SHIMADZU
Excellence in Science

Application News

No. C167

Liquid Chromatography Mass Spectrometry

Targeted screening with Library Searching of drugs of abuse by MTS measuring cocaine and related stimulants, amphetamines, opioids & benzodiazepines

Abstract

A multi-residue assay was developed measuring 90 drugs of abuse in addition to deuterated internal standards (32 compounds). Compounds measured included benzodiazepines, cocaine and related stimulants, amphetamines and opioids; in addition antipsychotic compounds were screened for also. The Multi-Targeted Screening (MTS) method was developed using the Shimadzu Forensic & Toxicology method package containing over 1200 compounds.

Blood samples were prepared by QuEChERs and measured by MTS using a Shimadzu LCMS-8060 and results compared to validated MRM analysis from a Shimadzu LCMS-8050. MTS operates by acquiring data with 2 MRMs per compound with the addition of threshold triggered product ion scan MS/MS at three different collision energies. Each product ion spectrum is then combined into a single merged spectrum for automated library searching.

There are 2 clear advantages of acquiring MRM triggered product ion spectrum data, the first is the higher confidence in compound identification as a result of library searching and the second is the quantitative data is near identical to a conventional 2 MRM method. This approach was evaluated in a routine clinical toxicology laboratory to detect and identify compounds in unknown samples.

Keywords. Spectral Library; Reduced false negative reporting; Toxicological screening; LCMS-8060; higher specificity

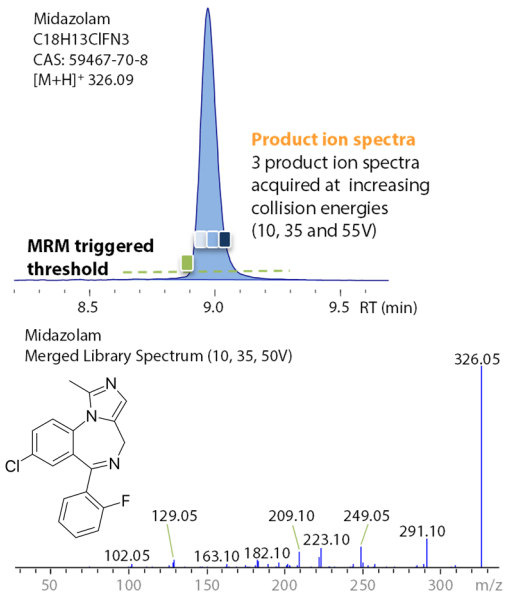

Fig. 1 MRM threshold triggered product ion scans acquired at low, medium and high collision energies (corresponding to 10, 35 and 55 V) are merged together to create a library searchable spectrum for midazolam. This approach increases the fragment ion information for each target and helps reduce false defect reporting.

Introduction

In forensic and toxicological environments, the increased use of both illicit and legalised recreational drugs has created a considerable challenge in sample measurement and LC-MS/MS analysis. Increasing the scope and context of the assay also increases the complexity of the assay and may compromise the likelihood of false positive and false negative reporting in routine clinical toxicology. To minimize the possibility of false defect reporting without compromising the accuracy, precision and limits of detection, methods were developed to combine the sensitivity of MRM detection with the identification power of a full scan product ion spectrum. The method has the capability of simultaneously using both precursor and product ion information enabling precise, accurate quantitation and library searchable compound identification. In a similar approach to previously published methodologies[(1)], threshold triggered product ion scan events were included at three collision energies (10, 35 and 55 V) to create an absolute intensity merged spectrum representing all three collision energies.

In this study, the method was designed not only to reduce false defect reporting by using product ion scan library matching but also considered the needs of a routine clinical toxicology and forensic laboratory by taking into account sample preparation and component separation across a diverse chemical space.

To develop a generic sample preparation method in clinical toxicology analysis, QuEChERS (an acronym for "quick, easy, cheap, effective, rugged, and safe") were used. QuEChERS are widely used in other application fields most notably food safety and pesticide analysis, however, few studies have used this approach in clinical toxicology. The method requires an initial extraction of the blood sample with acetonitrile, followed by liquid–liquid partitioning using salts such as $MgSO_4$/NaCl/NaOAc. Component separation was also optimized with a view to developing a single method capable of resolving targets in four panels of compounds normally measured in separate assays; these included cocaine and related stimulants, amphetamines, opioids and benzodiazepines in addition to complementary deuterated internal standard compounds for select compounds.

To test the viability of this approach and to quantify and identify targets in the four test panels, the MRM triggered product ion spectrum acquisition method was applied to a series of patient blood samples and compared against a validated LC-MS/MS method using 2 MRM's for each target compound.

Alan J. Barnes[*1], Tiphaine Robin[*2], Neil Loftus[*1], Pierre Marquet[*2], Souleiman El Balkhi[*2], Sylvain Dulaurent[*2], Franck Saint-Marcoux[*2]

*1 Shimadzu Corporation, Manchester, UK
*2 CHU Limoges, France

Experimental

Patient blood samples were analysed from a diverse range of backgrounds commonly encountered in a routine pathology laboratory including emergency overdose, routine drug testing, driving under the influence of drugs (DUID) and samples provided by patients undergoing psychiatric treatment. All blood samples were extracted using an established QuEChERS based method[2, 3].

