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Development and comparison of reversed-phase ultra high-performance liquid chromatography (RP-UHPLC) and hydrophilic interaction liquid chromatography (HILIC) approaches to the analysis of regioisomeric fluorofentanyl derivatives and related compounds

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ABSTRACT

This study describes the development and comparison of low, intermediate and high pH gradient RP-UHPLC-MS/MS with that of gradient HILIC-MS/MS analysis for a range of fluorofentanyl derivatives including four families of *ortho*-, *meta*- and *para*-regioisomers. High pH RP-UHPLC-MS/MS using an ammonium hydroxide and methanol gradient on a high pH stable SuperC18 column at low temperature was demonstrated to be the most successful chromatographic mode for separating 26 analytes including: regioisomeric fluorofentanyls (n = 10); fentanyl analogues (n = 10), despropionyl precursors (n = 4) and two commonly encountered related substances (heroin and xylazine). Low and intermediate pH RP-UHPLC failed to afford separation of many of the fluorofentanyl regioisomers on stationary phases possessing complementary selectivity with either acetonitrile or methanol over a wide temperature range. HILIC on a bare silica column using an acetonitrile and ammonium acetate / acetic acid gradient provided good separation of fluorofentanyl regiosiomers except for the despropionyl series. High pH gradient RP-UHPLC was demonstrated to provide orthogonal chromatographic selectivity to that of HILIC in the gradient analysis of 18 fentanyl and related substances. Seven isobaric fluorofentanyl structural isomers could be readily discriminated from the unique fragmentation ions obtained using positive electrospray ionization MS/MS. The optimum high pH RP-UHPLC chromatographic conditions for the separation of the fluorofentanyls was equally successful for the rapid separation of a wide range of fentanyl regio- and structural isomers.

1. Introduction

Over the past fifteen years, there has been a marked increase in novel (or new) psychoactive substances (NPS) seized by law enforcement agencies globally [1]. NPS in their pure form, or within a formulation, are not covered by the United Nations Single Convention on Narcotic Drugs (1961), as amended by the Protocol (1972), or by the United Nations Convention on Psychotropic Substances (1971) but can potentially lead to negative health or social risks like those posed by the substances covered by these international treaties [2]. Within this

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context, the terms "novel" or "new" does not necessarily refer to original inventions but to substances that have recently become available within the illicit market. Psychoactive substances prohibited under the international drug control conventions produce their effects through a small number of pharmacological mechanisms and can have significant chemical diversity within each family of psychoactive substances [1,2]. Current convention uses a functional "effect group" categorization to define NPS within six broad overlapping groups: (i) cannabinoid receptor agonists; (ii) classic hallucinogens; (iii) psychostimulants; (iv) opioid receptor agonists; (v) sedatives/hypnotics and (vi) dissociatives. The grouping is based on the features related to their chemical structure, psychopharmacological desired and unwanted effects [1,2].

Since the 1980s, opioid receptor agonist abuse has grown to pose a considerable threat to public health [3–6]. Specifically, fentanyl (**1**, Fig. 1) use has been linked to a significant increase in drug-related overdoses, especially in North America, and this risk of overdose is compounded by the occurrence of the substance as an adulterant within heroin and other drugs of abuse [7–10]. The rise in fentanyl abuse has been associated with the appearance of novel structural and isomeric fentanyl analogues, in 2008–2024 the United Nations Office on Drugs and Crime (UNODC) reported more than 80 New Psychoactive Substances (NPS) with opioid effect (including fentanyl analogues) in its Early Warning Advisory [1].

The emergence of regioisomeric derivatives of known synthetic drugs is a constant challenge in forensic casework. The availability of pure, isomeric starting materials renders the synthesis of regioisomeric derivatives extremely simple. These compounds tend to exhibit similar chemical and chromatographic properties, and their mass spectra are often equivalent. This complicates the identification of specific drug regioisomers, hence there is a requirement for selective analytical methods to identify regioisomers in prevalent NPS groups including: synthetic cannabinoids [11], fluoroamphetamines [12], chloroamphetamines [13], cathinones [14] and diphenidines [15,16]. In some cases, identification of drug regioisomers may also require the use of multivariate analysis in conjunction with mass spectral data [17–19]. The same challenge applies to the identification of regioisomeric fentanyls, which cannot be discriminated by conventional mass spectral databases, without more sophisticated multivariate approaches [19].

Previous studies have reported the analysis of structural and/or isomeric fentanyls [20]. Sisco et al. have reported a very sensitive direct analysis in real-time mass spectrometry [DART-MS, LOD = 0.08–0.35 ng] and ion mobility spectrometry [IMS, LOD = 1.0–10.0 ng] screening methods, but neither of these techniques facilitated efficient separation of the 18 analogues within the study [21]. High performance liquid chromatography (HPLC) has been applied by several groups [22,23] including one validated method, which has been developed and utilized to quantify (**1**) within bulk forensic samples of heroin (**18**) [23]. Hyphenated techniques (LC–MS, LC-MS/MS and UPLC-MS/MS) have also been applied to detect fentanyls and their metabolites in blood [24,25], urine [24] and wastewater [26]. Although these methods are rapid, they were not optimized to chromatographically resolve the target analytes, which can lead to ion suppression when analyzing low-concentration, adulterated street samples [27]. A fully validated GC–EI-MS method (employing SIM mode) has been developed, by Gilbert et al., allowing the separation and identification of 18 fentanyls, five commonly encountered controlled substances and four adulterants within 20 min. When applied to seized samples, the validated method allowed sensitive screening and quantitative analysis of the illicit (and potentially harmful) ingredients at trace levels (LOD = 0.007–0.822 μg/mL and LOQ = 0.023–2.742 μg/mL respectively) [20]. Gilbert et al. further developed their approach to fluorinated fentanyl derivatives ("fluorofentanyls") and reported a GC–EI-MS method (employing SIM mode) for the

