Examining the Roles of Species, Solubility, and Ionization in Contributing to Divergence in a Series of Structurally Similar Small Molecules

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Introduction

Early-stage drug discovery studies often evaluate a large number of structurally similar novel compounds, varying by as little as a singular functional group or atom, with the goal of identifying promising candidates to move on to later-stage development. Common sense and practice dictate that these closely related compounds will behave relatively similarly in their bio-analysis, leading to the development of a singular method designed to rapidly quantify such candidates without the need for full method development of each analog.

These initial methods, usually developed in rodents during early-stage development, look to take into account, and correct for, any bio-analytical issues observed that might impede accurate quantitation, such as divergence, potentially caused by, but not limited to, insolubility, non-specific binding (NSB), endogenous interference, or compound instability. However, we have discovered that as some candidates progress from rodent studies to more complex species, such as canine and primate, well-established methodologies dealing with the bio-analytical issues of the aforementioned candidates can become obsolete, as problems of divergence believed to have been solved for with the initial method development reemerge as hurdles.

When dealing with issues of divergence, such as those mentioned above, some possible solutions include: LC separation from any endogenous interference or manipulation of the extraction conditions to stabilize the compound of interest or eliminate the interference. However, LC separation of endogenous interference is not always possible, especially with highly hydrophilic compounds that do not allow for long retention or separation on conventional columns.

Therefore, in this examination we look to take a selection of analog compounds from a larger catalog, define the type(s) of divergence observed in each species, determine the root cause(s) for each, and thereby create a robust method designed to correct for the observed issues across all species.

Methods

EXTRACTION METHOD

Due to the desire for a rapid, cost effective extraction when performing early-stage discovery quantitation, only protein precipitation (PPT) and a 3 minute LC-MS/MS run time were considered.

TEST COMPOUNDS

In order to evaluate this trend, we chose to look at five structurally similar small molecules with slightly varying functionality off of a commonly shared backbone, made up of a Benzimidazole and an Indazole, that are part of a larger catalog in development at Biogen Idec. All compounds in this catalog have been shown to exhibit divergence across all tested species and are highly hydrophilic, which means that a solution by means of chromatographic separation is difficult and unlikely. A 10-point standard curve (front and back end) and 6-level QCs (n=2) were run to determine divergence.

VARIABLES: SPECIES, SOLUBILITY, NON-SPECIFIC BINDING, AND IONIZATION

Compounds were run in cassette against a generic Internal Standard (IS) of similar retention in mouse, rat, dog, and monkey plasma with varying Plasma:Acetonitrile ratios (1:3 and 1:6), with and without formic acid (FA) added (1:1 Plasma:5% FA in water) either before or after the plasma, in untreated and silanized plates and tubes, and under APCI and ES ionization.

EVALUATION OF ENDOGENOUS INTERFERENCE

A post-column infusion of the cassette against extracted blanks for each species and extraction condition was conducted to evaluate endogenous interference at the retention time of each analyte.

LC-MS/MS CONDITIONS

LC Column: Phenomenex Synergi™ Hydro-RP 50 x 2.0mm - Mobile Phase A: 0.1% FA in 95:5 Water:Acetonitrile - Mobile Phase B: 0.1% FA in 50:50 Methanol:Acetonitrile - LC Run: 3 minute gradient from 0% B to 100 % B in 1.4 minutes - Platform: AB Sciex API 4000™, 2 Shimadzu LC10-ADvp Pumps, LEAP Autosampler, Analyst® 1.4.2 - All compounds were analyzed under positive mode

TABLE 1.
LC-MS/MS Transitions (m/z)

LC-MS/MS Transitions (m/z)		
Compound ID	APCI	ESI
Compound A	509.5 → 427.3	509.5 → 427.4
Compound B	496.4 → 345.3	496.4 → 411.4
Compound C	526.4 → 444.4	526.5 → 441.4
Compound D	578.5 → 345.2	578.5 → 493.4
Compound E	513.5 → 356.3	513.5 → 356.3
Carbutamide (IS)	272.1 → 156.1	272.1 → 156.1

Results and Discussion

GENERIC PPT - DIFFERENT DIVERGENT TRENDS

First, a generic PPT (1:6 Plasma:Acetonitrile and 1:3 Plasma:Acetonitrile) was conducted for each species and run under both APCI and ES ionization. All compounds diverged in all species, with both PPT ratios and ionizations, except Rat plasma, 1:3 PPT, APCI. However, the 1:6 PPT and the 1:3 PPT showed two markedly different divergent trends. The 1:3 PPT showed a more classic divergence, while the 1:6 PPT showed a trend that also included an upward bending aspect.

