Quantitation of Tetracycline and 3 Derivatives In Human Urine by LC/MS/MS

Authors

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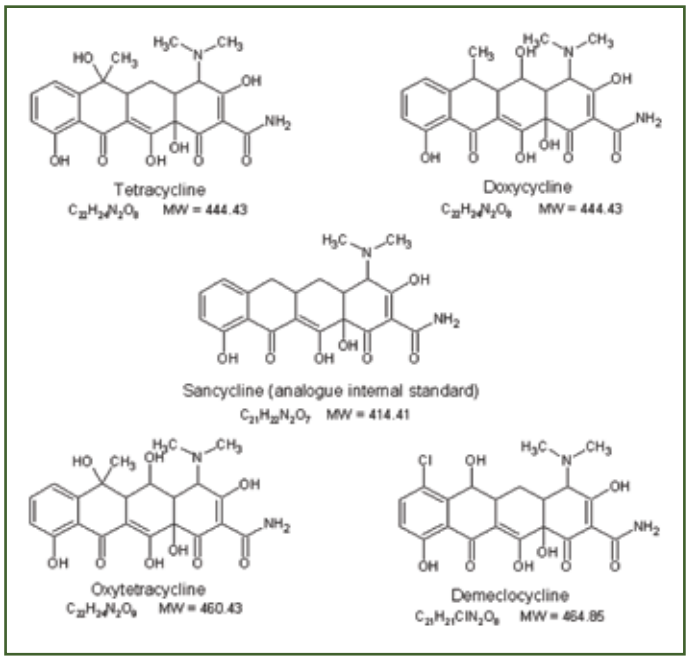
Introduction

Developing a multi-analyte, single (analogue) internal standard assay for quantitative GLP bioanalysis can present multiple challenges. This poster addresses two specific challenges encountered while developing a method for the quantitation of tetracycline, doxycycline, demeclocycline and oxytetracycline; 1-chromatographic separation and 2-recovery losses due to container adsorption. Problem 1 was addressed with changes to the HPLC system. Problem 2 was addressed by treating the samples with acid. The assay is designed to designate incurred samples as positive (greater than LLOQ) or fail (less than LLOQ); hence only a small dynamic range of 0.200 - 10.0 µg/mL was needed.

FIGURE 1–**Structures**



Challenge 1–Surviving the "One-Pass" Test

In a regulated quantitative environment ease and consistency of peak integration is a primary concern. The ideal method should allow for all peaks of interest to be integrated (accurately) with one set of parameters. In this assay, the resonance effects observed in doxycycline represented a road block to achieving this goal. Figures 2-5 represent XIC of doxycycline (MRM+ 445/428) injected on the system detailed below with variations to the column temperature. Figures 6-7 represent 'extracted' LLOQ (0.200 µg/mL) and ULOQ (10.0 µg/mL).

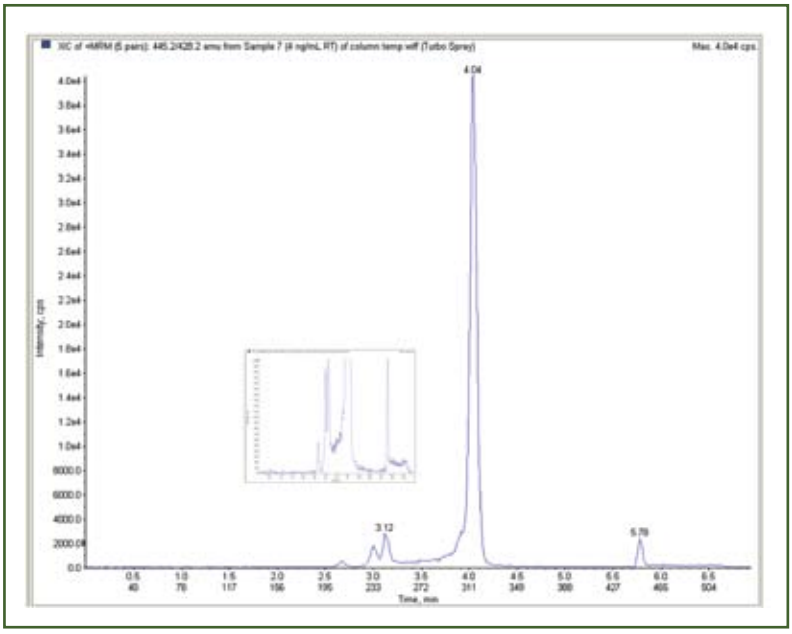
CHROMATOGRAPHIC CONDITIONS:

