Application Note: 350

Rapid Quantitative and Confirmational Screening for Drugs in Race Horse Urine by ESI-LC-MS/MS and MS^3

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Key Words
- LTQ™
- Confirmation
- Drug Screening
- MS^3
- Quantitation

Introduction

Drugs of abuse in the horse racing industry encompass a variety of chemical classes and are typically analyzed from a complex urine matrix. These factors render the rapid and effective diagnostic screening of these drugs at low levels difficult. Traditionally, quantitation has been performed by triple quadrupole mass spectrometry using reaction monitoring (SRM) mode. However, this method does not monitor structurally diagnostic fragmentation. Thus, a second step involving derivatization and GC/MS confirmation was required.

Goal

To develop a simple and fast, yet rugged LC/MS based method capable of simultaneous qualitative and quantitative analysis. We have evaluated the application of the Thermo Scientific LTQ linear ion trap mass spectrometer for providing low levels of detection, good reproducibility, and wide linear dynamic range required for reliable quantitation, with simultaneous structural confirmation using diagnostic full-scan MS/MS or MS^3 mass spectrometry.

Experimental Conditions

Sample Preparation

Standards and Unknowns: Standards of the compounds listed in Table 1 were prepared neat and in urine. Urine standards and unknowns were spiked, dried, and reconstituted with 90% water and 10% acetonitrile with 0.1% acetic acid. Typical Instrument Setup settings are shown in Figure 1.

HPLC

HPLC System: Thermo Scientific Surveyor™ LC system

Column: Thermo Scientific BETASIL™ C18, 3 µm, 100 × 2.1 mm

Flow Rate: 350 µL/min

Injection Volume: 10 µL (full loop)

Mobile Phase: (A) Water with 0.1% acetic acid
(B) Acetonitrile with 0.1% acetic acid

Gradient: 92% A to 90% B.

MS

Mass Spectrometry: Thermo Scientific LTQ linear ion trap mass spectrometer

API Source: Thermo Scientific Ion Max™ source with electrospray ionization (ESI) probe

Ion Transfer Capillary: 220°C; Sheath Gas: 30 units

Auxiliary Gas: 0 units; Sweep Gas: 20 units

Spray Voltage: 4.5 kV; Isolation Width: 3 amu

Normalized Collision Energy™: 28%

WideBand Activation™: Applied as needed (see Table 1)

Ion Polarity Mode: positive or negative (see Table 1)

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Results

Quantitation

Calibration curves were established using neat standards based on ion intensities from full-scan MS/MS chromatograms. Chromatograms for all the compounds listed in Table 1 were obtained in a single chromatographic run at each concentration. Figure 2 shows reconstructed ion chromatograms (RICs) from the analysis of the 50 pg/µL standards. The MS/MS spectra for all the drugs, with the exception of ketoprofen, are shown in Figure 3.

The MS/MS spectra were generated using a Normalized Collision Energy of 28%. The use of Normalized Collision Energy alleviates the necessity to optimize the collision energy for each compound as is necessary in traditional triple-quadrupole analysis, thus making this method extremely easy to set up and run. Compounds that underwent a non-specific water loss were additionally fragmented using WideBand Activation (see Table 1). This mode of fragmentation results in information-rich spectra enabling structural confirmation without requiring an additional MS^3 transition. The compound ketoprofen undergoes a neutral loss outside of the WideBand Activation window and was selected for an MS/MS to MS^3 comparison study and is discussed later.

Chromatographic and mass spectrometric methods were validated using the neat standards; subsequently the experiments were repeated using standards in horse urine. The RICs from these experiments are shown in Figure 4. Using the RICs, calibration curves were created for each of the compounds either neat (Figure 5) or in urine matrix (Figure 6). The calibration curves were linear over the three orders of magnitude assayed. In addition to demonstrating linearity, the quantitative results shown in Tables 2 and 3 demonstrate excellent reproducibility.

SEGMENT	RT	ID#	COMPOUND	*M/Z*	WB	RIC
1	3.40	416	Theobromine	181.0		137 + 163 + 181
	4.44	417	Theophylline	181.0		124 + 137
	4.56	152	Dyphylline	255.1		181
2	5.58	071	Caffeine	195.1		138
	5.71	089	Chlorothiazide	-293.9		214 + 215
	6.02	107	Cromolyn-Na	469.2	✔✔✔	245
	6.20	198	Hydroclorothiazide	-295.8		205 + 232 + 269
	6.49	311	Pemoline	177.0		106
3	7.20	614	Petoxifyline	279.1		181
4	8.95	117	Dexamethasone	393.1	✔	355 + 337 + 319
	9.60	481	Boldenone	287.1	✔	121 + 135 + 173
	10.16	499	Ketoprofen†	255 (209)		209 (105 + 194)
5	11.28	216	Indomethacin	358.0		139 + 174
	11.33	130	Diclofenac	295.9	✔	215 + 250
	11.93	175	Flufenamic Acid	282.1		264
	12.05	235	Meclofenamic Acid	295.9	✔	242 + 243

† *Ketoprofen was analyzed by both MS/MS and MS^3 for comparison study.*

4 *WB denotes use of wideband activation during MS/MS fragmentation.*

Table 1: List of target compounds; corresponding RT (retention time), segment (method segment see Figure 1), *m/z* denotes isolation mass and Ion Polarity, WB–WideBand Activation, RIC–masses used in generation of Reconstructed Ion Chromatograms for quantitation.

