Application Note: 346

MS/MS as an LC Detector for the Screening of Drugs and Their Metabolites in Race Horse Urine

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Key Words

- LCQ Advantage MAX™
- Surveyor™
- Data Dependent™
- Drug Screening
- Metabolite ID
- Toxicology

Introduction

Imipramine is a tricyclic antidepressant drug that is not a Drug Enforcement Administration controlled substance but has been classified by the Association of Racing Commissioners International Inc. as a class two drug in horses. Desipramine is a major metabolite of imipramine. These two analytes were analyzed on-line by LC–PDA MS/MS from extracts of horse urine. The urine sample was first treated with β-glucuronidase to hydrolyze glucuronide conjugates of imipramine and desipramine. This was followed by solid phase extraction. The concentration of imipramine and desipramine in the sample was determined by the internal standard method using the peak area ratio and linear regression analysis.

This application note presents a rapid method for quantitation of imipramine and desipramine in horse urine. It illustrates the advantages of MS/MS detection in terms of specificity, sensitivity and unambiguous identification, for the analysis of drugs and their metabolites.

Goal

1) Develop a rapid method to identify and quantitate tricyclic antidepressant imipramine and its major metabolite desipramine in horse urine.
2) Demonstrate the advantages of using MS/MS to identify and confirm the detection of imipramine and its metabolites.
3) Determine presence and structure of minor metabolites using Data Dependent LC-MS/MS analysis.

Experimental Conditions HPLC

LC system: Thermo Scientific Surveyor MS Pump, Surveyor Autosampler and Surveyor PDA Detector

Mobile phase: A: water containing 0.2% formic acid
B: Acetonitrile containing 0.2% formic acid

Column: 50 × 2.1 mm, 5 µm Thermo Scientific Hypersil™ C18 Column

Injection volume: 1 µL

Flow rate: 200 µL/min

Gradient:

Time (min)	% A	%B
0	98	2
0.2	98	2
8	25	75
9	10	90
10	10	90
10.01	98	2
15	98	2

Mass Spectrometer

Mass spectrometer: Thermo Scientific LCQ Advantage MAX
Ionization mode: Positive electrospray ionization (ESI)
Capillary temperature: 275 °C
Spray voltage: 4.5 kV
Sheath gas: 30 units
Sweep gas: 8 units

Analyte	*MH^+*	*Isolation Width*	*Collision Energy %*	*Scan Range*	*Quantifying MS/MS Product Ions*
Imipramine	281.2	1.5	30	75-285	86
Desipramine	267.2	1.5	30	70-290	236
Clomipramine (internal standard)	315.2	4	35	85-320	270

Table 1: MS parameters for imipramine, desipramine, and clomipramine (internal standard)

Standards

Calibration standards were prepared as follows:

Calibration level	*Volume of Imipramine and Desipramine working standard solution (µL)*	*Volume of Clomipramine working standard solution (µL)*	*Equivalent to Imipramine in the urine (ng/mL)*	*Equivalent to Clomipramine in the urine (ng/mL)*
C1	1:1 Dilution of C2	10	15.6	500
C2	1:1 Dilution of C3	10	31.3	500
C3	1:1 Dilution of C2	10	62.5	500
C4	1:1 Dilution of C2	10	125	500
C5	1:1 Dilution of C2	10	250	500
C6	1:1 Dilution of C2	10	500	500
C7	1:1 Dilution of C2	10	1000	500
C8	1:1 Dilution of C2	10	2000	500
C9	1:1 Dilution of C2	10	4000	500
C10	160	10	8000	500

Imipramine, desipramine and clomipramine working standard solutions were 50 ng/mL

Samples and Internal Standard

Imipramine was administered to the horse and a urine sample drawn after 0, 2, 4, 8 and 24 hours, post dose. One mL of the urine sample was spiked with 10 µL of 50 ng/µL clomipramine internal standard.

Sample Preparation

The calibration standard and urine samples were treated with β-glucuronidase to hydrolyze glucuronide conjugates of desipramine and imipramine, followed by solid phase extraction.

