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Introduction

LC/MS applications are filling an analysis gap and making valuable contributions to the clinical and toxicology laboratory. We will demonstrate how LC/MS has become crucial for our routine operations. As an example, an LC/MS assay that monitors compliance of drug rehabilitation patients using a transdermal patch, administering Fentanyl, will be shown.

Clinical/toxicological regulatory guidelines have started to address the appearance of the recent introduction of LC/MS and LC/MS/MS instrumentation into a routine analysis environment. Compound identification criteria for single quadrupoles based on ion ratios must make use of at least two ratios or one ratio of two characteristic ions if few ions exist. The unknowns must have ion ratios within $\pm 20\%$ of the extracted standards. The internal standard identification must be monitored by at least one ion ratio. In addition, selected ion monitoring (SIM) spectra identification must be based on retention time. These and other acceptance parameters require specialized quantitation and reporting features to be available within the mass spectrometry processing software.

We will present an LC/MS method that allows the use of multiple fragment ions for confident confirmation (as many as five ions). Analytical results for Fentanyl and its metabolite, Norfentanyl, are presented. The identification of each analyte is based on the routine assessment of one precursor ion and two product ions formed by in-source collision-induced dissociation (CID).

Methods and Instrumentation

Urine samples are from patients being tested for mandatory drug compliance.

Standards and two quality control samples were prepared by dilution of stock standards stored in methanol into pooled, drug-free urine. Five standard levels, 10, 50, 100, 200, 500 and 1000 ng/ml were used to generate a calibration curve.

Method development included the use of Norfentanyl-d₅, Normeperidine-d₄ as internal standards. External standard quantitation was also performed.

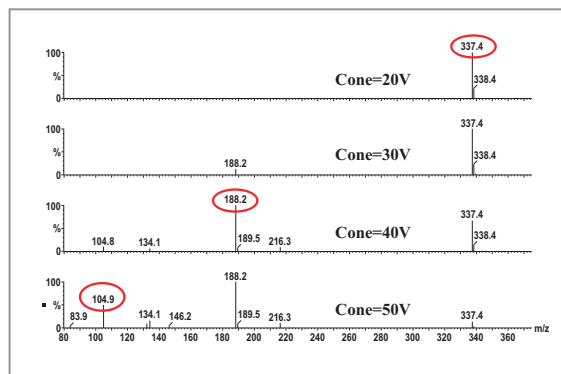
The drugs of interest were extracted by solid phase extraction (SPE), using an Oasis® 1cc/30mg MCX cartridge, prior to analysis.

Two LC/MS methods were developed. An initial HPLC method, using an existing amphetamine analysis template, was developed for Fentanyl/Norfentanyl. This 25-minute method used a pH tolerant, hybrid Waters® XTerra® MS C₁₈, 2.1x100mm, 3.5µ column. Mobile phases were 20mM ammonium bicarbonate buffer, pH 9.6(A) and methanol(B). The second method was developed to allow a desirable analyte selectivity change, shortened analysis time (8 minutes) and additional opiate family members to be incorporated into the analysis. A Waters Atlantis™ dC₁₈ IS (Intelligent Speed™), 2.1x20mm, 3µ shows promise. The mobile phases for the 20mm long column were 10mM ammonium formate buffer, pH 3.7(A) and methanol(B). Eight ions were monitored for each MS method. See Figures A and B for details.

Detection was accomplished with a single quadrupole MS detector. The ionization mode was positive ESI. Data collection used SIM for quantitation and full scans to monitor for other compounds of interest.

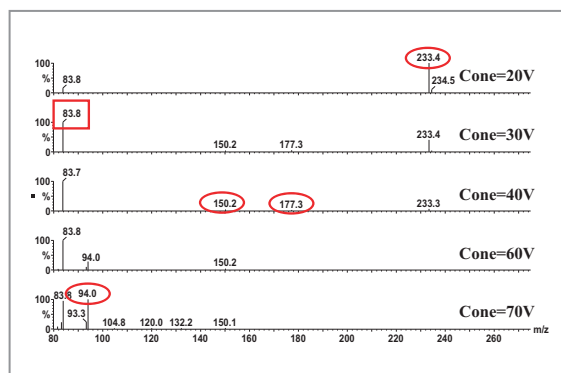
Masslynx™ 4.0 Software controlled the LC/MS system. Targetlynx™, a Masslynx Application Manager, accomplished multi-ion quantitative confirmation.