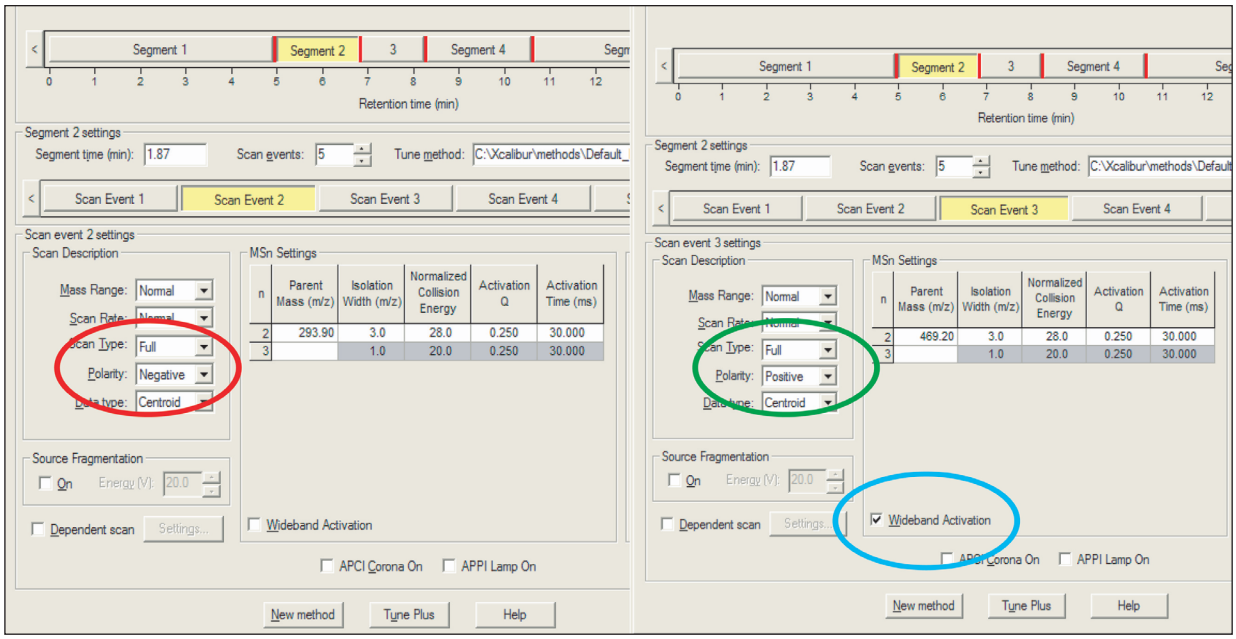


Figure 1: Instrument Setup settings for chlorothiazide (segment 2, scan event 2) and cromolyn-Na (segment 2, scan event 3)