Results and Discussions

LC-UV-MS/MS analysis of imipramine and desipramine

Figures 1 and 2 show the analysis of tricyclic antidepressant imipramine, its major metabolite desipramine, and the internal standard clomipramine by LC with MS/MS and UV detection, respectively. Figure 1 shows base peak and extracted ion chromatograms for the three analytes along with MS and MS/MS spectra. The MS and MS/MS spectra help in unambiguous identification of these analytes and represent the high specificity that can be obtained from such an analysis. Further, the MS/MS spectra can be stored in a library and used for rapid confirmation of the drug and its metabolite. Figure 2 shows total spectra obtained from a PDA detector as well as UV trace at 254 nm and 280 nm. The position of elution of the three compounds had to be determined by sequential injections of individual analytes. As illustrated in Figure 2, the UV spectra for these compounds appear almost identical, making their unambiguous identification difficult.

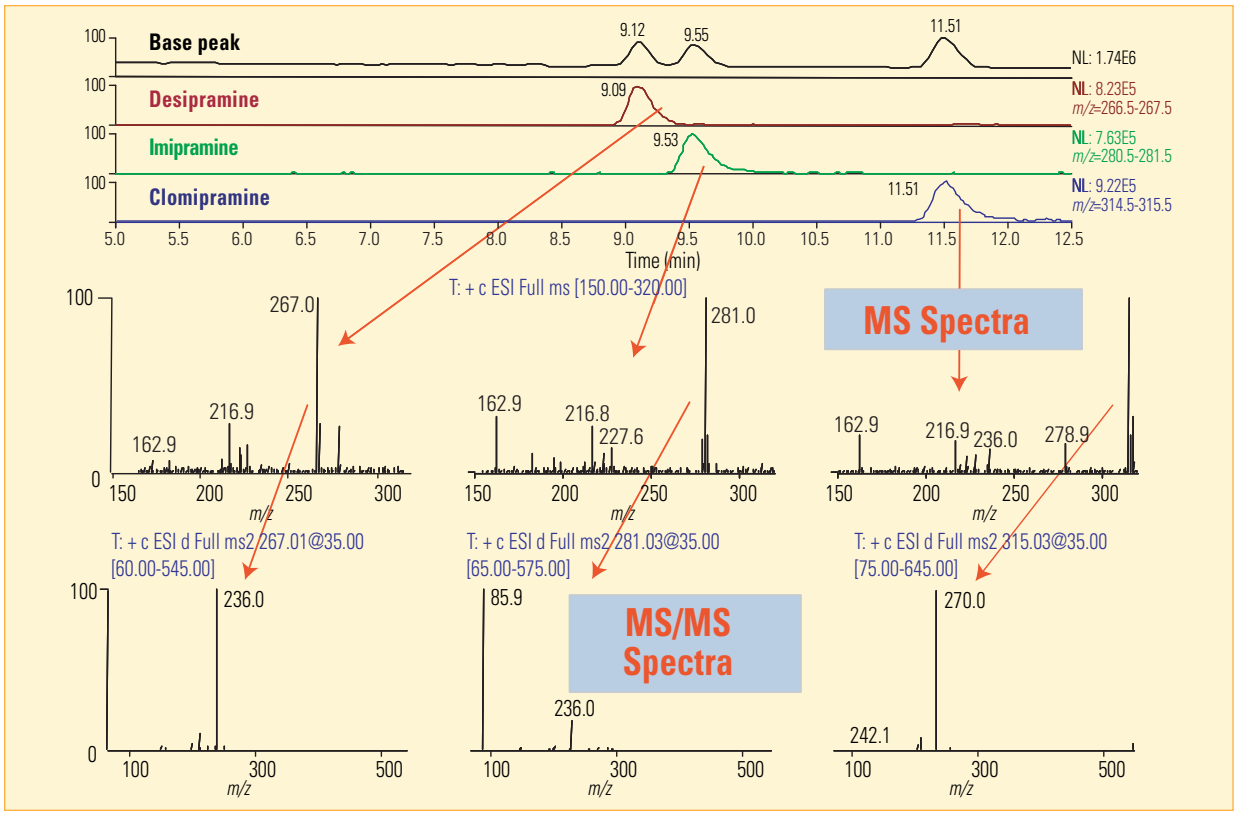


Figure 1: LC-MS/MS analysis of imipramine, desipramine and clomipramine (internal standard)

