Waters
THE SCIENCE OF WHAT'S POSSIBLE.™

Tof-MRM for the Confirmation of Fentanyl Analogues for Forensic Toxicology

Michelle Wood[1] and Tom Rosano[2]
[1]Waters Corporation, Wilmslow, UK, [2]Albany Medical Center, Albany, NY, USA

APPLICATION BENEFITS

- Tof-MRM for superior specificity and enhanced sensitivity of fentanyl analogues
- Novel analytical approach to compensate for matrix effects
- Simplified in-well sample preparation
- Differentiation of structural isomers
- Method is adaptable and can be easily updated as new fentanyl analogues emerge

WATERS SOLUTIONS

Xevo™ G2 XS QTof Mass Spectrometer

ACQUITY™ UPLC™ I-Class System (FTN)

ACQUITY HSS C_{18} Column

96-well Sample Collection Plate

KEYWORDS

HRMS, Tof-MRM, fentanyl analogues, Threshold Accurate Calibration, TAC, matrix effects

INTRODUCTION

High resolution mass spectrometry (HRMS) using quadrupole time-of-flight (QTof) instrumentation is increasingly used within the field of forensic toxicology as a comprehensive screening technique. Typically it is used in a non-targeted mode of data acquisition i.e., Tof-MS^E, which provides a highly specific identification based on a combination of precursor and fragment ions generated under low and high-energy conditions, respectively.[1-4] The Tof-MS^E approach has also been applied recently as the confirmatory step in a dual-definitive workflow protocol for commonly analyzed drug substances in forensic urine drug testing.[5]

QTof instruments can also be used in targeted acquisition mode, when the goal is to identify and quantify a more limited panel of analytes of interest.[6]

In recent years, the availability and use of illicit fentanyl and, in particular fentanyl analogues, has become increasingly evident in forensic toxicology. Consequently updated methods, which enable the sensitive detection and confirmation of these emerging substances, are urgently required.

The objective of this current analytical work was to evaluate the performance of Tof-MRM – a targeted mode of acquisition, which is also available on the same HRMS platform and to develop a confirmatory method for the fentanyl class. A secondary aim was to utilize a previously reported novel technique i.e., **T**hreshold **A**ccurate **C**alibration (TAC), for matrix normalization without the requirement for deuterated internal standards.[7-9]

EXPERIMENTAL

Reference analytes

Reference material for the fentanyls (fentanyl, norfentanyl, and all analogues) were obtained from Cerilliant and/or Cayman Chemical at a concentration of 1 mg/mL. A mixed fentanyl stock solution was prepared by dilution in methanol, to give a concentration of 10 µg/mL, and stored at -20 °C until use.

TAC spiking solutions

A TAC spiking solution was prepared by dilution of the mixed fentanyl stock with water to give a concentration of 30 ng/mL. A corresponding blank spiking solution was prepared in water.

Calibrators, controls and case samples

Analyte-free urine was enriched with the mixed fentanyl stock solution to yield a single calibrator at a concentration 2 ng/mL. A lower limit of detection (LLD) control was prepared at 0.8 ng/mL urine.

Quality control samples were prepared by enriching analyte-free urine with a mixed stock of fentanyls from a separate source, to yield the following QCs: 1.5, 3.0, 10 ng/mL urine.

Authentic samples were obtained from routine casework.

Sample preparation

Fifty microlitres of each sample (calibrator, control or case urine) were added to duplicate, adjacent wells of a 96-well plate for analysis of 'neat' and 'spiked' samples (Figure 1).

Fifty microlitres of the TAC spiking solution was added to all 'spiked' wells and 50 µL of blank spike was added to corresponding 'neat' wells.

After addition of 500 µL mobile phase (87% MPA:13% MPB) to all wells, the plate was transferred to the ACQUITY UPLC autosampler for the analysis of 5 µL of sample using UPLC-Tof-MRM.

