

Making the Leap to LC/MS/MS: Enhancing and Accelerating Clinical Research and Forensic Toxicology Applications

Introduction

The resolving power of chromatography combined with the sensitivity and selectivity of mass spectrometry results in an extremely useful analytical technique. GC/MS has long been the technique of choice for identification and quantification of analytes in complex mixtures. Because of its utility, extreme efforts are often employed in sample preparation to make difficult compounds amenable to GC/MS analysis. This sample preparation often includes liquid-liquid extraction (LLE), solid-phase extraction (SPE), and/or derivatization.

Liquid chromatography interfaced to mass spectrometry was the natural progression of chromatographic/mass spectral techniques. However, early attempts at interfacing LC to mass spectrometry had very limited capabilities. The introduction of electrospray ionization helped LC/MS/MS develop from a research tool into a routine technique. Further developments in electronics and computers have resulted in the economical benchtop instruments that are present in a majority of analytical labs today.

LC/MS/MS is a very complementary technique to GC/MS. Many of the compounds that are difficult to analyze by GC/MS — such as amines and semi-volatile compounds — are ideal candidates

for LC/MS/MS. By switching assays from GC/MS to LC/MS/MS, many advantages can be realized. First of all, GC is most commonly interfaced to single quadrupole mass analyzers. By moving from single MS to tandem MS/MS, selectivity is greatly enhanced, and therefore improved detection and quantitation limits can be achieved. Secondly, two limitations with GC/MS are that the sample had to be in an organic injection solvent and derivatization is often necessary to improve peak shape, ionization, and/or volatility. LC/MS/MS typically does not have these two requirements, and therefore sample preparation can be greatly simplified. Although sample preparation can include solid-phase extraction (SPE) or liquid-liquid extraction (LLE), it can be as simple as direct injection, dilution, or protein precipitation. By minimizing the amount of sample handling required, savings of both time and money can be attained. Additionally, decreasing the number of preparation steps can minimize areas where errors or contamination can be introduced. Several assays were transferred from GC/MS to LC/MS/MS and the methods and data compared and contrasted. Unless otherwise stated, all data were collected using an AB SCIEX API 3200™ LC/MS/MS system.