Flow Injection Analysis (FIA): Fentanyl CID Fragments



Precursor and product ions were determined by FIA of the stock standards. The MS method was created using this information.

FIA: Norfentanyl CID Fragments



Two CID fragments, 150.2 and 177.3 m/z, are easily detected at a cone voltage that would be appropriate for other drug candidates that we have an interest in monitoring. 94 m/z is also available for use in the method. While 83.8 m/z appears to be an attractive choice to monitor, the internal standard, Norfentanyl-d5, shares this CID fragment as can be seen in the following slide.

College of American Pathologists (CAP) Guidelines: Chemistry/Toxicology – March 2003

- Are the identification criteria for single stage mass spectrometry (i.e., GC/MS, LC/MS) in compliance with recommendations?

NOTE: An acceptable criterion for compound identification using ion ratios is that the unknown result must have ion ratios within $\pm 20\%$ of the extracted calibrator(s). Identification using ion ratios typically requires the use of at least 2 ion ratios. However, one ion ratio of 2 characteristic ions may be acceptable if there are only a few characteristic ratios. The internal standard's identification should be monitored with at least one ion ratio. An acceptable criterion for compound identification using total spectra is that the unknown result must have a "spectral match" quality or fit that is within the defined limits that the laboratory has set and validated. Ion ratios determined from total spectra analysis are an acceptable identification method, and should fulfill the same criteria as given above for ion ratio identifications.

COMMENTARY:

Mass spectral identification criteria must comply with recommendations.

- When applicable, are TIC identification criteria based on retention time and MS Library?

COMMENTARY:

The mass spectral identification criteria must be consistent with retention times and library.

Are SIM spectra identification criteria based on retention time?

COMMENTARY:

SIM spectra identification criteria must be based on retention time.

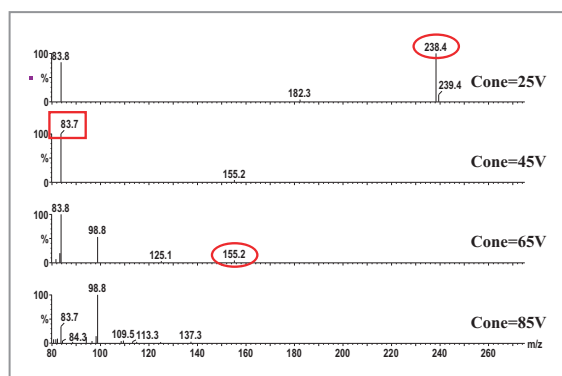
CAP Guidelines: Chemistry/Toxicology – March 2003

- For LC/MS, does the laboratory's assay procedure include an evaluation for possible ion-suppression?

COMMENTARY:

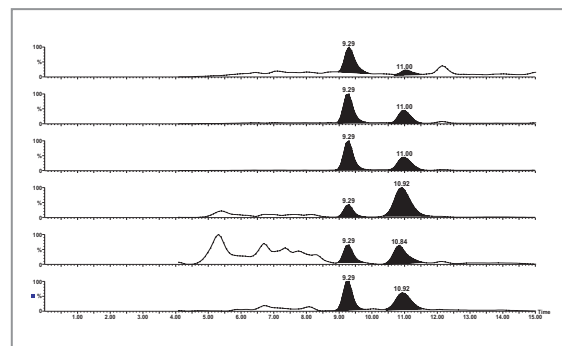
Ion suppression is a recognized analytical anomaly in LC/MS experiments. Such suppression can lead to false negative results or poor quantitative analyses. While difficult to predict and observe from specimen to specimen, certain precautions should be used to try to recognize when ion suppression occurs. As an example, for isotopically-labeled internal standards, if there is poor recovery of the internal standard, a signal to noise ratio greater than 3:1 should still suffice for acceptance of the specimen in question. If recovery of the isotopically-labeled internal standard is considered poor, then an alternate analysis should be considered, e.g., the method of standard addition. For analogue-type internal standards, internal standard recovery may be used as a guide for identification of ion suppression, although the method of standard addition would be better. For unique matrices, the method of standard addition is recommended.

FIA: Norfentanyl-d5 CID Fragments (Initial IS)



The identification of the internal standard is monitored with one CID fragment from those available.