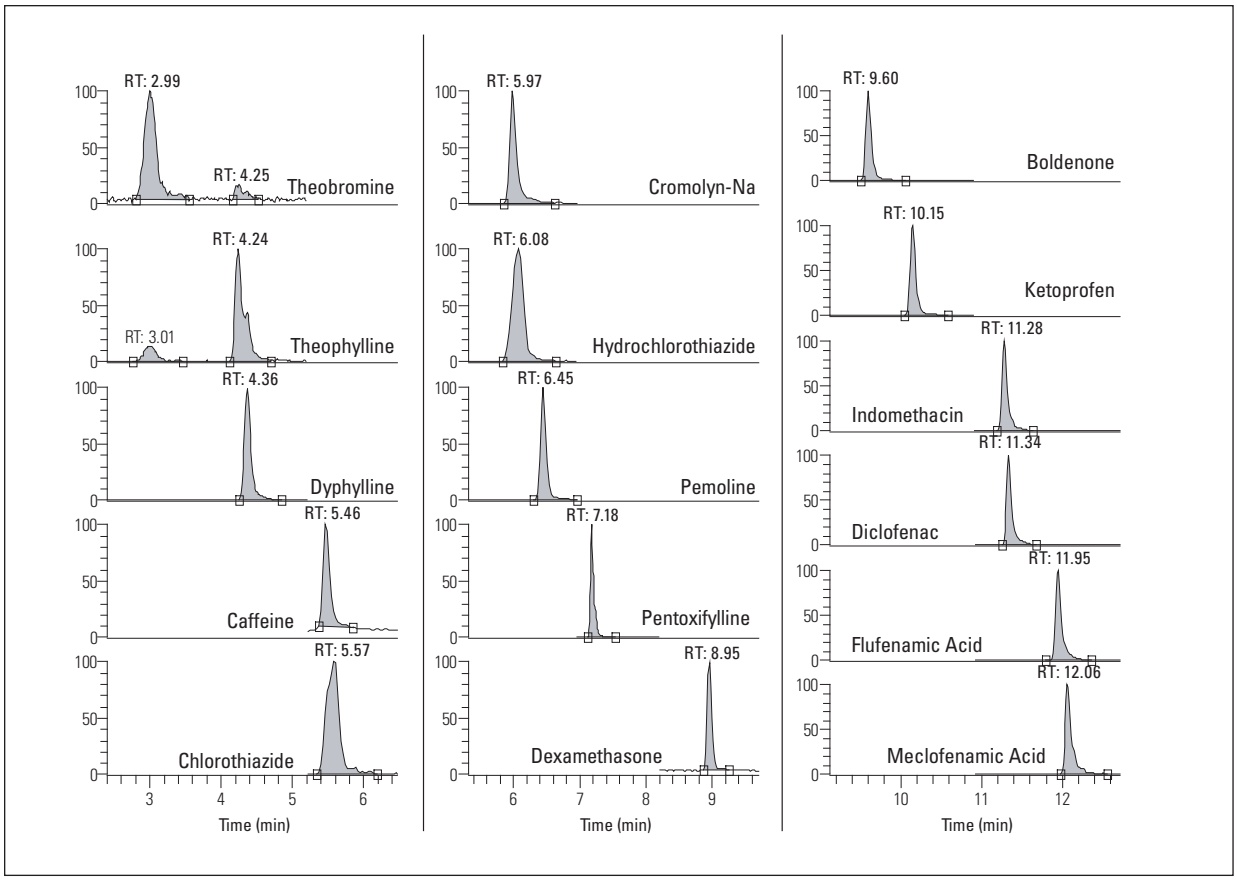


Figure 2: Reconstructed ion chromatograms, using the product ions listed in Table 1 (RIC column), from the analysis of 10 µL of the 50 pg/µL standards in solvent

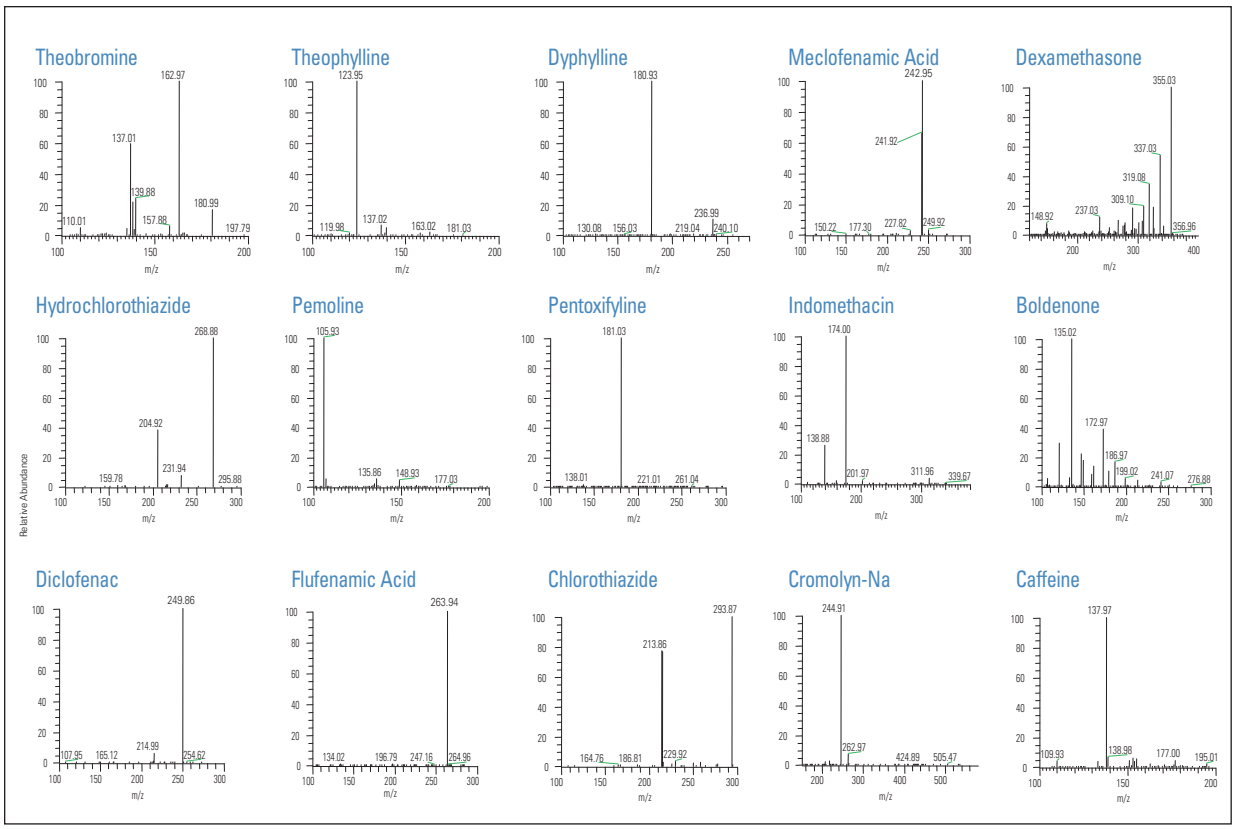


Figure 3: Full-scan MS/MS spectra corresponding to compounds depicted in Figure 2