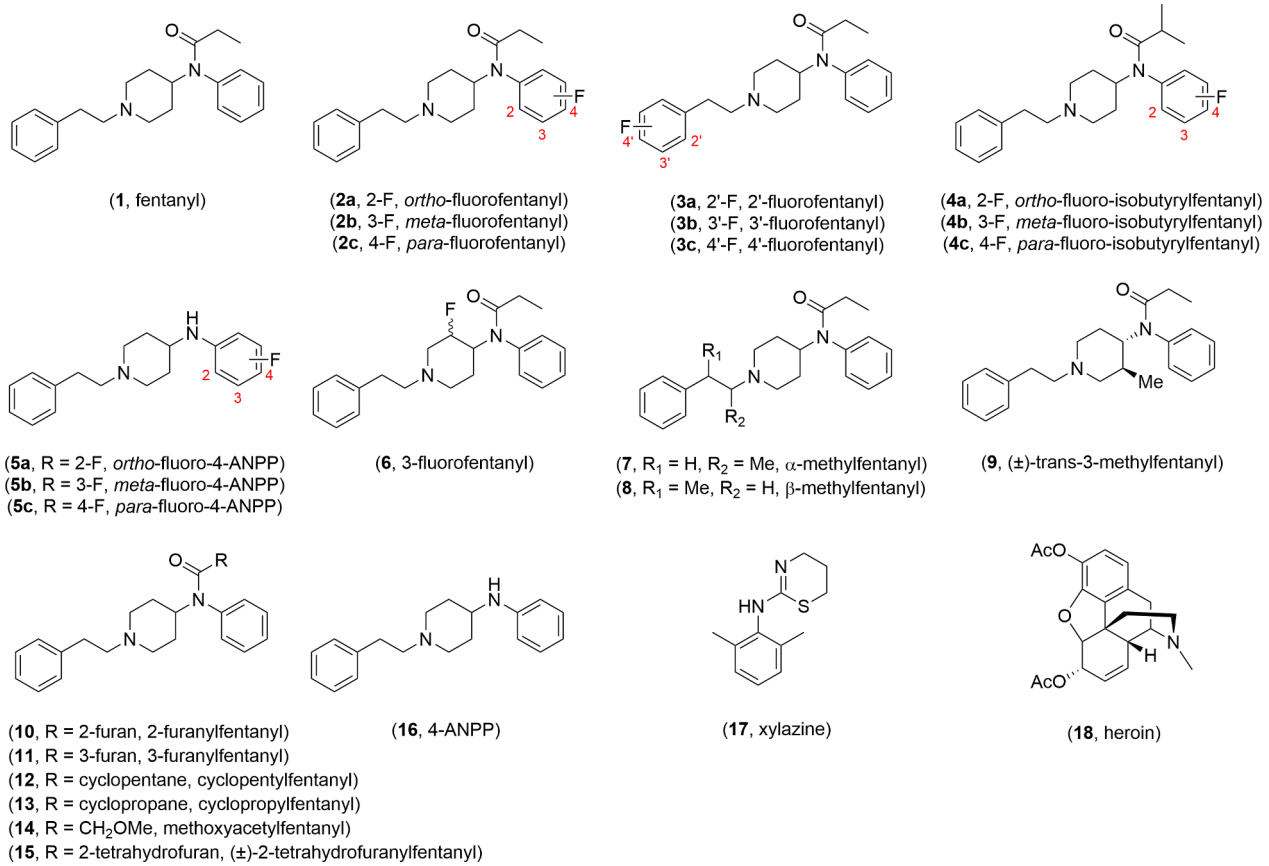

Fig. 1. Structures of fentanyl (**1**); regioisomeric fluorofentanyls (**2–4**, **6**); despropionyl precursors (**5**, **16**); fentanyl analogues (**7–15**), xylazine (**17**) and heroin (**18**) utilized in this study.

quantification of seven regioisomeric fluorofentanyls (**2a** – **2c**, **3a** – **3c** and **6**) (LOD = 9–20 ng/mL, LOQ = 31–67 ng/mL), fentanyl (**1**), (**18**), acetaminophen and caffeine, within 13 min [28]. In most cases, the fluorofentanyls were resolved from each other except for the 3′- and 4′-fluorinated derivatives (**3b** and **3c**). To achieve full separation, an orthogonal low-field (60 MHz) ^{19}F NMR method was developed, allowing the identification and quantification of target analogues (LOD = 74–400 μg/mL, LOQ = 290–1340 μg/mL) thereby facilitating their detection at low concentration (2.4 % *w/w*) within heroin [28].

Due to the lack of suitable ultra high-performance chromatography (UHPLC) methodologies to separate fluorofentanyl regioisomers this research provides a comparison of the analysis of a range of derivatives and related compounds under low, intermediate, high pH reversed-phase ultra high-performance chromatography (RP-UHPLC) and hydrophilic liquid chromatography (HILIC) conditions. Specifically, this study focuses on analytical approaches to discriminate regioisomeric fluorofentanyls (**2–4**, **6**); fentanyl analogues (**1**, **7–15**), despropionyl precursors (**5**, **16**) and commonly encountered related substances [e.g. xylazine (**17**) and heroin (**18**)] (see Fig. 1). The 2-, 3- and 4-fluorofentanyls (**2a – 2c**, **4a – 4c**) and fluoro-despropionyl precursors (**5a – 5c**) possess a fluoro substituent on the aniline ring and are commonly referred to as *ortho*-, *meta*- and *para*-regioisomers, whereas a fluorine situated on the phenylethyl ring gives rise to the isomeric 2′- 3′- and 4′-fluoro series (**3a – 3c**). The influence of the amide moiety on the separation of the regioisomers was evaluated by comparison of the parent fluorofentanyl series (**2a – 2c)** with the fluoroisobutyrylfentanyl series (**4a – 4c**) – possessing a larger amide moiety – and the fluoro-despropionyl precursors (**5a – 5c**) which lack the amide moiety. 3-Fluorofentanyl (**6**) differed from the other regioisomeric series in that it possesses a fluorine substituent on the central piperidine ring. Previously, there have been no systematic evaluations investigating the resolution of these forensically important fluorofentanyl regioisomers [29,30].

Low, intermediate and high pH mass spectrometric (MS) friendly mobile phase conditions in combination with RP-UHPLC stationary phases of complementary chromatographic selectivity [31] as well as temperature / gradient retention modelling were employed to evaluate the chromatographic behaviour and separation of these regioisomeric fluorofentanyls. The optimum RP-UHPLC methodology was then compared to a generic gradient HILIC methodology [32] which used a bare silica column and MS friendly mobile phase conditions. The successful LC conditions were then applied to the analysis of three structural isomers of methylfentanyl (**7–9**) and the separation of 2- and 3-furanylfentanyl (**10** and **11**). The chromatographic selectivity of low pH, high pH RP-UHPLC and HILIC conditions was compared using a mixture of 18 fentanyl and related substances. The MS fragmentation of the isobaric fluorofentanyl derivatives (**2a – 2c**, **3a – 3c** and **6**) was investigated to establish if MS could be used in combination with chromatographic separation to unambiguously confirm the presence or absence of these forensically important compounds.

2. Materials and methods

2.1. Synthesis of standards and procurement of forensic samples

Acetonitrile (ACN), methanol (MeOH) and water were of LC-MS grade and supplied by either Romil Limited (Cambridge, UK) or Honeywell (Calibre Scientific, USA). All mobile phase additives and buffer salts were supplied by Sigma-Aldrich, Inc., (St. Louis, MO, USA) or Merck Ltd., (Gillingham, UK). Fentanyl certified reference materials (**2a – 2c**, **4a – 4c**, **5a – 5c**, **6–9**, supplied as 100 μg/mL solutions in MeOH) were purchased from Cayman Chemical Company (Ann Arbor, MI, USA) or synthesized by MANchester DRug Analysis and Knowledge Exchange (MANDRAKE) (**2a – 2c**, **3a – 3c**, **6**, **9–18** as 1000 μg/mL solutions in 20:80 or 10:90 *v*/v ACN/water). To ensure the authenticity of the materials utilized within this study, synthesized samples were structurally characterized by ^{1}H NMR, ^{13}C{^{1}H}-NMR, GC–MS and ATR-FTIR [28]. Samples were further diluted into 50:50 v/v ACN/MeOH to produce a 100 ng/mL mixed solution for HILIC and 20:80 v/v ACN/water for RP-UHPLC.

2.2. Chromatographic systems

2.2.1. Ultra high-performance liquid chromatography tandem mass spectrometry (UHPLC-MS-MS) system 1

LC-MS/MS analysis was performed on a Shimadzu Nexera XS UHPLC (Shimadzu UK Ltd., Milton Keynes, UK) equipped with two binary pumps (LC-40D XS) with LPGE proportionating valves, degassers (DGU-405), autosampler (SIL-40C XS), column oven (CTO—40C), system controller (SCL-40). The MS source parameters were as defined in the Supplementary Material 1. The software used for data acquisition was LabSolutions LCMS (v5.120), and the software used for data processing including sample qualification and quantification was LabSolutions Insight LC-MS (v4.0). The injection volume was 2 μL unless otherwise stated. A minimum of two multiple reaction monitoring (MRM) transitions were identified by flow-injection analysis using the LabSolutions LC-MS MRM Optimization Tool to automatically optimize collision energy, prerod bias, and identify the most abundant product ions generated from Collision-induced dissociation (CID) fragmentation. The transitions used are specified in Supplementary Material 2. The interface voltage was optimized between 0.5 kV to 3 kV in 0.5 kV increments to maximize precursor ion generation. This was followed by optimization of the focus plate voltage on the LCMS-8060NX to ensure effective ion transfer from the ion source into the instrument. A single voltage for both interface and focus plates was used for all compounds.

2.2.2. Ultra high-performance liquid chromatography / high-resolution tandem quadrupole time-of-flight mass spectrometry (UHPLC-QTOF-MS/MS)

LC-MS/MS analysis was performed on a Shimadzu Nexera X3 UHPLC (Shimadzu UK Ltd., Milton Keynes, UK), equipped with a single, dual binary pump with in-built controller module and five channel degasser (LC-40B X3 and CBM-40lite), autosampler (SIL-40C X3), column oven (CTO—40S), and high-resolution tandem quadrupole time-of-flight mass spectrometer (LCMS-9050). The source parameters were as defined in Supplementary Material 2. The instrument was tuned prior to each use with sodium iodide solution to ensure mass accuracy less than 5 ppm and mass resolution greater than 45,000 FWHM at *m/z* 1273. The software used for data acquisition was LabSolutions LCMS (v5.120), and the software used for data processing was LabSolutions Insight LCMS Explore (v4.0). Data independent acquisition (DIA) MS/MS was used for the data acquisition, consisting of a full MS scan from 100 to 500 *m/z* at a speed of 10 Hz, followed by sequential MS/MS scans from precursor *m/z* 110 to 500, with a width of 20 Da, and TOF mass range set to *m/z* 40 to 500 for product ion acquisition and a CE centre of 30 V and spread of +/− 25 V for each step, carried out at 25 Hz. Structural identification was carried out through LabSolutions Insight Explore Assign feature, using the predicted formula (crosschecked with compound structure) ChemSpider and Pubmed to aid in identification. A tolerance of minimum ±10 ppm mass accuracy was also applied to structural identification of both precursor and fragment ions. The injection volume was 1 μL unless otherwise stated.

