



# Overcoming Challenges in Development of a Single Extraction LC/MS/MS Method for an Unstable Metabolic Compound Forming Two Chemically Different Metabolites

## Authors

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## Introduction

- TL3287 was designed to contain a metabolically unstable ester.
- When metabolized, two compounds of interest are formed, one aromatic and one aliphatic.
- This rapid metabolic instability creates a challenge in developing a rugged bioanalytical method.
- All 3 compounds have vastly different polarities and ionization properties.
- Early Discovery work was performed at Tandem Labs prior to the GLP project.
- From this work, many of the challenges were identified and most solved.

## Method

- 500.0 µL of precipitated whole blood supernatant (treated with PMSF) was extracted.
- The HPLC system consisted of Shimadzu LC-10AD HPLC pumps used in conjunction with a LEAP autosampler.
- Sciex API 5000™ mass spectrometers were operated in both the positive and negative ion modes using ESI.
- Suitable chromatography for injection 1 (TL3287 and the aromatic metabolite) was achieved using 2 dimensional chromatography on a phenyl phase column and a pentafluoro-phenyl phase column. A polarity switch from positive mode to negative mode was required midway through the acquisition of data for each sample.



## Method (continued)

- Suitable chromatography for injection 2 (the aliphatic metabolite) was achieved using 2 dimensional chromatography on a Polar RP column and a C8 phase column.
- The mobile phases consisted of 1% formic acid in water and 50/50 methanol/acetonitrile.
- 25  $\mu$ L of the final extract was injected for each method.

## Initial Challenges

(Determined during early Discovery work):

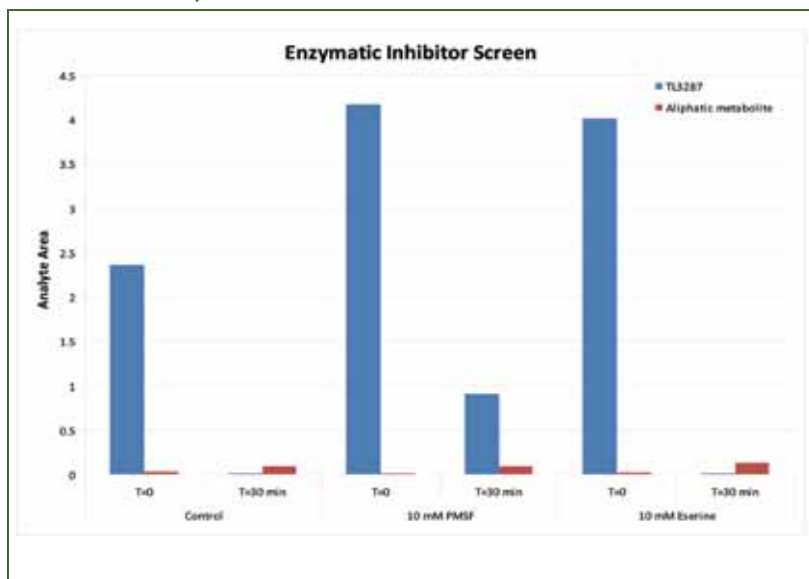
### 1. STABILITY

- Ester linkage in parent was observed to be extremely unstable *in vitro*.
- Careful attention to pH of sample, in order to preserve ester linkage.

Experiments:

1. Screen of multiple enzymatic inhibitors (Figure 1)
2. Test whether denaturing proteins in sample increases stability

FIGURE 1: Screen of enzymatic inhibitors





## Initial Challenges (continued)

### 2. IONIZATION

- TL3287 ionizes in only **positive** mode
- The aliphatic metabolite ionizes in both **positive and negative** mode
- The aromatic metabolite ionizes in only **negative** mode