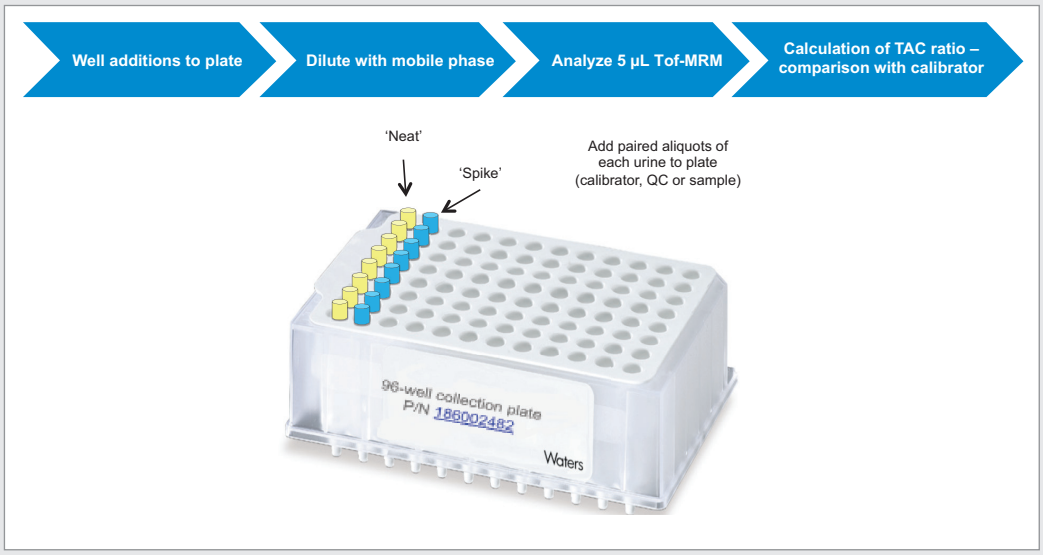

Figure 1. Summary of the analytical procedure: in-well sample preparation followed by Tof-MRM analysis.

LC conditions

LC system:	ACQUITY UPLC I-Class (FTN)
Column:	ACQUITY UPLC HSS C_{18} 100 Å, 1.8 µm, 2.1 mm × 150 mm (p/n 186003534)
Column temp.:	50 °C
Sample temp.:	10 °C
Injection volume	5 µL
Flow rate:	0.4 mL/min
Mobile phase A:	5 mM Ammonium formate pH 3.0
Mobile phase B:	0.1% Formic acid in acetonitrile
Gradient program:	Table 1

MS conditions

MS system:	Xevo G2-XS QTof
Ionization mode:	ESI positive
Capillary voltage:	0.8 kV
Cone voltage:	25 V
Desolvation temp.:	500 °C
Desolvation gas flow:	1000 L/hr
Cone gas:	20 L/Hr
Acquisition mode:	Tof-MRM (Table 2)

Table 1. UPLC Gradient Program.

UPLC gradient table			
Time (min)	**Flow (mL/min)**	**% MPA**	**% MPB**
0.0	0.4	87	13
0.5	0.4	87	13
10.0	0.4	50	50
10.75	0.4	5	95
12.25	0.4	5	95
12.5	0.4	87	13
15.0	0.4	87	13

Data management

UNIFI™ Scientific Information System was used for instrument control and data processing.

Table 2. Retention times (RT) and dual Tof-MRM conditions including optimized collision energy (CE).

Name	RT	Precursor mass (*m/z*)	Product ion 1 quantifier (*m/z*)	CE (eV)	Product ion 2 qualifier (*m/z*)	CE (eV)
4-ANPP	6.16	281.2012	188.1434	15	105.0699	40
Acetyl fentanyl	5.11	323.2118	188.1434	32	105.0699	40
Acryl fentanyl	5.97	335.2118	188.1434	26	105.0699	40
Butyrfentanyl (BF) Isobutyrfentanyl (iBF)	7.21 7.08	351.2431	188.1434	25	132.0808	40
Carfentanil	7.03	395.2329	335.2118	20	246.1489	20
Fentanyl	6.20	337.2274	188.1434	38	105.0699	40
4-Fluorobutyrfentanyl (4-BF) 4-Fluoroisobutyrfentanyl (4-FiBF)	7.53 7.42	369.2337	188.1434	26	105.0699	40
Furanyl fentanyl	6.44	375.2067	188.1434	22	105.0699	40
Beta-hydroxy fentanyl	5.40	353.2224	204.1385	26	335.2118	20
Methoxy acetyl fentanyl	4.81	353.2224	188.1434	22	105.0699	40
3-Methyl fentanyl	7.03	351.2431	204.1385	25	146.0964	30
Norfentanyl	3.22	233.1648	177.1386	28	150.0913	20

