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Development of a New UPLC-MS Method for Systematic Toxicological Analysis

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For forensic toxicology use only.

Abstract

A method has previously been described for the systematic toxicological analysis (STA) of biological specimens. This method comprised a 26 minute HPLC separation, in combination with the collection of full scan mass spectral data and has been successfully applied for the analysis of routine samples in laboratories worldwide over the last 5 years.

Since this method was first described, there have been some significant advances in the available technology. In 2004, the revolutionary separation technique, UPLC was introduced. We now present our latest STA method. This technique exploits the rapid separation afforded by UPLC combined with the ultra-fast scanning capabilities of the Waters TQ Detector, providing a comprehensive analysis in only 15 minutes – a time-saving of 40%.

Introduction

Broad screening techniques are routinely applied to biological samples for the identification of toxicants. In the field of forensic toxicology, the analysis of ante and postmortem specimens may be necessary to investigate cases of alleged chemical submission, to identify the use of illicit compounds and to ascertain the cause of death. In emergency toxicology, analysis may be required for the investigation of accidental poisoning, suspected overdose or following an adverse reaction to prescriptive or over-the-counter medication. In these latter situations, in particular, analytical speed and assay turnaround time can be a critical element.

Previously, we have described a screening method based on LC-MS.[1] The method comprised chromatographic separation (26 minutes) combined with full scan detection. Resultant data were collected and matched against a spectral database which had been created under identical analytical conditions.

The database contained information for approximately 500 toxicologically-relevant analytes. Since its release more than 4 years ago, this method has been successfully used in laboratories worldwide.

One of the main challenges facing forensic laboratories these days, is a need to increase service whilst holding costs to a minimum. The laboratory can now play a major role by providing greater sample throughput and expanding analytical capability whilst maintaining, or if possible improving, the data quality.

We present our latest STA method which utilises the newest state-of-the-art LC and MS technologies.

Innovative Technologies

ACQUITY UPLC

2004 saw the advent of UltraPerformance LC (UPLC); a major breakthrough in separation science which has provided scientists, from all disciplines, with vast improvements over their traditional HPLC techniques. The smaller particle size (sub-2 µm) of the UPLC columns (Figure 1) leads to enhanced chromatographic peak resolution; sharper and narrower peaks with increased signal to noise.

Figure 1. The Waters ACQUITY TQD system and ACQUITY UPLC column featuring eCord technology. The eCord electronically stores all the information for full traceability of your experiments including; date of column installation, certificate of analysis, number of injections, maximum temperature and pressure - a full column history.

This novel technique also allows a dramatic reduction of the sample run time. These enhancements ultimately result in the provision of superior analyte detection combined with increased sample throughput.

The Waters TQ Detector

UPLC systems can generate peak widths as narrow as one second at half-height. Consequently, this can pose a significant challenge for peak detection. To fully exploit the increased analytical capabilities afforded by UPLC, an appropriate detection system is also required. This system needs to have a sampling rate high enough to provide sufficient definition of the chromatographic peaks to allow reproducible detection and integration. The Waters TQ Detector has been designed to provide higher speed data acquisition whilst maintaining data quality with a maximum MS scan-speed of 10,000 amu/s. Ultra-fast polarity switching in only 20 ms means that both positive and negative ionising compounds can be detected in the same run.

Overview of Screening Methodology UPLC-MS Library

I. Library concept

This latest library method utilises the same library concept as previously described by Humbert1 i.e., for each analyte, mass spectra are collected under multiple fragmentation conditions. The degree of fragmentation is controlled by varying the cone voltage in the source of the mass spectrometer.

This process, known as in-source collision-induced dissociation (in-source CID), can be performed simultaneously in both ES+ and ES- modes, hence library entries can be created for positive and

negatively ionising compounds (Figure 2). Retention time (RT) information is also recorded for each analyte which provides additional confidence in the result. Data for authentic samples are collected under exactly the same UPLC-MS conditions as those used for library creation.

