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The Continuing Search for a General On-line Extraction Method for LC/MS/MS Sample Preparation

Tazeen Shahid & Robert T. Cass
Theravance, Inc., South San Francisco, CA 94080

901 Gateway Blvd.
South San Francisco, CA 94080
Phone (650) 808.6000
FAX (650) 827.8690
tshahid@theravance.com

Overview

- Using on-line extraction with column switching increases bioanalytical throughput.
- Depending on the application, online extraction method development should include evaluation of more than one manufacturers bonded phase.
- Depending on the application, on-line extraction method development should include evaluation of more than one column dimension.
- Extraction column efficiency is bonded phase as well as compound class specific.

Abstract

The need for lower limits of quantitation (LOQ) as well as higher throughput, led us to investigate on-line extraction method development and optimization to develop a general guideline for sorbent choice based on an analyte's physicochemical properties. We optimized LC/LC/MS/MS conditions for compounds that are commonly assayed from biological matrices. We chose a variety of classes including: β-lactams, anesthetics, opiates and muscarinic receptor antagonists. We evaluated different bonded phases ranging from C-18 to aminopropyl on silica and/or polymeric supports, as well as varying column dimensions and manufacturers for each chemical class. A total of 10 different extraction columns were tested, including commercially available and custom packed columns. Across the different chemical classes and columns evaluated, compound dependent extraction efficiencies were observed. These observations reinforce the known fact that every compound has a unique affinity for a certain type of sorbent. Of the 20 compounds investigated for on-line extraction from biomatrices, we have discovered the claim "general" or "generic" to be relative to the class of compound being analyzed. Although optimizing for these online extraction methods requires more development time, the increased extraction efficiency coupled with the decrease in the analysis time results in an overall five-fold time savings with respect to our traditional off-line sample preparation methods.

Introduction

The pursuit of a general method to perform on-line extraction of raw plasma samples for quantitative purposes is an on-going exercise in our laboratory (1). Although some groups have previously reported this achievement for their specific compound set (2)(3), for a laboratory that deals with many different chemotypes, one type of column is not the panacea for optimal extraction of multiple analytes from a biological matrix. As pharmaceutical companies become more competitive, the number of compounds that are synthesized by medicinal and/or combinatorial chemists have increased significantly. Every compound needs to go through numerous analyses before decisions can be made. To speed up the decision making process, results need to be ready in an efficient timeframe. High-throughput laboratories should be continuously updating their techniques to improve their turnaround time without compromising the quality of the results. The amount of time that is spent on traditional sample preparation techniques (Protein precipitation, Liquid Liquid Extraction etc. Figure 1) is the rate limiting factor as to how many samples can be analyzed. To reduce this time most companies turn to capital intensive hightech automation. Our approach was to investigate a cost effective, simple yet efficient technique that would enhance the quality of work done in our lab.