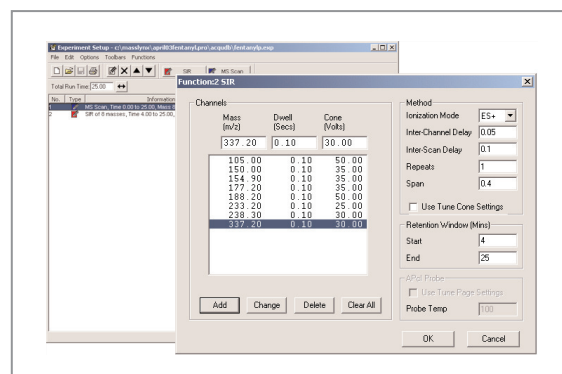
Fentanyl LC/MS Method



The LC/MS method was successfully implemented in the laboratory. Interference from other matrix components was not noted during the period that the method was developed.

MS: Waters Micromass® ZQ™ Mass Spectrometer (Initial Method)

Ionization: ESI+
 Source (C°): 125°C
 Desolvation (C°): 300°C
 Capillary Voltage: 3.0kV
 Data Acquisition: SIM
 (Centroid Scan 80 to 475m/z: Sample Review)



Initial Method

HPLC Waters Alliance® 2695 Separations Module

Column: Waters XTerra MS C₁₈,
2.1x100mm, 3.5µColumn (C^o): 40 °CMobile Phase: A. 20mM NH₄HCO₃ in Water,
pH 9.6

B. Methanol

Flow Rate: 0.25 ml/min

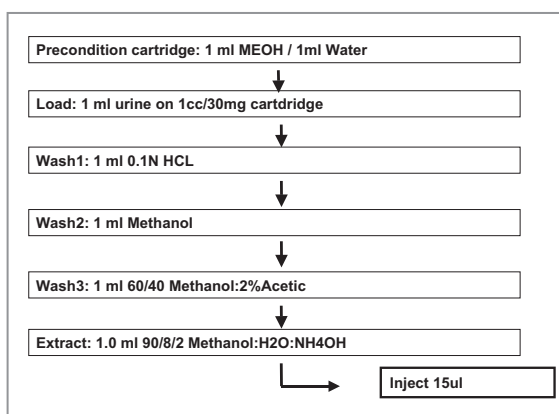
Inject volume: 15µl

Gradient:

Time	A%	B%	Curve
0.0	85	15	1
3.0	25	75	6
14.0	25	75	6
15.0	10	90	6
18.0	10	90	6
20.0	85	15	6
25.0	85	15	6

Sample Preparation: SPE

Oasis MCX (cation-exchange and RP sorbent)

**TargetLynx Overview**

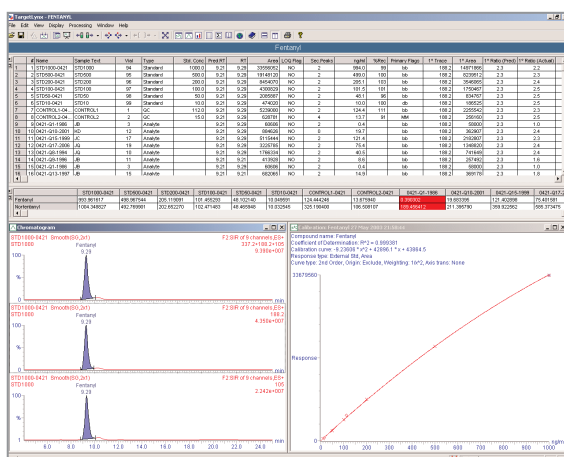
Mass spectrometry is an analytical tool that has proven utility in the identification and quantification of compounds. The spectral information supplied during an acquisition can indicate the identity of an analyte. In addition to the precursor m/z obtained, other information such as product ion m/z, product ion relative abundance and chromatographic retention time provide invaluable criteria to enable the confident identification of a compound.

The importance of developing a robust analytical method has a special emphasis placed upon it when the results are used in areas such as human health, environmental monitoring, and food analysis. Complex matrices necessitate sample preparation procedures. For complex cleanup strategies, recovery may vary. During analysis other sample components may be present that disturb chromatographic retention time. Compounds may exist in greater abundance and suppress detection of the analyte of interest. Monitoring the performance and cleanliness of the analytical system for each assay is a concern. In addition to these issues, there are other sources of variation that require ongoing review. TargetLynx is a specialized MassLynx Application Manager that provides tools that aid in the identification and quantification process. An assortment of routine tasks is automatically executed within the quantitation method.

