Separation and quantification of novel synthetic regioisomeric fentanyls in clinical extracts by high pH reverse-phase LC-MS/MS

[1]Benjamin Barrett; [2]Melvin Euerby; [3]Jennifer Field; [4]Nicholas Gilbert; [5]Stephane Moreau; [4]Oliver Sutcliffe

[1]Kingston University London, London, UK; [2]University of Strathclyde, Glasgow, UK; [3]Shimadzu UK Limited, Milton Keynes, UK; [4]MANchester DRug Analysis and Knowledge Exchange (MANDRAKE), Manchester Metropolitan University, Manchester, UK; [5]Shimadzu Europa GmbH, Duisburg, Germany.

Overview

- Fluorofentanyl exists in several regioisomeric forms, all with similar LogD values and chromatography, resulting in difficulty by MS/MS analysis to confirm the regioisomeric form.
- A newly developed, high pH separation method demonstrating separation was used following a mini-validation according to ISO 17025 and LAB51 guidelines. Nineteen samples collected from emergency departments in Philadelphia, PA, were quantified for a panel of novel fentanyl and fluorofentanyl derivatives.

1. Introduction

The growing impact of new psychoactive substances (NPS) in a constantly evolving drug market represents an increasing challenge in forensic toxicology. There have been multiple reports in recent years for the increase in detection of para-fluorofentanyl in postmortem samples in the US and EU. *Para*-fluorofentanyl is one of several regioisomeric fluorofentanyl compounds, found to be significantly more toxic than fentanyl by LD50 values [1][2]. At present, the *para*-isomer appears to be the most prevalent in seized street samples, however, recent studies have detected the ortho-isomer in post-mortem cases. The potency of the para- and meta-isomers has been reported to be like fentanyl, whereas the ortho-isomer appears to exhibit twice the potency [3]. The danger associated with illegally taking these fluorofentanyl analogues is very significant since their lethal dosage is extremely low. ‘Conventional’ methods that test for wide panels of opiates or drugs of abuse [4] may not properly separate out these compounds which can be key information for both research and areas in forensic toxicology, such as postmortem, has not been successfully completed for all regioisomers without coelution by both LC and GC [5][6]. This poster presents a partially validated separation method that was effectively applied for the confirmation and quantification of seven fluorofentanyls and additional drugs, metabolites, and fentanyl derivatives of relevance, such as xylazine, *para*-fluoro-4-ANPP, furanylfentanyl, and methoxyfentanyl, within clinical toxicological extracts.

2. Materials and Methods

Clinical toxicology samples were extracted by a simple liquid-liquid method and screened by high-resolution QTOF MS/MS and samples positive for fluorofentanyl were triaged for confirmation and quantification. These samples were then analysed with a full calibration, quality control samples, and blanks following validation.

- **LC Separation**
  - Column: Two Avantor ACE SuperC18, 2µm, 2.1 x 150mm, coupled with 50mm 0.005” i.d. stainless steel tubing, column temp. 35 ºC, flow rate: 0.6 mL/min, 20 min total analysis time.
  - A: water + 0.1% ammonium hydroxide (25% solution w/v)
  - B: methanol + 0.1% ammonium hydroxide (25% solution w/v)
- **MS/MS Analysis**
  - LCMS-8060NX with electrospray ionisation (Shimadzu Corporation, Japan).
  - A minimum of two MRM transitions were optimised automatically for each compound by flow-injection analysis and LabSolutions software, selected based on intensity and selectivity to avoid interference.

3. Development

The regioisomeric families of fluorofentanyls were successfully separated based on mass through unique fragments generated in CID or by chromatography within a high pH, reversed phase chromatographic method. This method was compared with and selected against a hydrophilic interaction chromatographic method and low pH reversed phased method based on resolution, sensitivity, and ease of implementation.