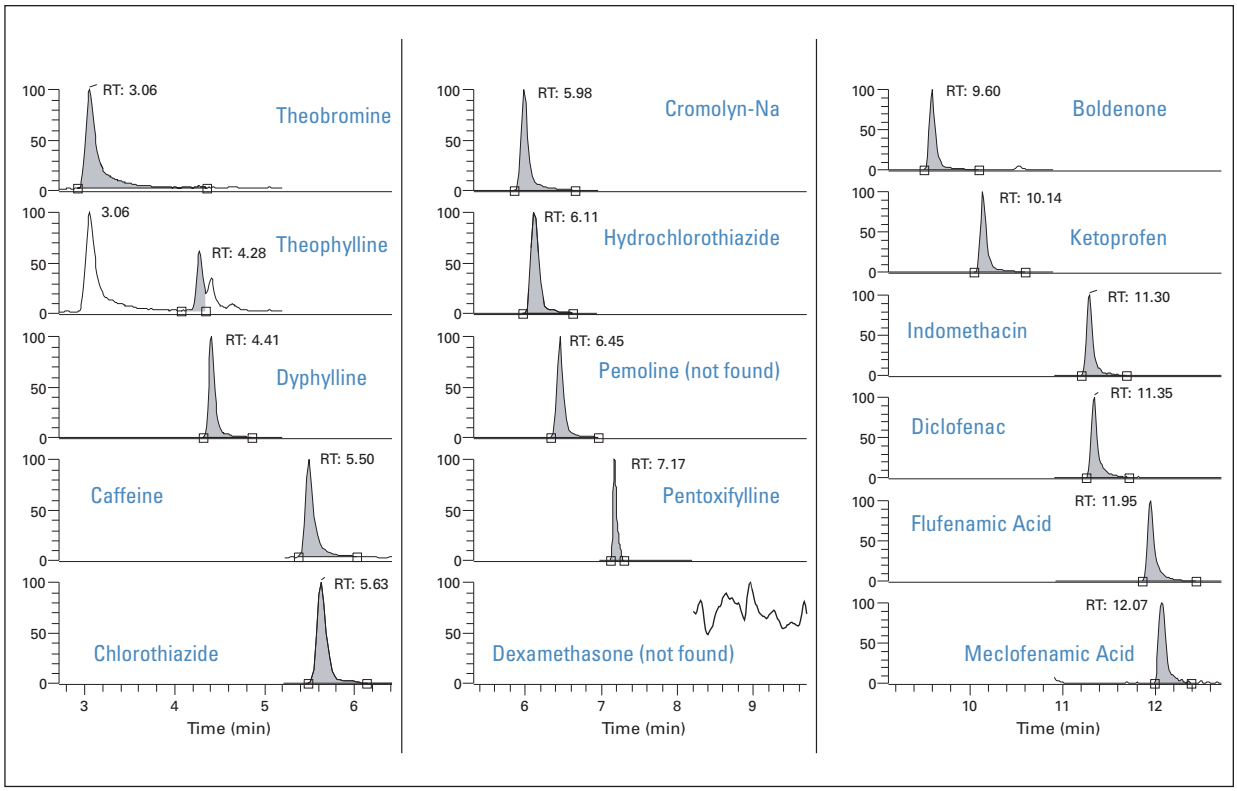


Figure 4: Reconstructed ion chromatograms, using the product ions listed in Table 1 (RIC column), from the analysis of a 10 µL injection of the 50 pg/µL standard in horse urine