2.2.3. Ultra high-performance liquid chromatography tandem mass spectrometry (UHPLC-MS-MS) system 2

Analysis of the methylfentanyls (**7 – 9**), fluorinated depropionylfentanyl precursors (**5a – 5b**) and fluoroisobutyrylfentanyl isomers (**4a – 4c**) was carried out using the ThermoFisher UltiMate 3000 UHPLC coupled with the ThermoFisher TSQ Vantage LC-MS/MS, operating parameters are as defined in Supplementary Material 3 and MRMs in Supplementary Material 4. Data was acquired using Thermo Chromeleon™ Chromatography Data System (CDS). The software used for

data processing including sample qualification and quantification was Thermo Xcalibur™.

2.3. Retention modelling using ultra high-performance liquid chromatography photodiode Array spectrometry

Shimadzu Nexera X2 UHPLC (Shimadzu UK Ltd., Milton Keynes, UK) consisted of two binary pumps (LC-30 CE) and LPGE proportionating valves, degassers (DGU-405), autosampler (SIL-30 AC), column oven (CTO-20 AC), diode array detector (SPD-M30A) with a 1 μL/10 mm pathlength flow cell, 40 μL mixer installed and system controller (CMB-20 A). The software used for data acquisition was LabSolutions LCMS (Shimadzu UK Ltd., version 5.120), and the software used for data processing, including sample qualification and quantification, was LabSolutions Insight LCMS (v4.0). ACE Excel SuperC18, ACE Excel C18-Amide, ACE Excel C18-AR and ACE Excel C18-PFP (2 μm, 100 Å, 150 × 3 mm i.d.) were as supplied by VWR International (Reading, UK). 2 × 2 gradient time (10–30 min) versus temperature (30–60 °C) retention models with appropriate validation points were constructed using either mobile phases A: 20 mM ammonium formate (pH 3.0) in water, 20 mM ammonium acetate (native pH) in water, or 0.1 % *v*/v of 25 % *w*/w ammonium hydroxide solution in water (SuperC18 column only) and mobile phases B: 20 mM ammonium formate (pH 3.0) in water/ACN or MeOH 20:80 v/v, 20 mM ammonium acetate (native pH) in water/ACN or MeOH 20:80 v/v, or 0.1 % v/v of 25 % w/w ammonium hydroxide solution in water/ACN or MeOH 20:80 v/v (SuperC18 column only). The initial and final %B was 60 and 100 % respectively. A flow rate of 0.43 mL/min was employed. At least 20 column volumes of the appropriate mobile phase were flushed through the columns prior to commencing the testing or on changing the mobile phase conditions. The photodiode array (PDA) detector was set to monitor a wavelength of 254 nm (bandwidth 8 nm) with a reference at 360 nm (bandwidth 100 nm). The data sampling rate was set at 40 Hz. Chromatographic values reported are the average of duplicate injections.

2.4. Low pH RP-UHPLC evaluation

2.4.1. Optimized low pH RP-UHPLC chromatographic conditions for the separation of 2-, 3- and 4-fluorodespropionyl precursors (**5a – 5c**)

A HALO C18, 2.7 μm, 90 Å,100 × 2.1 mm (supplied by Advanced Materials Technology, Wilmington, DE, USA) or an ACME Plus C18, 1.9 μm, 200 Å, 100 × 2.1 mm (supplied by Phase Analytical Technology, State College, PA, USA) column was employed with mobile phase A consisting of 0.1 % *v*/v formic acid in water and mobile phase B corresponding to 0.1 % v/v formic acid in ACN. A linear gradient from 5 to 80 % B over 12 min with a flow rate of 0.4 mL/min and a column temperature of 25 °C was employed.

2.4.2. Generic low pH RP-UHPLC

A conventional generic low pH RP-LC screening methodology was employed. Mobile phase A consisted of 0.02 % v/v formic acid and 2 mM ammonium formate in water and mobile phase B of 0.02 % v/v formic acid and 2 mM ammonium formate in MeOH. The rinse solution used for the measuring pump and rinse port was isopropanol. The column used was a Acquity CSH C18 1.8 μm, 130 Å, 100 mm × 2.1 mm, supplied by Waters Ltd. (Wilmslow, UK). The following gradient conditions were employed: 5 % to 50 % B in 8.5 min, followed by 50 % to 100 % B in 8.9 min, plus a hold at 100 % B for 1.1 min, before dropping to 5 % for 3 min, resulting in a total analysis time of 13 min excluding pre-treatment. The flow rate was 0.6 mL/min with an oven temperature of 55 °C. On the Shimadzu LCMS-8060NX the injection volume was 1 μL, with coinjection of 10 μL water before and after sample injection, and on the ThermoFisher TSQ Vantage the injection volume was 3 μL.

2.5. Optimized high pH RP-LC chromatographic conditions for the separation of fluorofentanyls (2a – 2c, 3a – 3c and 6), fluoroisobutyrylfentanyls (4a – 4c), fluorodespropionyl precursors (5a – 5c) and fentanyl analogues (10−11)

An ACE Excel SuperC18 (2 μm, 100 Å, 150 × 3 mm i.d.) column was employed with mobile phase A 0.1 % *v*/v of a 25 % *w*/w ammonium hydroxide solution in water and mobile phase B 0.1 % v/v of a 25 % w/w ammonium hydroxide solution in MeOH at a flow rate of 0.43 mL/min and a temperature of 25 °C. A gradient of 80 % to 90 % organic in 10 min, followed by an isocratic hold at 90 % organic for 1 min, before dropping to 80 % in 0.1 min with a equilibration phase for an additional 5.9 min, giving a total analysis time of 17 min was employed. A flow rate of 0.43 mL/min and an oven temperature of 25 °C were employed with an injection volume of 3 μL. Alternatively, two ACE Excel SuperC18 columns were coupled together with 50 mm, 0.005″ i.d., stainless steel tubing and Restek EXP 2 fittings to ensure minimal additional system volume. A translated gradient was used to maintain the same (t_G x FR)/V_m and hence the same selectivity. A gradient from 80 % to 90 % organic in 14.3 min, followed by an isocratic hold at 90 % organic for 1 min, before dropping to 80 % with an equilibration phase for an additional 4.7 min at a flow rate 0.6 mL/min was employed resulting in a total analysis time of 20 min. An oven temperature of 35 °C was employed with a typical pressure maximum of 840 bar.

2.6. HILIC chromatographic conditions

Mobile phases A and B consisted of 0.1 % *v*/v acetic acid and 5 mM ammonium acetate (native pH) in water and ACN respectively. The rinse solution used for the measuring pump and rinse port was isopropanol. A HALO SiO_2 HILIC column (2.7 μm, 90 Å, 150 mm × 2.1 mm i.d.,) was supplied by Advanced Materials Technology, Inc. (Wilmington, DE, USA). The mobile phase was held isocratically at 98 % B for 1 min and then ramped down from 98 to 87 % B with a gradient time of 17 min at a flow rate of 0.4 mL/min. The flow rate and %B were then changed to 1 mL/min and 98 %B over 1 min. This was held for 6 min then the flow was reduced to 0.4 mL/min over 1 min before the next injection. An injection volume of 1 and 3 μL and an oven temperature of 40 and 25 °C was employed on the Shimadzu LCMS-8060NX and ThermoFisher TSQ Vantage systems, respectively. The fentanyl samples were prepared at a concentration of 100 ng/mL in ACN.