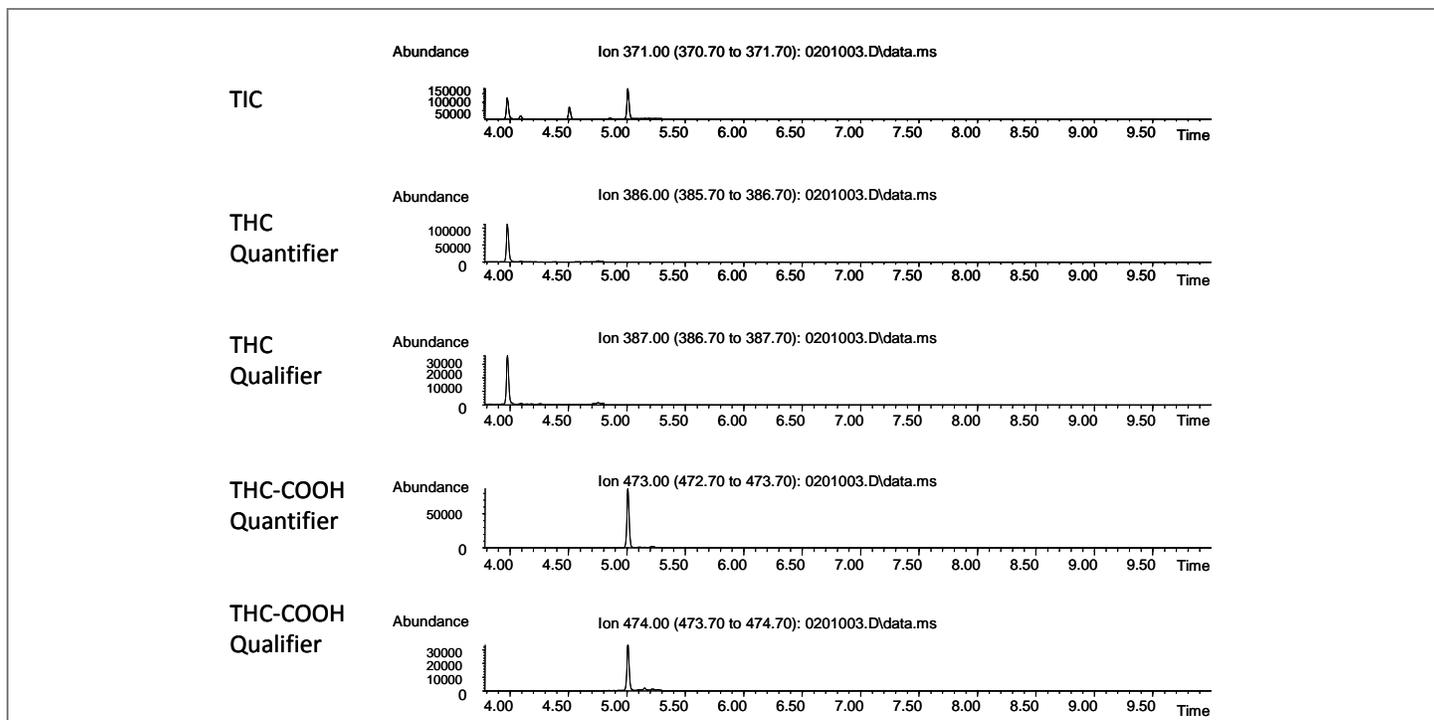


Figure 1. Chromatogram of a low QC (10 ng/mL) analyzed using GC/MS. The total run time was 10 minutes.

THC in blood

Cannabis is one of the most commonly used drugs in the world and is illegal in most countries. The active ingredient is Δ^9 -THC, which is the target analyte along with its major metabolite, 11-nor-9-carboxy- Δ^9 -tetrahydrocannabinol (THC-COOH). An LC/MS/MS method for analysis of THC and THC-COOH in blood was developed to replace an existing GC/MS method.

For GC/MS, sample preparation consisted of solid-phase extraction followed by derivatization and sample concentration. Use of LC/MS/MS allowed omission of the derivatization step. Representative GC/MS and LC/MS/MS data from a low QC (10 ng/mL) are shown in Figures 1 and 2, respectively. Note the reduction in run times: 10 minutes for the GC/MS analysis and 4.5 minutes for the LC/MS/MS analysis. The long GC/MS run time was necessary to bake out the GC column of excess derivatizing reagent. The LLOQ for LC/MS/MS was also better at 0.5 ng/mL, versus 2 ng/mL observed for GC/MS analysis.

By switching the analysis of THC and its metabolite from GC/MS to LC/MS/MS, sample preparation was simplified and run times shortened, resulting in significant time savings. Furthermore, the LLOQ improved by a factor of 4x.