FIGURE 1. Divergent curve: Compound A, Monkey plasma, 1:3 PPT, ES

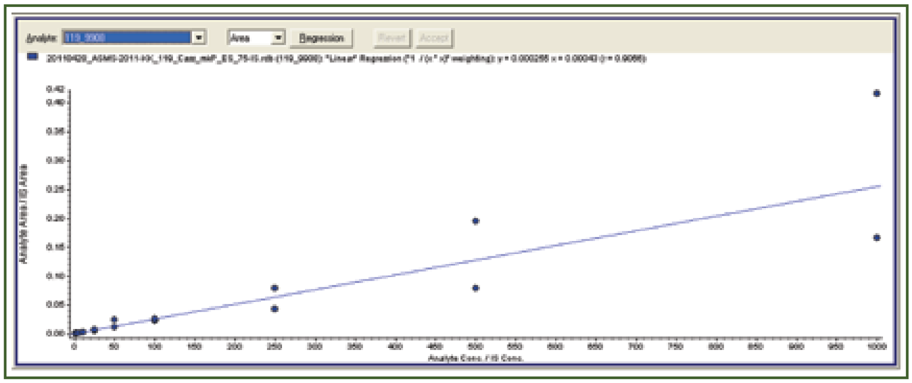
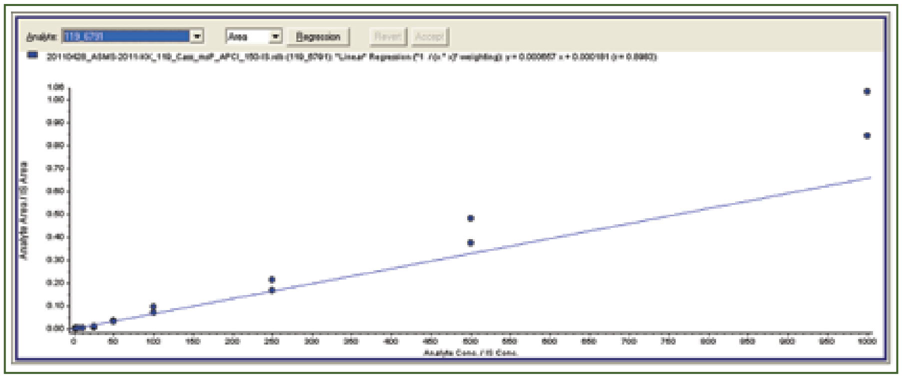


FIGURE 2. Divergent, upward-bending curve: Compound E, Mouse plasma, 1:6 PPT, APCI



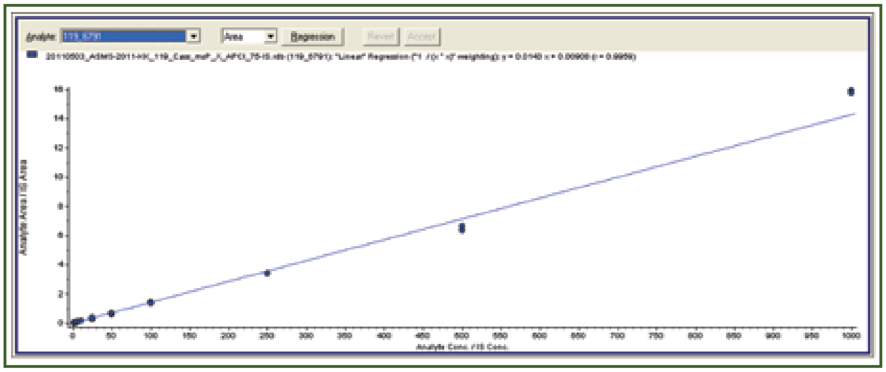
This led us to conclude that we were dealing with multiple issues, one of which was likely low solubility in organic due to the hydrophilic nature of the compounds, and that we would need to maintain as high an aqueous content as possible.