2.7. Software

Log *D* and pK_a values were predicted using ACD/Percepta (Toronto, Canada, version 2019.1.3). Resolution value (R_s) and tailing factor (t_f) was calculated as defined in the United States Pharmacopoeia:

$$R_s = 1.18\left(t_r^2 - t_r^1\right)/(w_1 + w_2)$$

where t_r^1 and t_r^2 are the retention times in minutes of, respectively, the first and the last eluting peak of a pair, while w_1 and w_2 are the widths at half height in minutes of these peaks.

$$t_f = w_{0.05}/(2d)$$

$w_{0.05}$ = width of the peak at 5 % of the peak height.

d = distance between the perpendicular dropped from the peak maximum and the leading edge of the peak at 5 % of the peak height.

3. Results and discussion

3.1. RP-UHPLC separation

The fluorinated analytes (**2a – 2c**, **3a – 3c**, **4a – 4c**, **5a – 5c** and **6**) possessed estimated pK_a values in the range of 8.0 to 9.0. Furthermore, the fluoro-despropionyl precursors (**5a – 5c**) possessed an additional

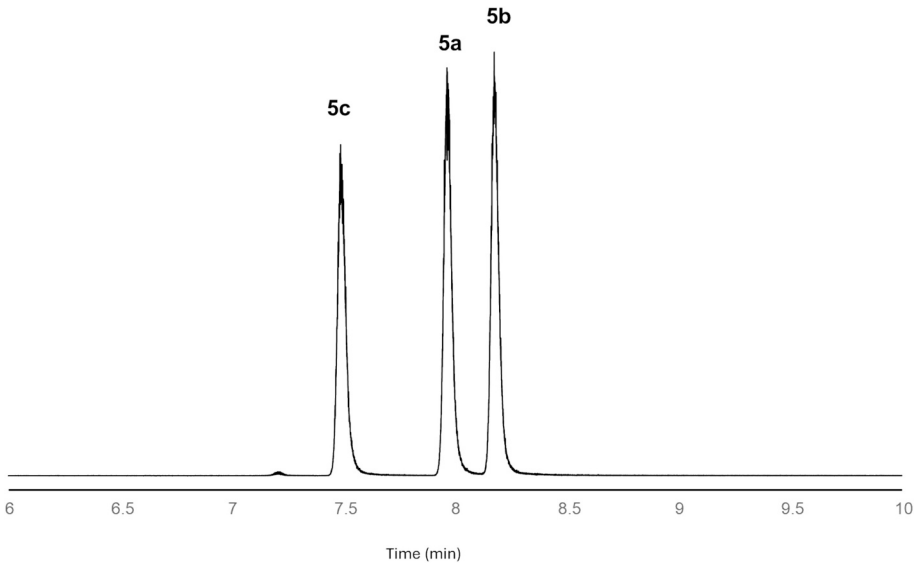

Fig. 2. RP-UHPLC/MS/MS separation of **5a – 5c** (TIC, MRM *m/z* 299.15 > 105.08, 299.15 > 188.14, and 299.15 > 77.07) under low pH mobile phase conditions, mobile phase A: 0.1 % *v*/v formic acid in water and mobile phase B: 0.09 % v/v formic acid in ACN, ACME Plus C18 3 μm, 100 × 2.1 mm, 5 to 80 % B in 12 min, 0.4 mL/min, 5 μL injection with each compound at a concentration of 500 ng/mL, see Section 2.4.2 for experimental conditions.

weak amino functionality with a pK_a value of 4.3. Log *D* values were estimated to range from 0.2 to 1.4, 1.8–2.8 and 3.6–4.5 at pH 3, 6.5 and 10.7 respectively. Due to the difference in their ionization state and that of the silica based reversed phase columns as a function of mobile phase pH, the RP-UHPLC separation of these fluorinated analogues (**2a – 2c**, **3a – 3c**, **4a – 4c**, **5a – 5c** and **6**) was evaluated at low, intermediate and high pH.

3.1.1. Low and intermediate pH RP-UHPLC separations

Typical toxicological LC-MS/MS screening conditions [33–35] employing new generation silica based C18 columns in conjunction with either formic acid, or, in combination with ammonium formate pH 3, and either MeOH or ACN gradients, failed to resolve the regioisomeric fluorofentanyl derivatives (**2a – 2c**, **3a – 3c** and **4a – 4c**, see Supplementary Material 5). The *ortho*-isomer was observed to elute after the *para*- and *meta*- isomers which inevitably co-eluted. The lack of resolution of fluorofentanyl regioisomers when chromatographed at low pH using RP-UHPLC conditions is well documented [29,30,36,37]. Recently, serially coupled columns with differing chromatographic selectivity (i.e., phenyl hexyl and cyano) have been employed to achieve resolution of *ortho*-, *meta*- and *para*-fluoroisobutyrylfentanyl isomers (**4a – 4c**) at low pH [38].

In contrast, the fluorinated despropionyl precursors (**5a – 5c**) were easily separated under low pH mobile phase conditions with a conventional C18 phase yielding $R_{s\ (5a/5c)} = 6.9$ and $R_{s\ (5b/5a)} = 3.2$ (see Fig. 2). The rationale for the separation may reside in the fact that **5a – 5c** possess two amino functionalities compared to **2a – 2c**, **3a – 3c** and **4a – 4c** which only possess one. The elution order for these regioisomers was observed to be *para*- < *ortho*- < *meta*-.

Previously, intermediate pH and a SuperC18 column had been successfully employed to separate a variety of regioisomeric diphenidine derivatives [15,39]. However, similar UHPLC conditions failed to yield baseline separation of the regioisomeric fluorofentanyls (**2a – 2c**). The elution order in most cases was observed to be similar to that observed at low pH (i.e., *para*- ≤ *meta*- ≤ *ortho*-).

Temperature and gradient time retention modelling [40] using a range of differing RP-UHPLC columns specifically designed for their complementary selectivity (i.e., SuperC18, C18-Amide, C18-AR and C18-PFP [31]) with either ACN or MeOH at low (i.e., 10 mM ammonium formate pH 3.0) and intermediate pH (i.e., 20 mM ammonium acetate native pH) failed to afford baseline separation of compounds **2a – 2c**.

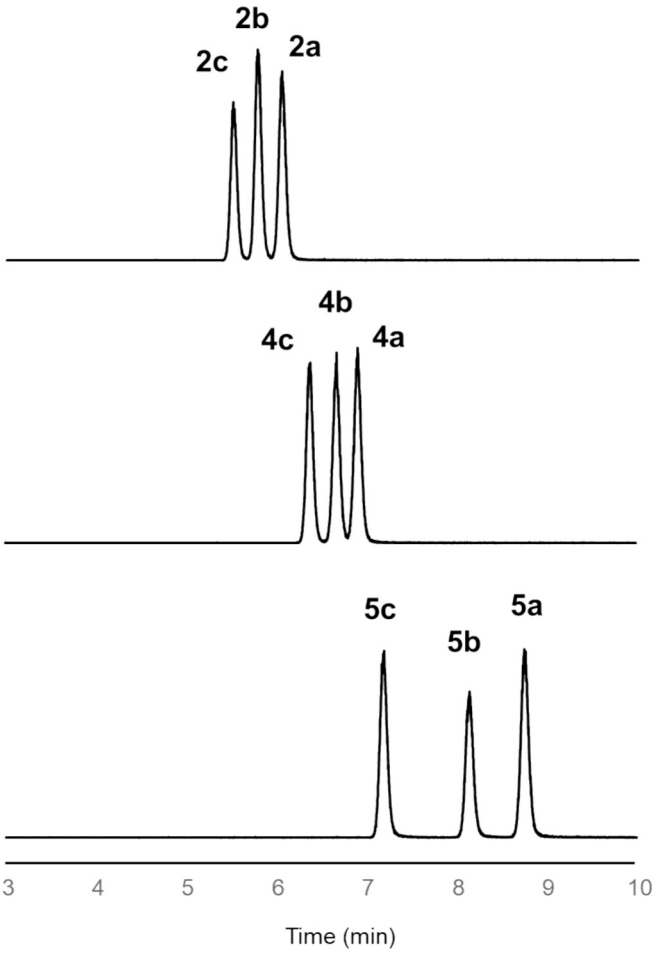

Fig. 3. RP-UHPLC-MS/MS separation of **2a – 2c** (TIC, MRM *m/z* 355.20 > 188.35 and 355.20 > 150.30), **4a – 4c** (TIC, MRM *m/z* 369.19 > 188.14, 369.19 > 105.08 and 369.19 > 103.07) and **5a – 5c** (TIC, MRM *m/z* 299.15 > 105.08, 299.15 > 188.14, and 299.15 > 77.07) under high pH mobile phase conditions, 150 × 3 mm SuperC18 2 μm column at 25 °C, for experimental UHPLC conditions see Section 2.5. R_s (**2b/2c**) = 1.8 and R_s (**2a/2b**) = 1.4, R_s (**5b/5c**) = 6.3 and R_s (**5a/5b**) = 3.9, R_s (**4b/4c**) = 2.0 and R_s (**4a/4b**) = 1.8.