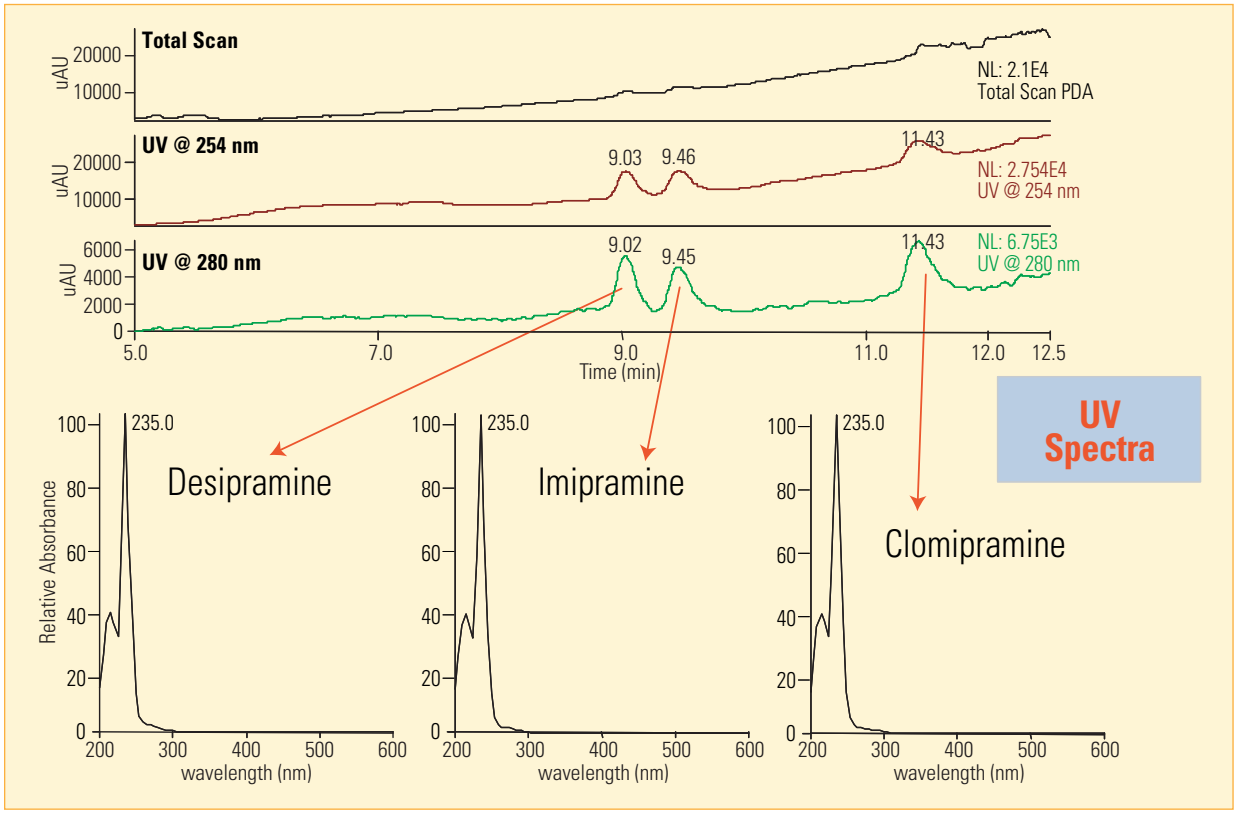


Figure 2: LC-UV analysis of imipramine, desipramine and clomipramine (internal standard)

Figure 3 shows chromatograms obtained for the analysis of imipramine, desipramine and clomipramine (IS) with MS and UV detection at levels of 5 and 0.5 ng on-column. At 0.5 ng on-column, both imipramine and desipramine could be easily identified when MS was used as a detector whereas these analytes were hardly visible in the UV trace. The concentration of clomipramine is the same at both these levels. This illustrates the excellent sensitivity that can be obtained during analysis by LC-MS/MS.