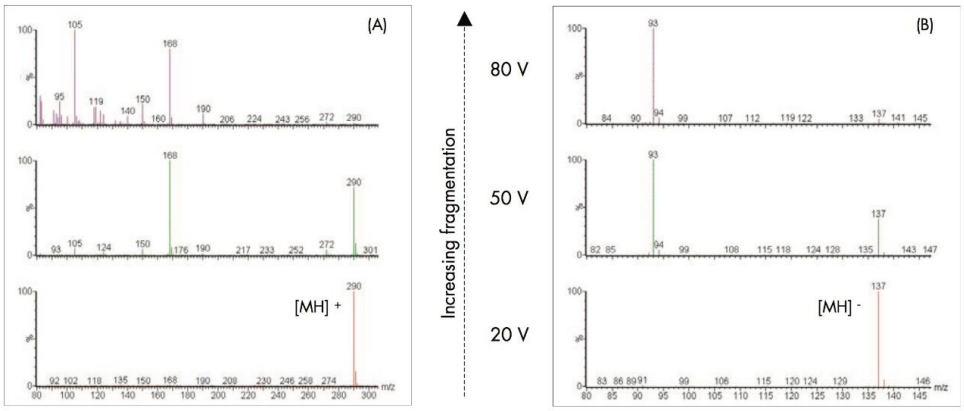

Figure 2. Fragmentation patterns for benzoylecgonine in positive ionisation mode (A) and salicylic acid in negative ionisation mode (B). Only spectral data acquired at 20, 50 and 80 V are shown for simplicity. However, typically the library contains 6 mass spectra (recorded at 6 cone voltages) for each analyte, in addition to RT.

II. Library content

A new database has been created and contains data for 500 of the most commonly-encountered toxicants including illicit drugs and metabolites, and prescribed drugs.

The library constitutes a powerful and reliable tool for the toxicology laboratory. It has been extensively investigated for accuracy of RT and spectral data within both Waters and collaborator's laboratories. The library is also easy to maintain and fully appendable by the user.

III. Utility of full scan data

The collection of full scan MS data provides a more comprehensive screening for true unknowns than any targeted LC-MS/MS approach. The result is a more complete (rather than a targeted/restricted) dataset. As the acquired data files remain unaltered, the data may be interrogated retrospectively if required; this can be performed even without the need to re-analyse the sample.

The flexibility of the Waters TQ Detector allows the user to collect full scan data for broad screening but also to make use of the LC -MS/MS capabilities to confirm the presence of proposed analytes without the need for additional instrumentation. Confirmation assays are typically performed by using the instrument in multiple reaction monitoring (MRM) mode and require the ion ratio of qualifier and quantifier ions to be determined.

Experimental

LC Conditions

LC System:	ACQUITY UPLC System
Column:	ACQUITY UPLC HSS C_{18} Column 2.1 x 150 mm, 1.8 µm
Column Temp:	50 ˚C
Flow Rate:	400 µL/min.
Mobile Phase A:	5 mM ammonium formate, pH 3.0
Mobile Phase B:	Acetonitrile with 0.1 % formic acid
Initial Conditions:	87 % Mobile Phase A
Gradient:	Gradient increasing to 95 % Mobile Phase B
Analysis Time:	15 Minutes
Weak wash:	10 % acetonitrile in water (600 µL)
Strong wash:	95 % acetonitrile in water (200 µL)

MS Conditions

MS System:	Waters TQ Detector
Capillary Voltage:	3.5 kV
Cone Voltage:	20 V to 95 V (in 15 V increments)
Desolvation Temp:	400 ˚C
Desolvation Gas:	800 L/Hr
Source Temp:	150 ˚C
Acquisition Range:	*m/z* 80 – 650

Software

Waters MassLynx software v4.1 was used for data acquisition and the ChromaLynx application manager[2] was used for data processing. ChromaLynx is a unique data processing software based on deconvolution techniques.

The application manager automatically examines the chromatograms produced at each cone voltage, detects the components and calculates the average spectral match factor (MF) against the library (maximum MF = 1000). Candidates are assigned with the following symbols according to the total accuracy of the match:

These are user-definable criteria (typically MF >700, 500-700 and <500 respectively, are utilised).

