A Strategic Approach for Dealing with Variable Adduct Formation & Irreversible Protein Binding for LC-MS/MS Applications

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Outline

In bioanalysis there can be *many* challenges, and *many* are not easy to overcome

Today, I am going to tell you a story about a situation where two such challenges were faced, and how I dealt with them.

The picture shows you how I felt, but let me tell you the story

The story starts…

- Analyte: ester, includes a long hydrocarbon chain
  - Hydrolyses under basic conditions
- Proposed therapy is as a pulmonary vasodilator
- Administered via inhalation
  - Target LLOQ of 25 pg/mL
- Pre-clinical studies
  - Sample volume available – 25µL

- Or, to put it another way…
  - A high sensitivity assay is required for a very hydrophobic and unstable entity with restricted sample volume

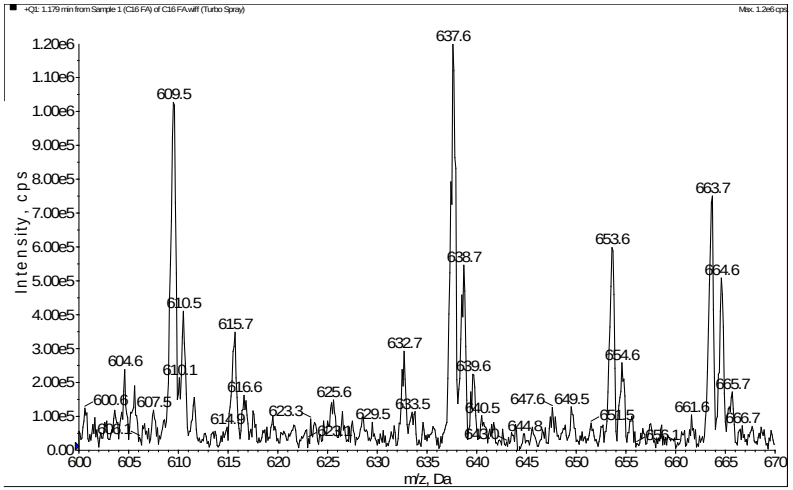
Overview

The two challenges to address:

- variable adduct ion formation
  - understand, eliminate, control?
- Irreversible binding with plasma proteins
  - is it the case?

1st Challenge: Variable Adduct Ion Formation

- The analyte carries a negative charge…
  - adduct formation with cations
- Sodium is a cation…
  - adducts are very stable
  - problematic for mass spec
- APCI
  - No adduct formation
  - Poor signal/sensitivity
- ESI
  - most abundant ion: $(M+Na)^+$
  - low abundance: $(M+NH_4)^+$



- $(M+Na)^+$ - Imprecise and inaccurate results
- $(M+NH_4)^+$ - Precise and accurate results, but low sensitivity

- Challenge – how to limit and control the $(M+Na)^+$ adduct, and generate the $(M+NH_4)^+$ adduct.

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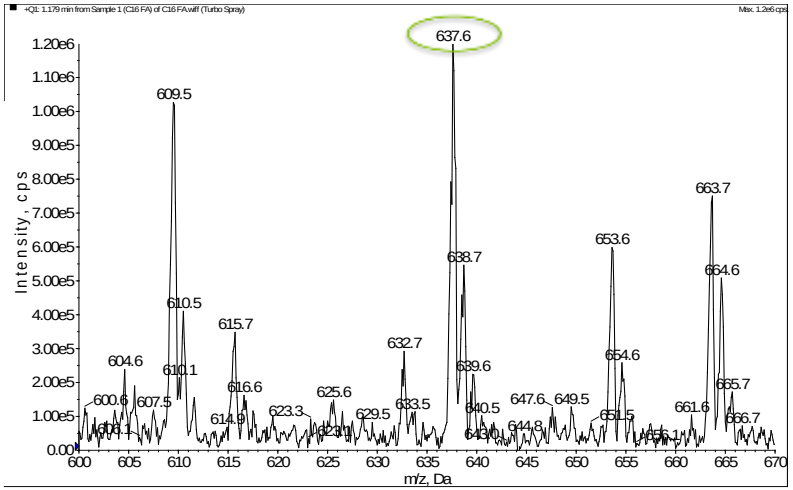