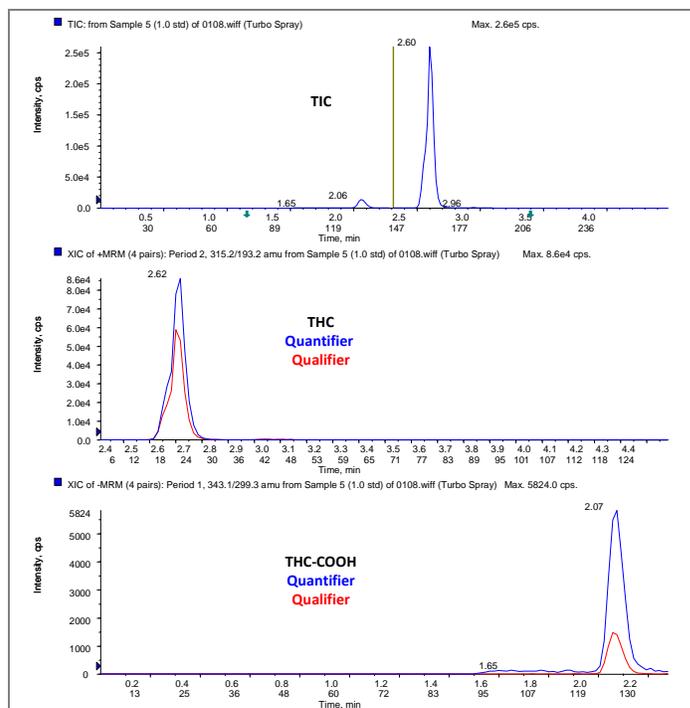


Figure 2. Chromatogram of a low QC (10 ng/mL) analyzed using LC/MS/MS. The total run time was 4.5 minutes, which is less than half the time required for GC/MS analysis.

Opiates in urine

Opiates are a class of drugs frequently prescribed for pain relief. However, these drugs are often illegally obtained and abused. Because of the high prevalence of illicit use, frequent testing may be required. This method analyzes urine for the presence of morphine, oxycodone, hydromorphone, codeine, oxycodone, hydrocodone, and 6-monoacetylmorphine (6-MAM).

A comparison of the sample preparation protocols for GC/MS and LC/MS/MS is shown in Figure 3. For GC/MS, sample preparation consisted of hydrolysis, derivatization, and an extensive SPE cleanup procedure. The steps highlighted in blue were omitted for LC/MS/MS analysis and sample preparation was reduced to hydrolysis, centrifugation, and sample dilution. Also note that GC/MS required a sample volume of 1 mL that was subsequently concentrated 10x for analysis. For LC/MS/MS, the sample volume was reduced to 0.250 mL with a dilution factor of approximately 5x, demonstrating the improved selectivity and sensitivity of LC with tandem mass spectral detection. By eliminating SPE clean-up, significant reductions in both time and consumable costs were achieved.

Time savings were not limited to the front-end sample preparation, but also to the instrumental analysis. Total run time was reduced by 40%, dropping from 10 minutes for the GC/MS method to 6 minutes for the LC/MS/MS method. Also, due to the

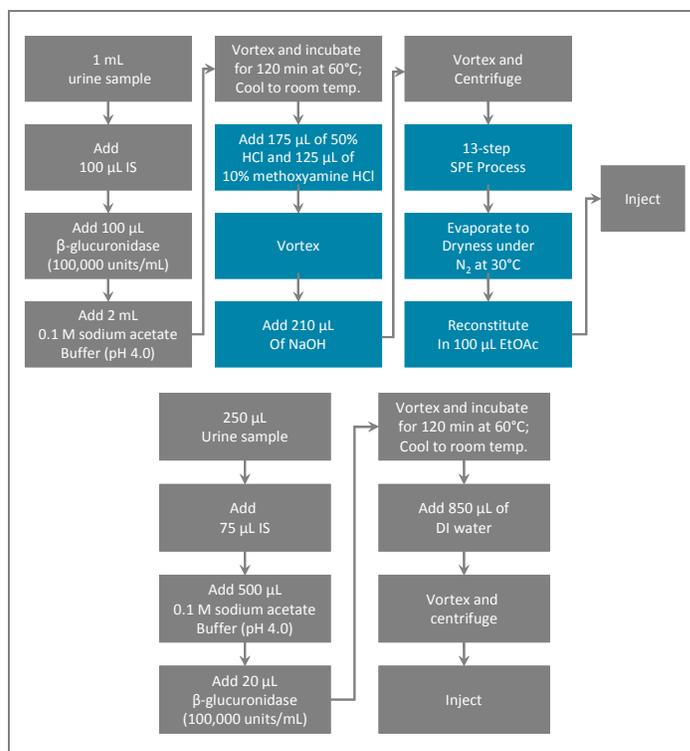


Figure 3. Sample preparation for analysis of opiates in urine. (Top) GC/MS sample preparation protocol. The steps highlighted in blue could be omitted when LC/MS/MS analysis was used. (Bottom) Sample preparation protocol for LC/MS/MS analysis uses a smaller sample volume and is much simpler than the GC/MS preparation.