Analytical Column: Fortis Phenyl, 5µm, 2.1x50mm (Fortis Technologies)
Mobile Phase A: 0.1% Formic Acid (aq)
Mobile Phase B: 0.1% Formic Acid in Methanol
Post-Elution column wash with Acetone

TABLE 1–**API 4000 Positive ESI Operating Conditions:**

Analyte	Q1 Mass (±0.3 amu)	Q3 Mass (±0.3 amu)	Dwell (msec)	DP (V)	EP (V)	CE (V)	CXP (V)
Tetracycline	445.2	410.2	150	50	10	28	15
Doxycycline	445.2	428.2	150	50	10	28	15
Demeclocycline	465.2	430.2	150	50	10	28	15
Oxytetracycline	461.2	426.2	150	50	10	25	15
Sancycline (IS)	415.2	309.2	150	50	10	45	15

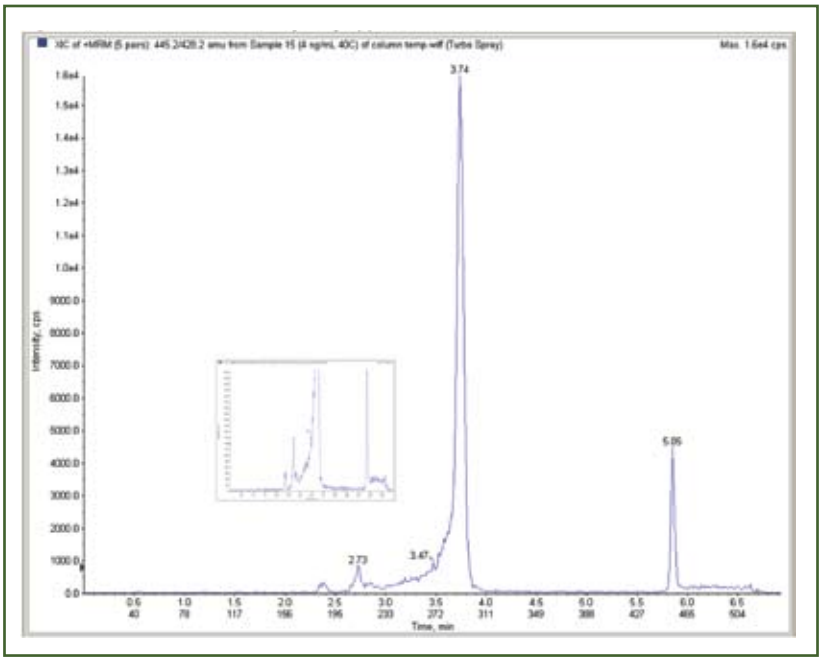
FIGURE 2–**Production Approach:** (minimal equipment)-column is used under ambient conditions



Problem: Inadequate separation of tautomers is a challenge to "one-pass" integration.

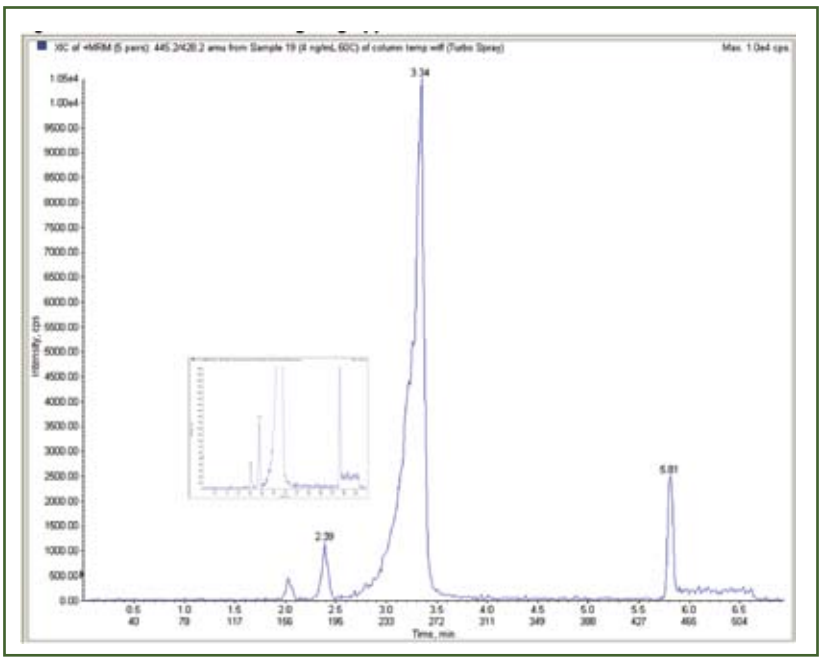
Challenge 1–Surviving the "One-Pass" Test continued

