Forensic Science International xxx (2012) xxx–xxx

Contents lists available at SciVerse ScienceDirect

Forensic Science International

journal homepage: www.elsevier.com/locate/forsciint

Profiling of illicit fentanyl using UHPLC–MS/MS

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ARTICLE INFO

Article history:
Received 14 July 2011
Received in revised form 22 February 2012
Accepted 28 February 2012
Available online xxx

Keywords:
Fentanyl
UHPLC–MS/MS
Profiling

ABSTRACT

Methodology is presented for the profiling of fentanyl in seized drugs using ultra high performance liquid chromatography in combination with tandem mass spectrometry (UHPLC–MS/MS). Target analysis was performed for 40 fentanyl processing impurities, several of which are markers for a specific synthetic route (Siegfried or Janssen). For the separation of these solutes, an Acquity BEH C18 1.7 μm particle column (150 mm × 2.1 mm) with a binary 1% formic acid (pH 2.0)/acetonitrile gradient was used. For MS/MS detection, an atmospheric pressure positive electrospray source was employed with selected reaction monitoring (SRM). The coupling of the high separation power of UHPLC with the highly selective and sensitive detection of MS/MS is amenable to the determination of synthetic route and linking of drug seizures. The technology is also applicable to exhibits containing trace levels of fentanyl in the presence of significantly excess amounts of heroin and/or adulterants.

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1. Introduction

Fentanyl, an opioid analgesic with potency approximately one hundred times that of morphine [1], was patented by Janssen Pharmaceutica (Belgium) in 1964. Due to its potential for abuse, fentanyl is a Schedule II drug under the U.S. Controlled Substances Act. Over the past several years, in the United States there have been over a thousand reported deaths attributed in part or whole to fentanyl overdoses. In many instances, the abuse of heroin containing fentanyl was responsible for the fatalities.

The profiling of fentanyl is important for legal and intelligence purposes. In this vein, it is desirable to determine the synthesis route and whether or not whether two or more seized exhibits came from a common source. Fentanyl has been clandestinely manufactured by both the Janssen [2] and the Siegfried synthesis routes [3]. Pharmaceutical fentanyl is legitimately produced by the Janssen synthesis. Manufacturing impurities that are unique to one of these synthetic routes can be used as identification markers for legal and intelligence purposes. Previous studies have identified markers that are unique for either the Janssen and Siegfried syntheses, as well as other markers that are common to both routes [4].

For fentanyl profiling, highly sensitive and specific methodologies for the analysis of manufacturing impurities are desirable. UHPLC–MS/MS, which combines the high separation power of ultra high performance liquid chromatography (UHPLC) with the excellent sensitivity and specificity of selected reaction monitoring (SRM) detection, is well suited for this purpose [5,6]. UHPLC utilizes sub 2 μm particle columns to generate good peak capacities [7]. SRM, which monitors a specific precursor-product ion combination, offers high specificity of detection, and excellent detection limits are obtained due to low chemical noise. UHPLC–MS/MS has been previously utilized for heroin profiling [5] and for the identification of fentanyl homologs and analogs [6].

In this study, the use of UHPLC–MS/MS for the profiling of seized fentanyl exhibits is discussed. Forty potential fentanyl manufacturing impurity markers are targeted using dual SRM detection.

2. Materials and methods

2.1. Materials

The structure of fentanyl, and the associated manufacturing impurities, including abbreviations used throughout the manuscript, are shown in Fig. 1. Fentanyl was obtained from the drug reference collection of the Drug Enforcement Administration, Special Testing and Research Laboratory. The impurities aniline, benzylamine, phenethylamine, 1-benzyl-4-piperidone, 1-benzyl-4-hydroxypiperidine, 1-phenethyl-4-piperidone and 1-benzoyl-4-piperidinone were obtained from Aldrich (Milwaukee, WI, USA). The origins of the additional impurities in Fig. 1 are described by Berrier and Casale [4]. High-purity deionized water was obtained from a Millipore Milli-Q-Gradient A10 water system (Bedford, MA, USA). HPLC-grade acetonitrile was obtained from Burdick and Jackson (Muskegon, MI, USA). Formic acid, 96%, was obtained from Sigma–Aldrich (St. Louis, MO, USA).