Innovative analytical approaches: TAC

A previously-described approach (**T**hreshold **A**ccurate **C**alibration; TAC)[1-3] was employed in this study to prepare the samples and to normalize matrix effects without the use of deuterated internal standards.

Briefly, calibrator, QC and unknown samples were analyzed without ('neat'), and with ('spiked'), addition of a cut-off amount of reference analytes (Figure 1).

The TAC ratio of 'neat' to 'spiked' peak-area response was determined for each specimen and compared with the ratio obtained for the urine calibrator containing drugs at the cut-off threshold concentration (in this assay, 2 ng/mL) for a simplified qualitative presentation of results e.g.:

- Analytes with a TAC ratio exceeding the calibrator TAC ratio are **POSITIVE**
- Analytes with a TAC ratio below that of the calibrator are **NEGATIVE**

$$\text{TAC ratio} = \frac{\text{'Neat' peak-area response}}{\text{'Spiked' peak-area response} - \text{'Neat' peak-area response}}$$

Enabling technologies: Tof-MRM

Time-of-flight-mass spectrometers (Tof-MS) are typically used in a non-targeted acquisition mode such as MS^E to facilitate broad forensic screening.[4-6] Although this mode already provides a sensitive assay with limits of detection in the low ng/mL range, the same instrument can also be used in an alternative, mode e.g., Tof-MRM (Figure 2), this targeted mode can offer further increases in sensitivity; indeed, enhancements ranging from 2 to 200-fold have been reported.[7] Improved sensitivity can be useful where analytes are likely at very low concentrations and/or where simplified sample preparation techniques such as dilution-only protocols, are being utilized.

In this study, Tof-MRM was applied to diluted urine samples urine samples to analyze fentanyls – due to their high potency, these substances are often encountered in samples at low, or sub-ng/mL concentrations.

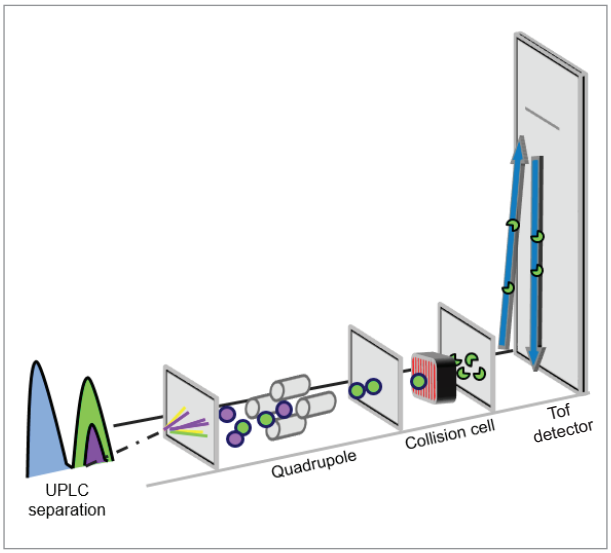

Figure 2. Schematic showing Tof-MRM analysis – in this mode the quadrupole of the QTof is used to isolate a specific precursor mass. Only this mass is permitted to enter the collision cell where it undergoes fragmentation. In this example, the quadrupole is set to isolate the m/z of the green peak. The Tof detector records the masses of all fragment ions – typically a quantifier and qualifier ion is monitored (Table 2).

RESULTS AND DISCUSSION

An initial evaluation of Tof-MRM mode, using calibrators, revealed an increased sensitivity over Tof-MS^E; increases ranged from 5 to 20-fold. Owing to the aim to use a simple sample preparation protocol (effectively a 10-fold dilution of the sample) the targeted acquisition mode was utilized for the remaining study.