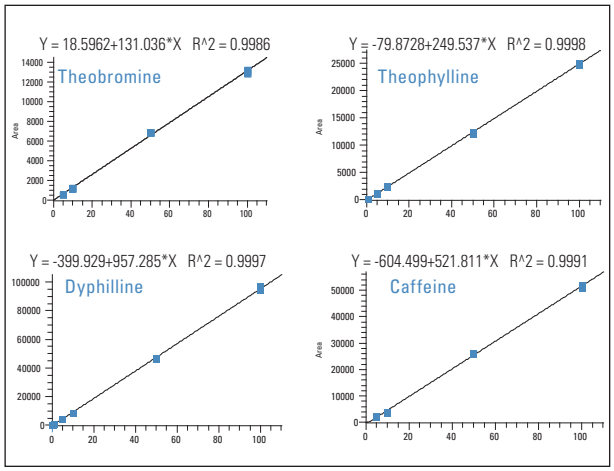


Figure 5: Representative calibration curves from standards prepared in solvent

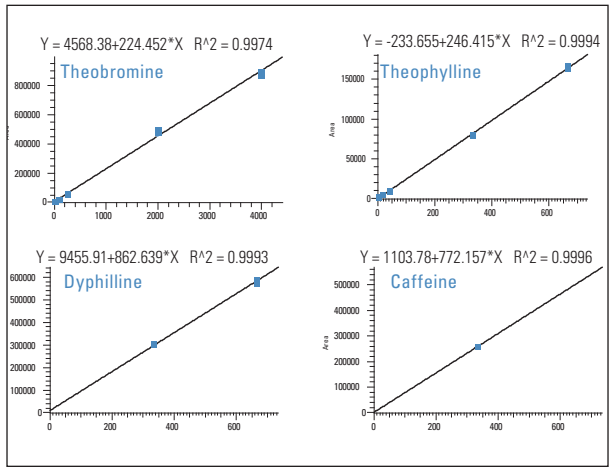


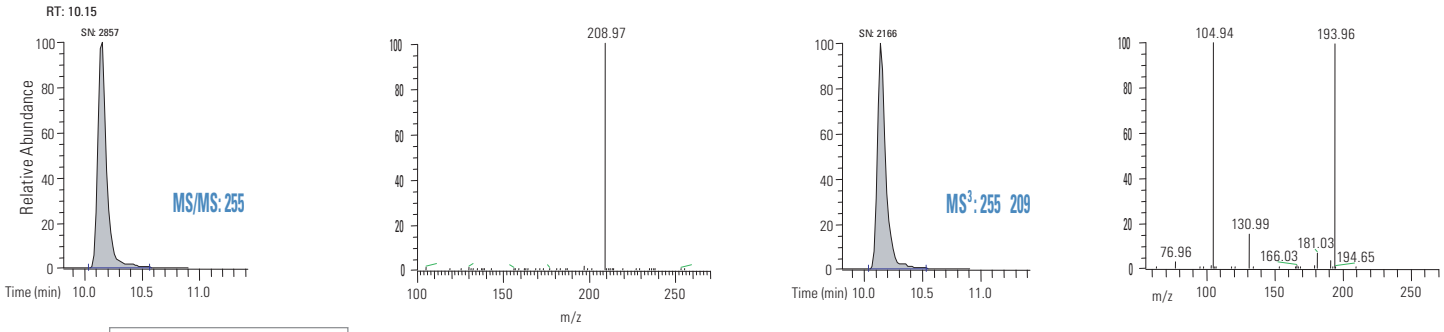
Figure 6: Representative calibration curves from standards prepared in horse urine

The %RSD for three replicate injections is less than 10% for all neat standards at the 1 pg/µL level and higher (see Table 2). The results for standards in urine were also excellent. The %RSD, five replicate injections, for the lowest level assayed in urine was less than 10% for most analytes (see Table 3), and commonly less than 3% for mid- and high-concentration samples. To complete the quantitative study, two QC urine samples were analyzed. The results shown in Table 4 demonstrate a high level of quantitation accuracy, with a deviation of less than 10% for most analytes. In addition, excellent reproducibility was demonstrated with the %RSD being less than 8% for all but two compounds (see Table 4).

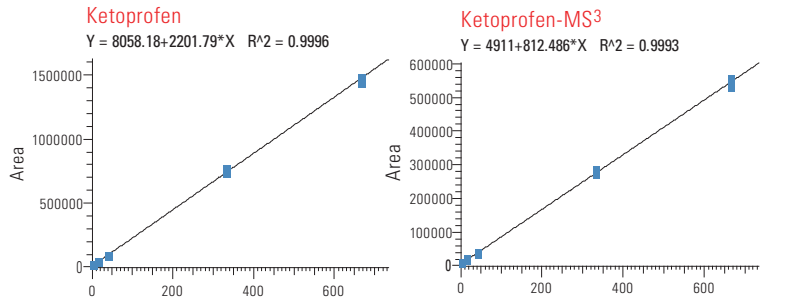
Ketoprofen – MS/MS vs. MS³: Ketoprofen undergoes a neutral loss of a 46 amu fragment in MS/MS mode due to the loss of the carboxyl group (see Ketoprofen structure). This is outside of the mass window for WideBand Activation and thus, an MS³ experiment was performed to generate additional diagnostic ions without sacrificing sensitivity or reproducibility. To demonstrate this, standards and two urine QC samples were analyzed in both MS/MS and MS³ mode, with results shown in Figure 7. There is no loss of sensitivity, accuracy, or reproducibility in obtaining this additional information. The %RSD from the MS/MS and MS³ data are virtually identical. While the sensitivity remains unchanged, the accuracy in the analysis of the unknowns is actually improved in the MS³ experiments (see Figure 7).

Robustness

To assess the ruggedness of the method, a 166 pg/µL standard in horse urine was assayed over 100 consecutive injections. The results are displayed in Figure 9. The mean and coefficient of variation (%CV) for four compounds: theobromine, caffeine, pentoxyphylline, and ketoprofen were determined to be less than 4% for all four compounds.