FIGURE 3–**Traditional Resonance Fighting Approach:** column heated to 40°C



Problem: Resonance forms have neither been averaged nor resolved.

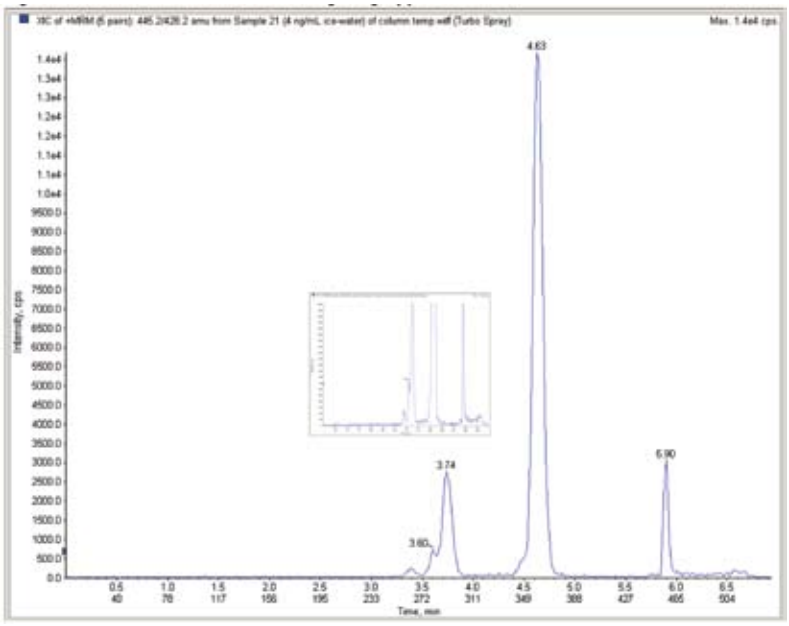
FIGURE 4–- **Traditional Resonance Fighting Approach:** column heated to 60°C



Problem: Resonance forms have neither been averaged nor resolved.

Challenge 1–Surviving the "One-Pass" Test continued

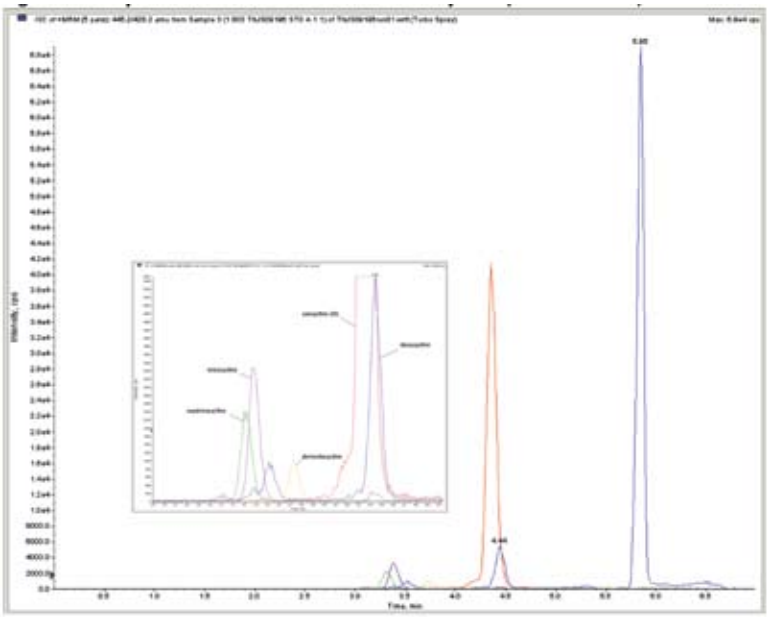
FIGURE 5–**Anti-Traditional Resonance Fighting Approach:** column chilled in ice-water bath



Problem: Tautomers have been completely resolved.