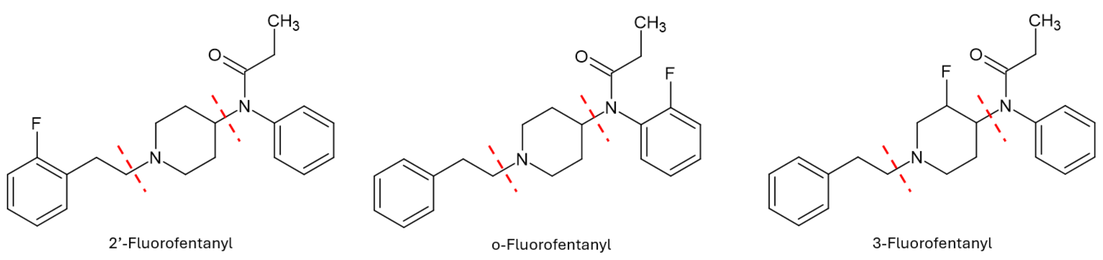

Figure 1. Structures of isomeric forms of fluorofentanyl analysed, showing the location of primary and secondary fragmentation by CID. Note the fragment masses for these change depending on the location of fluorine (source: ACD Labs).

Table 1. MRM transitions and voltages used for quantification and qualification of isomeric and regioisomeric compounds analysed.

Compound	Precursor m/z	Product m/z	Q1 Prerod Bias	Collision Energy	Q3 Prerod Bias
2-/3-Furanylfentanyl	375.10	188.30	-19.0	-23.0	-17.0
		105.30	-19.0	-32.0	-15.0
ortho-/meta-/para-Fluorofentanyl	355.20	188.35	-18.0	-24.0	-17.0
		150.30	-18.0	-30.0	-21.0
3-Fluorofentanyl	355.20	299.35	-18.0	-20.0	-30.0
		186.30	-18.0	-23.0	-17.0
2’-/3’-/4’-Fluorofentanyl	355.20	123.40	-18.0	-32.0	-17.0
		152.30	-18.0	-26.0	-14.0

The high pH, reversed phase method was compared with and selected against a hydrophilic interaction chromatographic method and low pH reversed phase chromatographic method based on resolution, sensitivity, and ease of implementation.

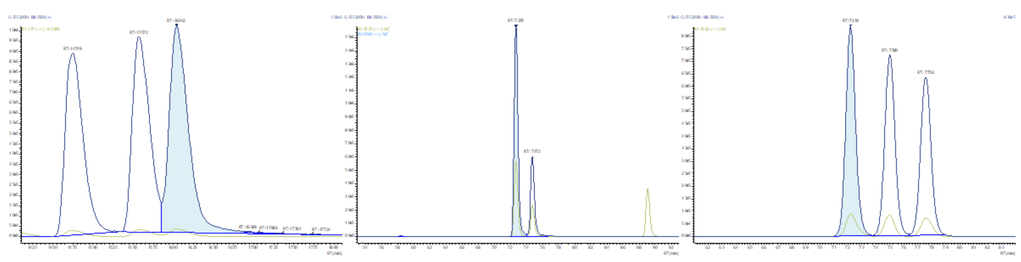

Figure 2. Chromatograms of ortho, para, and meta-Fluorofentanyl (10 pg on column) by different chromatographic methods (left – HILIC, middle – low pH/conventional, right – high pH) with para-fluorofentanyl highlighted in blue.

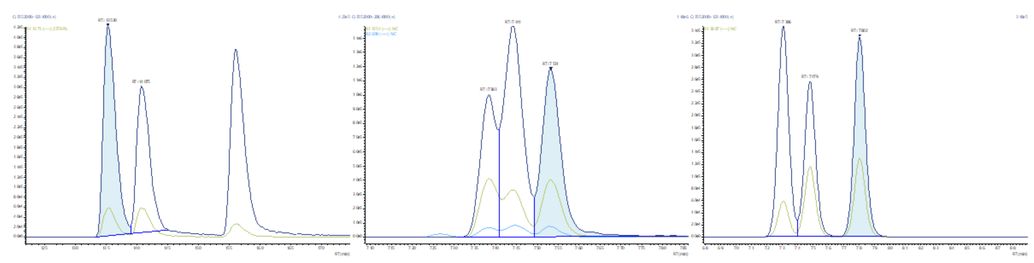

Figure 3. Chromatograms of 2’, 3’, and 4’-Fluorofentanyl (10 pg on column) by different chromatographic methods (left – HILIC, middle – low pH/conventional, right – high pH) with 2’-fluorofentanyl highlighted in blue.