SAMPLE	KETOPROFEN AREA	KETOPROFEN CAL. CONC.	KETOPROFEN %RSD	KETOPROFEN - MS³ AREA	KETOPROFEN - MS³ CAL. CONC.	KETOPROFEN - MS³ %RSD
3.3 pg/µL	20350	6.073	3.75%	8205	3.874	4.05%
	19635	5.751		8012	3.636	
	20281	6.042		8201	3.87	
	20046	5.936		7928	3.531	
	19185	5.548		8137	3.79	
16.6 pg/µL	45386	17.362	3.86%	17551	15.425	2.82%
	43015	16.293		18007	15.988	
	44934	17.158		17120	14.892	
	42855	16.221		17380	15.214	
	42111	15.885		17830	15.769	
41.6 pg/µL	90353	37.637	2.23%	37454	40.022	2.09%
	87917	36.539		36469	38.805	
	87285	36.254		37773	40.417	
	91348	38.086		36210	38.485	
	87594	36.393		37289	39.818	
33.3 pg/µL	762536	340.72	1.67%	279903	339.663	1.97%
	760825	339.948		278910	338.436	
	755161	337.394		270466	328.	
	762369	340.644		282912	343.382	
	733111	327.452		284341	345.147	
650 pg/µL	1447518	649.573	1.72%	554232	678.703	2.86%
	1482572	665.378		519271	635.495	
	1505908	675.901		551821	675.723	
	1505854	675.876		552697	676.806	
	1466808	658.27		533792	653.441	



	Unknown 1 Conc. (pg/µL)	Unknown 1 Cal. Conc.	Unknown 1 Difference	Unknown 1 %RSD	Unknown 2 Conc. (pg/µL)	Unknown 2 Cal. Conc.	Unknown 2 Difference	Unknown 2 %RSD
Ketoprofen	41.6	38.0	91.4%	1.22%	104.1	104.6	100.5%	1.53%
Ketoprofen - MS³	41.6	41.7	100.2%	1.26%	104.1	106.1	101.9%	2.05%

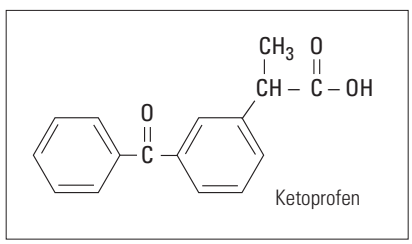


Figure 7: Comparison of MS/MS to MS³ quantitation of Ketoprofen

	AVERAGE AREA	AVERAGE CALC. CONC	%RSD	AVERAGE AREA	AVERAGE CALC. CONC	%RSD	AVERAGE AREA
		0.1 pg/µL			0.5 pg/µL		
Theobromine							
Theophylline							234
Dyphylline	72	0.49	0.52%	427	1	2.40%	784
Caffeine							
Chlorothiazine				75	0	24.53%	149
Cromolyn-Na	176	0.25	42.33%	456	1	1.28%	917
Hydroclorothiazide							255
Pemoline	102	0.34	18.81%	400	1	11.86%	780
Petoxifyline	793	-0.32	3.79%	4308	0	1.86%	8319
Dexamethasone				479	0	2.45%	1017
Boldenone	219	-0.01	14.44%	1261	0	1.97%	2471
Ketoprofen	315	0.60	7.64%	1191	1	9.43%	2381
Indomethacin				103	1	11.11%	212
Diclofenac	64	0.30	11.55%	281	1	8.84%	475
Flufenamic Acid	310	0.22	13.97%	892	1	2.52%	1780
Meclofenamic Acid	14	0.18	17.51%	61	1	41.26%	113

Table 2: Quantitation results for standards prepared in solvent

	AVERAGE AREA	AVERAGE CALC. CONC	%RSD	AVERAGE AREA	AVERAGE CALC. CONC	%RSD	AVERAGE AREA	AVERAGE CALC. CONC	%RSD	AVERAGE AREA	AVERAGE CALC. CONC	%RSD
	3.3 pg/µL			16.6 pg/µL			41.6 pg/µL			333.3 pg/µL		
Theophylline (A)	1129	6	10.48%	2540	17	5.53%	5185	38	2.82%	45170	334	2.11%
Dyphylline (A)	9832	5	1.94%	22461	17	2.73%	44917	39	0.98%	322434	334	2.24%
Caffeine (A)	6330	6	6.86%	15401	18	4.94%	30016	37	2.14%	258749	336	1.20%
Chlorothiazine (A)	1798	7	5.99%	3834	17	5.48%	7967	37	0.94%	70426	334	1.24%
Hydroclorothiazide (A)	2487	7	3.07%	5684	18	2.05%	11276	38	2.02%	92748	331	1.39%
Pentoxifylline (A)	122524	-2	-6.01%	296023	16	3.35%	605430	49	2.36%	3152583	332	0.97%
Boldenone (A)	13426	7	2.77%	33942	19	1.55%	58628	35	2.21%	593649	334	1.24%
Ketoprofen (A)	19899	6	3.75%	43660	17	3.86%	88899	37	2.23%	754801	337	1.67%
Ketoprofen – MS^3 (A)	8097	4	4.05%	17578	15	2.82%	37039	40	2.09%	279306	339	1.97%
Indomethacin (A)	1087	2	28.26%	2382	13	6.78%	6442	48	4.05%	35792	332	2.61%
Diclofenac (A)	2577	3	2.37%	5355	13	5.46%	14471	46	4.62%	78597	332	2.94%
Meclofenamic Acid (A)	290	7	10.78%	447	10	22.75%	2130	44	2.92%	6086	122	16.84%
	6.6 pg/µL			33.4 pg/µL			83.3 pg/µL			333.3 pg/µL		
Cromolyn-Na (B)	11298	9	2.70%	30183	27	1.08%	88171	83	0.80%	698392	675	2.40%
Flufenamic Acid (B)	3442	15	3.59%	7763	21	1.84%	55407	88	2.44%	135790	200	13.92%
	20 pg/µL			100 pg/µL			250 pg/µL			2000 pg/µL		
Theobromine (C)	6293	51	1.86%	17288	92	3.23%	51615	222	1.58%	471898	2009	0.77%