Results and Discussion

The minimised volumes and optimised flow paths of the UPLC instrumentation allow a precise and rapid delivery of mobile phase gradients and column equilibration. The total analytical time for the new STA method has been reduced from 26 min to 15 min as seen in Figure 3.

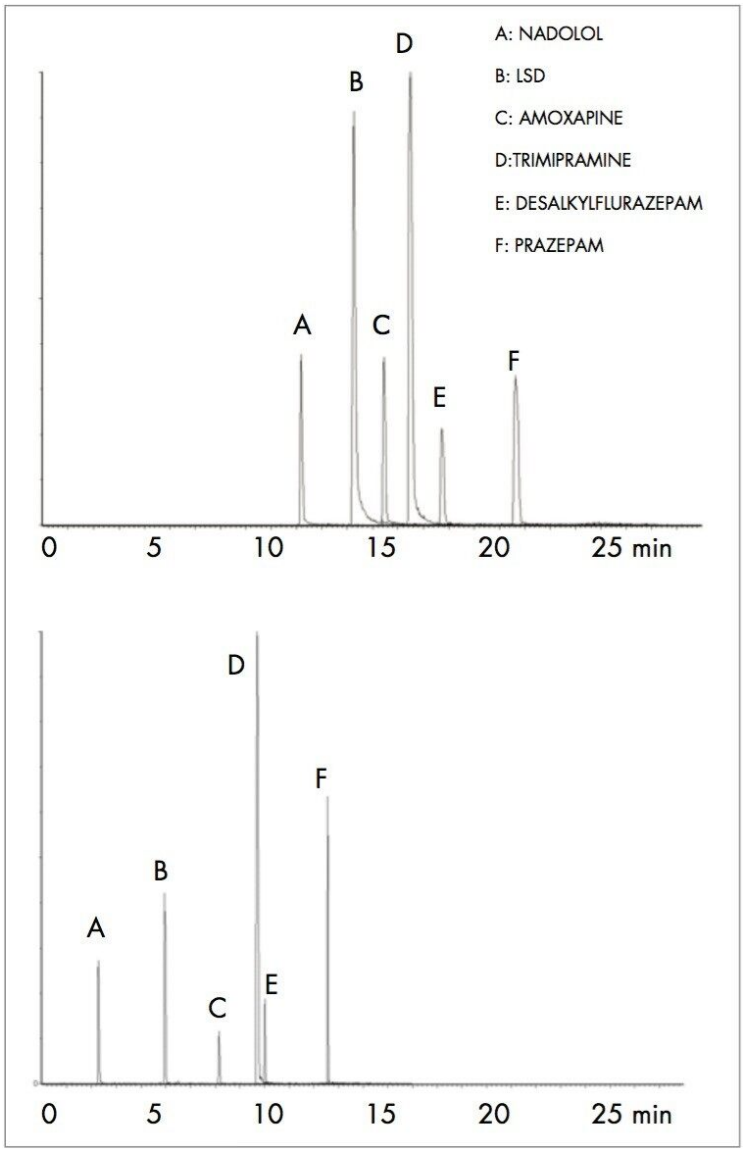

Figure 3. Extracted ion chromatograms of a mixture of standards analysed using the original screening configuration i.e., Alliance 2695 plus Quattro micro (top trace) versus the latest instrumentation i.e., ACQUITY TQD system (bottom trace). Total analysis time has been reduced from 26 to 15 min.

The increased speed and resolution associated with UPLC results in a significant reduction in peak width.

Figure 4 shows an example of the analysis of colchicine; peak widths (half-height) are reduced from 8.4 seconds with HPLC to 2.1 seconds with UPLC.

Such narrow peaks would pose a potential problem for any ‘normal’ MS detector and could compromise data quality by producing insufficient or poor reproducibility of spectral data for a qualitative analyses and even poor sensitivity and reproducibility for quantitative analyses. In this STA method, scans rates of >7000 amu/sec are used. The figure below demonstrates that when coupled to the ultrafast scanning Waters TQ Detector, data quantity and quality is maintained.