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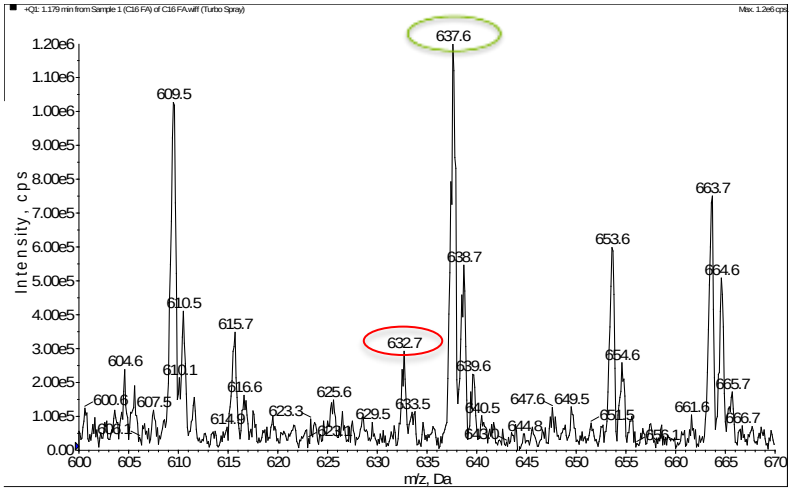

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1st Challenge: Variable Adduct Ion Formation

Control of Na^+ Adduct Formation and Sensitivity Enhancement – $(M+NH_4)^+$

- Consideration of the reaction potentials of components:
  - Na^+ strong positive
  - NH_4^+ weak positive
  - Analyte weak negative
  - TFA strong negative

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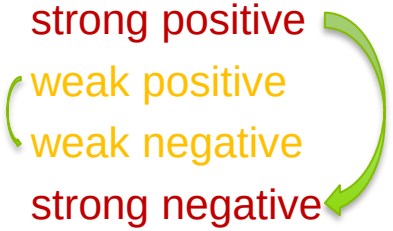

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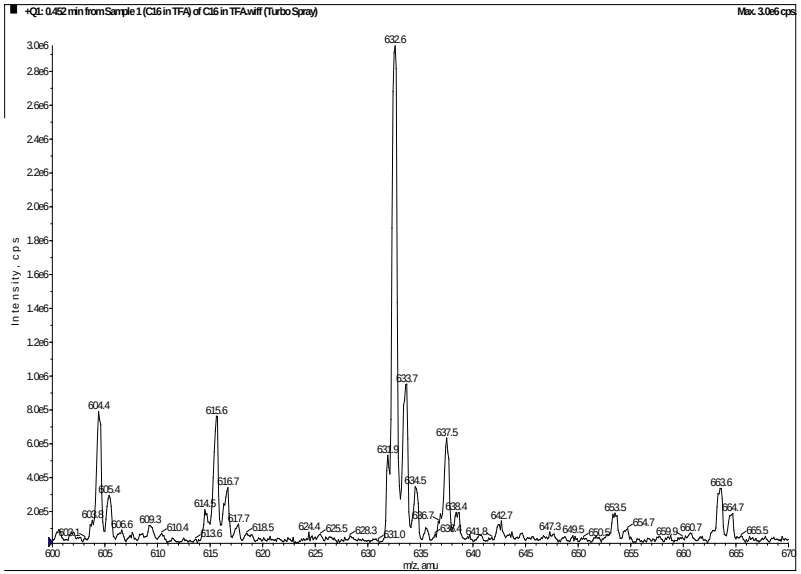
- 20 mM Ammonium Acetate for excess NH_4^+ ions
- Resultant CV at LLOQ: 4.8%
- Possible mechanism of action
  - Cation scavenger through ion-pair formation?
  - Other?



