifar et. al. J. Chromatogr. A 1187 (2008) 103-110.

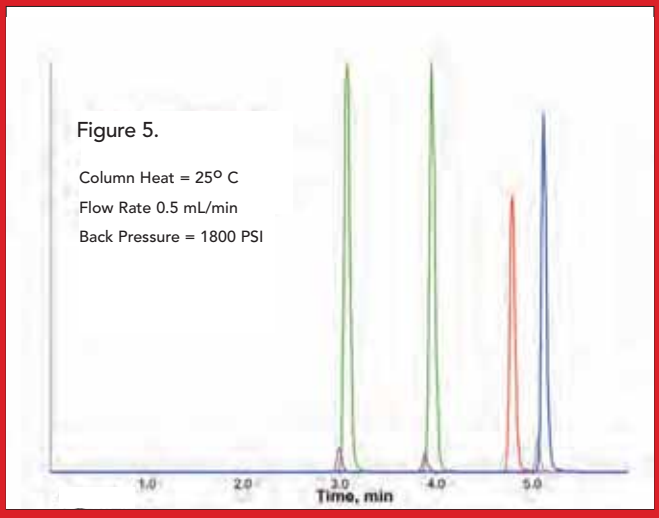
Current practices in fast and ultra-fast chromatography using sub-2 µm particles

ASMS 2008 LC/MS users workshop summary:

At ASMS 2008 in Denver, Dr. Shane Needham from Alturas Analytics, Inc. co-chaired the combined LC/MS and Pharmaceutical Interest Group Workshop with Dr. Chris Petucci from Wyeth. The workshop was a discussion led by brief presentations from leaders in the LC/MS and pharmaceutical industry. The presentations included a theoretical presentation, ultra fast LC/MS in Drug Discovery, ultra fast LC/MS in regulated bioanalysis and ultra high performance LC/MS separations for proteins and peptides. The overall summary was that improved speed, sensitivity, resolution and efficiency can be obtained with the use of sub 2µm packing particles. However, due to the need for special equipment and decreased column longevity, the industry has not fully adopted this technology. Additionally, the reduction in performance due to extra column effects and other issues related to "real world" samples often minimize the improved performance of sub 2µm particles compared to 3-5µm particles. The presenters also showed data that with careful attention to extra column effects and minimization of extra column volumes that similar performance can be obtained with 3µm particles. Several of the presentations also showed comparable sub 2µm particle performance on 3-5µm particles by simply heating the mobile phase to >50 °C. It should be noted that the majority of the sub 2µm methods currently in-place have the column heated to >50 °C to improve performance and reduce the column back pressure. The presenters summarized that improved performance is usually obtained from optimized systems and not just smaller particles. Also discussed was the current use of 2.5µm Fused Core™ particles where researchers have found comparable performance to sub 2µm particles yet at only ½ the back-pressure. See Figures. 4 and 5 that show the improvement in the speed and resolution for the LC/MS/MS analysis of a drug and three metabolites and their stable labeled internal standards from human plasma by the use of a column heater set to 50 °C.

For more information regarding Alturas Analytics visit our website at **www.alturasanalytics.com**.





STAFF PROFILE: Matthew Pollard

Matthew Pollard serves as a Senior Scientist at Alturas Analytics. Matt's primary responsibility is acting as Study Director and Principal Investigator, though he acts as Alturas' SOP Manager as well. He also manages the peer-based QC directive, and oversees all in-house instrumentation maintenance, service, troubleshooting and repair.

As a Study Director and Principal Investigator, Matt is committed to clear and responsive communication with the study Sponsor, ensuring that the Sponsor is informed on study progress and findings. As a QC manager, Matt ensures that validations and studies are performed according to Alturas' SOPs, the Bioanalytical Protocol, and to current GLP Guidelines and established industry practices.

In addition to project management, Matt assumes responsibility for instrument maintenance and repair. His extensive background with instrumentation theory, design, troubleshooting and repair has minimized Alturas' instrument down-time. Matt acquired his instrumentation skills while building mass spectrometers, ion mobility spectrometers, and infrared spectrophotometers during his graduate and post-graduate career.

Matt has a B.S. in Chemistry from Cal Poly, San Luis Obispo and a Ph.D. in Physical Chemistry from the University of Idaho. Prior to grad school, Matt was a bench chemist in a GMP laboratory where he developed HPLC and GC methods in support of the manufacturing process. He came to Alturas in November 2006 after completing a 2-year post doctorate, during which he developed new mass spectrometers for the detection of warfare agents, biologics, and explosives.

Matt lives in Moscow, Idaho with his wife Jen (also a Ph.D. in Chemistry) three children, 9 chickens, and an old dog, J.J. Matt enjoys bicycling, rock climbing, backpacking, and mountaineering.