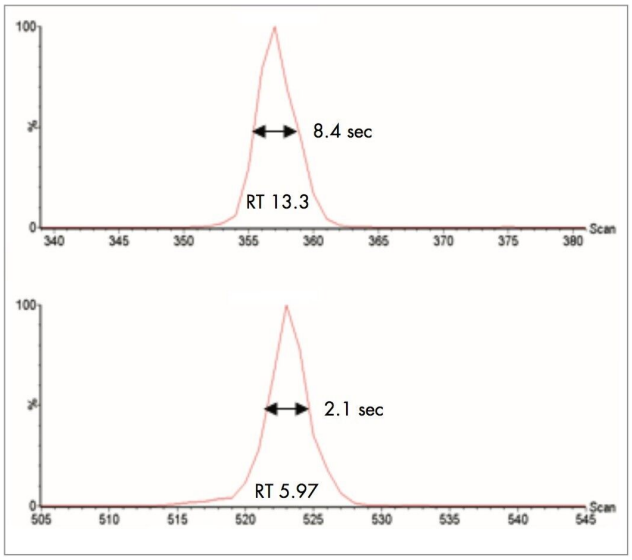

Figure 4. Analysis of colchicine. The fast scanning capability of the TQ Detector ensures that a sufficient number of scans is maintained (11 scans in each case), even though UPLC separation leads to much narrower, peaks i.e., peak width (at half height) for colchicine has been reduced from 8.4 sec (HPLC) to 2.1 sec (UPLC).

Sharper chromatographic peaks typically leads to increased signal to noise ratios and consequently improved detection limits. The increased chromatographic resolution also provides enhanced deconvolution of the data and peak identification by the ChromaLynx application manager.

Figure 5 shows a typical results browser. The data was obtained following the analysis of an authentic urine sample. The sample was prepared using liquid:liquid extraction (LLE) prior to analysis by the STA method. Several compounds and metabolites were identified. The candidate listing includes the name of th proposed compound followed by the observed retention time (RT), the reference/library RT (within the parentheses) and the average match factor.

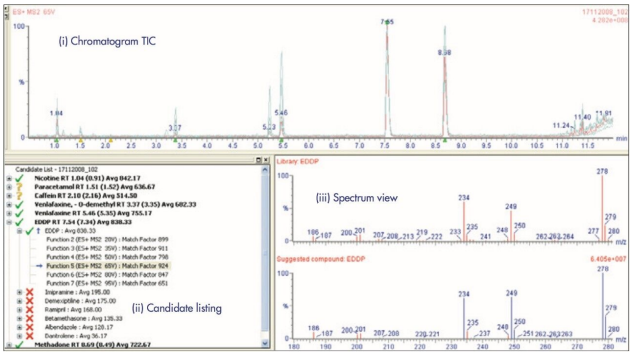

Figure 5. Analysis of an authentic urine sample. The browser shows; (i) the total ion chromatogram (TIC); (ii) the list of proposed candidates; (iii) the spectral match for function 5 (cone voltage 65 V) of one of the proposed candidates (EDDP). The spectrum view window allows a direct visual comparison of the acquired spectral data with the library data. In this example an excellent average MF was observed i.e., 924 out of a possible max. 1000.

The results viewed in the browser can be reported using the report generator option. An example of one available report format is shown in Figure 6.