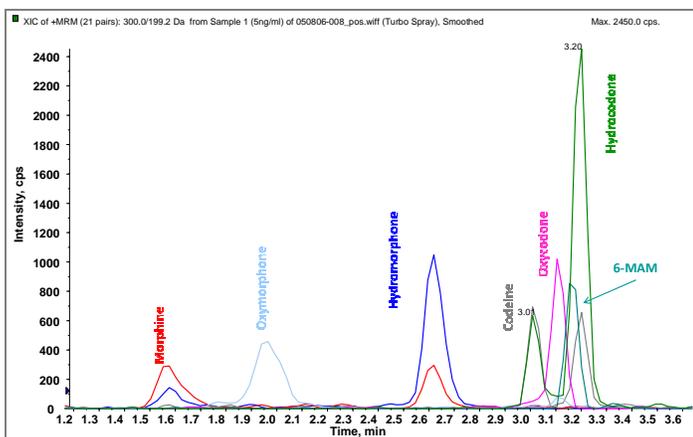


Figure 4. Representative chromatogram for opiates in urine. Data shown are from a 5 ng/mL calibrator, which is the LLOQ for this assay. The linear range was 5 – 10,000 ng/mL.

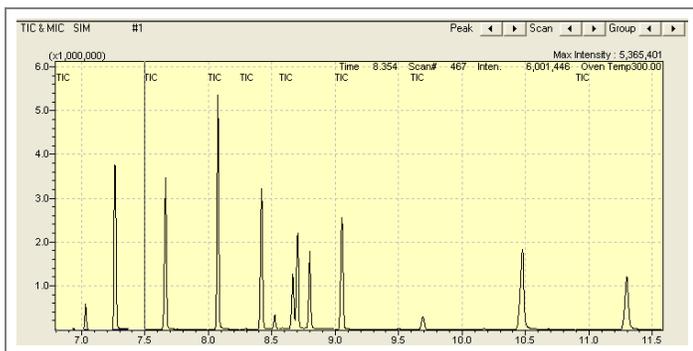


Figure 5. Total ion chromatogram for GC/MS analysis of ten benzodiazepines. Total run time was 12 minutes per sample.

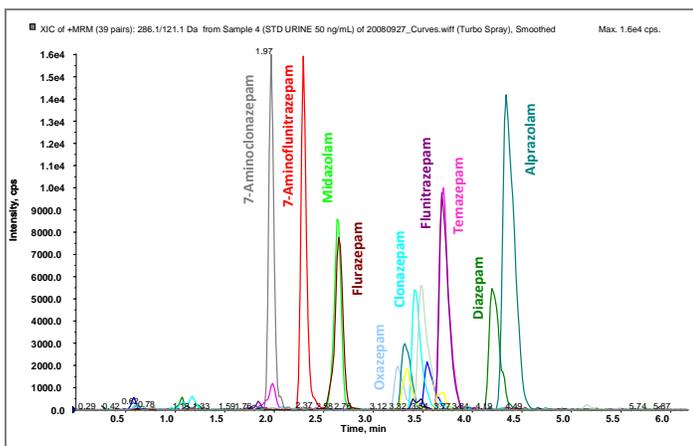


Figure 6. Extracted ion chromatograms for analysis of 14 benzodiazepines using LC/MS/MS. Not all analytes are labeled in the figure. Data shown were obtained from a 50 ng/mL calibrator, and the LLOQ for all analytes was 10 ng/mL or better. Total run time for this analysis, including column equilibration, was about 8 minutes.

sensitivity and derivatization requirements, 6-MAM was a separate, dedicated GC/MS method. However, all opiates, including 6-MAM, were included in the LC/MS/MS extraction and analytical method. Figure 4 shows a representative chromatogram at 5 ng/mL, which was the working LLOQ for this method.