Quantitation of imipramine and desipramine in horse urine

Figures 4 and 5 show calibration curves obtained for imipramine and its major metabolite desipramine in horse urine with clomipramine used as an internal standard. The coefficient of correlation is 0.9896 for calibration curve of imipramine and 0.9836 for the calibration curve of desipramine. The % CV values are less than 7% for the imipramine calibration curve and 15% for the

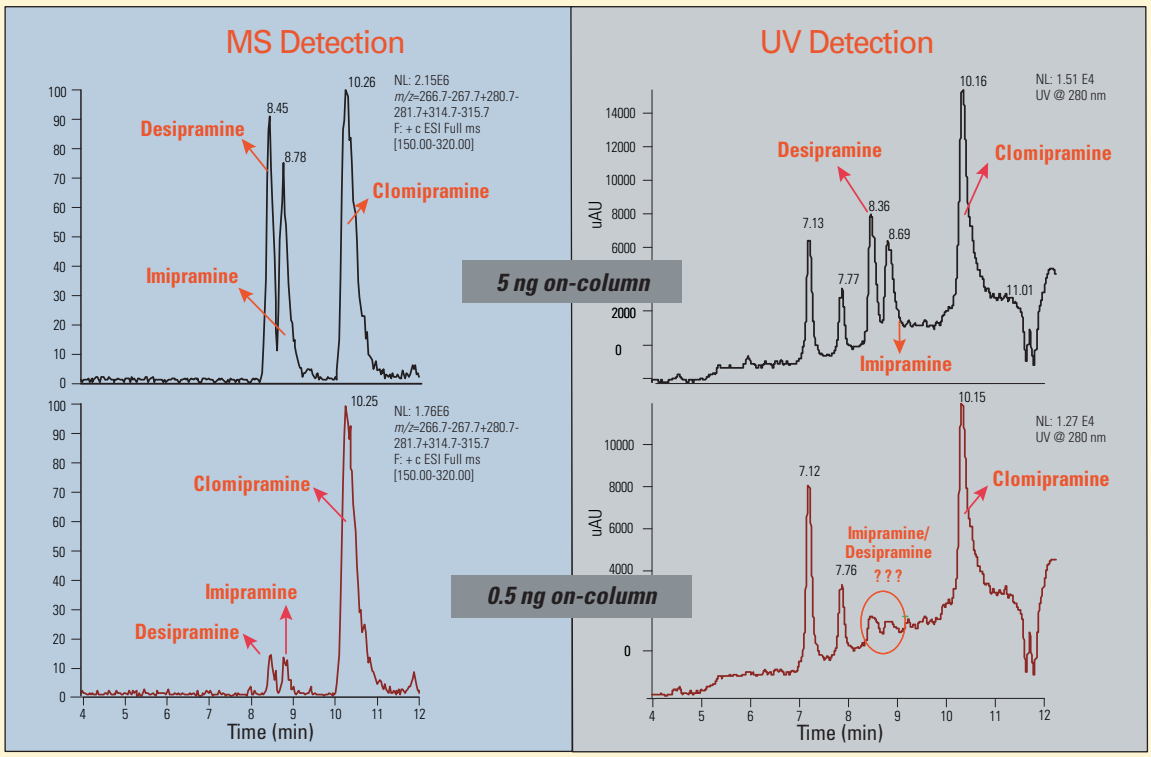
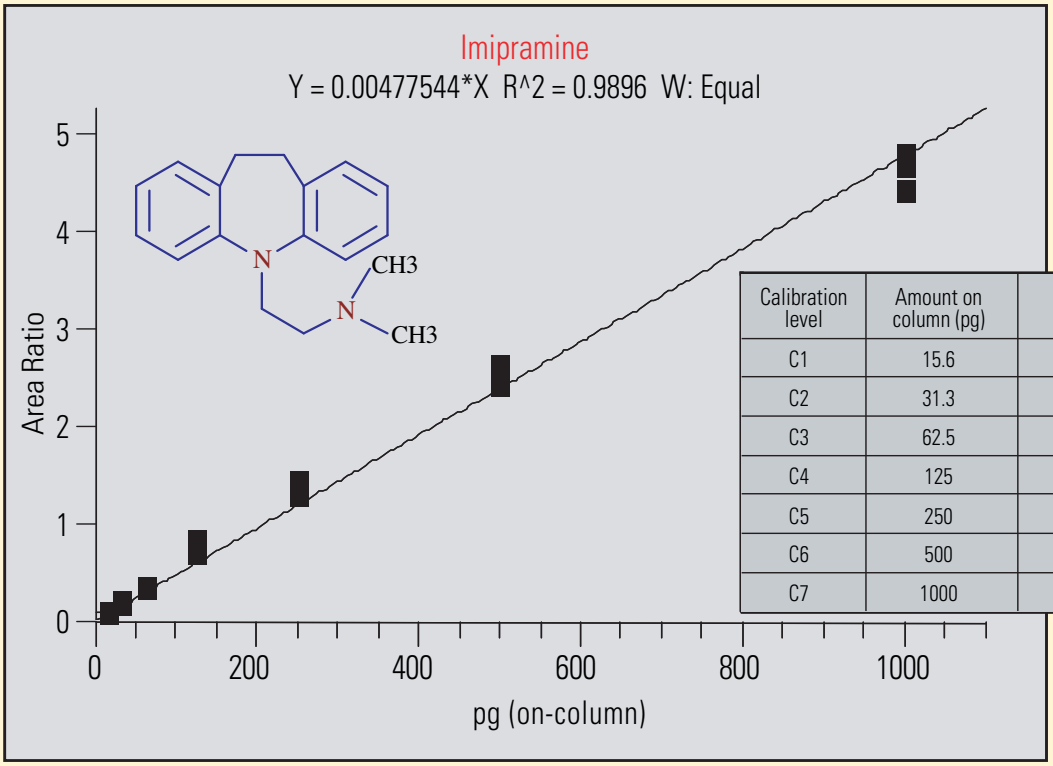
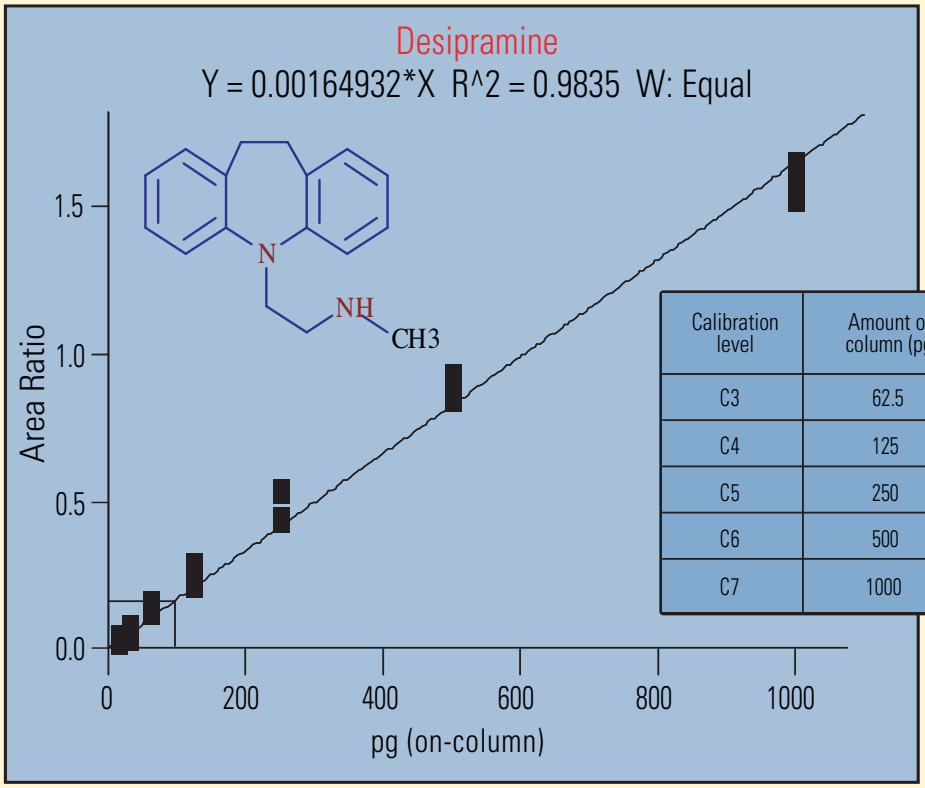