EXPLORING NON-SPECIFIC BINDING

Next, we tested the 1:3 PPT extraction in silanized plates and tubes to determine whether NSB was responsible for the observed divergence. Mouse and Rat plasma showed no divergence under either APCI or ES ionization, however Dog and Monkey still diverged under both. Therefore something more than just NSB to the plastic was causing the divergence, potentially an endogenous interference or binding to something present in the Monkey and Dog plasma that was not present in the Mouse or Rat plasma.

Results and Discussion (continued)

FIGURE 3. Successful curve: Compound E, Mouse plasma, 1:3 PPT, APCI, Silanized (r2=0.9959



ASSESSING ENDOGENOUS INTERFERENCE THROUGH POST-COLUMN INFUSION

An examination of Post-column infusions of the compounds with extracted blanks under each extraction condition showed considerably more interference in the retention window (1.57 – 1.84 min) for the 1:3 PPT than the 1:6 PPT. The 1:3 PPT in the silanized plates also showed considerably less interference than the 1:3 PPT, but slightly more than the 1:6 PPT.

FIGURE 4. Compound A (RT – 1.60 min), Monkey plasma, 1:3 PPT, ES

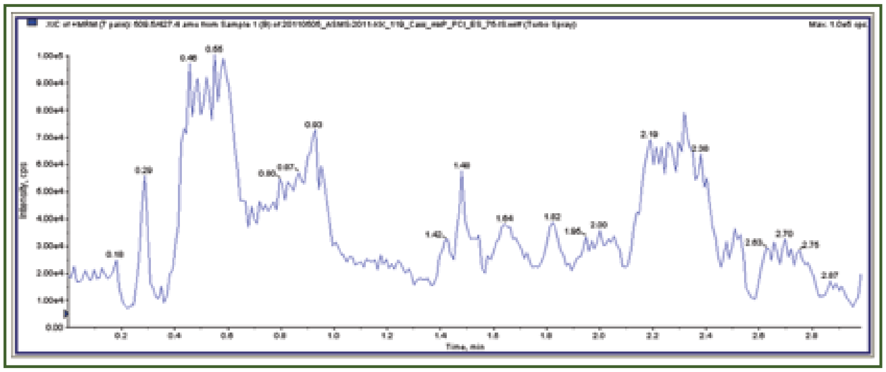
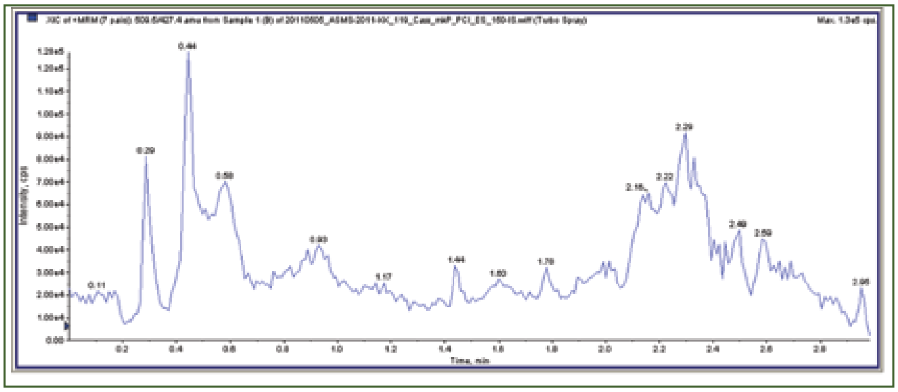
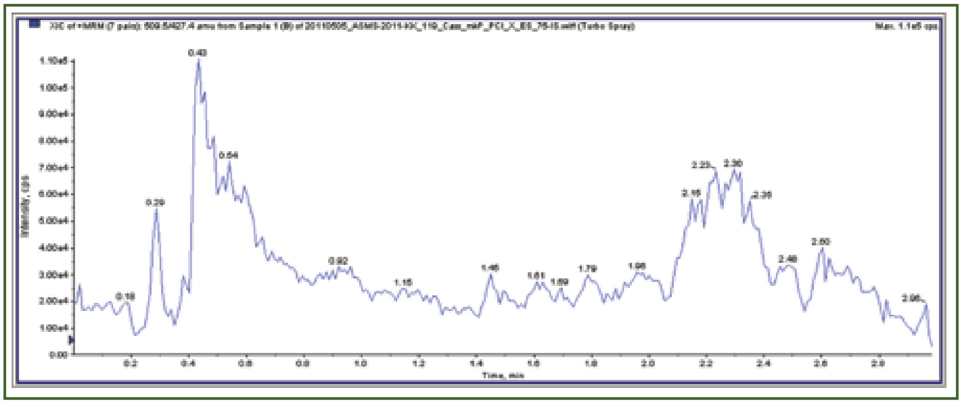