When results from the LC/MS/MS method were compared with the validated GC/MS method, there was a correlation of 90%. The inter- and intra-day CVs were <10% for both the high and low QCs. The data quality was also greatly improved, as measured by a reduction in the sample re-run rate, which dropped from an average of 5 samples per batch of 30 samples to 1 sample per batch of 30.

By transferring the analysis of opiates in urine to an LC/MS/MS method, significant advantages were realized while maintaining—and even improving—data quality, which greatly reduced the number of samples requiring repeat analysis. Previous use of GC/MS required two runs and long, extensive sample preparation protocols for complete panel analysis. When analyzed using LC/MS/MS, sample size was reduced, sample preparation simplified, run time shortened, and only one run was necessary. These benefits of LC/MS/MS resulted not only in time savings but also significant reductions in consumable and labor costs.

Benzodiazepines in urine

Benzodiazepines are frequently prescribed for sleep disorders, anxiety, stress, and several other conditions. Because of the high prevalence of these drugs, they are often involved in illicit use and forensic cases. Traditionally, benzodiazepine screening has been performed utilizing immunoassay with GC/MS confirmation and quantification. GC/MS analysis of benzodiazepines requires hydrolysis followed by extensive SPE clean-up and derivatization in order to detect the low concentrations, similar to the sample preparation for opiates.

For GC/MS analysis of benzodiazepines, 1 mL of urine was hydrolyzed, cleaned up using a multi-step SPE procedure, derivatized, and reconstituted in 0.100 mL (a 10x concentration) prior to analysis. Sample preparation for LC/MS/MS analysis started with 0.500 mL of urine and consisted of hydrolysis, centrifugation, and 10x dilution, similar to the preparation procedure outline in Figure 3. Total analytical run time was again significantly reduced, as illustrated in Figures 5 and 6, which show chromatograms from GC/MS and LC/MS/MS analyses, respectively. When GC/MS analysis is utilized, total analytical run time is about 12 minutes, versus 8 minutes using LC/MS/MS. As with the opiate analysis, transferring the method from GC/MS to LC/MS/MS resulted in several advantages: simplified sample

preparation, including omission of time-consuming SPE and derivatization steps, and shorter analytical run time.

Analysis across drug classes

One of the most significant advantages of being able to use a simple, relatively generic sample preparation is that it allows analysis of drugs across multiple compound classes in a single method. An example of this multi-class drug analysis is demonstrated in Figure 7, which shows analysis of 9 opiates and 10 benzodiazepines in 6 minutes. Sample preparation was the same as depicted in Figure 3.

The ability to analyze across drug classes can be extended to confirmation panels or comprehensive drug screening. Figure 8 shows a representative chromatogram of a confirmation panel for 30 commonly abused illicit and prescription drugs. Even with a high number of analytes, precision and accuracy can be maintained by proper method setup and use of appropriate internal standards (Figure 9). Although Figure 9 presents statistics for morphine, statistics for other compounds were of similar quality. Figure 10 shows a multi-target drug screen for over 700 drugs using an AB SCIEX 3200 QTRAP[®] instrument.

The method takes advantage of the hybrid triple quadrupole/linear ion trap capabilities of the instrument by using the selective MRM mode to detect the target compounds, and the sensitive full-scan capabilities of the ion trap to obtain full-scan MS/MS spectra of the analytes. These spectra can be searched against a library for compound identification and confirmation. A representative report showing library confirmation for temazepam is presented in Figure 11.

Summary

Many toxicological analyses are being transferred from GC/MS to LC/MS/MS to take advantage of: reduced sample preparation and handling; improved detection limits; and faster analytical run times. This reduction in sample handling, consumables, and time helps reduce costs and minimize potential for errors or contamination. Furthermore, because the sample preparation protocols are relatively generic, it is easy to analyze drugs across multiple classes, resulting in even greater time savings. LC/MS/MS can be utilized to analyze multiple drug classes in a single assay for quantitation/confirmation, as well as comprehensive drug screening. Because of these advantages, as well as the versatility of hybrid instruments, the use of LC/MS/MS is rapidly increasing in the forensic toxicology field and is quickly becoming the analytical technique of choice.