Figure 3: Comparison of MS and UV detection at two different concentrations



Calibration level	Amount on column (pg)	% CV
C1	15.6	4.25
C2	31.3	1.25
C3	62.5	2.28
C4	125	7.34
C5	250	5.32
C6	500	3.65
C7	1000	4.00

Figure 4: Calibration curve for imipramine in horse urine



Calibration level	Amount on column (pg)	% CV
C3	62.5	14.96
C4	125	14.30
C5	250	11.24
C6	500	4.73
C7	1000	4.02

Figure 5: Calibration curve for desipramine in horse urine

desipramine calibration curve. Figure 6 shows analysis of imipramine and desipramine in horse urine sample drawn two hours post-administration of the drug. The amount of imipramine and its major metabolite desipramine was determined using the calibration curves shown in Figures 4 and 5. Table 2 shows the amount of these two analytes as determined in horse urine. For the sample drawn two hours post-administration of the drug, the amount of imipramine and desipramine was determined to be 28 and 1567 ng/mL, respectively. The amount of desipramine determined at this time is above the upper limit of quantitation for the calibration curve shown in Figure 5.

Time (hr)	Imipramine (ng/mL)	Desipramine (ng/mL)
0+	17.56	20.85*
2	28.12	1567.16**
4	4.25*	189.06
8	6.75*	96.56
24	6.11*	13.11*

Table 2: Determination of imipramine and desipramine in horse urine for samples drawn at different times post injection of the drug (*below lower limit of quantitation, **above upper limit of quantitation)

Identification of metabolites of imipramine

A urine sample from the race horse obtained two hours after administration of the drug was also analyzed by Data Dependent LC-MS/MS, with MS/MS on the top two most intense ions to determine the presence of other metabolites. Figure 7 shows the workflow for such an analysis. The extracted ion chromatograms in Figure 8 show the presence of four additional metabolites: desmethyl desipramine, OH desipramine, OH-imipramine, and N-Oxide of imipramine, as well as their MS/MS fragmentation pattern. As indicated by the two peaks in the extracted ion chromatogram for *m/z* 297.2, imipramine is metabolized to two metabolites that have the same *m/z*. In this case, the MS/MS fragmentation pattern enables unambiguous distinction between the two metabolites.