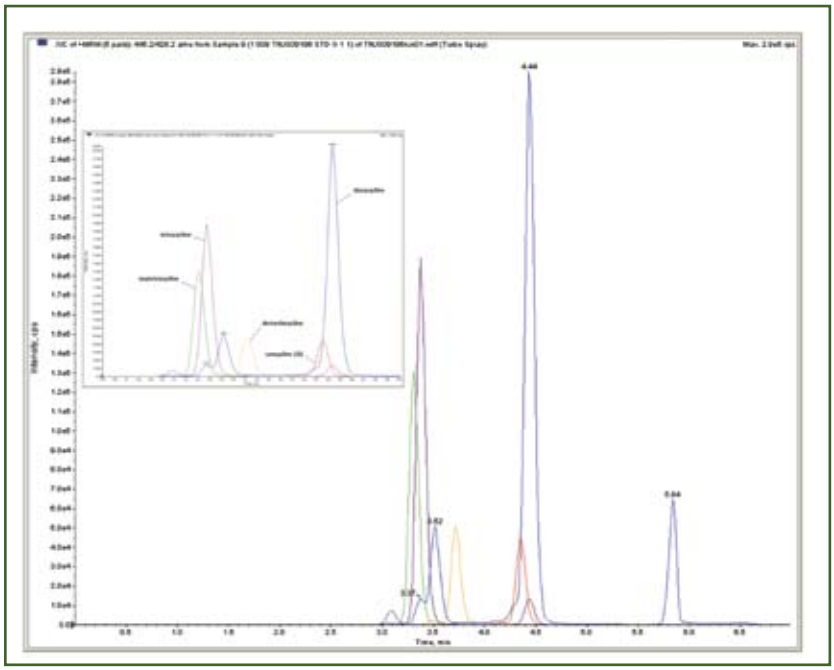
Production Bonus: Ice-water bath is inexpensive and easy to maintain.

FIGURE 6–**Analytes and Internal Standard on Final System** (chilled column): LLOQ level



Challenge 1–Surviving the "One-Pass" Test continued

FIGURE 6–**Analytes and Internal Standard on Final System** (chilled column): ULOQ level



Challenge 2–Recovery

Extracting analytes from urine is often complicated by recovery losses due to container adsorption. Early tests showed no loss to container adsorption but assay development was hindered by inconsistent day to day results. After some investigation into the possible cause (stability, solubility, matrix effects, etc.), the inconsistent results were determined to be a product of pH dependant adsorption losses. The greater the pH, the greater the loss. The data presented in Table 2 represents a spiked urine sample, pH ~8, exposed to exaggerated storage conditions (i.e. samples were transferred through a series of tubes). Table 3 reports the results of samples treated with acid (final pH ~1-2) to prevent recovery losses.

Challenge 2–Recovery continued

TABLE 2–**Spiked Urine Samples Exposed to Polypropylene**

# of transfers	untreated urine samples			
	tetracycline	doxycycline	demeclocycline	oxytetracycline
	% accuracy			
0x	100.0	100.0	100.0	100.0
1x	91.3	92.0	81.8	95.4
2x	79.1	82.8	65.9	91.0
3x	67.3	74.9	50.1	88.1
4x	58.1	69.5	39.9	85.8
5x	46.9	63.0	28.7	83.6
avg loss per transfer	**-10.6**	**-7.4**	**-14.3**	**-3.3**

TABLE 6A–**Accuracy and Precision of 3 Analytical Runs:** Tetracycline

		Tetracycline			
		LLOQ QC	QC1	QC2	QC3
		theorectical concentration (µg/mL)			
		0.200	0.600	4.00	8.00
Run 1	Found Conc. Mean	0.196	0.584	3.93	7.9
	%CV	1.9	1	2	1.3
	%Theorectical	99.6	98.4	93.0	90.0
	n	6	5	6	6
Run 2	Found Conc. Mean	0.19	0.582	4.07	8.03
	%CV	1.2	1.8	4.9	1.9
	%Theorectical	99.0	98.2	107.0	103.0
	n	6	6	6	6
Run 3	Found Conc. Mean	0.204	0.607	4.03	7.98
	%CV	3	2.1	3.6	2.4
	%Theorectical	100.4	100.7	103.0	98.0
	n	6	6	6	6
Inter-Run	Found Conc. Mean	**0.197**	**0.591**	**4.01**	**7.97**
	%CV	**3.6**	**2.4**	**1.8**	**0.8**
	%Theorectical	**99.7**	**99.1**	**101.0**	**97.0**
	n	**18**	**17**	**18**	**18**