3. Validation

Each compound was validated for several criteria including linearity, sensitivity, carryover, accuracy, precision, retention time stability, and ion ratio consistency. The acceptance for these criteria was based on UKAS LAB51 for accreditation of laboratories performing analysis of toxicology samples.

All compounds demonstrated stable retention time (calibration standards and QCs, n = 35) across batch analysis, linearity with R^2 > 0.99 between 0.05 and 100 ng/mL (8 points, bracket calibration, linear regression, 1/x weighting, no force through origin), accuracy and precision of 90% to 110% and RSDs of less than 15% (excluding heroin) in QC samples (n = 15 at 0.250, 15, and 75 ng/mL), low carryover for all compounds (<0.01% following 1 µg on column), consistent ion ratios, and high sensitivity (S:N ratio greater than 10 by root mean squared at 0.05 ng/mL).

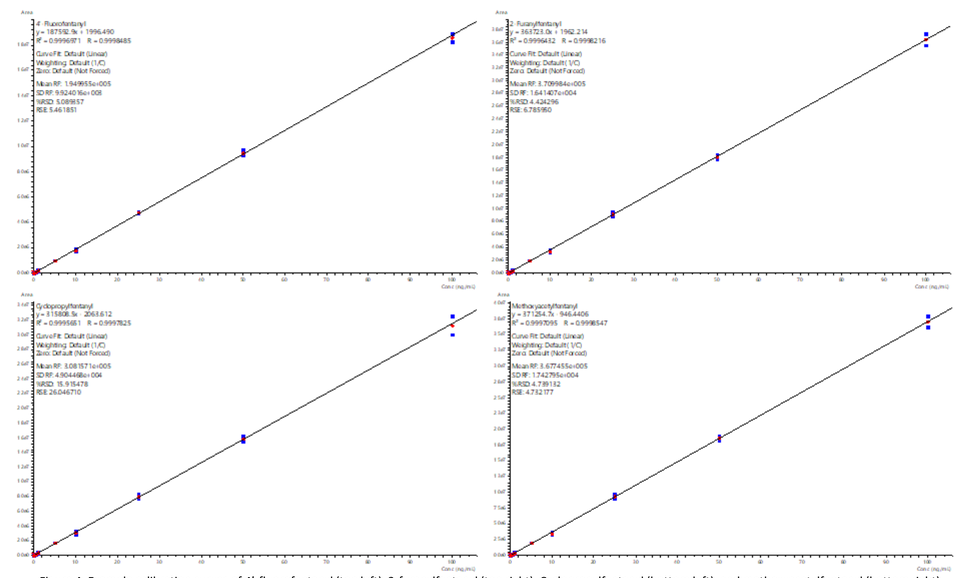

Figure 4. Example calibration curves of 4’-fluorofentanyl (top left), 2-furanylfentanyl (top right), Cyclopropylfentanyl (bottom left), and methoxyacetylfentanyl (bottom right).

Table 2. Validation results summary for all compounds within the analysed panel.