100 µL whole blood sample was added to 200 µL acetonitrile containing internal standard compounds. Samples were vortexed for ~5 seconds then incubated at room temperature for 10 min. 40 mg of QuEChERS salts were added, samples were then vortexed for ~5 seconds. Samples were then centrifuged (16,000 g, 10 min); 50 uL of supernatant was removed and added to 150 µL mobile phase A. Calibration curves were prepared in the range 5 to 2000 µg/L for benzodiazepines, and 5 to 500 µg/L for cocaine and related stimulants, amphetamines and opioids.

Data was acquired to monitor a predefined MRM transition for each compound. Once the MRM signal was above a set threshold intensity of 10,000 counts, a series of alternating product ion scans were acquired at three collision energies (10, 35, 55 V) with an automatic exclusion for 3 seconds after 2 consecutive MS/MS scans. Product ion spectrum data from each collision energy was merged together to create a fragment rich single spectrum for library searching.

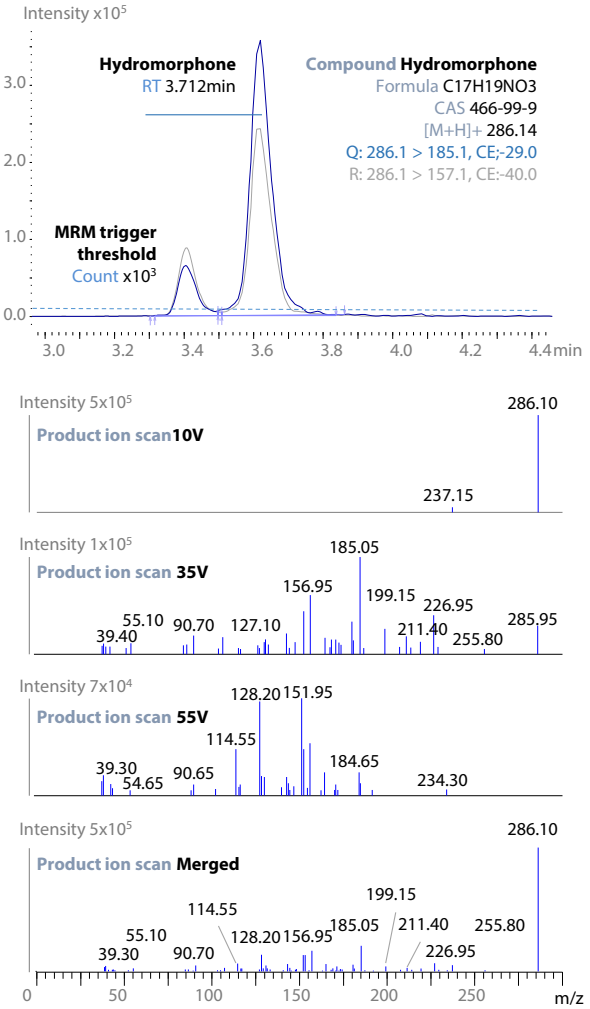

Fig. 2 MS/MS acquisition data for hydromorphone. Above a predefined MRM intensity threshold, product ion scans are triggered acquiring scan data at different collision energies (10, 35 and 55 V). For library based identification, the product ion scans from each collision energy are merged together.

Table 1 LC acquisition parameters

Liquid chromatography		
UHPLC	Nexera™ LC system	
Analytical column	Raptor® Biphenyl (Restek) (2.7 um 100 × 2.1 mm)	
Column temperature	40 °C	
Flow rate	0.3 mL/minute	
Solvent A	2 mmol/L ammonium formate and 0.002 % formic acid	
Solvent B	2 mmol/L ammonium formate and 0.002 % formic acid in methanol	
Binary Gradient	Time (mins)	%B
	1.0	5
	2.0	40
	10.5	100
	13.5	100
	13.51	5
	17.0	Stop
Column conditioning	11-16.2 min	0.5 mL/min
Injection volume	5 µL	

Table 2 LC-MS/MS method used to acquire a library searchable data

LC-MS/MS Mass spectrometry	MRM Spectrum mode generating library searchable spectra
Target number of compounds	122 (including 32 ISTDs)
Pause time/dwell time	1 msec./3 msec.
Ionisation mode	ESI +/-
Polarity switching time	5 msec
Interface temperature	300 °C
Heat block temperature	400 °C
Desolvation line temperature	250 °C
Nebulising gas	3 L/min
Heating gas	10 L/min
Drying gas	10 L/min

Table 3 In the MS/MS method, each target compound combined MRM and product ion scan acquisition's. The MRM threshold trigger was set to an intensity of 10,000 counts, above this threshold product ion scan data was acquired. As one example, MS/MS acquisition parameters for hydromorphone and morphine-6-glucuronide are shown below.