- A product ion and up to four precursor ions can be monitored for identification and quantification of an analyte.
- If targeted ions are determined to be present, they are integrated. The abundance ratios between the target ions must fall within the method's defined limits or the sample is flagged.

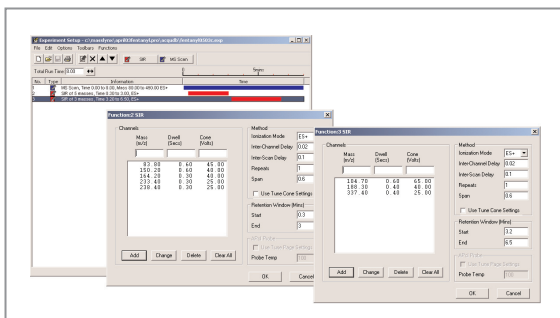
- Retention time, blank response, S/N ratio and coefficient of determination are additional acceptance criteria. Parameters outside the designated limits will be flagged in the report.
- LOD and LOQ requirements for target analytes must be met or the results will be flagged.
- QC samples are monitored.

TargetLynx Report: Ion Ratio Flags



**MS: Waters Micromass ZQ Mass Spectrometer
(Shortened Analysis)**

Ionization:	ESI+
Source (C°):	125 °C
Desolvation (C°):	300 °C
Capillary Voltage:	3.0kV
Data Acquisition:	SIM (Selected Ion Monitoring)
	Centroid Scan: Sample
	Review)



Shortened Analysis Method

HPLC:	Waters Alliance 2695 Separations Module
Column:	Waters Atlantis dC ₁₈ IS, 2.1x20mm, 3m
Column (C°):	45 °C
Mobile Phase:	A. 10mM NH ₄ Formate in Water, pH 3.7

Flow Rate: 1.0 mL/min, 1.25 mL/min at
6-8 minutes

Inject Volume: 12 μ L

Gradient:

Time	A%	B%	Curve
.0	100	0	1
1.0	100	0	6
3.0	75	25	6
4.0	60	40	6
5.0	0	100	6
6.0	0	100	6
6.5	100	0	6
8.0	100	0	6

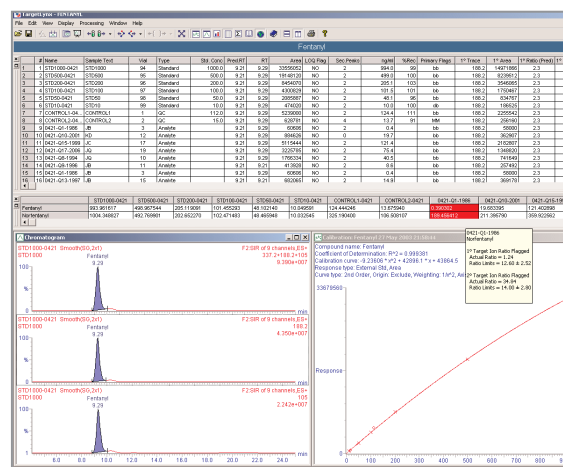
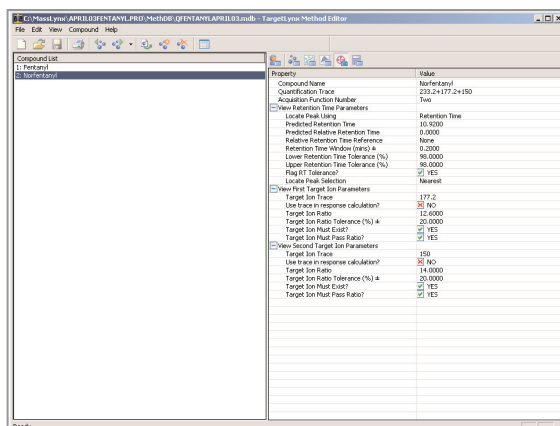
Two Analysis Goals: Confirmation and Quantitation

- Detect illegal drug use
- Monitor renewed episodes of drug use
- Confirm drug therapy regimens followed
- Useful indicator of effective metabolism with multiple drug regimens
- Metabolites monitored as test of compliance