3.1.2. High pH separations

RP-UHPLC employing high pH conditions in conjunction with high pH stable stationary phases is nowadays extremely popular [41] for the analysis of basic compounds since the latter compounds can be chromatographed in their ion suppressed mode. This often results in enhanced retention, improved peak shape and different chromatographic selectivity compared to that observed at low and intermediate pH. Retention modelling (i.e., temperature versus gradient time) of the fluorofentanyls (**2a – 2c**) with either MeOH or ACN using a high pH stable C18 stationary phase (i.e., SuperC18) indicated that the optimal separation could be achieved at temperatures ≤25 °C using MeOH as the organic modifier (see Supplementary Material 6). Optimum UHPLC conditions for the separation of **2a – 2c** also resolved the regioisomers of the additional fluoroisobutyrylfentanyl (**4a – 4c**) and fluorodespropionyl precursor (**5a – 5c**) series (see Fig. 3). Resolution of the **3a – 3c** series was less successful with $R_{s\ (3b/3c)} = 0.7$ and $R_{s\ (3a/3b)} = 1.5$ being obtained.

Baseline separation of the 2′-fluoro-, 3′-fluoro- and 4′-fluorofentanyls (**3a – 3c**) could be achieved using two serially coupled 15 cm columns operating at 0.6 mL/min with a gradient time of 14.3 min and an elevated temperature to reduce the operating back pressure to an acceptable 840 bar (see Fig. 4). The UHPLC conditions (see Section 2.5) were adjusted to maintain a constant chromatographic selectivity (i.e., a constant $(t_G \times FR)/V_m$ was employed). However, a slightly differing chromatographic selectivity was observed (same elution order), this may have been due to an elevated temperature being employed to reduce the back pressure or the fact that chromatography at elevated pressures (i.e., >800 bar), can affect the molar volume which then affects the retention of certain analytes to differing degrees [42].

Even though the isomers of **2a – 2c** and **3a – 3c** eluted in a similar retention window, the two types of classes fluorofentanyls could be differentiated based on their differing MS/MS fragmentation ions (see Section 3.3.1). The fentanyl derivatives typically generated symmetrical peaks with tailing factors in the range of 0.9–1.0 when chromatographed under these high pH conditions. This was attributed to the analytes running in their unionized form thereby reducing potential secondary interactions with the stationary phase.

As expected, the more hydrophobic fluoroisobutyrylfentanyl regioisomers (**4a – 4c**) were longer retained than their corresponding fluorofentanyl (**2a – 2c**) analogues when chromatographed at high pH. Interestingly, the corresponding fluorodespropionyl precursors (**5a – 5c**), which lacked the amide functionality, were retained longer than the corresponding fentanyl derivatives possessing an amide functionality (i.e., **2a – 2c** and **4a – 4c** see Fig. 3). The elution order of the regioisomeric fluorinated analytes (**2a – 2c**, **3a – 3c**, **4a – 4c** and **5a – 5c**) using high pH RP-UHPLC conditions was observed in all cases to be *para-* < *meta-* < *ortho-* which contrasts with that observed at low pH (i.e., elution order is *para-* < *ortho-* < *meta-* for the regioisomers **5a – 5c**).

3-Fluorofentanyl (**6**), which possesses the fluoro-substituent on the piperidine ring rather than the phenyl- or phenylethyl- moieties, was well separated from **2a – 2c**, **3a – 3c**, **4a – 4c** and **5a – 5c** and was retained longer at low pH compared to the other fluorinated derivatives (i.e., relative $t_{R\ (6/2a)} = 1.19$) however, with high pH conditions it eluted in a similar retention window (relative $t_{R\ (6/2a)} = 1.00$). If the presence of (**6**) is suspected in forensic samples when high pH RP-UHPLC conditions are employed, its presence can be unambiguously confirmed using low pH RP-UHPLC. In addition, it exhibited a unique MS/MS fragmentation pattern compared to compounds **2a – 2c** and **3a – 3c** possessing identical molecular formulae (see Section 3.3). The high pH RP-UHPLC method was demonstrated to be stable with respect to retention time (%RSD < 0.20 % $n = 18$ over a 20-h period) for the isobaric fluorofentanyls (**2a – 2c**, **3a – 3c** and **6**).

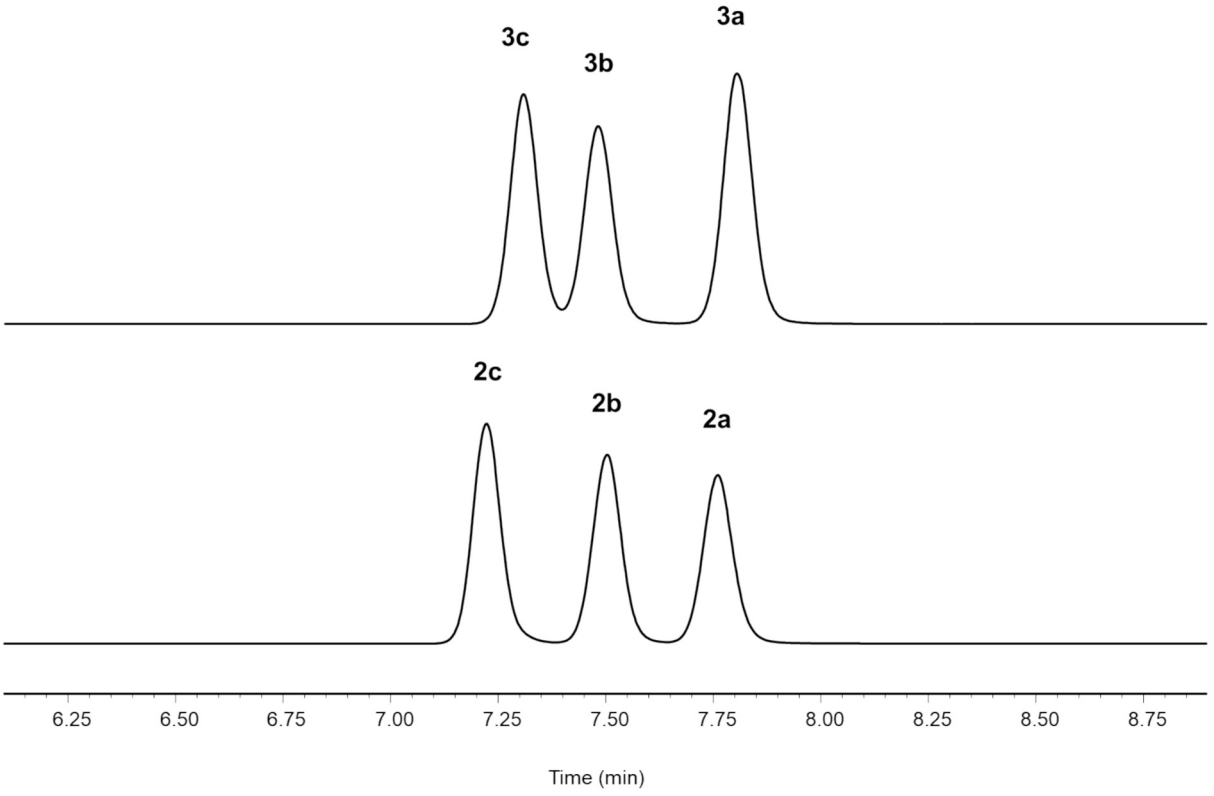

Fig. 4. RP-UHPLC-MS/MS separation of **2a – 2c** (TIC, MRM *m/z* 355.20 > 188.35 and 355.20 > 150.30) and **3a – 3c** (TIC, MRM *m/z* 355.20 > 123.40 and 355.20 > 152.30) under high pH mobile phase conditions, 2 × 150 × 3 mm i.d., SuperC18 2 μm columns at 35 °C using an 0.1 % *v/v* ammonia (pH 10.7)/MeOH gradient, (gradient profile was scaled to maintain a constant $(t_G \times FR)/V_m$). R_s (**2b/2c**) = 2.4, R_s (**2a/2b**) = 2.1, R_s (**3b/3c**) = 1.4 and R_s (**3a/3b**) = 2.6, see Section 2.5 for experimental conditions.