FIGURE 5. Compound A (RT – 1.60 min), Monkey plasma, 1:6 PPT, ES



Results and Discussion (continued)

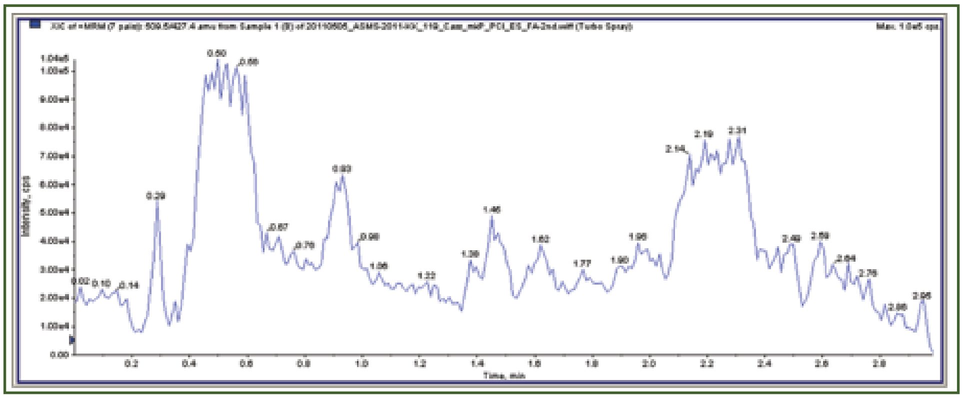
FIGURE 6. Compound A (RT – 1.60 min), Monkey plasma, 1:3 PPT, ES, Silanized



REMOVING ADDITIONAL INTERFERENCE BY ADDING ACID

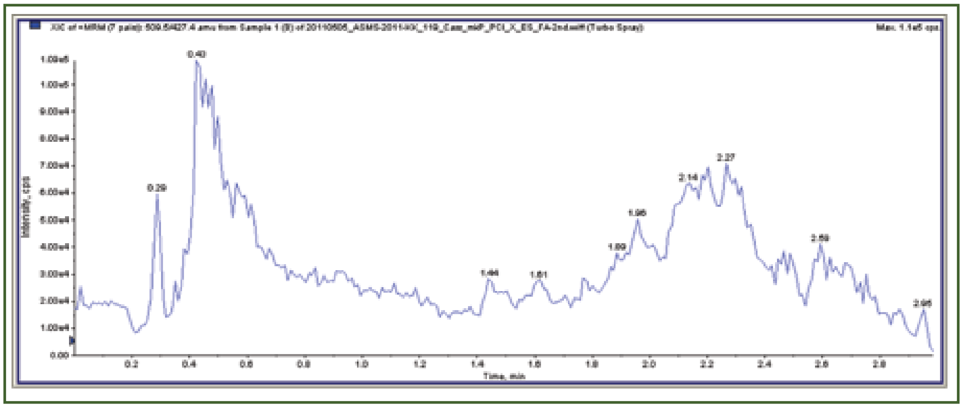
Due to the hydrophilicity of the compounds, but the need to precipitate additional background likely caused by residual protein and other endogenous interferences, we decided to add a 1:1 ratio of 5% formic acid in water to the plasma aliquot and to maintain a 1:3 Aqueous:Organic PPT. This should facilitate additional precipitation, and could also help prevent any plasma binding by the compounds. We varied the timing of the addition of the acid (either before or after addition of the plasma to the plate) and extracted in both plastic and silanized plates and tubes. What we found was that the addition of the acid did not actually reduce the endogenous interference significantly in the plastic plates, or improve upon the previously achieved removal in the 1:3 PPT silanized plates.