MS/MS method		
Acquisition time		2.962-4.462 (mins)
Compound name		Hydromorphone
MS/MS acquisition mode	Event	Acquisition parameters
MRM	36	
Channel 1		CE:-29.0, 286.15>185.10
Channel 2		CE:-40.0, 286.15>157.10
Product Ion Scan	37	CE:-10.0, 30.00:291.50
Product Ion Scan	38	CE:-35.0, 30.00:291.50
Product Ion Scan	39	CE:-55.0, 30.00:291.50
Acquisition time		2.971-4.471 (mins)
Compound name		Morphine
MS/MS acquisition mode	Event	Acquisition parameters
MRM	40	
Channel 1		CE:-31.0, 286.15>185.10
Channel 2		CE:-45.0, 286.15>157.10
Product Ion Scan	41	CE:-10.0, 30.00:467.20
Product Ion Scan	42	CE:-35.0, 30.00:467.20
Product Ion Scan	43	CE:-55.0, 30.00:467.20

Table 4 CAO compound summary: Library hit, accuracy and R2 for calibration standard 100 µg/L.

Compound	Library Hit	SI score	RT (min)	Accuracy (%)	R^2
Ecgonine methylester	Ecgonine methylester	77	1.06	102.7	0.9995
Norephedrine	Norephedrine	48	3.25	107.2	0.9967
Norpseudoephedrine	Norephedrine	81	3.32	98.2	0.9999
Anhydroecgonine methyl ester	Anhydroecgonine methyl ester	32	3.40	97.6	0.9925
Morphine	Morphine	74	3.41	102.3	0.9989
Ephedrine	Ephedrine	71	3.51	109.9	0.9992
Pseudoephedrine	Pseudoephedrine	80	3.58	95.8	0.9997
Hydromorphone	Hydromorphone	85	3.62	97.0	0.9956
Methiopropamine	Methiopropamine	82	3.68	103.2	0.9998
Amphetamine	Phenylpropylamine	82	3.70	100.1	0.9993
Methcathinone	Methcathinone	93	3.71	93.3	0.9995
Noroxycodone	Noroxycodone	73	3.93	120.5	0.9949
Methamphetamine	Methamphetamine	79	3.96	94.8	0.9981
MDA	MDA	76	3.98	110.3	0.9975
Naloxone	Naloxone	61	3.98	115.0	0.9960
Dihydrocodeine	Dihydrocodeine	76	4.09	110.9	0.9957
Naltrexone	Naltrexone	77	4.11	99.8	0.9973
Ritalinic acid	Ritalinic acid	84	4.21	98.2	0.9990
Codeine	Codeine	90	4.11	114.3	0.9948
Pholcodine	Pholcodine	80	4.47	98.4	0.9940
Oxycodone	Oxycodone	71	4.20	108.1	0.9988
MDMA	MDMA	89	4.24	92.2	0.9927
6-MAM	6-MAM	81	4.24	98.4	0.9972
Mephedrone	Mephedrone	84	4.43	89.5	0.9989
BDB	BDB	82	4.45	104.0	0.9983
Norfenfluramine	Norfenfluramine	94	4.51	93.9	0.9990
MDEA	MDEA	96	4.55	102.3	0.9987
Benzoylecgonine	Benzoylecgonine	94	4.64	99.1	0.9985
Hydrocodone	Hydrocodone	75	4.52	112.0	0.9919
MBDB	MBDB	97	4.74	96.4	0.9995
Ethylmorphine	Ethylmorphine	93	4.71	104.6	0.9998
4-MTA	4-MTA	84	4.89	101.2	0.9980
M-CPP (meta-Chlorophenylpiperazine)	M-CPP (meta-Chlorophenylpiperazine)	86	4.89	99.0	0.9969
2-CB	2-CB	42	5.03	103.9	0.9993
Methylphenidate	Methylphenidate	45	5.20	89.2	0.9977
Cocaine	Cocaine	93	5.53	97.4	0.9997
2-CI	2-CI	83	5.53	91.9	0.9989
3,4-Methylenedioxypyrovalerone	3,4-Methylenedioxypyrovalerone	92	5.81	89.7	0.9983
Cocaethylene	Cocaethylene	96	6.11	98.3	0.9998
Dextromethorphan	Dextromethorphan	90	7.35	92.9	0.9897
EDDP	EDDP	88	7.66	97.1	0.9990
Methadone	Methadone	90	8.32	100.4	0.9999