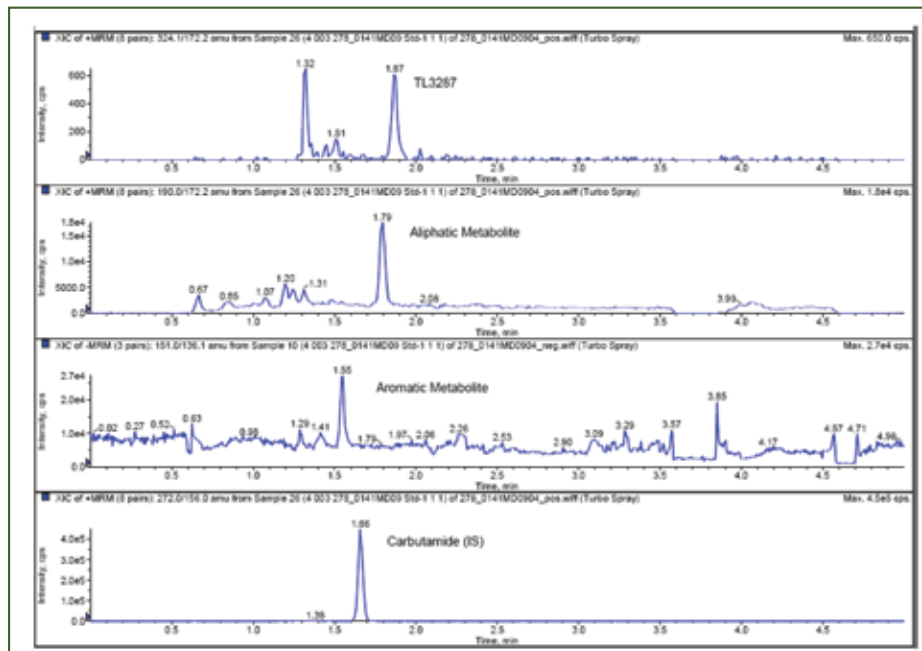
### 3. INTERNAL STANDARD

- During early discovery level work, one nonrelated compound (Carbutamide) was used as an internal standard for all 3 analytes.

### 4. POLARITY

- Steep gradient required to elute all compounds in one injection (Figure 2)
- Leads to severe carryover

FIGURE 2: Steep gradient to elute all analytes with good peak shape



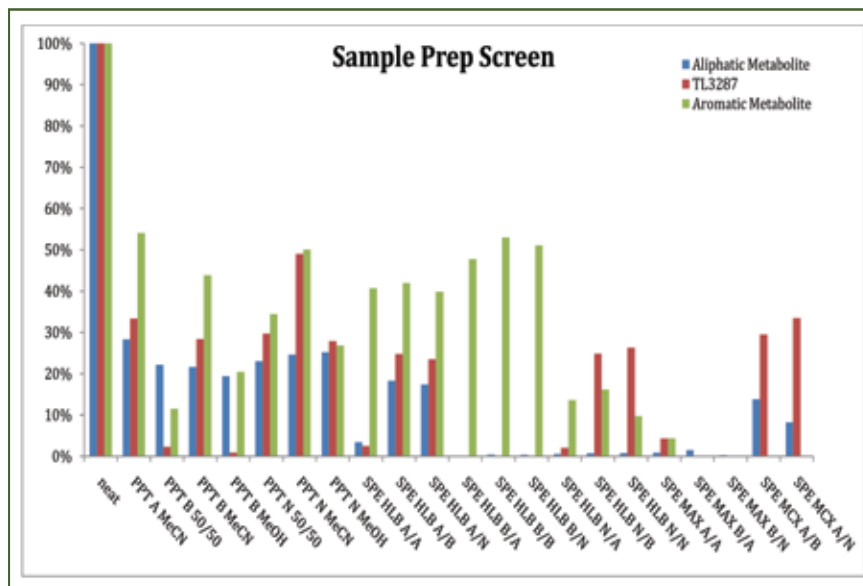


## Sample Preparation Method Development

### 5. SAMPLE PREPARATION SCREEN

- Early Discovery work used a protein precipitation method
- A complete sample preparation screen was carried out to help improve method precision and ruggedness (Figure 3)

FIGURE 3: Sample Preparation Screen



### 6. CHROMATOGRAPHY DEVELOPMENT

- Develop improved single injection method
- All compounds need baseline separation to use polarity switch.
- Tailing Peaks (Figure 4)
- Dual Injection method
- Due to elution order (Figure 5), we could not group positive mode analytes together