Samples were prepared using a simplified in-well sample protocol (Figure 1) and subsequently analyzed using a 15 minute chromatographic separation (Table 1), combined with optimized dual-transition Tof-MRM monitoring (Figure 3, Table 2). The method was highly sensitive and also permitted differentiation of structural isomers (Figure 4).

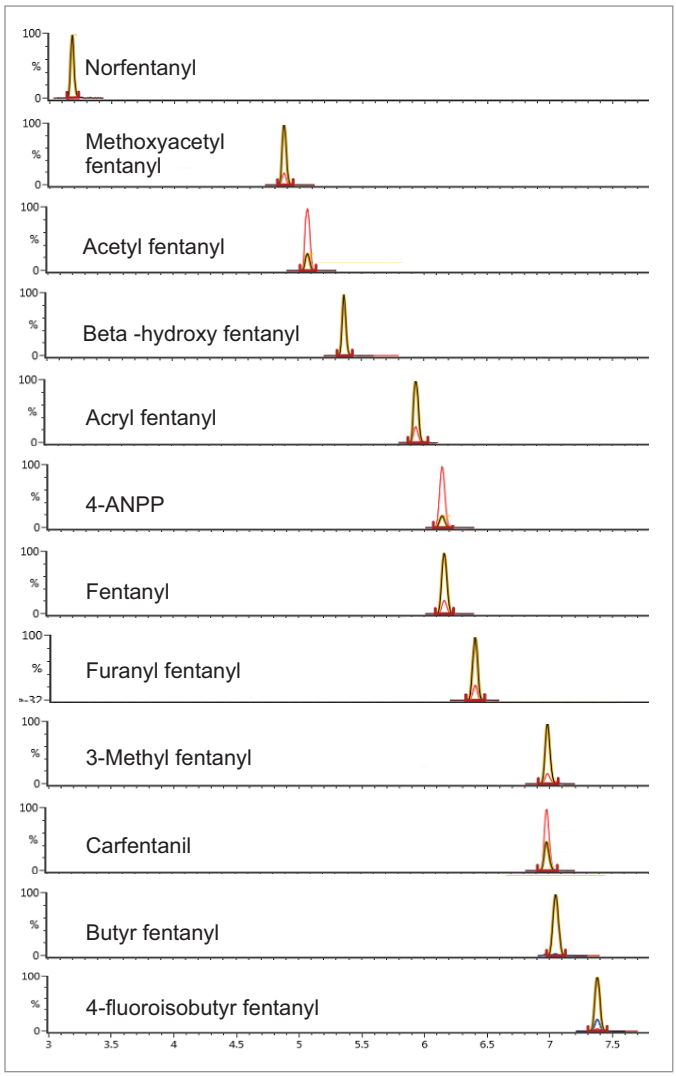

Figure 3. Tof-MRM mass chromatograms for an analyte-free urine sample enriched with the fentanyls at a 10 ng/mL; quantifier (yellow-trace) and qualifier (red-trace) are shown overlaid.