Figure 1: On-Line Extraction-Sample Preparation

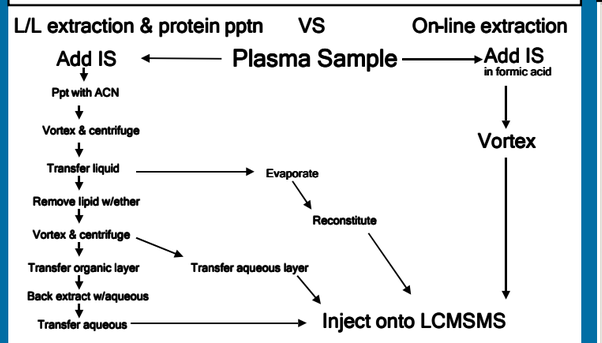

Figure 2: Representative TIC & XIC of one class on one phase

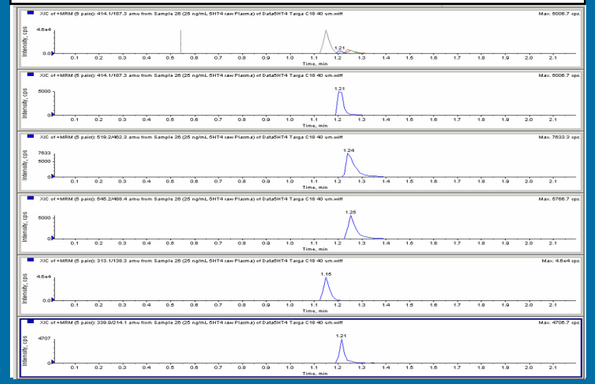

Figure 3: Representative Standard Curves in Crashed and Raw Plasma

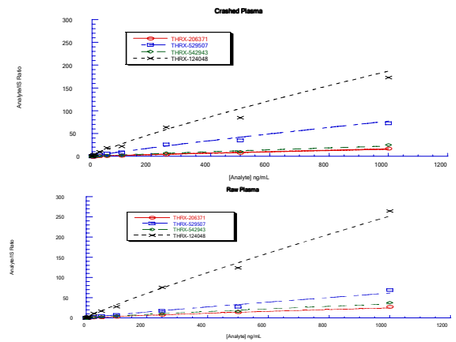

Figure 4: Extraction Columns vs Class of Compounds

RESULTS
Crashed Plasma

	Best	2nd	3rd	4th	5th	6th	7th	8th	9th
For 5HT4 compounds									
THRX-206371	Metasil Basic	Varian FOCUS	C-18 TARGA	Strata-X/YMC Basic	NEXUS 30x1.0	Cohesive Cyclone	APS Hypersil		
THRX-529507	Metasil Basic	YMC Basic	C-18 TARGA	Varian FOCUS	APS Hypersil	Strata-X	NEXUS 30x1.0	Cohesive Cyclone	
THRX-542943	Metasil Basic	C-18 TARGA	Strata-X/Varian FOCUS	YMC Basic	APS Hypersil	NEXUS 30x1.0	Cohesive Cyclone		
THRX-124048	Varian FOCUS	C-18 TARGA	NEXUS 30x1.0	YMC Basic/Strata-X	Metasil Basic	Cohesive Cyclone	APS Hypersil		
For NSMR1 compounds									
THRX-891619	C-18 Polymeric	Targa C18	Cyclone/Metasil	Focus	YMC Basic	Strata-X	Aps Hypersil	Cohesive C18	
THRX-654755	Nexus	Targa C18	C-18 Polymeric	Cohesive Cyclone	YMC Basic	Strata-X	Metasil Basic	APS Hypersil	Varian FOCUS
For Anesthetics									
Bupivacaine	Targa C-18	Varian FOCUS	Nexus 30x1.0	YMC Basic	Metasil Basic	Strata/Cyclone	Cohesive C-18	APS Hypersil	
Prilocaine	Targa C-18	Varian FOCUS	Nexus 30x1.0	Strata-X	Cohesive Cyclone	Metasil Basic	Cohesive C-18	YMC Basic	APS Hypersil

Raw Plasma

	Best	2nd	3rd	4th	5th	6th	7th	8th	9th
For 5HT4 compounds									
THRX-206371	Targa C-18	YMC Basic	Varian FOCUS	Cyclone/APS	Nexus 30x1.0	Strata-X			
THRX-529507	Targa C-18	YMC Basic	APS Hypersil	Strata-X	Varian FOCUS	Metasil Basic	Nexus 30x1.0	Cohesive Cyclone	
THRX-542943	Targa C-18	YMC Basic	Strata/Metasil	APS/FOCUS	Nexus 30x1.0	Cohesive Cyclone			
THRX-124048	Cohesive Cyclone	Nexus 30x1.0	Targa C18	YMC Basic	APS Hypersil	FOCUS	Metasil Basic	Strata-X	
For NSMR1 compounds									
THRX-891619	Targa C-18	Nexus	Cyclone/YMC	Metasil Basic	FOCUS	Strata-X	Cohesive G-18	APS Hypersil	
THRX-654755	Targa C-18	Nexus	Cyclone	YMC Basic	Metasil Basic	FOCUS	Strata-X	Cohesive G-18	APS
For Anesthetics									
Bupivacaine	Targa C18	Cyclone	Nexus	YMC Basic	FOCUS	Strata-X	Metasil Basic	Cohesive C18	APS
Prilocaine	Cyclone	Nexus	Targa C-18	Metasil Basic	Strata-X	FOCUS	Cohesive C-18	YMC Basic	APS