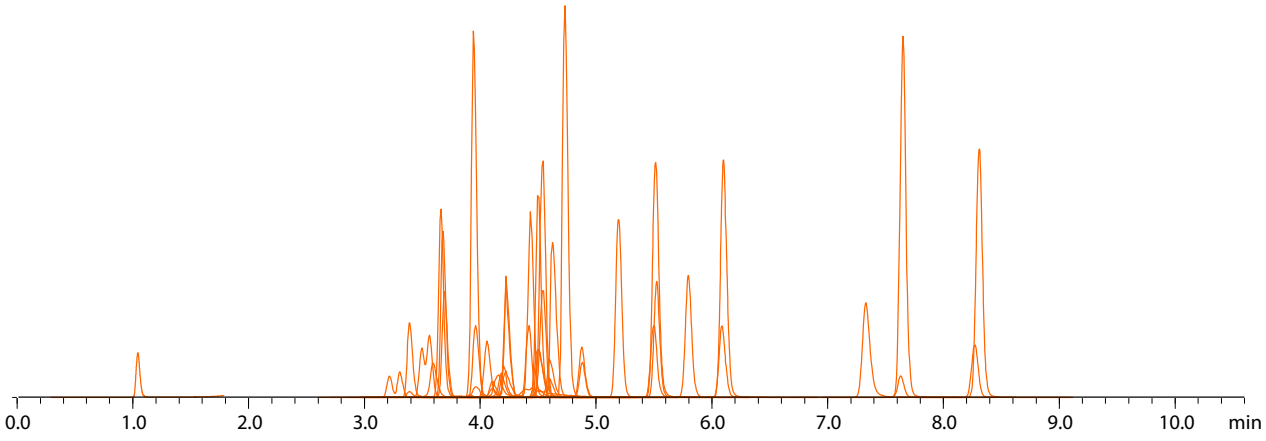

Fig. 3 MRM chromatograms for the CAO panel of drug targets (Table 3) separated using a biphenyl column.

Table 5 Benzodiazepine compound summary: Library hit, accuracy and R^2 for calibration standard 100 µg/L.

Compound	Library Hit	SI score	RT (min)	Accuracy (%)	R^2
7-aminonitrazepam	7-aminonitrazepam	96	5.27	95.3	0.9969
7-aminoclonazepam	7-aminoclonazepam	79	5.32	103.8	0.9986
7-aminoflunitrazepam	7-aminoflunitrazepam	95	5.99	99.8	0.9989
3-Hydroxybromazepam	3-Hydroxybromazepam	54	6.19	103.1	0.9994
Zopiclone	Zopiclone	75	6.72	107.6	0.9992
Desmethylflunitrazepam	Desmethylflunitrazepam	58	7.16	112.9	0.9988
Bromazepam	Bromazepam	68	7.19	105.1	0.9969
Flurazepam	Flurazepam	85	7.22	87.7	0.9974
N-desmethylclobazam	N-desmethylclobazam	63	7.25	108.3	0.9972
Lorazepam	Lorazepam	70	7.26	100.0	0.9997
3-hydroxy-flunitrazepam	3-hydroxy-flunitrazepam	61	7.41	118.8	0.9943
Oxazepam	Oxazepam	63	7.44	102.9	0.9941
Clonazepam	Clonazepam	62	7.46	92.2	0.9943
2-(2-amino-5-bromobenzoyl)pyridine	2-(2-amino-5-bromobenzoyl)pyridine	89	7.47	112.4	0.9961
Nitrazepam	Nitrazepam	81	7.49	102.9	0.9996
Zolpidem	Zolpidem	87	7.56	93.9	0.9990
Desalkylflurazepam	Desalkylflurazepam	63	7.65	112.3	0.9852
Hydroxyalprazolam	Hydroxyalprazolam	83	7.85	86.1	0.9985
Chlordiazepoxide	Chlordiazepoxide	70	7.94	102.5	0.9993
4-hydroxymidazolam	4-hydroxymidazolam	64	8.07	91.3	0.9973
1-hydroxymidazolam	1-hydroxymidazolam	88	8.07	89.1	0.9967
Clobazam	Clobazam	87	8.11	100.6	0.9990
Nordiazepam	Nordiazepam	79	8.13	112.4	0.9951
Flunitrazepam	Flunitrazepam	83	8.23	100.7	0.9991
Lormetazepam	Lormetazepam	80	8.25	97.3	0.9953
Estazolam	Estazolam	88	8.30	104.3	0.9992
Triazolam	Triazolam	85	8.33	99.1	0.9994
Temazepam	Temazepam	76	8.40	104.5	0.9998
Ethyl loflazepate	Ethyl loflazepate	86	8.49	100.3	0.9994
Alprazolam	Alprazolam	97	8.53	112.1	0.9972
Midazolam	Midazolam	96	8.94	94.2	0.9973
Diazepam	Diazepam	92	9.07	96.1	0.9993
Clotiazepam	Clotiazepam	94	9.49	90.5	0.9959
Tetrazepam	Tetrazepam	90	9.62	90.0	0.9992
Loprazolam	Loprazolam	76	9.68	97.3	0.9987