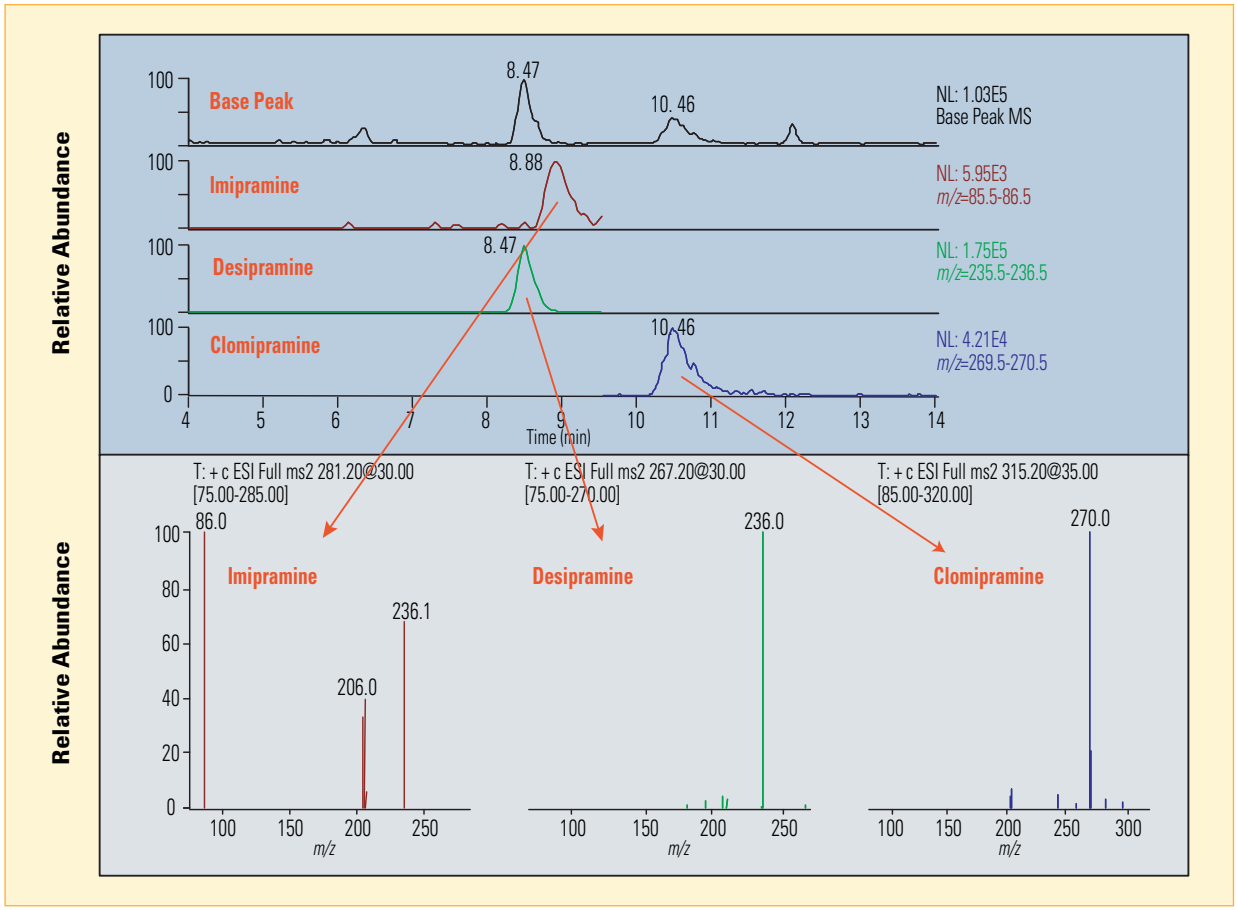


Figure 6: Analysis of imipramine and desipramine in horse urine for sample drawn two hour post-administration of drug

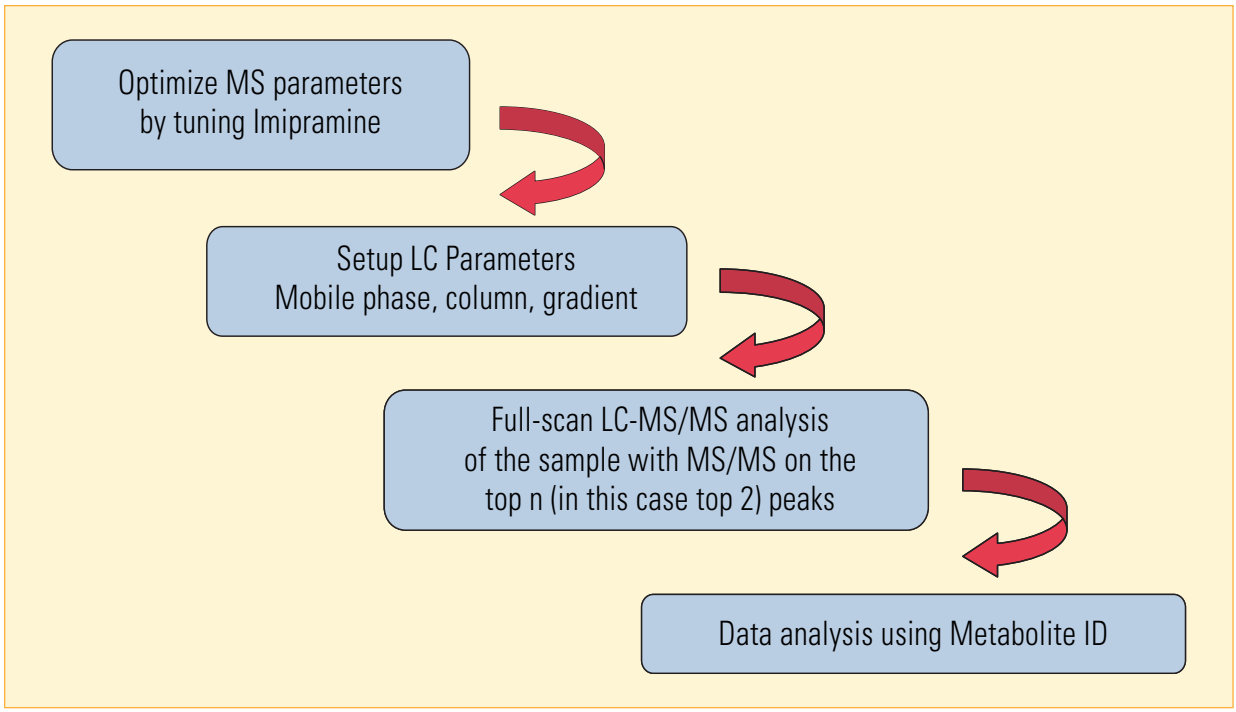


Figure 7: Workflow for the identification of imipramine and its metabolite in horse urine