Date	Fent (ng/ml)	Norfent (ng/ml)	Comments	Date	Fent (ng/ml)	Norfent (ng/ml)	Comments
3/20	42	293	100 ug/h	4/16	261	548	100 ug/h
3/21	114	279	100 ug/h	4/17	129	499	100 ug/h
3/24	27	336	100 ug/h	4/18	130	362	100 ug/h
3/28	77	491	100 ug/h	4/19	41	516	100 ug/h
3/29	31	412	100 ug/h	4/21	76	596	100 ug/h
3/31	52	410	100 ug/h	4/22	267	538	100 ug/h
4/1	32	73	100 ug/h	4/23	36	320	100 ug/h
4/3	40	44	clarithromycin	4/24	11	113	100 ug/h
4/4	151	60	(Biaxin)	4/26	34	816	100 ug/h
4/5	13	31	therapy	4/28	59	479	100 ug/h
4/8	164	55	****	4/29	151	260	100 ug/h
4/9	224	69	****	4/30	68	740	100 ug/h
4/10	118	63	****	5/1	4	210	100 ug/h
4/11	318	163	****	5/2	38	587	100 ug/h
4/12	70	243	100 ug/h	5/3	164	393	100 ug/h
4/14	211	824	100 ug/h	5/6	178	532	100 ug/h

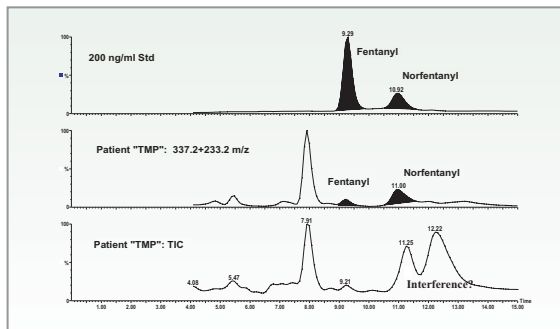
TargetLynx Method: Targeting Properties

TargetLynx Report: Reviewing Results Aided by Pop-Up Summary



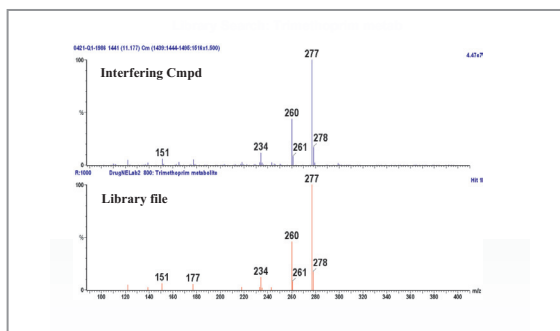
- Possible to "target" four ions
- Ratio and Tolerance monitored
- Reporting method uses Acceptance Parameters (S/N, retention time, ion ratio) to detect and report compound to fulfill regulatory guidelines

Investigation: Ratios Fail Acceptance Parameters



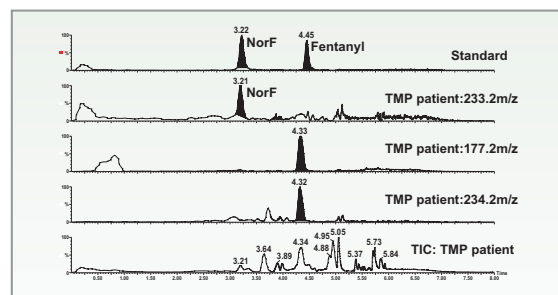
Investigation of the full scan data, from the patient that failed the TargetLynx acceptance parameters, showed an unknown compound eluting in the retention time window for norfentanyl.

Library Search: Trimethoprim Metab



A user-built library quickly identifies the unknown as a metabolite of trimethoprim.

Opiate Method Optimization: Shortened Analysis and Selectivity Change



- Atlantis dC₁₈ IS Column, 3.5m, 2.1x20 mm
- 10mM Ammonium Formate, pH 3.7/MEOH, Opiate resolution
- 0 to 40% methanol from 0 to 4 minutes, 100% 4 6 minutes

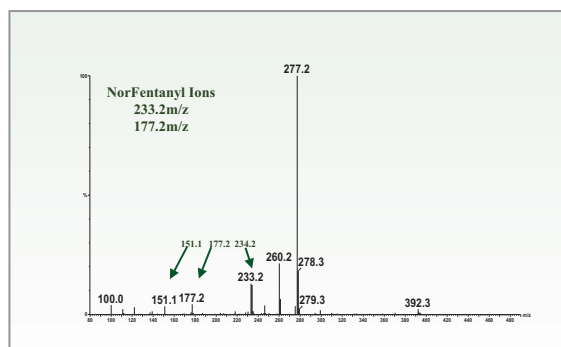
This modified method has shortened the analysis time, as well as, provided a selectivity change. The trimethoprim and metabolite elute well outside the retention time window for norfentanyl.