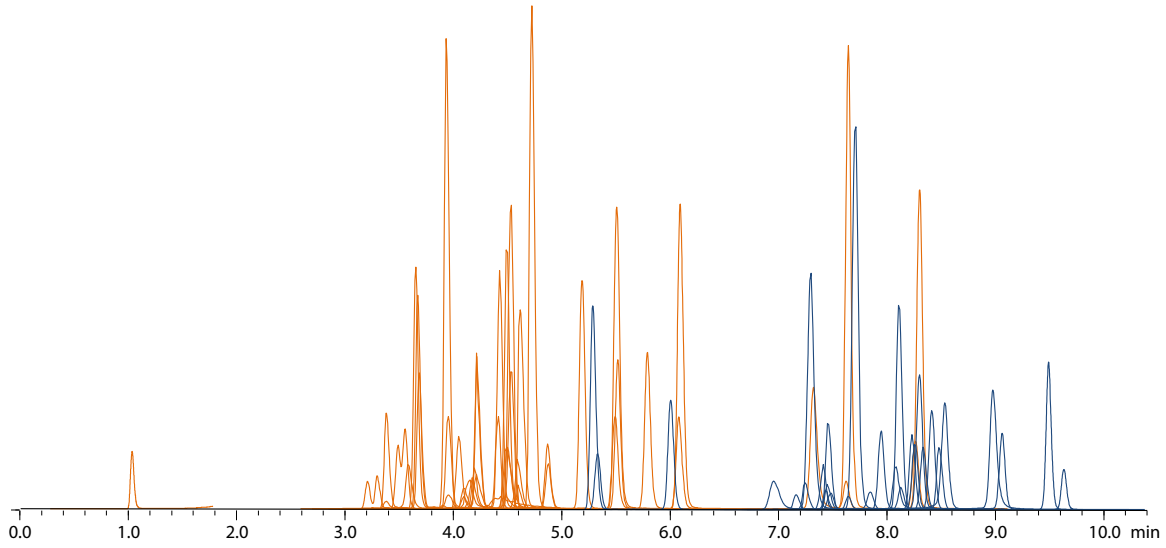

Fig. 4 MRM chromatograms for 35 Benzodiazepines (blue trace) separated in under 10 min (Table 4 lists each benzodiazepine in the test panel). The mobile phase composition was based on a previously published method with the aim of accommodating a broad chemical space for both positive and negative modes of ionisation[4] (the orange trace includes the CAO compounds by way of a comparison).