Challenge 2–Recovery continued

TABLE 6B–**Accuracy and Precision of 3 Analytical Runs:** Doxycycline

		Doxycycline			
		LLOQ QC	QC1	QC2	QC3
			theorectical concentration (µg/mL)		
		0.200	0.600	4.00	8.00
Run 1	Found Conc. Mean	0.201	0.598	4	7.99
	%CV	4.2	2.3	2.4	1.4
	%Theorectical	100.1	99.8	100.0	99.0
	n	6	5	6	6
Run 2	Found Conc. Mean	0.194	0.579	4	7.9
	%CV	1.1	1.2	4.1	1.7
	%Theorectical	99.4	97.9	100.0	90.0
	n	6	6	6	6
Run 3	Found Conc. Mean	0.21	0.598	3.92	7.84
	%CV	3.1	2.1	3.3	2.4
	%Theorectical	101.0	99.8	92.0	84.0
	n	6	6	6	6
Inter-Run	Found Conc. Mean	**0.202**	**0.592**	**3.97**	**7.91**
	%CV	**4.0**	**1.9**	**1.2**	**1.0**
	%Theorectical	**100.2**	**99.2**	**97.3**	**91.0**
	n	**18**	**17**	**18**	**18**

TABLE 6C–**Accuracy and Precision of 3 Analytical Runs:** Demeclocycline

		Demeclocycline			
		LLOQ QC	QC1	QC2	QC3
			theorectical concentration (µg/mL)		
		0.200	0.600	4.00	8.00
Run 1	Found Conc. Mean	0.204	0.594	3.98	7.93
	%CV	2	2.5	1.8	2
	%Theorectical	100.4	99.4	98.0	93.0
	n	6	5	6	6
Run 2	Found Conc. Mean	0.191	0.59	4.09	8.13
	%CV	6.9	2.5	5.8	1.7
	%Theorectical	99.1	99.0	109.0	113.0
	n	6	6	6	6
Run 3	Found Conc. Mean	0.203	0.615	4.13	8.25
	%CV	3.5	1.6	3.2	1.8
	%Theorectical	100.3	101.5	113.0	125.0
	n	6	6	6	6
Inter-Run	Found Conc. Mean	**0.199**	**0.600**	**4.07**	**8.10**
	%CV	**3.6**	**2.2**	**1.9**	**2.0**
	%Theorectical	**99.9**	**100.0**	**106.7**	**110.3**
	n	**18**	**17**	**18**	**18**

Challenge 2–Recovery continued

TABLE 6D–**Accuracy and Precision of 3 Analytical Runs:** Oxytetracycline

		Oxytetracycline			
		LLOQ QC	QC1	QC2	QC3
			theorectical concentration (µg/mL)		
		0.200	0.600	4.00	8.00
Run 1	Found Conc. Mean	0.202	0.6	3.99	7.95
	%CV	3	1.3	1.7	1.5
	%Theorectical	100.2	100.0	99.0	95.0
	n	6	5	6	6
Run 2	Found Conc. Mean	0.201	0.606	4.17	8.23
	%CV	5.2	2.9	5.1	2.1
	%Theorectical	100.1	100.6	117.0	123.0
	n	6	6	6	6
Run 3	Found Conc. Mean	0.222	0.611	4.16	8.25
	%CV	1.7	2	2	2.1
	%Theorectical	102.2	101.1	116.0	125.0
	n	6	6	6	6
Inter-Run	Found Conc. Mean	**0.208**	**0.606**	**4.11**	**8.14**
	%CV	**5.7**	**0.9**	**2.5**	**2.1**
	%Theorectical	**100.8**	**100.6**	**110.7**	**114.3**
	n	**18**	**17**	**18**	**18**

Discussion

Resonance effects of doxycycline were overcome by cooling the analytical column, resulting in rugged and reproducible peak shape. Lot to lot and day to day assay performance was guaranteed by treating urine samples with acid to prevent adsorption losses.

Conclusion

A GLP assay for the quantitation of tetracycline, doxycycline, demeclocycline and oxytetracycline was successfully validated.