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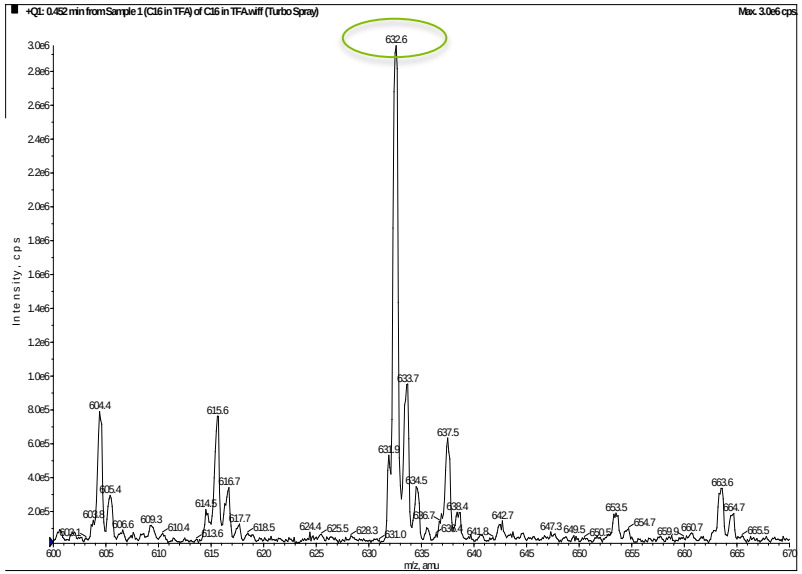

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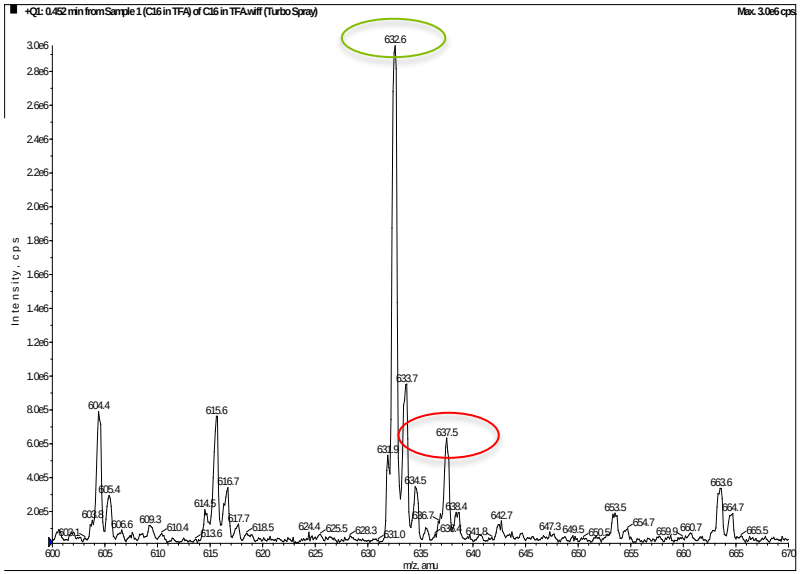

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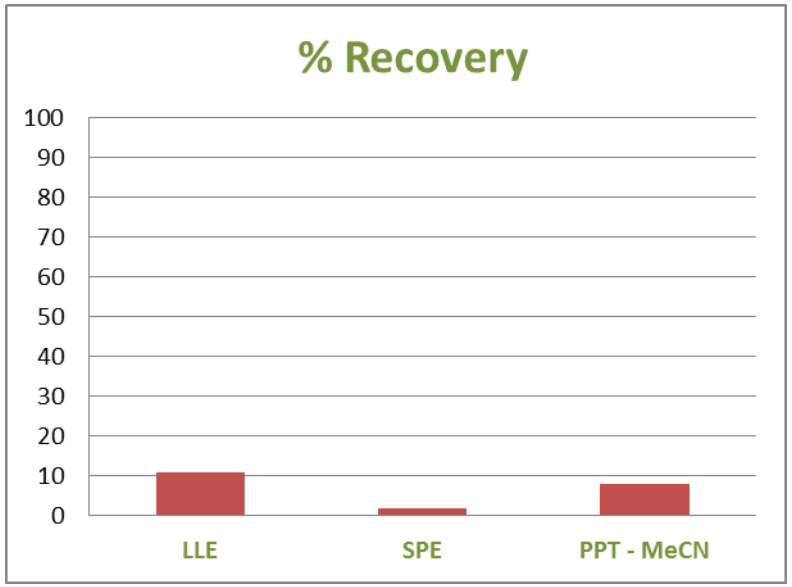
2nd Challenge: Irreversible binding with plasma proteins?