2.2. Preparation of solutions

Formic acid (1% (w/v), pH 2.0) was prepared by adding 10 g of formic acid into 1 L of water.

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0379-0738/$ – see front matter. Published by Elsevier Ireland Ltd.
doi:10.1016/j.forsciint.2012.02.024

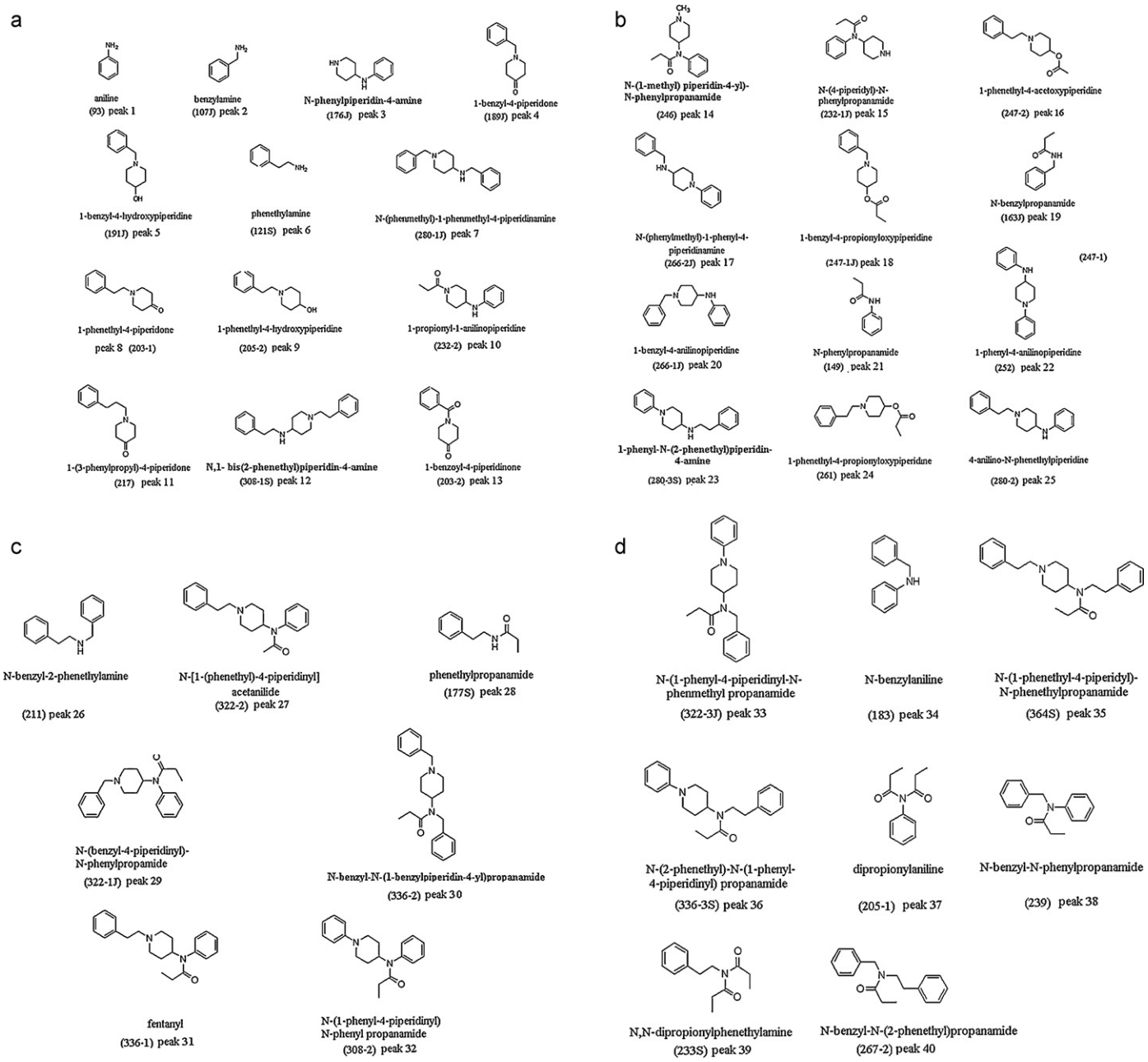

Fig. 1. Structure of fentanyl, and manufacturing impurities. Abbreviated names for the processing impurities are based on the nominal mass of the solute, with, if appropriate a J or S designation depending on whether the compound is a Janssen or Siegfried synthesis-specific marker.

The injection solvent consisted of a solution of 5% acetonitrile and 95% of 1% formic acid (pH 2.2).

A mixture of standard solutes and isolated from synthesis and seized exhibits [4] containing standard compounds were dissolved in injection solvent in order to obtain concentrations between ~0.1 and 20 μg/mL.

The powdered fentanyl samples were weighed according to the most abundant analyte[1] in order that its maximum concentration was 200 μg/mL, and the fentanyl concentration was ≤20 μg/mL after dilution with injection solvent. The resulting fentanyl concentration was 20 μg/mL unless the ratio of the most abundant analyte to fentanyl was ≥10.

All standard and sample solutions were filtered with Daigger (Vernon Hills, IL, USA) 17 mm regenerated cellulose syringe filters prior to UHPLC–MS/MS analysis.

2.3. Instrumentation