QC: External Standard Method

Fentanyl/Norfentanyl Quality Control

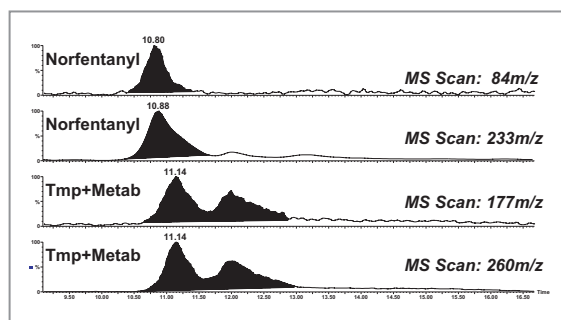
Date	Fent (ng/ml)	Norfent (ng/ml)	Date	Fent (ng/ml)	Norfent (ng/ml)
Level 1	Level 1	Level 2	Level 2	Level 2	Level 2
3/24	118	300	3/24	16	102
3/24	94	253	3/24	14	93
3/25	115	322	3/25	16	121
3/28	106	289	3/28	23	97
3/28	106	395	3/28	11	115
3/31	119	376	3/31	17	121
4/1	104	338	4/1	16	113
4/1	110	311	4/1	16	102
4/1	94	266	4/1	15	89
4/5	110	384	4/5	16	122
4/4	104	357	4/4	18	145
4/7	115	364	4/7	16	118
4/7	116	372	4/7	16	121
4/12	106	322	4/12	13	101
4/15	126	337	4/15	11	110
4/16	121	302	4/16	13	114
4/21	115	299	4/21	13	100
4/22	125	333	4/22	14	107
4/23	126	312	4/23	10	101
4/25	120	300	4/25	13	92
MEAN	112.1	328.0		14.9	110.1
SD	9.6084	39.5221		2.9340	13.2157
CV	8.6	12.0		19.6	12.0

MS Method: Spectrum from Scan. Retention time 11.0 minutes.



The spectrum of the unidentified compound shows several ions that are also used for the analysis of norfentanyl.

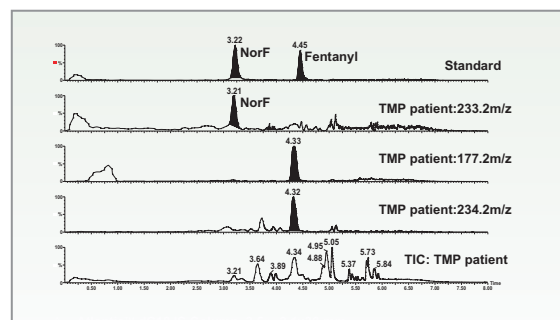
Interference: Modify MS Method (Dwell on 84 m/z)



- SIM function: 84 m/z replaces 177.1 m/z
- Choose New IS: Norfentanyl-d5 shares 84m/z
- External standard method substitution when dealing with this interference
- Modify HPLC method: Goals include selectivity change and shorten time of analysis

A quick modification of the analysis method resolved the problem within 45 minutes. The 84 m/z ion was substituted for the 177 m/z. The ion ratio had failed because of this 177 m/z interference. The norfentanyl-d5 was removed as the internal standard since the 84 m/z is also a product of the IS. An external standard method was substituted while the fentanyl assay was modified.

Opiate Standard: LC Separation ...Importance of Retention Time



In addition to fentanyl, several other opiates are of importance in this patient group. This new LC/MS analysis, in the ammonium formate buffer, allows their simultaneous detection. Among the acceptance parameters monitored by TargetLynx, the use of the retention time window flag allows positive identification of the drugs that have the same molecular weight within this closely related family.

Conclusions

- Confident ID is possible with single quadrupole MS and automated TargetLynx monitoring of Retention Time, CID fragments, Ion Ratios (up to four) and S/N
- LC separation with MS detection provides needed specificity to detect and confirm drugs in a clinical setting
- Sample preparation is reduced with LC/MS
- LC/MS is robust and requires little daily maintenance

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