FIGURE 7. Compound A (RT – 1.60 min), Monkey plasma, 1:3 PPT + FA, ES



Results and Discussion (continued)

FIGURE 8. Compound A (RT – 1.60 min), Monkey plasma, 1:3 PPT + FA, ES, Silanized



COMPOUND STABILIZATION WITH FORMIC ACID

Although the addition of formic acid did not seem to improve removal of interferences, combined with the silanized plates, it did seem to stabilize the compounds, likely preventing plasma binding and providing solid linear results. Addition of acid after the plasma provided linearity in Mouse, Rat, Dog, and Monkey under both APCI and ES ionization. Also, addition of acid after the plasma in plastic plates provided linearity in Rat, Dog, and Monkey under both APCI and ES ionization, but not Mouse. However, it was observed that by introducing the formic acid to the plates first, before the plasma, regardless of plate type or ionization, all species showed divergence. This is likely caused by an interaction between the coating and the formic acid so that the coating is no longer viable as a barrier between the compound and the plastic, and so that the formic acid is no longer available to provide a barrier against plasma binding of the compound.

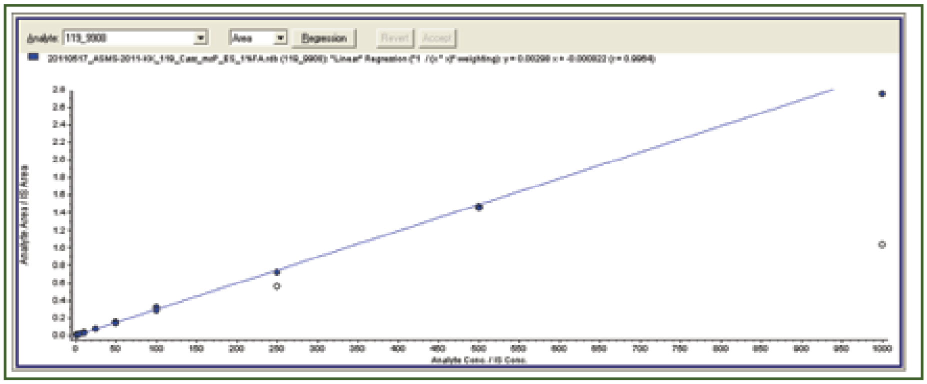
		PLASTIC PLATES				SILANIZED PLATES			
		Mouse	Rat	Dog	Monkey	Mouse	Rat	Dog	Monkey
APCI	Acid 1st	Diverged	Diverged	Diverged	Diverged	Diverged	Diverged	Diverged	Diverged
	Acid 2nd	Diverged	Linear	Linear	Linear	Linear	Linear	Linear	Linear
ES	Acid 1st	Diverged	Diverged	Diverged	Diverged	Diverged	Diverged	Diverged	Diverged
	Acid 2nd	Diverged	Linear	Linear	Linear	Linear	Linear	Linear	Linear

Results and Discussion (continued)

PREVENTING PLASMA BINDING

A final examination was run to determine the role plasma binding plays in causing the compound divergence as opposed to binding to the plastic of the plates and tubes. Formic acid was added to blank Mouse plasma (which exhibited divergence across all conditions in untreated plates) to give a final concentration of 1%. This acidified plasma was then used to create the standard curve and QCs in untreated tubes and plates. The resulting runs showed complete linearity across all compounds, lending credence to the hypothesis that it is indeed plasma binding that is at the root of the divergence, and that variability in species (variability in plasma composition) determines the strength of the binding.

FIGURE 9. Successful curve: Compound A, Acidified Mouse plasma, 1:3 PPT, ES (r2=0.9964)



Conclusion

- The strong hydrophilic nature of this catalog of compounds is at the root of its linear quantitation issues, causing divergence due to insolubility and non-specific binding to plasma under generic PPT extraction conditions.
- The shape of a divergent curve can give you insight into what is actually causing its divergence.
- Timing when a modifier is added, either before or after the samples, as in this case the 5% Formic acid, can have a large affect on the effectiveness of it.
- Species, and more specifically greater numbers of proteins that appear in the plasma of higher species, can play a large role in the effectiveness of a method.
- Ionization source does not seem to play a prominent role in causing divergence in this category of compounds.
- Addition of acid to the plasma before compound introduction seems to prevent plasma binding.

Acknowledgements

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