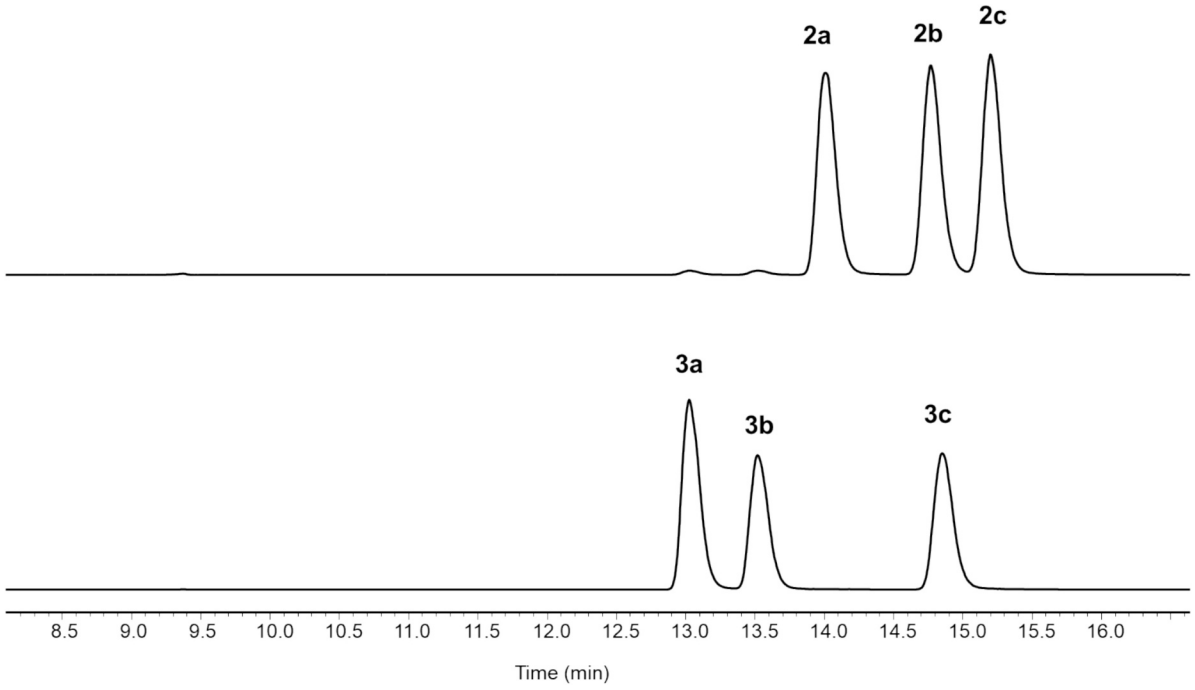

Fig. 5. HILIC-MS/MS separation of **2a – 2c** (TIC, MRM *m/z* 355.20 > 188.35 and 355.20 > 150.30) and **3a – 3c** (TIC, MRM *m/z* 355.20 > 123.40 and 355.20 > 152.30), HALO HILIC column, 2.7 µm, 150 mm × 2.1 mm i.d., mobile phase A: 0.1 % acetic acid and 5 mM ammonium acetate in water, mobile phase B: ACN, 40 °C, 0.4 mL/min., gradient programme 98–87 % B in 17 mins, see Section 2.6 for experimental conditions, R_s (**2b/2a**) = 2.8 and R_s (**2c/2b**) = 1.8 and R_s (**3b/3a**) = 1.9 and R_s (**3c/3b**) = 5.0.

3.2. *Hydrophilic interaction LIquid chromatography (HILIC)*

A validated LC-MS/MS methodology using Hydrophilic Interaction LIquid Chromatography (HILIC) for the quantitative separation of 19 novel opioids in whole blood and serum and 17 qualitatively in urine [32] and for the analysis of xylazine (**17**), *para*-fluorofentanyl (**2c**), fentanyl (**1**) and fentanyl related compounds in postmortem blood have been recently reported [43]. The HILIC conditions employed a bare silica stationary phase chromatographed at 40 °C using isocratic or gradient mobile phase conditions composed of differing amounts of ammonium formate and formic acid in water and ACN. The former methodology was reported to separate the regioisomers (**2a**) and (**2c**), however, the separation of (**2b**) was not investigated. In addition, the method failed to separate (**4a**) from its structural isomer 4-fluoro-*n*-butyrylfentanyl. The latter methodology described the partial separation of the *ortho*-, *meta*- and *para*-fluorofentanyls (**2a – 2c**) for qualitative monitoring purposes.

Using similar gradient HILIC conditions with a superficially porous 2.7 µm bare silica column chromatographed at either 25 or 40 °C, in conjunction with a rapid gradient composed of 5 mM ammonium acetate in water with 0.1 % acetic acid (native pH 4.5) and ACN, the excellent separation of the regioisomeric fluorofentanyl (**2a – 2c**, **3a – 3c**) and fluoroisobutyrylfentanyl (**4a – 4c**) series was achieved (see Fig. 5 and Fig. 6). Fig. 5 highlights the separation of **2a – 2c**, **3a – 3c** at 40 °C whereas Fig. 6 highlights the separation of **2a – 2c**, **4a – 4c** at 25 °C undertaken at a different laboratory.

The elution order of all the derivatives (**2a – 2c**, **3a – 3c** and **4a – 4c**) under HILIC conditions was demonstrated to be orthogonal (i.e., elution order of *ortho*- < *meta*- < *para*-) to that observed with high pH RP-LC chromatography (i.e., *para*- < *meta*- < *ortho*-, see Fig. 3–6) and in agreement with the elution order observed previously in HILIC analysis of **2a – 2c** [43].

Interestingly, the separation of the precursors **5a – 5c** which were readily separated at low or high pH RP-UHPLC conditions could not be resolved using HILIC conditions (i.e., **5c** and **5b** co-eluted). The same rationale (i.e., **5a – 5c** possess two amino functionalities compared to **2a**

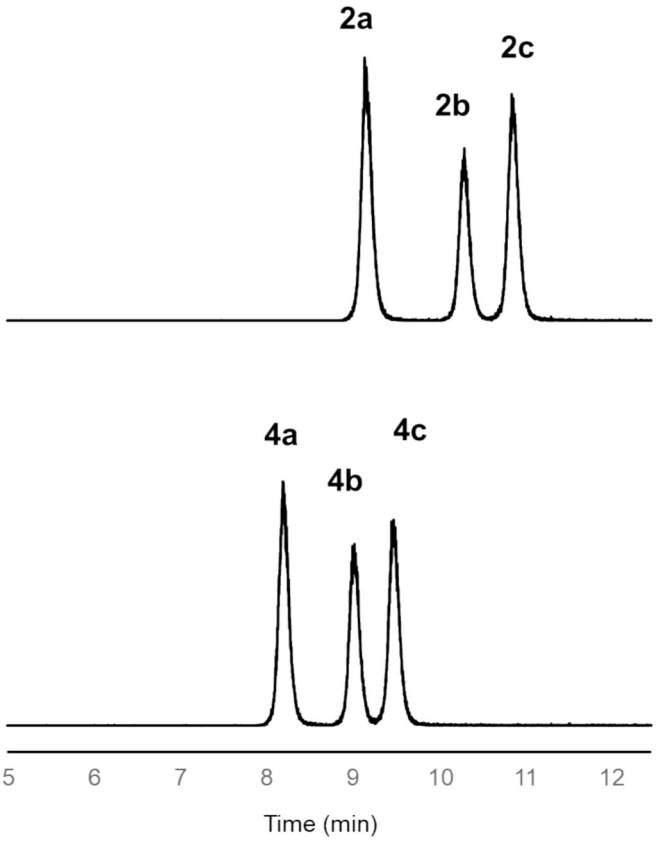

Fig. 6. HILIC-MS/MS separation of **2a – 2c** (TIC, MRM *m/z* 355.20 > 188.35 and 355.20 > 150.30) and **4a – 4c** (TIC, MRM *m/z* 369.19 > 188.14, 369.19 > 105.08 and 369.19 > 103.07). HALO HILIC column, 2.7 µm, 150 mm × 2.1 mm i.d., mobile phase A: 0.1 % acetic acid and 5 mM ammonium acetate in water, mobile phase B: ACN, 25 °C, 0.4 mL/min., gradient programme 98–87 %B in 17 mins, R_s (**2b/2a**) = 3.8 and R_s (**2c/2b**) = 1.9 and R_s (**4b/4a**) = 2.8 and R_s (**4c/4b**) = 1.6., see Section 2.6 for experimental conditions.