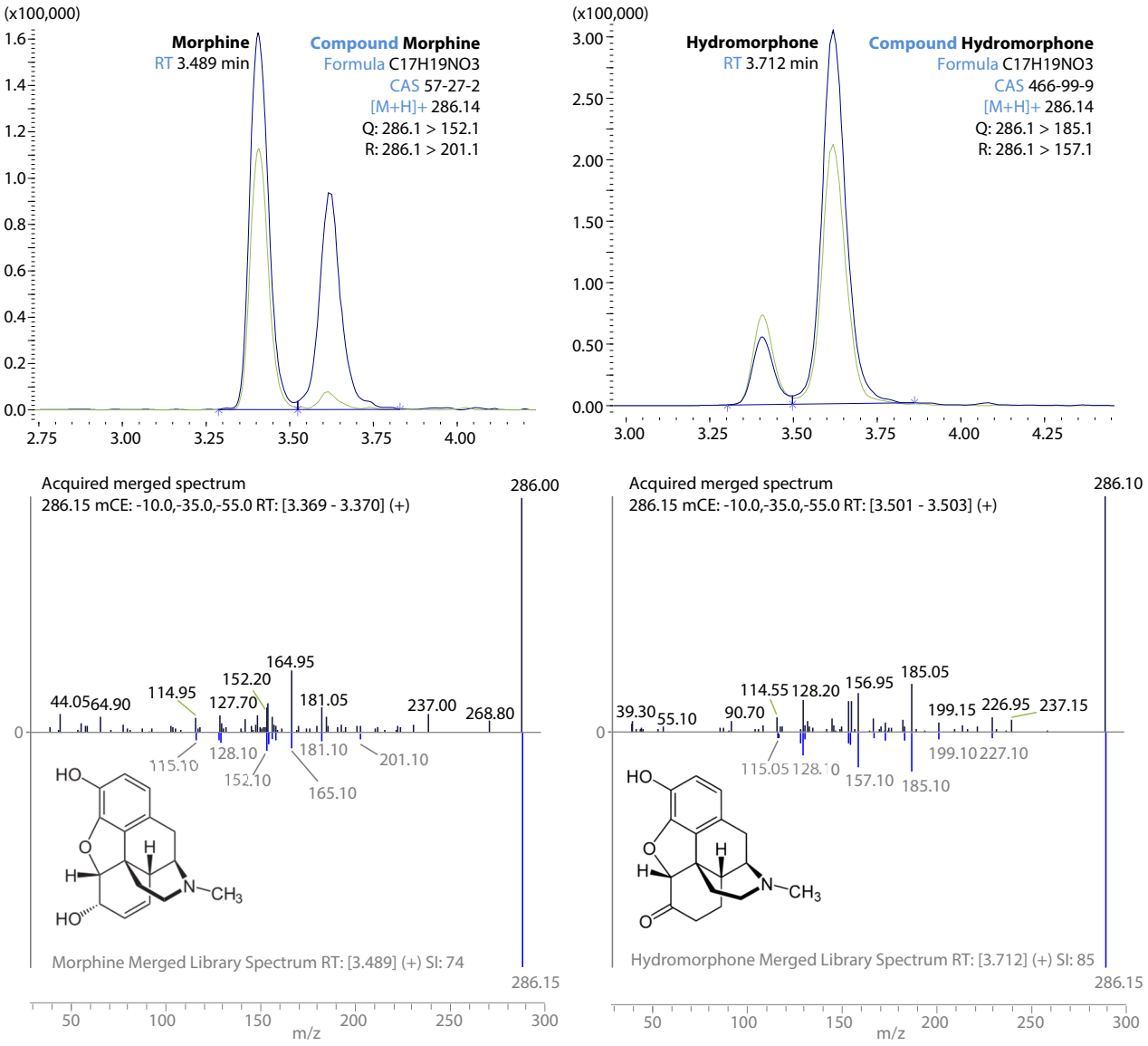

Fig. 5 MRM triggered product ion scan MS/MS data for morphine and hydromorphone extracted from blood samples using a QuEChERs extraction protocol. The identification of isobaric compounds such as morphine and hydromorphone in blood samples was confirmed by matching acquired product ion spectrum data with a reference library generated using certified materials.

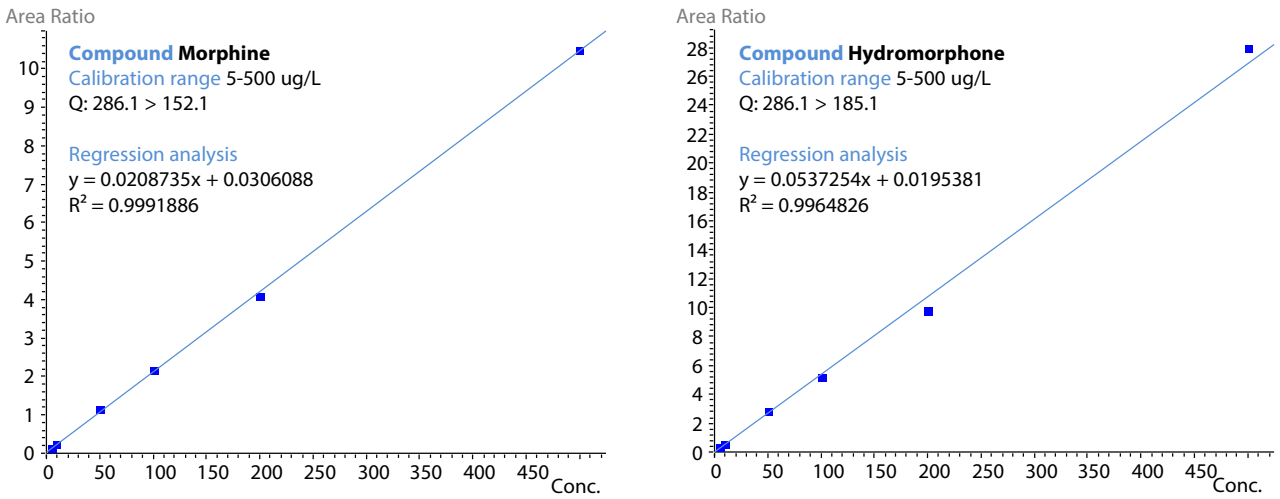

Fig. 6 Calibration curve data for morphine and hydromorphone extracted from blood samples (calibration range 5-500 ug/L). As the MS/MS method uses MRM triggered product ion scans the quantifier ion was used to generate the calibration curve for target compounds.