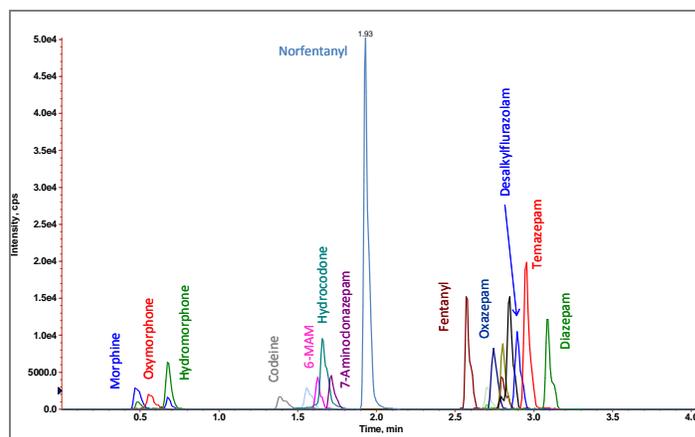


Figure 7. Combined benzodiazepine and opiate analysis. Nine opiates and ten benzodiazepines were analyzed in a single method. Data shown are from a 10 ng/mL calibrator (1 ng/mL for fentanyl), and total run time was 6 minutes. The LC conditions used for this analysis are different from the conditions used for the benzodiazepine and opiate analyses previously discussed.

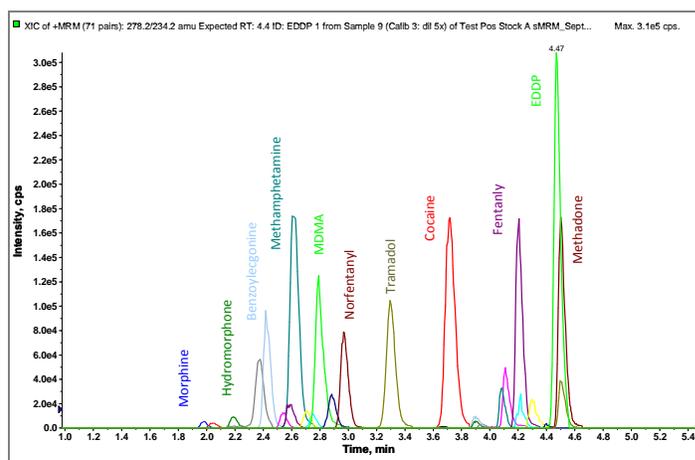


Figure 8. Analysis of a 50 ng/mL calibrator for 30 illicit and prescription drugs in urine. Total run time was 6.5 minutes. Not all drugs are labeled in this figure.