## Sample Preparation Method Development (continued)

FIGURE 4: Injection of all analytes using polarity switch

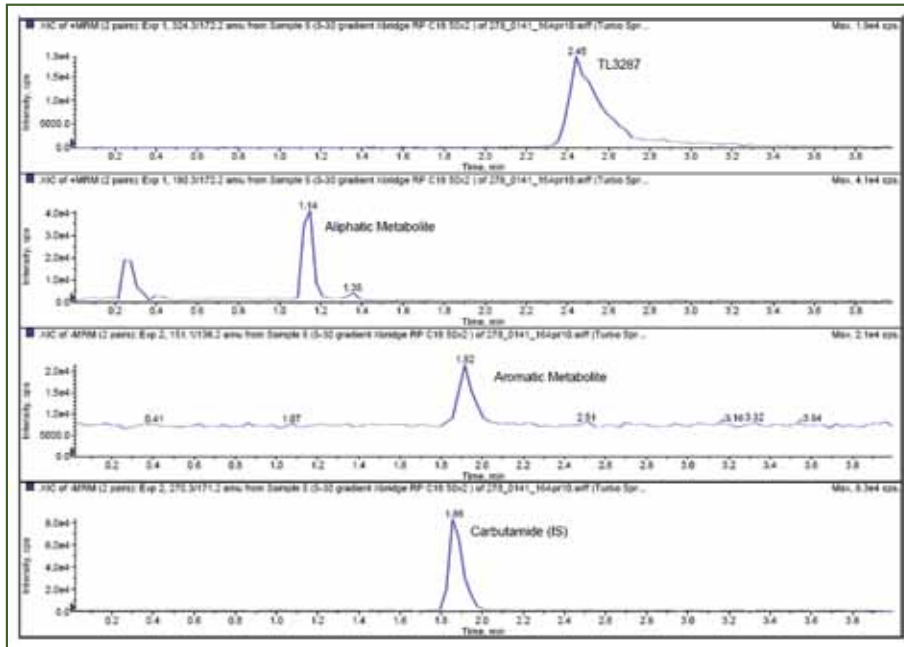


FIGURE 5: Elution order of analytes

Compound	SRM/Positive ion mode	SRM/Negative ion mode	LC Elution order using C18 column (Polarity order)
Aliphatic metabolite	190 → 172	188 → 111	1
Carbutamide	272 → 156	270 → 171	2
Aromatic metabolite	None	151 → 136	3
TL3287	324 → 172	None	4

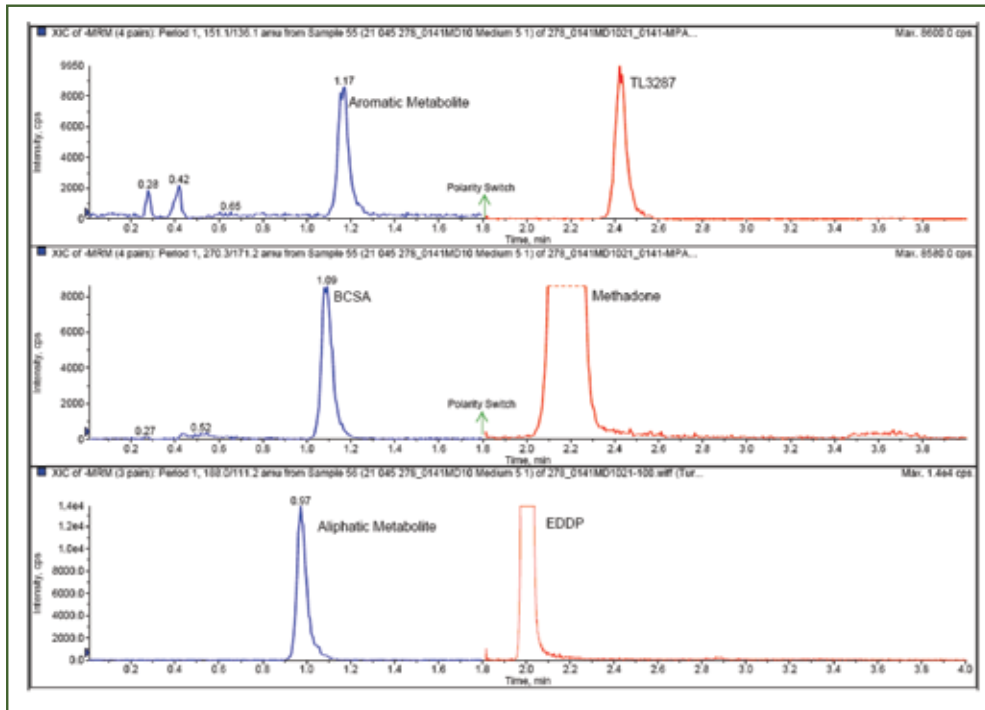
### 7. LINEARITY AND CARRYOVER ASSESSMENT

- A range finding batch was extracted and injected to assess linearity from 0.01 ng/mL to 1000 ng/mL
- Optimization of LC conditions to reduce LLOQ from 1 ng/mL for each analyte to 0.25 ng/mL for TL3287 and 0.500 ng/mL for the aromatic metabolite (Figure 6)
  - 1st injection on a PFP column
  - 2nd injection on a C8 column



## Sample Preparation Method Development (continued)

FIGURE 6: Dual injection method to obtain lower LLOQ (Analyte/IS pairs: Aromatic Metabolite/BCSA; TL3287/Methadone; Aliphatic Metabolite/EDDP)