Materials and Methods

Standard Curve Preparation in Raw Plasma

45 μL of Blank Plasma
+ 5 μL of Standard curve
+ 5 uL of Internal Standard (100 ng/mL)
+ 250 μL of 0.1% FA in water

Standard Curve Preparation Crashed Plasma

45 μL of Blank Plasma
+ 5 μL of Standard curve
+ 5 uL of Internal Standard (100 ng/mL)
+ 400 μL of Acetonitrile to precipitate the proteins
- 450 μL of Supernatant
dry down under nitrogen; reconstitute with 300 μL of 5% Methanol

Analytical Column

Higgins TARGA, C18 (20 x 2.0mm, 5 μm) (Higgins Analytical, Mountainview CA)
Inject 10 μL

Representative Method

Analytical Column: Initial 0.5 minute hold followed by a linear gradient of 10-90 % B over 1.1 minutes
Flow rate was 500 - 1000 μL/minute depending on class & column
Mobile Phase A: 0.1% Formic Acid in Water
Mobile Phase B: 0.1% Formic Acid in Acetonitrile

Extraction Column: Sample Loaded onto the column during the initial hold of 0.5 minutes
Flow rate dependent on the dimensions of the extraction column
Loading/wash Solvent: HPLC grade Water

Instrumentation and Hardware

PE Sciex API 3000 using TIS
HTS PAL LEAP Autosampler
Shimadzu Pumps and System Controller
Data processed with Analyst version 1.3

Results

- Separation coupled with online extraction helps focus analytes and give better resolution (Figure 2).
- Peak shapes for all the extraction columns were symmetric, with the exception being the cohesive C-18, which did not give a clean chromatogram (Figure 2).
- For overall extraction efficiency and selectivity, the TARGA C-18 performed well with all the classes followed next by the Basic columns and the Varian FOCUS and Nexus (Figure 4).
- The performance of the Basic and FOCUS columns was class dependent. The robustness of these columns is yet to be tested in our lab (Figure 4).
- For overall robustness, the Strata-X and the Oasis HLB have been loaded with more than a thousand injections and shown to give excellent resolution and recovery. For normal screening usage these guarantee a long life.
- Curves in raw plasma gave steeper slopes than curves in crashed plasma for every extraction column tested (Figure 3).
- Both matrices had a differential effect on the IS compared to the analyte. This will be further investigated in our lab by comparing structural analogs of the analyte as IS.

Discussion/Conclusions

Our preliminary results and experiments reinforce the theory that there isn't a general method that would work with every compound that goes through a PK screen. Although the TARGA C-18 gave the overall best result, other extraction columns such as the Basics and the FOCUS can work well for certain compounds if low LOQs are required. These results have opened up options for chromatographic cleanup and separation.

Mismatching the analytical and extraction column bonded phases introduces degrees of differential cleanup and separation. By taking advantage of this orthogonality, analysts have the ability to tailor their methods for their specific analyte(s) of interest with minimum effort spent on sample preparation and method development. (4)

Figure 4 shows a general guideline to help in selecting the best column for a particular compound and then working through the remaining columns in the list.

Further work such as optimization of chromatographic parameters and sample environment is being carried out on these columns. More column phases will be evaluated and added to the list in Figure 4.

In conclusion:

- Employing on-line extraction as the primary form of preparation and analysis for screening PK samples has increased throughput five fold.
- Different manufacturers bonded phases were tested and show very significant differences in column behaviors with similar sorbent chemistry (e.g. TARGA – C18 vs Cohesive C-18)
- Different compounds within a particular class behaved differently on similar sorbents proving extraction column efficiency to be phase as well as compound and class dependent.

References

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