	R2	LLOQ (ng/mL)	Rt Stability (%RSD)	Accuracy (%)	Precision (%RSD)	Carryover (%)
2-Furanylfentanyl	0.9996	0.05	0.19	103.1	3.35	0.0030
3-Furanylfentanyl	0.9996	0.05	0.18	99.2	11.80	0.0044
4-ANPP	0.9996	0.05	0.17	98.8	8.35	0.0054
Cyclopentylfentanyl	0.9994	0.05	0.16	103.4	2.62	0.0008
Cyclopropylfentanyl	0.9995	0.05	0.33	100.2	6.81	0.0005
Fentanyl	0.9993	0.05	0.23	98.8	12.54	0.0004
Heroin	0.9993	0.05	0.14	94.8	10.25	0.0023
Methoxyacetylfentanyl	0.9996	0.05	0.20	102.4	3.74	0.0039
THF fentanyl	0.9945	0.10	0.19	105.5	2.56	0.0073
Xylazine	0.9990	0.05	0.13	102.6	4.63	0.0001
p-Fluoro-4-ANPP	0.9996	0.05	0.17	100.8	7.84	0.0078
o-Fluorofentanyl	0.9996	0.05	0.17	100.7	10.50	0.0029
m-Fluorofentanyl	0.9994	0.05	0.17	102.2	8.22	0.0023
p-Fluorofentanyl	0.9995	0.05	0.19	102.2	3.29	0.0036
2'-Fluorofentanyl	0.9995	0.05	0.19	100.4	7.82	0.0023
3'-Fluorofentanyl	0.9993	0.05	0.18	103.3	6.88	0.0038
4'-Fluorofentanyl	0.9995	0.05	0.19	102.8	3.47	0.0019
3-Fluorofentanyl	0.9993	0.05	0.19	103.1	4.57	0.0006

4. Sample Results

All samples found *para*-fluorofentanyl at a concentration between **0.43 ng/mL and 31.16 ng/mL** with a median result of **0.91 ng/mL**. *Ortho*- and *meta*-fluorofentanyl were not detected in any samples. Many of the samples demonstrated polydrug use by individual's tests, specifically xylazine, fentanyl, and potential precursors or metabolites of 4-ANPP and *p*-F-4-ANPP.

Fentanyl, was detected and quantified in the range of 0.45 – 119.64 ng/mL, with a median result of 14.36 ng/mL, xylazine in the range of 0 – 14.91 ng/mL, median result of 1.42 ng/mL, 4-ANPP in the range of 0.12 – 76.62 ng/mL, median result of 1.65 ng/mL, and *p*-F-4-ANPP in the range of 0.07 – 28.13 ng/mL, median result of 0.35 ng/mL, being present in multiple samples reported below. These results are similar to previous findings of fentanyl and fentanyl analogues in clinical blood samples [7].

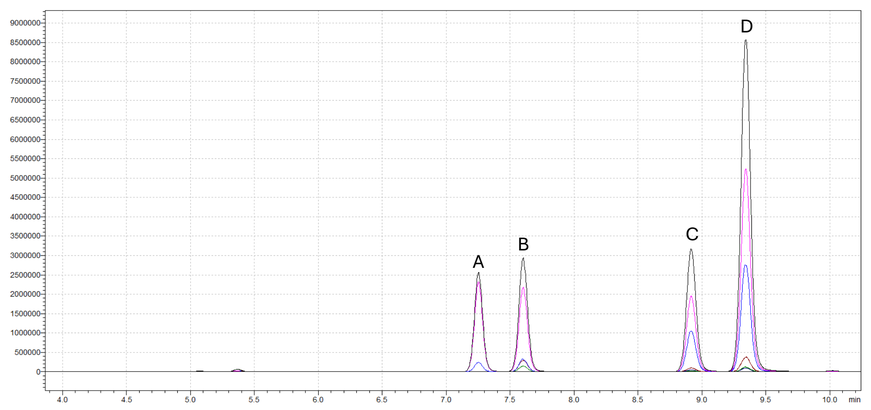

Figure 5. TIC chromatogram of sample testing positive for p-fluorofentanyl (A; 15.40 ng/mL), p-fluoro-4-ANPP (C; 28.13 ng/mL), 4-ANPP (D; 76.62 ng/mL), and fentanyl (B; 119.64 ng/mL)

5. Conclusion

A method has been successfully developed and partially validated for the separation and quantification of seven regioisomeric fluorinated fentanyl derivatives, with additional novel fentanyls and other substances that may be present within polydrug users. This method can also be practically adapted by a laboratory with standard HPLC-MS/MS equipment and applied to real casework samples to accurately identify regioisomeric fluorinated fentanyl derivatives.

Acknowledgement

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