### 8. SELECTIVITY EVALUATION

- Formal QC pools were tested and selectivity low QCs were tested in 10 different lots of matrix
- Accuracy and precision good for formal pools, poor for selectivity (Figure 7).
- New internal standards screened and 3 new ones were selected



## Sample Preparation Method Development (continued)

FIGURE 7: Selectivity Evaluation

Sample ID	TL3287 % Deviation	Aromatic Metabolite % Deviation	Aliphatic metabolite % Deviation
Low	3.5	1.9	5.3
Low	3.3	8	7.3
Low	2	11.1	6.7
Low	1.1	*24.3	-2.7
Low	2.8	-1.1	1.3
Low	-2.7	5.2	-0.7
Sel-Low	-14.4	12.4	-11.3
Sel-Low	-4.8	8.9	-4
Sel-Low	-1.2	9.7	*-20.0
Sel-Low	-14.4	9.6	-14
Sel-Low	-4.8	10.8	*-18.7
Sel-Low	-7.6	13.6	-12
Sel-Low	-13.2	14.4	*-18.0
Sel-Low	*-21.5	7.5	*-18.7
Sel-Low	1.5	*16.7	7.3
Sel-Low	-11.2	12.8	*-19.3
Medium	4	12	-1
Medium	4	*15.0	4.5
Medium	6	*19.0	2.5
Medium	6	12	-2.5
Medium	9	12	-1.5
Medium	3	14	1.5
High	0	7	-1.5
High	1	2	-3.5
High	0	6	-3.5
High	-2.2	9	-3.5
High	-4.3	9	-2
High	-3.3	2	-6



## Sample Preparation Method Development (continued)

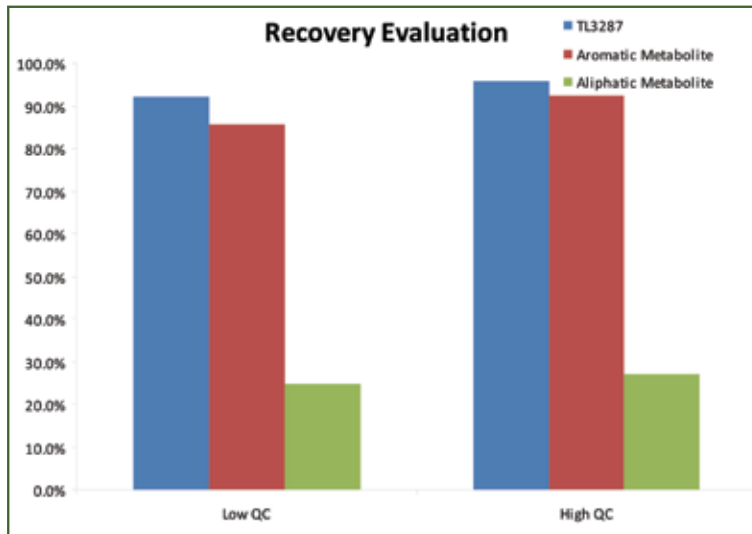
### 9. PROBLEMS WITH ALIPHATIC METABOLITE

- Multiple QC pool preparations gave inconsistent results (Figure 8)
- No preparation error proved by very consistent results for TL3287
- Evaluated recovery (Figure 9)
- Evaluated whole blood stability (Figure 10)
- Precision improved by development of 2d chromatography for each injection (Figure 11)

FIGURE 8: Comparison of multiple pool preparations for the Aliphatic Metabolite

	High QC	High LTS	Dilution QC	Medium QC	Low QC	Low LTS	LLOQ QC
Average (Aliphatic Metabolite)	108.3	93.5	119.9	101.2	109	150.5	94.6
Average (TL3287)	97.2	101.3	98.1	105	105.3	97.8	97.6

