Utilization of on-line dilution techniques to improve quantitative and qualitative LC/MS^n analyses for drug discovery.

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Overview

• On-line dilution of samples containing high levels of organic solvent were evaluated using LC mixers for drug discovery LC gradient conditions.

•Ten in-house compounds were evaluated in solution and plasma extracts at different injection volumes.

• *In vitro* metabolic profile of one compound was examined at large injection volumes.

• The use of LC mixers was found to improve peak shape and enabled the injection of higher volumes than usual.

Introduction

Generic assay conditions are the standard in drug discovery LC/MS^n analyses.[1] Typical assay conditions involve the precipitation of an *in vivo/vitro* sample with an organic solvent followed by ESI LC/MS/MS analysis using a reverse phase gradient. Samples need to be frequently diluted further after precipitation in order to reduce the organic content of the sample thus ensuring adequate chromatography. This may not be an option given the sensitivity requirements of the assay.

On-line dilution is an excellent alternative as it increases sensitivity by eliminating the offline dilution step and improves chromatography by enabling polar compounds to "trap" at the head of the column prior to gradient elution. In the past, on-line dilution has been performed in two ways:

1. Increasing the tubing volume between the injector and column (e.g., reverse flushing sample through large sample loop)[2]
2. Using an aqueous make-up from another pump.[3,4]

Another option for 1 is the use of an LC mixer instead of a tubing volume increase. The objective of this work is to evaluate the use of LC mixers for on-line dilution of precipitated plasma and hepatocyte extracts using discovery LC/MS/MS conditions.

Experimental

Design

• 10 proprietary AZ compounds of various polarities were pooled into two groups for analysis.

• Each group was prepared in 3:1 ACN:water (0.1% FA total) and extracted in rat plasma by 3:1 0.1% FA in ACN.

• Group 1 and 2 solutions were injected in increments from 10-50µL to assess the maximum amount of injection volume possible to ensure peak integrity for most of the analytes (4/5).

• Group 1 and 2 plasma extracts were injected at 10µL assay volumes and maximum solutions volumes.

• *In vitro* samples for one compound were precipitated by 1:1 0.1% FA in ACN and analyzed with 10µL and 100µL injections.

• The following dilutions conditions were tested:

1. no dilution
2. LC in-line mixer bypass (i.e., 50µL in-line mixer removed from LC to in between injector and column)
3. 50 µL sample loop dilution
4. LC mixers @ 50, 150 and 250µL volumes (250µL mixer was used only for profiling injections).

Note: *ASI HyperShear™* LC cartridge mixers were used.

Figure 1: Assessment of chromatography using solutions with 10µL injection volumes

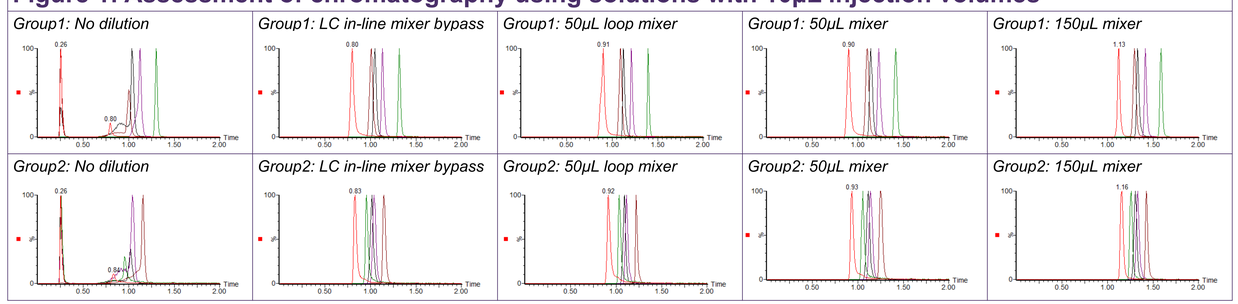


Figure 2: Maximum solution injections (4/5 pass)

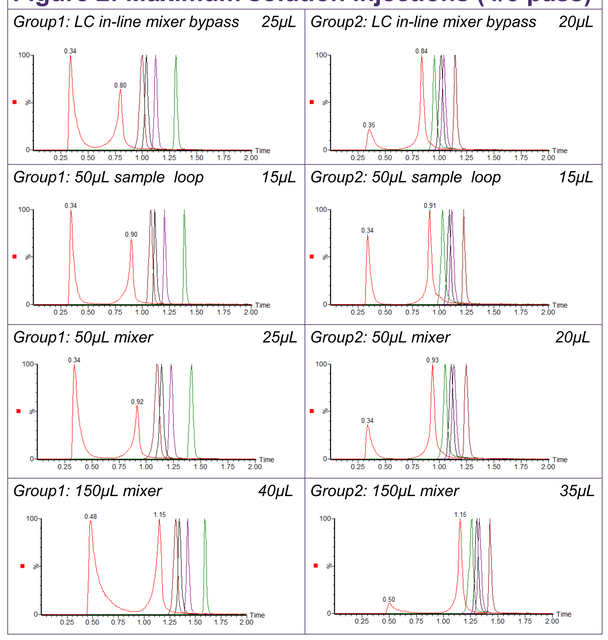
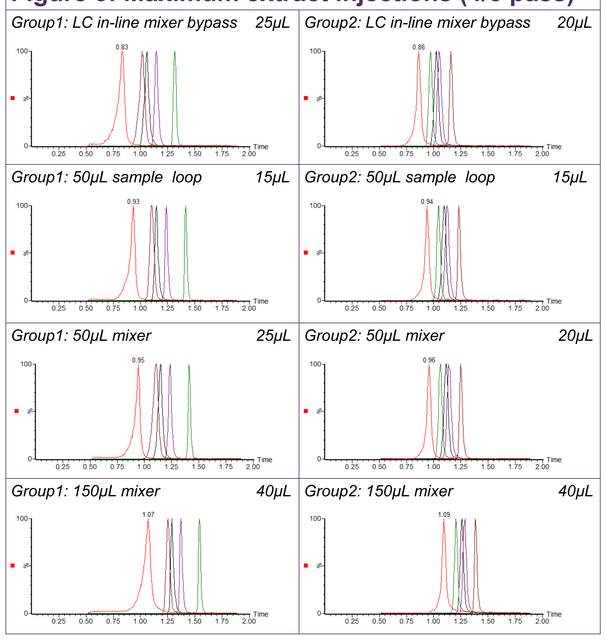