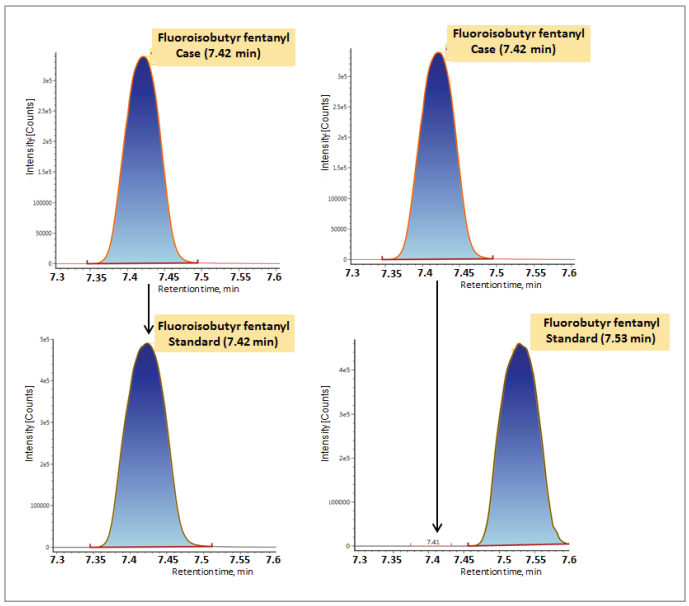

Figure 4. Tof-MRM analysis of 4-fluoroisobutyr fentanyl in a forensic case (upper) compared with reference standard analysis of 4-fluoroisobutyr fentanyl (lower-left) and 4-fluorobutyr fentanyl (lower-right).

Validation studies were conducted over 17 analytical runs and were designed in accordance with New York State Department of Health guidelines. The innovative TAC approach normalized the matrix effect and allowed consistent, threshold-accurate detection of all fentanyls investigated. Acceptable precision and accuracy was demonstrated for analyte concentrations around the cut-off i.e., at 0.8, 1.5, 3.0, and 10.0 ng/mL (Figure 5).

The confirmatory method was applied to 25 forensic case samples that had been also analyzed by a separate, fast, UPLC-MS/MS based screening method and the Tof-MRM demonstrated 100% concordance with the independent screen.[8] All case findings are summarized in Table 3, with fentanyl, norfentanyl, beta-hydroxy fentanyl, and 4-ANPP among the most commonly found. 4-ANPP is an intermediate, used in the manufacture of fentanyls and as such can be found as an impurity in fentanyl preparations; it is also understood to be a metabolite of fentanyl and some of the analogues including furanyl fentanyl, acetyl fentanyl, and acryl fentanyl.

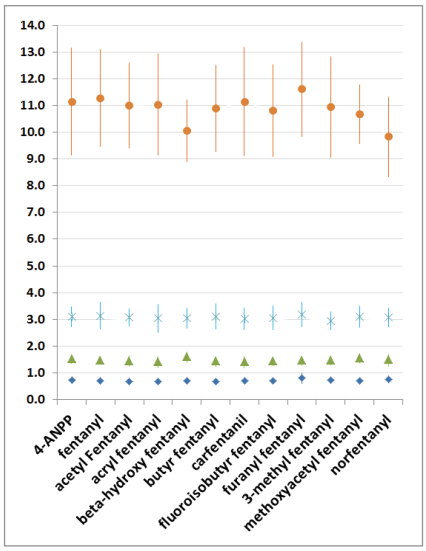

Figure 5. Accuracy and precision data for the QCs, over 17 analytical runs. Mean ± SD is shown for the target concentrations of 0.8, 1.5, 3.0, and 10.0 ng/mL.

Table 2. Retention times (RT) and dual Tof-MRM conditions including optimized collision energy (CE).

Case no.	Fentanyls
1	Furanyl fentanyl, 4-ANPP
2	Furanyl fentanyl, fluoroisobutyr fentanyl, 4-ANPP
3	Norfentanyl, 4-ANPP
4	Methoxyacetyl fentanyl, 4-ANPP
5	Fluoroisobutyl fentanyl, fentanyl, norfentanyl
6	Fentanyl, norfentanyl, beta-hydroxyfentanyl
7	Fentanyl, norfentanyl, 4-ANPP
8	Fentanyl, norfentanyl, methoxyacetyl fentanyl
9	Fentanyl, norfentanyl, 4-ANPP
10	Methoxyacetyl fentanyl, 4-ANPP
11	Methoxyacetyl fentanyl, 4-ANPP
12	Fentanyl, norfentanyl, beta-hydroxyfentanyl
13	Fentanyl, norfentanyl, beta-hydroxyfentanyl, acetyl fentanyl, fluoroisobutyr fentanyl, 4-ANPP
14	Fentanyl, norfentanyl, beta-hydroxyfentanyl, acetyl fentanyl
15	Fentanyl, norfentanyl, beta-hydroxyfentanyl, 4-ANPP
16	Fentanyl, fluoroisobutyr fentanyl
17	Fentanyl, norfentanyl, beta-hydroxyfentanyl
18	Fentanyl, norfentanyl, beta-hydroxyfentanyl, methoxyacetyl fentanyl, 4-ANPP
19	Fentanyl, norfentanyl, beta-hydroxyfentanyl
20	Fentanyl, norfentanyl, beta-hydroxyfentanyl, 4-ANPP
21	Fentanyl, norfentanyl, beta-hydroxyfentanyl
22	Fentanyl, norfentanyl, 4-ANPP
23	Fentanyl, norfentanyl, beta-hydroxyfentanyl
24	Fentanyl, norfentanyl, fluoroisobutyr fentanyl
25	Fentanyl, norfentanyl, acetylfentanyl