- Now that we can see the analyte at the levels needed, how do we get it out of the plasma?
- Liquid/Liquid extraction
  - Since the Log P is ≥ 4.0
  - Interference & low recovery
- Solid Phase Extraction
  - Pure standard is retained
  - Spiked material is un-retained – even at a higher Log P
- Protein Precipitation
  - Acetonitrile ~ 8%
  - Methanol ~ 5%



- The three most commonly used techniques do not work…
  - …this is not good!

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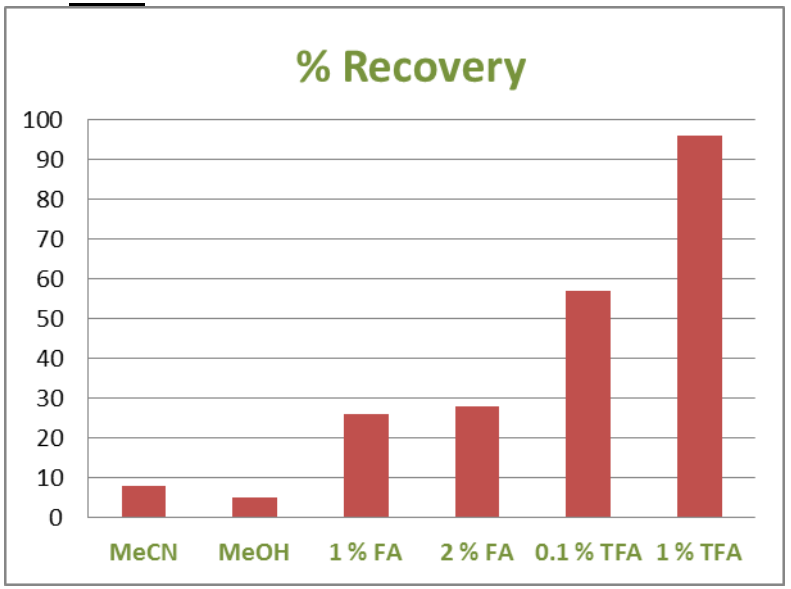
2nd Challenge: Irreversible binding with plasma proteins?

Analyte protein binding – a hypothesis:

- Analyte: anionic & hydrophobic
- Plasma proteins: carriers
- The analyte binds to plasma proteins via anionic **and** Van der Waals

- To release the analyte BOTH links have to be broken
  - Solvent and formic acid:
    - ~ 28% recovery, insufficient protein disruption to expose analyte
  - TFA to break ionic interactions and disrupt tertiary structure
  - 1% TFA and solvent: ~95% recovery



- Analyte fully exposed, anion fully neutralised, Van der Waals overcome

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Finally… Implementation of the Method

- Achieved LLOQ -25 pg/mL with S/N ratio >5 using protein precipitation
- BMV Validation in Rat and Dog Plasma using 25 µL sample volume
- Calibration range 25 - 5000 pg/mL
- Successfully supported toxicokinetic studies (~600 samples)
- ISR analysis successfully performed in each species

Conclusions

- Bioanalysis can be challenging, but generally follows clear principles, and recognises that there may be more than one *'issue'* with a method
  - The *understanding*, elimination or *control* of variables – such as Na^+ adducts – improves assay performance
    - Because variable levels of sodium ions come from many sources
  - The disassociation of the analyte from matrix components
    - Mixed mode protein binding requires a dual-mechanism of release
- Best practice approach for future studies

But these are just my thoughts, I'd be interested to hear of your views, and I'm at this conference all week!

The End…(other stories running in parallel)

Acknowledgements

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  + David Bakes, Director Bioanalytical and Translational Sciences
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++++Thank you