Figure 3: Maximum extract injections (4/5 pass)



Dilution configurations

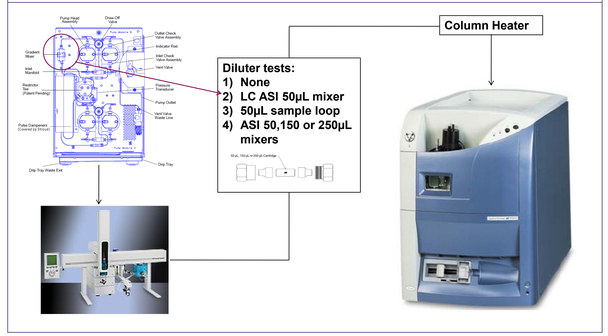
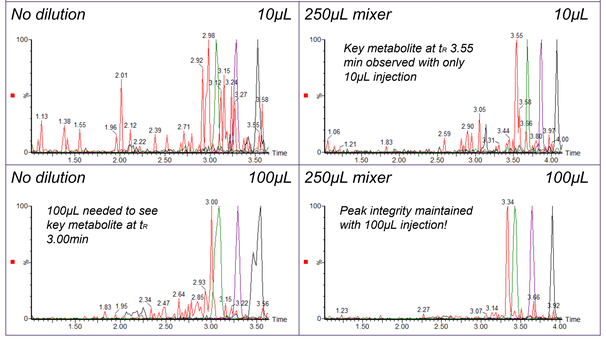


Figure 4: *In vitro* metabolite profiling injections



Experimental (cont.)

LC/MS/MS Conditions

• MS: Waters Quattro Premier™

• LC: Waters 2777 injector with Waters 1525µ binary pumps

• Column: Thermo HyPurity 50x2.1mm, 3µm @50ºC

• Quantitative gradient: runtime 2.50 min

Time (min)	% 0.1% FA in H2O	% 0.1% FA in ACN	Flow (µL/min)
0.00	95	5	750
2.00	5	95	750
2.01	95	5	750

• In vitro profiling gradient: runtime 9.00min

Time (min)	% 0.1% FA in H2O	% 0.1% FA in ACN	Flow (µL/min)
0.00	95	5	750
1.00	95	5	750
6.00	45	55	750
6.01	5	95	750
7.00	5	95	750
7.01	95	5	750

Results

• Chromatography was not acceptable in solution without any on-line dilution. (Fig. 1)

• Chromatography was acceptable for all compounds in solution using all diluters with 10µL injection volumes. (Fig 1)

• Only 5µL more volume could be injected with the loop dilutor in solution and plasma extracts.

• Using a 50µL mixer, either from the LC or added externally, enabled the increase of the typical injection volume to 20-25µL for most compounds in solution and extracts. (Fig 2-3)

• Using a 150µL mixer enabled another increase of the injection volume to 35-40µL in solutions and extracts.

• Using a 250µL mixer enabled the injection of 100µL for *in vitro* profiling without compromising peak shape integrity. (Fig 4)

Conclusions

• Using LC mixers enables the injection of higher than normal volumes for samples with high organic content.

• Even though dilution through a sample loop allows adequate dilution at 10µL, it does not increase injection volume as significantly as with the use of LC mixers.

• Moving the in-line LC mixer between the injector and column provides the simplest method to improve chromatography and increase injection volumes.

• The largest increases in volume result from the use of large volume LC mixers but the volume is limited by the speed necessary for the analysis. A volume mixer too large will result in an unacceptable LC gradient delay.

• Large volume mixers are ideal in metabolite profiling assays where gradient delay time and flow rates are not an issue.

• This *simple low cost* technique is amendable to other scenarios where samples contain high amounts of organic solvent (e.g., SPE elution extracts, DBS sample extracts, etc.)

References

1. Korfmacher WA, Using Mass Spectrometry for Drug Metabolism Studies, 2004.
2. Hendricks et al, 55th ASMS Conference, 2005.
3. Li et al., *Rapid Commun. Mass Spectrom.* 2010; 24:2575–2583.
4. Liu et al., *Journal of Chromatography A*, 2008; 1198–1199 :87–94.

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