The UHPLC–MS/MS instrumentation consisted of a Waters Acquity Ultra Performance LC system (Milford, MA, USA) equipped with a Waters Micromass Quattro Premier Tandem Quadrapole Mass Spectrometer (Manchester, UK). The chromatographic separation was carried out with 20 μL injections at 25 °C using a Waters Acquity UPLC BEH C18 1.7 μm particle column (150 mm × 2.1 mm) (Milford, MA, USA) and a 26 min convex binary gradient (curve 7) with a flow rate of 0.30 mL/min. The initial conditions consisted of 5% acetonitrile and 95% of 1% formic acid (pH 2.2), while the final conditions were 61.4% acetonitrile and 38.6% of 1% formic acid (pH 2.2). The final conditions were held for 3 min prior to a 1.9 min gradient re-equilibration. The mass spectrometer was operated in the positive ion electrospray mode with SRM detection. The capillary voltage in the electrospray ionization (ESI) probe was 3.50 kV. The source block and desolvation temperatures were set at 100 and 300 °C, respectively, while the cone gas (nitrogen) flow was 46 L/h, and the desolvation gas (nitrogen) flow was 597 L/h. Argon was used as the collision gas at a pressure of approximately 5.9×10^{-3} mbar. For each solute analyzed by infusion, cone voltages and collision energies were optimized for two precursor-product combinations. The first SRM (the most intense signal) is the measuring transition, while the second SRM is used for confirmation purposes. Individual cone voltages and collision energies are described in Table 1. Dwell times of 0.005 s were used for all analytes.

[1] Solute capable of retention on BEH C18 column.