Table 3: Quantitation results for standards prepared in horse urine

AVERAGE CALC. CONC	%RSD	AVERAGE AREA	AVERAGE CALC. CONC	%RSD	AVERAGE AREA	AVERAGE CALC. CONC	%RSD	AVERAGE AREA	AVERAGE CALC. CONC	%RSD	AVERAGE AREA	AVERAGE CALC. CONC	%RSD
1.0 pg/µL			5.0 pg/µL			10 pg/µL			50 pg/µL			100 pg/µL	
		625	5	0.47%	1222	9	2.55%	6856	52	0.41%	12993	99	1.43%
1	1.79%	1208	5	2.58%	2408	10	2.52%	12207	49	1.36%	24967	100	0.66%
1	3.10%	4359	5	0.91%	8593	9	0.72%	46667	49	1.47%	95782	100	1.13%
		2330	6	5.03%	3912	9	3.15%	26131	51	0.85%	51308	99	1.07%
1	22.14%	645	4	13.31%	1294	9	4.88%	7711	53	2.66%	14231	98	1.10%
1	5.66%	4672	5	7.11%	8889	9	2.63%	49369	51	3.40%	96148	100	1.12%
1	7.88%	1170	5	11.66%	2663	10	2.83%	13545	50	1.19%	26856	100	2.72%
1	3.65%	3774	5	2.79%	8117	10	1.35%	42321	50	0.62%	84890	100	1.79%
1	1.13%	44808	5	0.56%	93532	10	1.05%	474442	53	2.08%	877603	98	1.77%
1	5.52%	5758	5	1.14%	11952	10	0.47%	60483	51	2.69%	118294	100	1.68%
1	5.08%	12325	5	2.25%	23958	10	1.07%	120812	50	1.33%	238560	100	1.68%
1	7.15%	11906	5	1.22%	22945	10	3.13%	119082	48	1.17%	253903	101	0.46%
1	2.78%	212	1	2.78%	2259	10	2.80%	11565	50	1.77%	23189	100	0.18%
1	8.40%	2382	5	1.20%	4712	10	3.18%	24920	50	1.14%	50161	100	2.06%
1	6.92%	8546	5	0.39%	17468	10	2.60%	90104	50	0.36%	178996	100	1.28%
1	7.84%	641	5	7.97%	1446	10	12.83%	7294	51	1.39%	14337	100	0.21%

AVERAGE AREA	AVERAGE CALC. CONC	%RSD
650 pg/µL		
96129	667	1.31%
575760	667	3.71%
511382	666	1.21%
143677	666	0.54%
186723	668	1.64%
5840616	667	0.95%
1301762	666	1.22%
1481732	665	1.72%
542362	664	2.86%
61378	667	1.74%
122821	668	2.18%
7065	142	10.74%
1350 pg/µL		
88842	84	2.10%
170496	249	10.77%
4000 pg/µL		
825323	3998	1.85%

	QC Sample				QC Sample 2			
	Conc. (pg/µL)	Cal. Conc.	Difference	%RSD	Conc. (pg/µL)	Cal. Conc.	Difference	%RSD
Theobromine (C)	250.0	231.7	92.7%	1.72%	625.0	615.0	98.4%	1.84%
Theophylline (A)	41.6	38.6	92.7%	1.96%	104.1	103.5	99.4%	2.58%
Dyphylline (A)	41.6	41.3	99.3%	2.02%	104.1	115.5	110.9%	3.38%
Caffeine (A)	41.6	42.4	101.9%	3.30%	104.1	109.6	105.3%	3.05%
Chlorothiazine (A)	41.6	43.0	103.3%	2.64%	104.1	114.4	109.9%	1.65%
Cromolyn-Na (B)	83.3	83.9	100.7%	2.10%	210.0	193.9	92.4%	1.77%
Hydroclorothiazide (A)	41.6	41.8	100.5%	2.64%	104.1	113.1	108.6%	2.27%
Pentoxifylline (A)	41.6	44.5	106.9%	2.23%	104.1	126.5	121.5%	1.43%
Boldenone (A)	41.6	38.8	93.4%	1.04%	104.1	102.4	98.4%	2.63%
Ketoprofen (A)	41.6	38.0	91.4%	1.22%	104.1	104.6	100.5%	1.53%
Ketoprofen – MS^3 (A)	41.6	41.7	100.2%	1.26%	104.1	106.1	101.9%	2.05%
Indomethacin (A)	41.6	49.7	119.5%	5.78%	104.1	116.4	111.8%	1.62%
Diclofenac (A)	41.6	48.4	116.3%	5.89%	104.1	124.1	119.2%	7.94%
Flufenamic Acid (B)	83.3	60.7	72.9%	2.21%	210.0	141.6	67.4%	19.10%
Meclofenamic Acid (A)	41.6	33.3	80.1%	6.74%	104.1	89.8	86.3%	21.76%