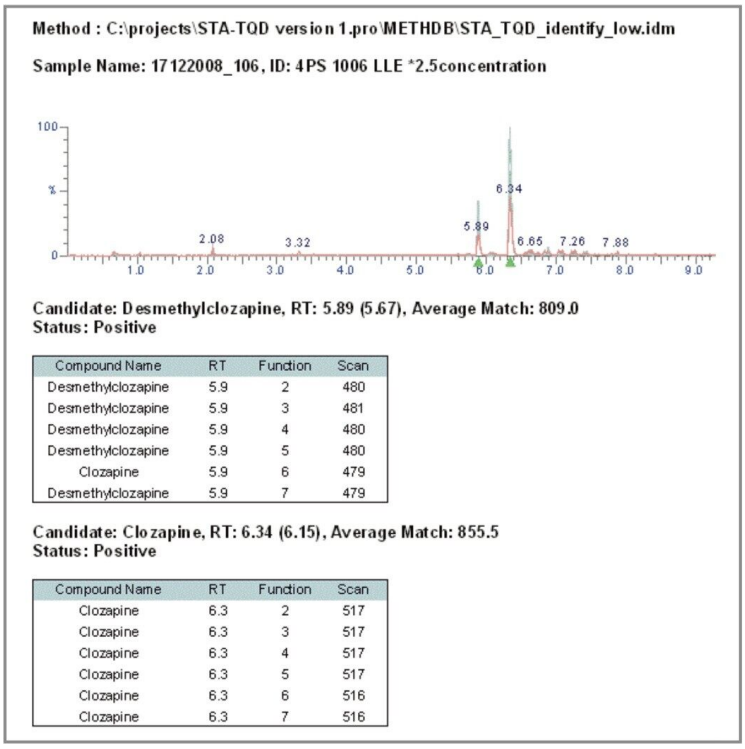

Figure 6. Example of a simplified report for a serum sample containing clozapine and its metabolite desmethylclozapine. Clozapine was the top hit (match) in all of the 6 cone voltage functions examined. The metabolite was the top hit in 5. Both showed excellent average MFs against the library.

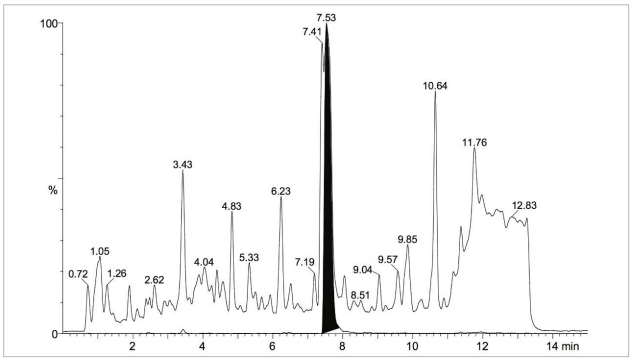

Figure 7. Positive identification of dosulepine in addition to several other toxicological compounds in a forensic sample. Dosulepine was not included in the routine targeted LC-MS/MS-based screen and therefore was not initially identified. Methyl clonazepam was added to the sample prior to analysis and used as an internal standard to verify chromatographic performance.

Conclusion

Toxicology laboratories require the ability to perform STA to screen and identify unknown compounds in a variety of complex biological specimens. In addition, these laboratories face an increased demand in sample throughput and the need to analyse a greater number of samples in a shorter time.

The superior speed and resolution afforded by the use of UPLC, combined with the ability of the TQ Detector to match UPLC performance with rapid polarity switching and ultra-fast scanning, ensure the laboratory can perform prompt, efficient and thorough analyses.

The method described in this application note utilises full scan spectra and retention time to identify toxicants. Analytical time is just 15 minutes thus maximising sample throughput and optimising workflows. The comprehensive features of ChromaLynx deconvolution and automatic data processing software ensure that the maximum number of possible compounds are detected, identified and reported.

A starter project (including library) is provided which contains everything the user needs to perform a comprehensive screen. The methods are supplied on DVD and are ready for immediate implementation within the laboratory with minimal user intervention. The DVD also contains supporting documentation and literature including a user manual and a ‘step by step’ workflow specifically designed with the new user in mind; a simple guide from initial instrument setup (including system verification using a system suitability mixture) through to the analysis of authentic samples.

A dedicated team of Waters applications specialists are also available worldwide to implement and provide training. The ability to add additional compounds to the already comprehensive library, in combination with retrospective analytical capabilities, ensure that this STA method will continue to remain versatile and relevant for the future.

References

1. General Unknown Screening for Drugs in Biological Samples by LC-MS. Luc Humbert, Michel Lhermitte, Frederic Grisel. Waters application note 720001552EN.

2. MassLynx 4.1 Brochure. Waters brochure reference: 720001408EN.

3. Targeted MRM Screening for Toxicants in Biological Samples by UPLC-MS/MS. Mark Roberts, Robert Lee and Michelle Wood. Waters application note 720002749EN.

Acknowledgements

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