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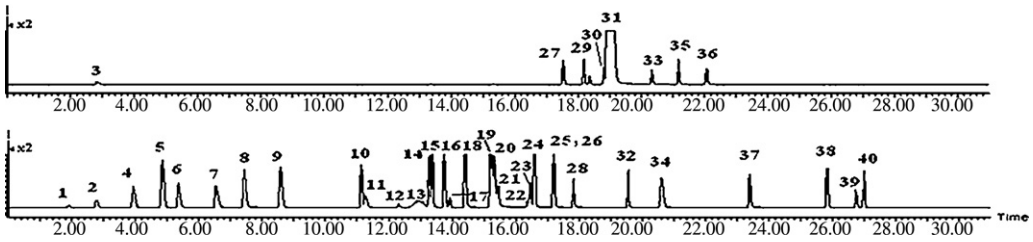

Fig. 2. UHPLC–MS/MS gradient separation of a standard mixture of fentanyl in process impurities (solute concentrations 0.1–20 μg/mL). Individual peak assignments for total ion SRM chromatogram are shown in Fig. 1. See Section 2 for UHPLC–MS/MS conditions.

3. Results and discussion

3.1. UHPLC–MS/MS separation of fentanyl and process impurity markers

For the separation of fentanyl and 40 manufacturing impurities, a 15 cm × 1.7 μm C18 column with mobile phase solvents containing formic acid (1% (w/v), pH 2.2) and acetonitrile was chosen with dual SRM detection in the positive electrospray mode. The use of an ammonium formate buffer (10 mM, pH 3.6) as a separation buffer was initially investigated. No peaks were obtained for solutes containing the piperidone moiety. In addition, the use of methanol as an organic modifier resulted in severely distorted peak shapes for solutes containing the piperidone moiety.

The separation is shown in Fig. 2. In spite of the relatively high peak capacity of UHPLC, extensive overlap still exists for certain solutes (see Fig. 2). The use of individual SRM provides selective detection for the overlapping peaks, as shown in Fig. 3. Three impurity markers (2-chloroethyl)benzene, (2-bromoethyl)benzene, and benzyl propionate gave no positive electrospray response, and therefore were not included in this study.

Table 1
SRMs, cone and collision energies of fentanyl and process impurity markers.

Compound	SRM #1	Cone voltage, collision energy	SRM #2	Cone voltage, collision energy
93	94>77	30, 20	94>51	30, 25
107J	108>91	20, 12	108>108	20, 5
176J	177>84	20, 20	177>177	20, 10
189J	190>91	38, 25	190>65	38, 45
191J	192>91	30, 25	192>84	30, 20
121S	122>105	20, 15	122>79	20, 20
280-1J	281>91	30, 40	281>174	30, 25
203-1	204>105	40, 25	204>134	40, 20
205-2	206>105	35, 30	206>188	35, 20
232-2	233>84	25, 30	233>140	25, 20
217	218>91	40, 35	218>114	40, 25
308-1S	309>105	30, 35	309>188	30, 25
203-2	204>105	30, 25	204>77	30, 40
246	247>98	30, 30	247>70	30, 40
232-1J	233>84	30, 25	233>94	30, 40
247-2	248>105	30, 35	248>188	30, 25
266-2J	267>160	25, 25	267>91	25, 35
247-1J	248>91	30, 30	248>174	30, 20
163J	164>91	25, 20	164>86	25, 15
266-1J	267>91	20, 40	267>174	20, 25
149	150>94	30, 20	150>77	30, 35
252	253>160	20, 20	253>106	20, 30
280-3S	281>160	25, 25	281>132	25, 35
261	262>105	35, 40	262>188	35, 25
280-2	281>105	20, 45	281>188	20, 25
211	212>91	25, 25	212>105	25, 25
322-2	323>105	35, 45	323>188	35, 30
177S	178>105	30, 20	178>122	30, 15
322-1J	323>91	35, 45	323>174	35, 30
336-2	337>91	35, 45	337>174	35, 30
Fentanyl	337>105	35, 50	337>188	35, 30
308-2	309>160	30, 30	309>106	30, 40
322-3J	323>160	35, 25	323>91	35, 45
183	184>91	20, 20	184>106	20, 10
364S	365>188	35, 30	365>105	35, 55
336-3S	337>160	30, 30	337>106	30, 40
205-1	206>150	15, 10	206>94	15, 30
239	240>91	25, 30	240>106	25, 20
233S	234>178	15, 10	234>105	15, 30
267-2	268>91	30, 30	268>105	30, 30