– 2c, **3a – 3c** and **4a – 4c** which only possess one) that explains why the regioisomeric separation of **5a – 5c** was achievable at low pH, may account for their lack of resolution under HILIC conditions.

3-Fluorofentanyl (**6**) elutes before the fluorofentanyl regioisomers (**2a – 2c** and **3a – 3c**) with a relative $t_{R\ (6/2a)} = 0.67$ which contrasted with that observed at low and high pH RP-UHPLC (i.e., relative $t_{R\ (6/2a)} = 1.19$ and 1.00 respectively). To reduce the column equilibrium time, and to shorten the analysis cycle while still flushing the column with 20 column volumes [44] of the initial gradient mobile phase conditions prior to the next injection, a flow ramp from 0.4 to 1 mL/min (6 min hold) to 0.4 mL/min was employed.

3.3. Mass spectrometry

3.3.1. Mass fragmentation of 2a – 2c, 3a – 3c, 4a – 4c, 5a – 5c and 6

Even though the individual regioisomers of the fluorofentanyls (**2a – 2c**, **3a – 3c** and **6**) could be resolved within each regioisomeric family, they all eluted in a similar retention window. Hence, it was necessary to be able to differentiate them by their differing MS/MS fragment ions. Multiple reaction monitoring (MRM) optimization for the fluorinated derivatives (**2a – 2c**, **3a – 3c**, **4a – 4c**, **5a – 5c** and **6**) using positive electrospray ionization was performed automatically using flow injection analysis (FIA)-MS/MS coupled to the LabSolutions MRM optimization tool on a triple quadrupole MS instrument. The fragment ions generated and selected were then confirmed by high resolution MS as shown in Fig. 7. The most abundant fragments resulting from collision-induced dissociation (CID) analysis of the analogues with the fluorine atom attached to the *N*-phenyl moiety (i.e., **2a – 2c**, **4a – 4c** and **5a – 5c**) corresponded to the ethylbenzene and 1-(2-phenylethyl)piperidine fragmentations, resulting in *m/z* values of 105 and 188 respectively. The fluorofentanyl series (**3a – 3c**) which possessed the fluorine atom on the 2-phenylethyl moiety generated corresponding fragments with *m/z* values of 123 and 206. In contrast, 3-fluorofentanyl (**6**) generated a unique *m/z* ion of 299 corresponding to cleavage of the amide bond. The preferred MS fragmentation patterns are highlighted in Fig. 7, with the dotted lines representing β-cleavage sites for each series. These results concur with those previously reported in the literature [38,45].

Analysis software was used for structural identification and confirmation of the *m/z* fragments generated by FIA-MS/MS. The fragments shown in Fig. 7 and Fig. 8 were successfully identified with mass errors less than 7 ppm. It also highlighted the clear abundance of these five ions as product ions that would allow high sensitivity UHPLC-MS/MS. Fig. 8

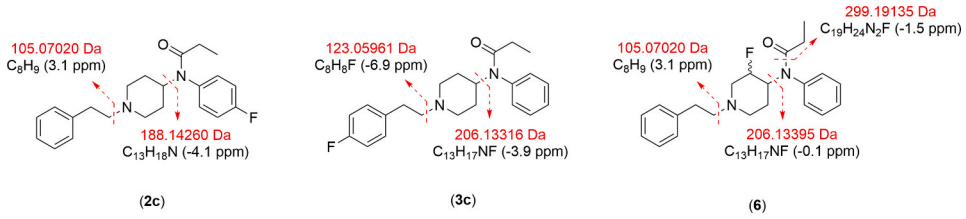

Fig. 7. Structures of isomeric fluorofentanyls, typified by derivatives **2c**, **3c** and **6** highlighting the location of primary and secondary fragmentation by CID.

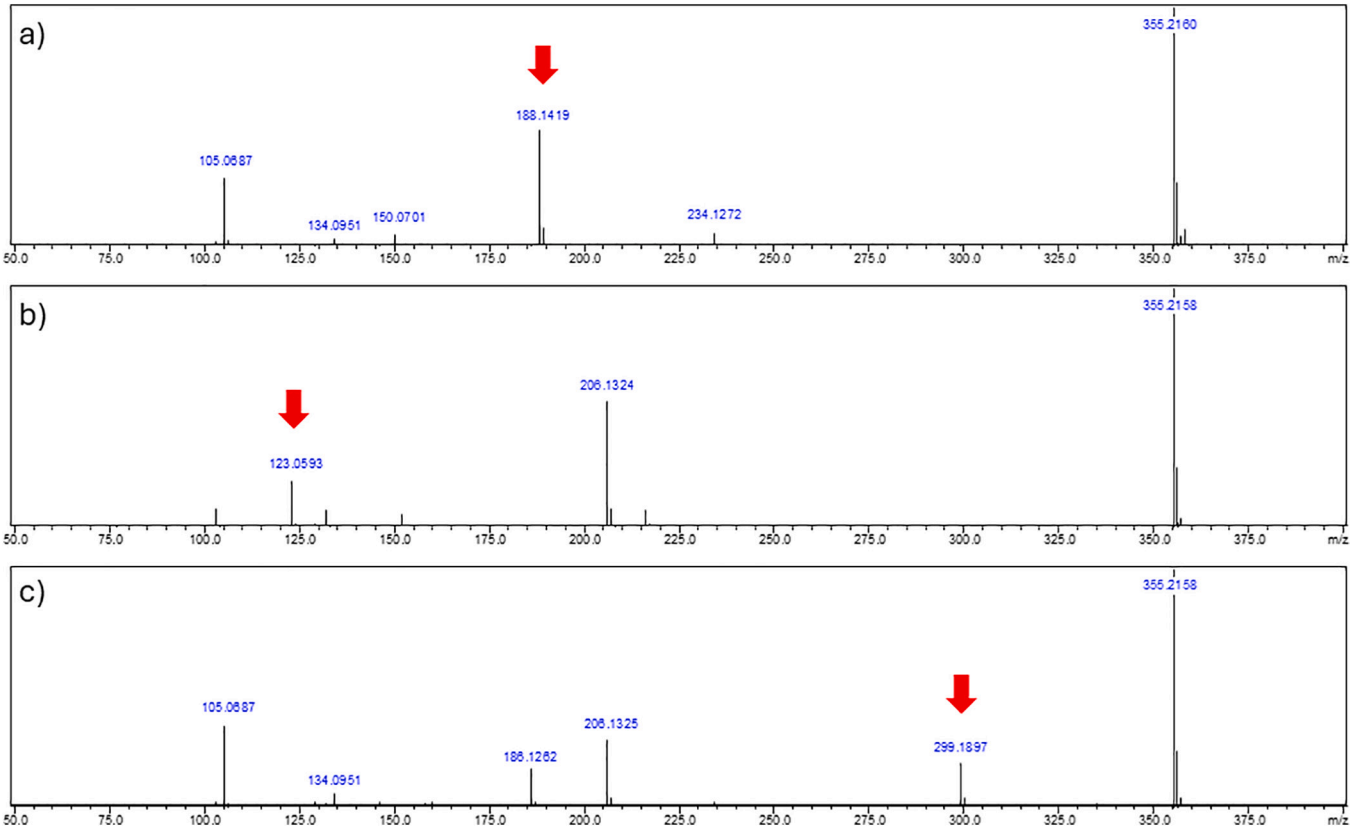

Fig. 8. Mass spectra of isomeric fluorofentanyls: (a) *ortho*-, *meta*-, and *para*-fluorofentanyl (**2a-2c**); 2′-, 3′-, 4′-fluorofentanyls (**3a-3c**) and (c) 3-fluorofentanyl (**6**).

highlights that the following *m/z* ions 188, 123 and 299 (marked with red arrows) are unique for the fluorofentanyls (**2a – 2c**, **3a – 3c** and **6**) respectively. A subsequent study (manuscript in preparation) will detail the comparison and the efficacy of using HILIC and high pH RP-UHPLC coupled to MS/MS for the analysis of fluorofentanyls and related substances within clinical samples [46].