Method performance

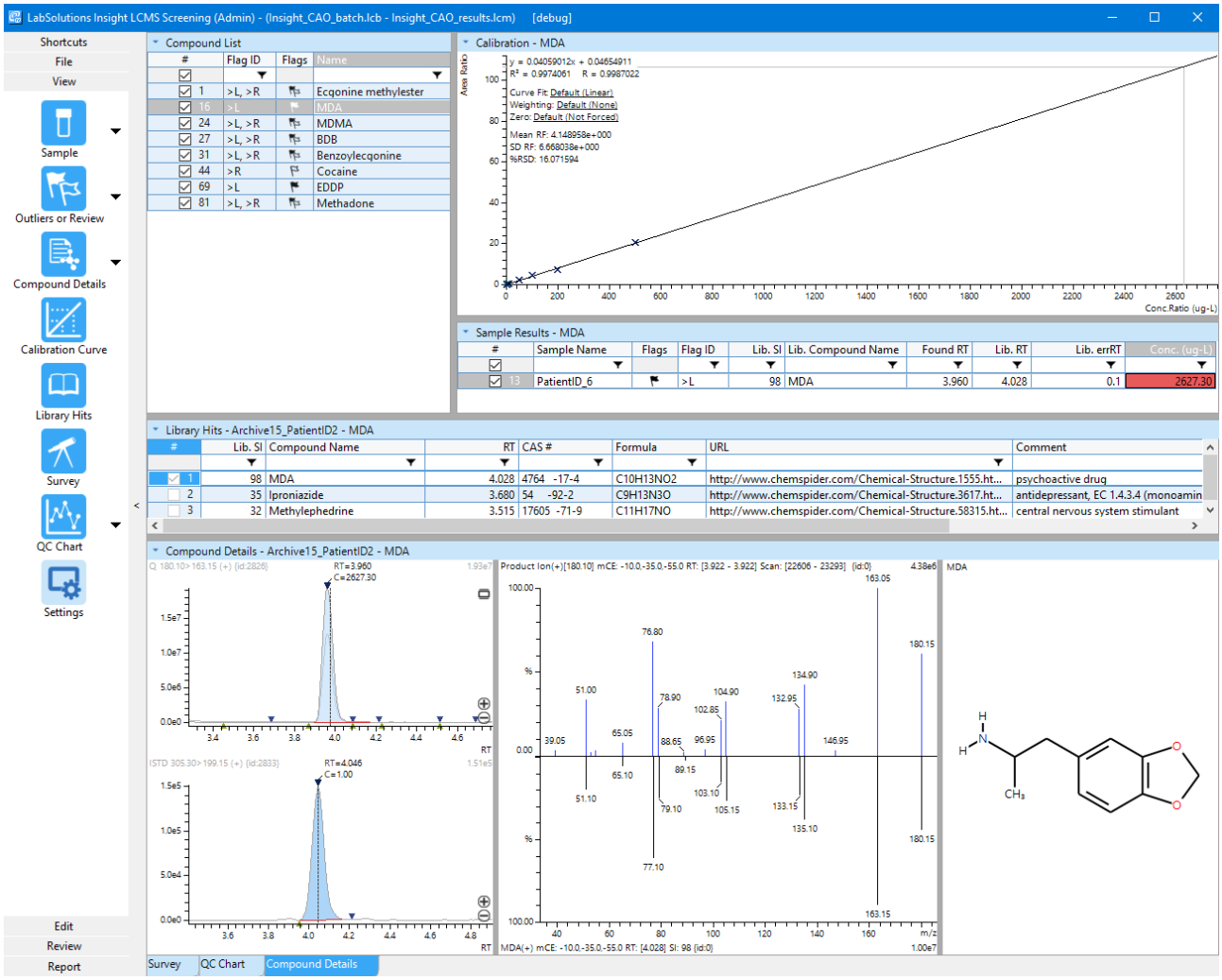

Fig. 7 LabSolutions™ Insight software helps to accelerate data review-by-exception, in this case a reporting flag was enabled to filter drug concentration when measured above a set value. MDA was identified by matching the merged product ion scan data and retention time with a reference library of spectra and retention times (similarity score of 98; retention time variance of 0.1 minute compared with the reference library).