Table 4: Quantitation results for the analysis of unknown levels of drugs in horse urine

Conclusions

Positive and negative ion detection of co-eluting drugs was accomplished in a single chromatographic run using automated polarity switching. Drugs that underwent a neutral water loss were further fragmented using WideBand Activation to provide a diagnostically rich MS/MS spectrum for structural confirmation. The compound ketoprofen underwent a prominent, non-specific neutral loss of formic acid and was further analyzed using an MS^3 transition. Full-scan MS^n data was reprocessed to quantify all 16 compounds by reconstructed ion chromatograms (RICs), or post-acquisition MRM, and provided results comparable to triple quadrupole SRM quantitation. It is possible to achieve the low % RSD required in quantitation due to the fast cycle time of the Thermo Scientific LTQ. In the case of non-specific neutral molecule losses, MS^3 experiments generated diagnostic spectra for confirmational purposes while providing quantitative results comparable to the MS/MS data. Results of the ruggedness study demonstrate no appreciable loss of sensitivity or reproducibility across 100 replicate urine injections. Thus, using the Thermo Scientific LTQ two-dimensional linear ion trap, we have demonstrated the development of a simple, rapid, and rugged method capable of confirmational screening and simultaneous quantitation of drugs in horse urine using both full-scan LC/MS/MS and MS^3 spectra.

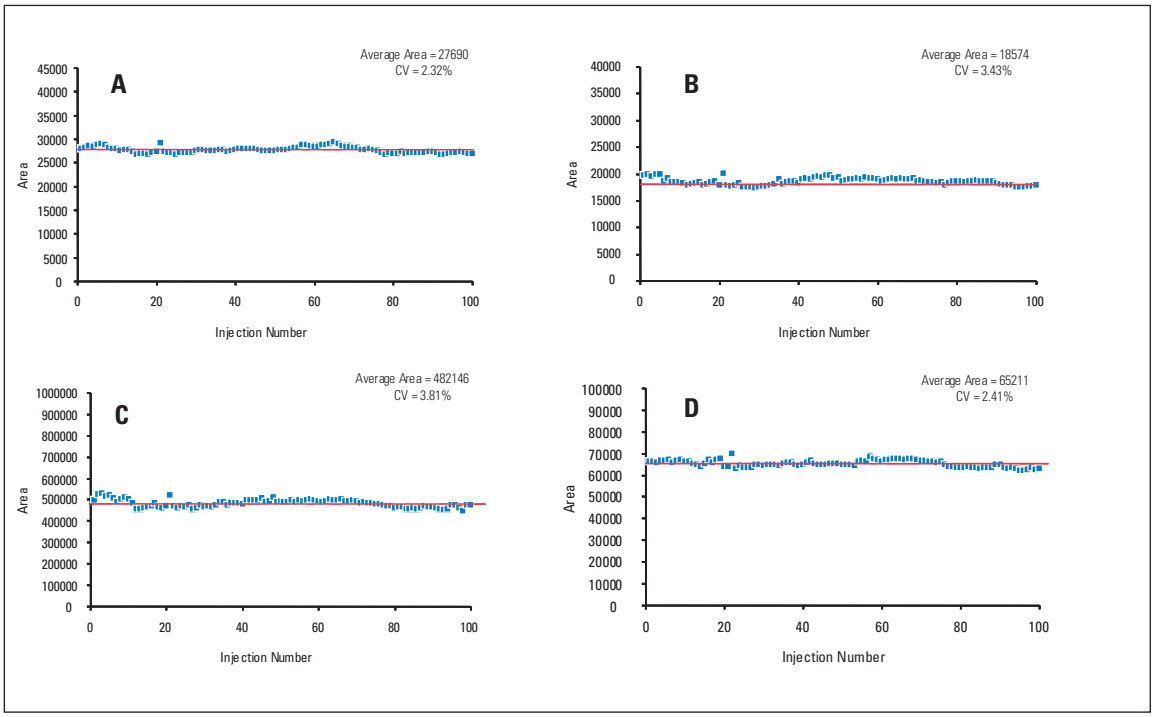


Figure 8: Ruggedness and reproducibility for 100 consecutive injections of a 166 pg/µL standard of theobromine, caffeine, pentoxyphylline, and ketoprofen in urine

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