3.4. Separation of additional fentanyl structural isomers

3.4.1. 2- and 3-Furanylfentanyl isomers (*10* and *11*)

The 2- and 3-furanylfentanyls (**10** and **11**) were readily separated using the described RP-UHPLC conditions (see Supplementary Material 7). Both high and low pH conditions generated good resolution of the structural isomers ($R_{s\ (11/10)}$ = 5.5 and 3.4 respectively). In contrast, chromatography under HILIC conditions failed to afford any separation between the 2- and 3-furanyl structural isomers (**10** and **11**).

3.4.2. Methylfentanyl structural isomers (*7–9*)

The described gradient HILIC methodology readily separated three structural isomers of methylfentanyl (**7–9**), $R_{s\ (9/8)}$ = 4.4 and $R_{s\ (7/9)}$ = 13.2. The elution order was observed to be β-methylfentanyl (**8**) < (±)-*trans*-3-methylfentanyl (**9**) < α-methylfentanyl (**7**) (see Supplementary Material 8).

3.5. Comparison of the chromatographic selectivity using HILIC, low and high pH RP-UHPLC

The chromatographic selectivity of the 18 fentanyl derivatives and related substances when chromatographed under HILIC, low and high pH RP conditions as expressed as selectivity correlations (S) was determined using Eq. 1. Where r^2 equates to the correlation between the retention times of two chromatographic conditions [47]. Selectivity correlation values of 0 and 100 signify columns of equal and orthogonal retention selectivity.

$$S = 100\sqrt{1 - r^2} \quad (1)$$

Correlation of retention times in HILIC compared to low and high pH RP-UHPLC conditions, highlighted the orthogonality in their elution order for the 18 fentanyl and related substances investigated (see Supplementary Material 9). Table 1 highlighted the high selectivity values for the chromatography performed under HILIC versus high pH RP conditions (i.e., selectivity correlation = 97). A moderate degree of chromatographic selectivity (i.e., selectivity correction = 65 and 67) was also observed between HILIC versus low pH RP and high versus low pH mobile phase conditions.

Table 1
Chromatographic selectivity under HILIC, low and high pH RP-UHPLC conditions as expressed as selectivity correlations (S). See Supplementary Material 10 for retention times run under each chromatographic mode.

Chromatographic mode 1	Chromatographic mode 2	Selectivity correlation (S)
HILIC	Low pH RP-UHPLC	65
HILIC	High pH RP-UHPLC	97
Low pH RP-UHPLC	High pH RP-UHPLC	67

4. Conclusion

Typical generic low pH gradient LC-MS/MS screening conditions employed in toxicological laboratories failed to resolve the regioisomers of the fluorofentanyl (**2a – 2c** and **3a-3c**) and fluoroisobutyrylfentanyl (**4a-4c**) families. Evaluation of low and intermediate pH RP-UHPLC with stationary phases of complementary chromatographic selectivity, when combined with temperature and gradient retention modelling, demonstrated the lack of separation of these regioisomers. In contrast, the corresponding regioisomeric fluorodespropionyl precursors (**5a – 5c**) were easily separated under low pH mobile phase conditions with a standard C18 column, $R_{s\ (5a/5c)}$ = 6.9 and $R_{s\ (5b/5a)}$ = 3.2.

Gradient RP-UHPLC using a high pH mobile phase, low temperature, MeOH and a new generation high pH stable C18 phase was shown to resolve the twelve fluorinated derivatives (**2a – 2c**, **3a – 3c**, **4a – 4c**, **5a – 5c**). High pH mobile phase conditions generated excellent peak shapes for the analytes when analysed in their ion-suppressed mode. Retention time stability was demonstrated to be excellent (%RSD <0.2 %). The elution order of the regioisomeric derivatives (**2a – 2c**, **3a – 3c**, **4a – 4c, 5a – 5c**) was observed in all cases to be *para-* < *meta-* < *ortho-* which contrasts with that observed at low pH (i.e., elution order is *para-* < *ortho-* < *meta-* for the regioisomeric precursors (**5a – 5c**).

HILIC using a bare superficially porous silica column in conjunction with a rapid gradient of 5 mM ammonium acetate in water with 0.1 % acetic acid (native pH 4.5) and ACN achieved the separation of the fluorofentanyl (**2a – 2c** and **3a-3c**) and fluoroisobutyrylfentanyl (**4a-4c**) groups. In contrast, **5a – 5c** failed to be resolved under HILIC conditions.

LC-MS/MS fragmentation under positive electrospray ionization of the regioisomers of the fluorofentanyls (**2a – 2c**, **3a – 3c** and **6**) – which possessed the same molecular formulae – were shown to generate unique fragmentation ions which enabled both MS fragmentation and chromatographic separation to unambiguously confirm the presence or absence of these forensically important synthetic opioids.

Both the high and low pH RP-UHPLC conditions were shown to resolve the 2- and 3-furanylfentanyl isomers (**10** and **11**), however, the former chromatographic mode generated better resolution. In contrast, chromatography under HILIC conditions failed to afford any separation between these structural isomers.

The HILIC methodology was shown to readily separate the three structural isomers of methylfentanyl (**7–9**). The elution order was observed to be β-methylfentanyl (**8**) < (±)-*trans*-3-methylfentanyl (**9**) < α-methylfentanyl (**7**).

A moderate degree of chromatographic selectivity for 18 fentanyl and related substances was observed between high and low pH mobile phase conditions and between HILIC and low pH mobile phase conditions. In contrast, when HILIC was compared to high pH RP-LC conditions, a high degree of orthogonality in the elution order was observed between the two differing modes of separation.

The C18 RP and HILIC columns both exhibited good lifetime stability when used, washed and stored appropriately with no discernible loss in column performance observed. The major concern regarding the HILIC methodology was the long equilibration that was required.

The research has shown that high pH RP-UHPLC conditions were able to separate each individual regioisomeric series and, when regioisomers from other series co-eluted, differing MS/MS fragments would allow the separation, detection and quantification of all the isobaric fluorofentanyls.

Whilst the method is not fully validated in this paper, a comparison and validation of the low versus high pH RP-UHPLC-MS/MS and the HILIC-MS/MS methodologies for the detection and quantification of

these forensically important fluorofentanyl analogues and related substances in clinical whole blood samples will be reported in a subsequent paper [46]. It is believed upon illustrating the validity of the methodology, that these complementary techniques could be implemented in a forensic illicit drug laboratory. The analyses have already shown good reliability and intervariability, where the methods were successfully transferred between different analysts, instruments and laboratories. The use of a triple quadrupole mass spectrometer as the detector in conjunction with the chromatographic separation obtained by the HPLC offers multi-specificity for peak confirmation, employing retention time, precursor *m/z* and product *m/z* to identify illicit drugs with greater certainty. It is envisaged that the high pH method would be implemented as a 'triage' strategy where samples that were found to contain fluorofentanyls by more comprehensive screening techniques such as high-resolution mass spectrometry were then analysed using this high pH RP-MS/MS methodology to differentiate between the regioisomeric forms of the fluorofentanyl *meta*, *para*, and *ortho*- isomers.

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CRediT authorship contribution statement

Jennifer K. Field: Writing – review & editing, Methodology, Investigation, Formal analysis. **Benjamin S. Barrett:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Erika Sitch:** Methodology, Investigation, Formal analysis. **Ryan E. Mewis:** Investigation, Formal analysis. **William H. Campbell:** Writing – review & editing, Supervision, Methodology, Conceptualization. **Melvin R. Euerby:** Writing – review & editing, Supervision, Methodology, Investigation, Formal analysis, Conceptualization. **Oliver B. Sutcliffe:** Writing – review & editing, Supervision, Methodology, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.forc.2025.100682.

Data availability

Data will be made available on request.

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