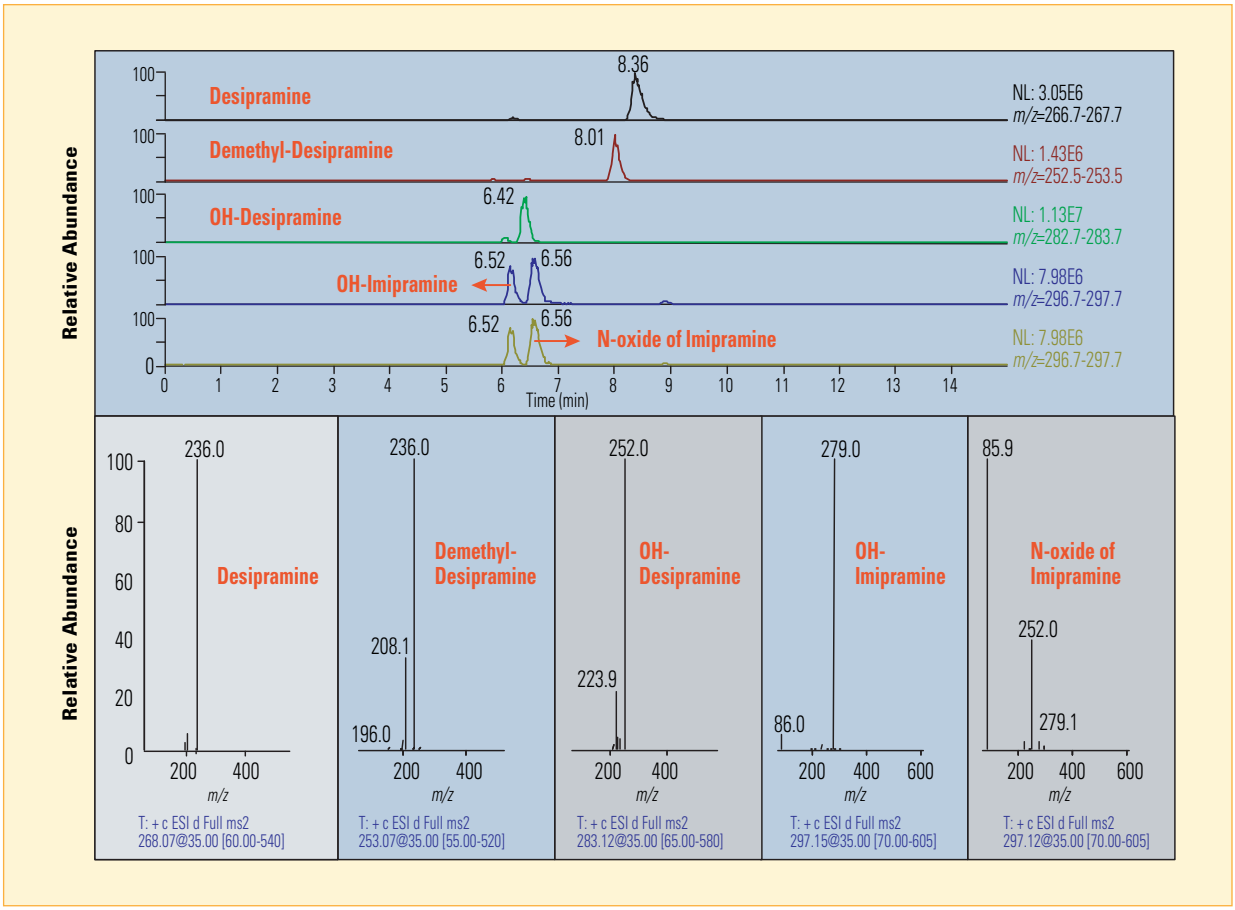


Figure 8: Data Dependent LC-MS/MS analysis of metabolites of imipramine

Conclusions

Full scan MS/MS analysis using a Thermo Scientific LCQ Advantage MAX ion trap mass spectrometer provides the selectivity and sensitivity necessary to support ADME/Tox studies of imipramine in horse urine. Analysis of drugs and their metabolites in complex biological samples using MS/MS detection enables unambiguous identification of these analytes. Data Dependent LC-MS/MS analysis facilitates presence and structural determination of several co-eluting minor metabolites. MS/MS information is invaluable in the identification of metabolites with the same *m/z* (e.g., OH-imipramine and N-oxide of imipramine).

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