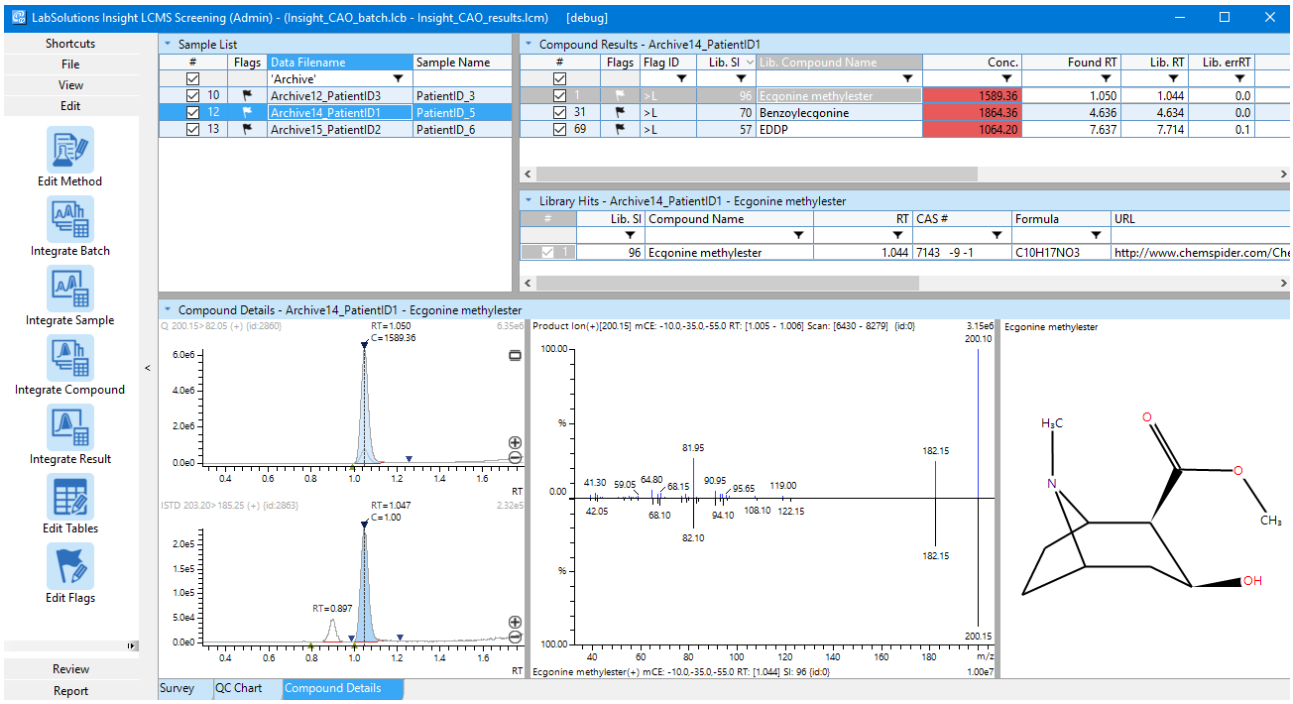

Fig. 8 To help differing needs in data review the data browser can be simply changed to show or hide alternative data views. In this blood sample, ecgonine methylester resulted in a similarity score of 96 with the reference library.

Comparison with conventional MRM methods

In Fig. 9, 24 patient blood samples were analysed by both MS/MS methods. For a number of patient samples more than one target compound was measured resulting in 54 sample points in the regression analysis. Regression analysis shows a close agreement between both methods (slope=0.9996; regression coefficient r2>0.99).

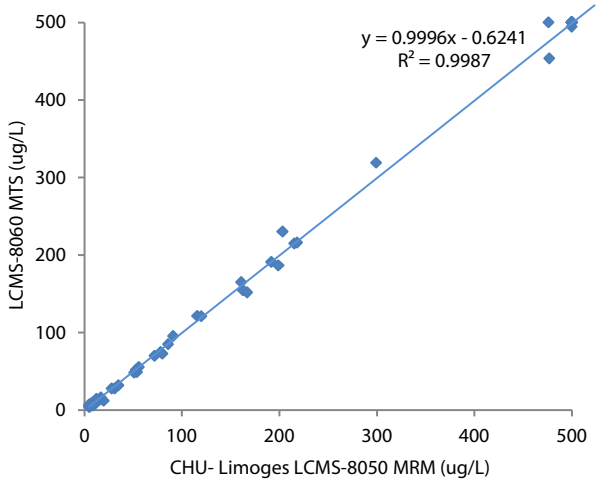

Fig. 9 Regression analysis comparing the results from 24 patient blood samples acquired using the LCMS-8060 MTS method with library searching to a conventional LCMS-8050 MRM method.

Conclusions

A generic method was developed for clinical toxicology and forensic analysis using a QuEChERS sample preparation method, a single LC analysis and an MRM triggered product ion spectrum acquisition method. The method was designed to meet the needs of a routine clinical toxicology and forensic laboratory by delivering a single approach to the analysis of a diverse range of target panels which is not only cost effective but also helps to reduce false defect reporting. By combining product ion scan data and MRM data acquisition, the MS/MS method results in higher confidence in compound identification as a result of library searching with robust quantitative data. Library identification added increased confidence to compound identification in situations where reference ion ratios were outside method tolerances or if concentrations measured were below or above LLOQ or ULOQ.

When the same samples were analysed by different methods (a conventional 2 MRM quantitative method compared to the MRM triggered product ion scan approach) the quantitative results were near identical (24 patient blood samples, 54 drug target compounds in total; regression analysis resulted in a slope=0.9996; regression coefficient r2>0.99).

This was implemented successfully to enable screening and quantitation by MTS for a broadly targeted drugs of abuse panel of compounds. Library identification added increased confidence to compound identification in situations where reference ion ratios were outside method tolerances or if concentrations measured were below or above LLOQ or ULOQ.

References

(1) Sauvage, F.L., Gaulier, J.M., Lacha tre, G., Marquet, P (2008) Pitfalls and Prevention Strategies for Liquid Chromatography–Tandem Mass Spectrometry in the Selected Reaction–Monitoring Mode for Drug Analysis, *Clinical Chemistry*, 54:9, 1519–1527

(2) Ponceleta L., El Bakhia, S., Dulaurenta, S,, Saint-Marcoux, F. (2016) QuEChERS sample preparation prior to LC–MS/MS determination of benzodiazepines, *Toxicologie Analytique et Clinique*, 28(3), 201–210

(3) Dulaurent, S., El Balkhi, S., Poncelet, L., Gaulier, J.M., Marquet, P., Saint-Marcoux, F. (2016) QuEChERS sample preparation prior to LC-MS/MS determination of opiates, amphetamines, and cocaine metabolites in whole blood, *Analytical and Bioanalytical Chemistry*, 408(5), 1467–1474

(4) Marquet, P., Venisse, M., Lacassie, É., Lachâtre, G. (2000) In-source ID mass spectral libraries for the “general unknown” screening of drugs and toxicants, *Analusis*, (28), 925-934

First Edition: May 2018

Shimadzu Corporation

www.shimadzu.com/an/