CONCLUSIONS

This application note describes a confirmatory method for use in forensic toxicology.

In line with previous reports, Tof-MRM provided increased sensitivity over the non-targeted Tof-MS^E approach.

The method utilizes some innovative approaches to produce a simple, yet accurate and precise qualitative method for the analysis of fentanyl and fentanyl analogues in urine.

The TAC approach, resulted in an accurate qualitative confirmation without the requirement of deuterated internal standards, which may not always be available, particularly for newer drug analogues.

The TAC approach also means that the method is adaptable and can be readily updated as new fentanyl analogues emerge.

References

1. Rosano TG, Wood M, Ihenetu K, and Swift TA. Drug Screening in Medical Examiner Casework by High-Resolution Mass Spectrometry (UPLC–MS^E-TOF). *Journal of Analytical Toxicology* 37(8), 1–14 (2013).
2. Dalsgaard P et al., Quantitatitive Analysis of Screening of 30 Drugs in Whole Blood by SPE-UHPLC-TOF-MS. *Forensic Science and Criminology* 1 (1), 1–6 (2013).
3. Bidny S, Gago K, Chung P, Albertyn D, and Pasin D. Simultaneous Screening and Quantification of Basic, Neutral and Acidic Drugs in Blood Using UPLC-QTOF-MS. *Journal of Analytical Toxicology* 41(3), 181–195 (2017).
4. Grapp M, Kaufmann C, Streit F, and Binder L. Systematic Forensic Toxicological Analysis by Liquid Chromatography-Quadrupole-Time-of-Flight Mass Spectrometry in Serum and Comparison to Gas Chromatography Mass Spectrometry. *Forensic Science International* 287, 63–73 (2018).
5. Rosano TG, Ohouo PY and Wood M. Application of High Resolution UPLC-MS^E/TOF for Confirmation in Forensic Urine Drug Screening by UPLC-MS/MS. In press *Journal of Analytical Toxicology,* bky106, https://doi.org/10.1093/jat/bky106.
6. Wood M, Barknowitz G, Goshawk JA, and Lee R. Evaluation of Various Tof Acquisition Strategies for the Analysis of Illicit Drug Substances. Poster presentation at the 56th TIAFT Conference, Ghent, Belgium (2018).
7. Rosano T, Ohouo P, and Wood M. Application of High Resolution Mass Spectrometry for Fentanyl Analog Confirmation and Discovery in Forensic Urine Casework. Poster presentation at the Annual Meeting for the Society of Forensic Toxicologists (SOFT) Minneapolis, MN, US (2018).
8. Rosano T, Ohouo P, LeQue J, Freeto S, and Wood M. Definitive Drug and Metabolite Screening in Urine by UPLC-MS/MS Using a Novel Calibration Technique. *Journal of Analytical Toxicology* 40(8), 628–638 (2016).
9. Rosano T, Ohouo P, and Wood M. Screening with Quantification for 64 Drugs and Metabolites in Human Urine using UPLC-MS/MS Analysis and a Threshold Accurate Calibration. *Journal of Analytical Toxicology* 41(6), 536–546 (2017).

For Forensic Toxicology Use Only.

Waters

THE SCIENCE OF WHAT'S POSSIBLE.™

Waters Corporation
34 Maple Street
Milford, MA 01757 U.S.A.
T: 1 508 478 2000
F: 1 508 872 1990
www.waters.com