FIGURE 9: Recovery Evaluation





## Sample Preparation Method Development (continued)

FIGURE 10: Whole Blood Stability Evaluation

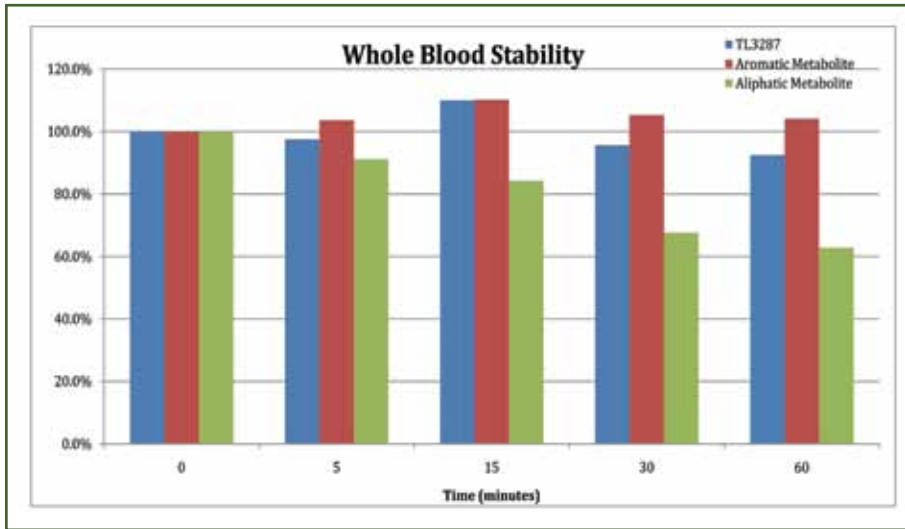
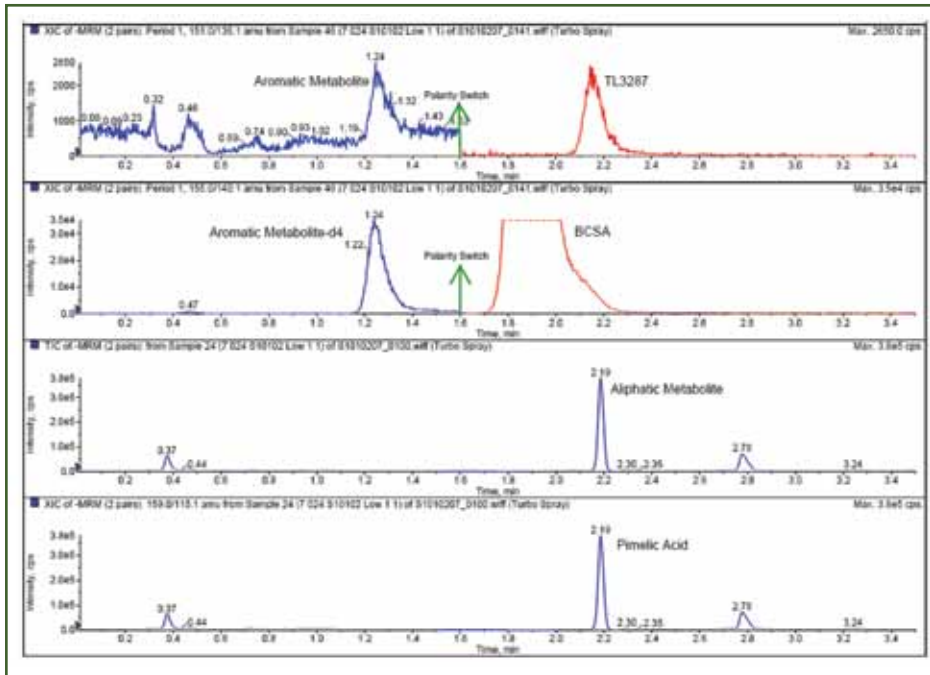


FIGURE 11: Final Conditions (Analyte/IS pairs: Aromatic Metabolite/Aromatic Metabolite-d4; TL3287/BCSA; Aliphatic Metabolite/Pimelic Acid)





## Results and Discussion (continued)

FIGURE 12: Accuracy and Precision statistics from final method

	TL3287				Aromatic Metabolite				Aliphatic Metabolite			
	LLOQ QC 0.250 ng/mL	Low QC 0.750 ng/mL	Medium QC 10.0 ng/mL	High QC 100 ng/mL	LLOQ QC 2.50 ng/mL	Low QC 7.50 ng/mL	Medium QC 100 ng/mL	High QC 1000 ng/mL	LLOQ QC 5.00 ng/mL	Low QC 15.0 ng/mL	Medium QC 200 ng/mL	High QC 2000 ng/mL
Mean Observed Conc.	0.241	0.744	10.3	98.5	2.55	7.85	105	1020	4.79	13.8	182	1880
%Bias	-3.6	-0.8	3	-1.5	2	4.7	5	2	-4.2	-8	-9	-6
Between Run Precision (%CV)	1.5	1.7	2.3	1.2	2.8	3.4	1.7	0	4.3	3.1	2.6	1.6
n	18	18	18	18	18	18	18	18	18	18	18	18
Number of Runs	3	3	3	3	3	3	3	3	3	3	3	3

## Conclusion

- Protein binding of the aliphatic metabolite complicated development.
- pH fluctuations would cause degradation of TL3287.
- A rugged method was validated with sufficient selectivity and stability in whole blood established (Figure 12).
- Study samples were received and successfully analyzed, with ISR meeting acceptance criteria.