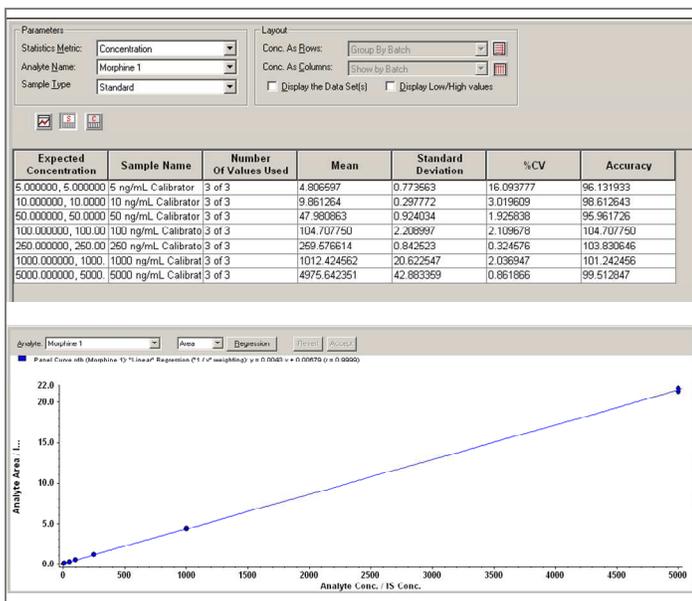


Figure 9. Data quality for a multi-analyte panel was maintained as supported by the precision, accuracy, and linearity. Results for morphine are displayed, and similar results were obtained for other analytes, though the upper limit of quantification was lower than 5,000 ng/mL for some compounds.

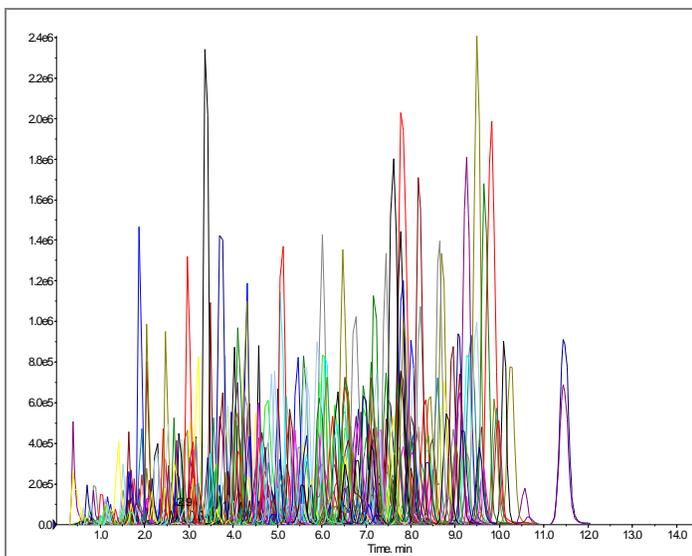


Figure 10. Multi-target LC/MS/MS screening for >700 drugs. MRM mode was combined with dependent full-scan MS/MS spectral acquisition. The MS/MS spectra were searched against a library for compound identification and confirmation.

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Publication number: 1037210-01

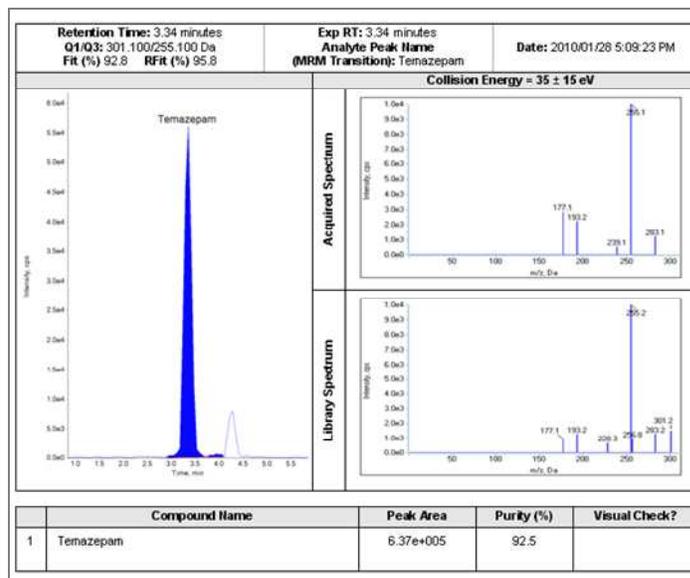


Figure 11. Identification of temazepam from a urine sample analyzed using the 700-compound multi-target LC/MS/MS screening method. The MS/MS spectrum from the unknown sample is on top and the library spectrum is on the bottom.

Acknowledgments

The author would like to thank Albert Elian, Massachusetts State Police; Timothy D. Dahn, AIT Laboratories; and Sumandeep Rana, Redwood Toxicology for their contribution of data and/or samples.