3.2. Method validation

Figures of merit, including linearity and limit of detection (LOD) for SRM1,[2] retention time (rt), precision, SRM1 peak area precision, and SRM1/SRM2[3] peak area precision, were obtained for 7 representative manufacturing impurities (see Table 2). As shown in Table 2, linearity was obtained for the various solutes, with dynamic ranges of at least 2 orders of magnitude and a correlation coefficient >0.997. In addition, LODs from the high pg/mL to the low ng/mL range were obtained (see Table 2). For the lower and upper limits of linearity, the %RSD of retention time was ≤0.2 (see Table 2). For the upper limits of linearity the area SRM1%RSD was ≤2.73, while the SRM1%RSD for the lower limits of linearity was ≤17.3 (six solutes ≤ 7.54). As shown in Table 2, the average SRM ratios for the individual solutes at the upper and lower limits of the linearity range are in reasonable agreement with %RSDs of ≤3.2 and ≤12.8, respectively.

Since many of the seized fentanyl exhibits contain adulterants (see Table 3), the analysis of the processing impurities in the presence of these solutes was investigated. The relative retention times (relative to 322-2) of the impurity markers, fentanyl, and the adulterants are shown in Table 3. In order to minimize peak overload, possible ion suppression, and source contamination, the most abundant adulterant and fentanyl were limited to 200 and 20 μg/mL, respectively. The effects of ion suppression were investigated by analyzing processing impurities contained in a fentanyl powder synthesized in-house by the Janssen method, before and after the addition of adulterants. For both experiments, the fentanyl concentration was 20 μg/mL, while the concentration of quinine, heroin, cocaine, and diphenhydramine was 200 μg/mL. For basic impurities either co-eluting or eluting near an adulterant, there is a small shift to lower retention times due to saturation of the retention sites by the major component. Ion suppression (up to 7× loss in signal) was observed for an impurity (basic or neutral) depending on its signal-to-noise and degree of overlap with the adulterant peak(s). Therefore, when comparing samples to ascertain whether they came from a common source, the possible effect of adulterants on the relative amounts of the impurities was considered.

[2] Transition for the product ion of highest intensity (i.e., product ion 1).
[3] Transition for the confirmatory product ion (i.e., product ion 2).

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Table 2
Figures of merit for selected target analytes.

Solute	Linearity range (ng/mL)[a]	Correlation coefficient, R^2	LOD (ng/mL)[b]	Concentration (ng/mL)	%RSD[c] RT	%RSD[c] area SRM1	Area SRM1/area SRM2 (%RSD[c])
107J	5–500	0.9983	1.0	5	0.20	7.09	1.26 (4.5)
107J				500	0.20	2.51	1.26 (1.0)
203-2	50–9000	0.9975	8.7	50	0.12	4.52	2.06 (1.0)
203-2				9000	0.12	2.73	1.97 (3.2)
205-2	1–500	0.9982	0.2	1	0.13	3.67	5.38 (5.8)
205-2				500	0.06	1.96	5.75 (1.0)
246	1–500	0.9987	0.2	1	0.03	5.19	4.49 (12.8)
246				500	0.04	2.62	4.32 (2.5)
247-2	1–500	0.9986	0.2	1	0.00	7.54	4.43 (5.5)
247-2				500	0.03	2.10	4.27 (1.1)
267-2	5–9000	0.9979	0.9	5	0.02	4.93	7.86 (10.8)
267-2				9000	0.02	0.62	6.35 (1.9)
280-2	5–1000	0.9974	1.2	5	0.05	17.3	1.12 (10.5)
280-2				1000	0.03	2.51	1.07 (2.8)

[a] External standard for SRM1 (transition for the product ion of highest intensity i.e., product ion 1).
[b] 2× signal-to-noise.
[c] $n = 5$.

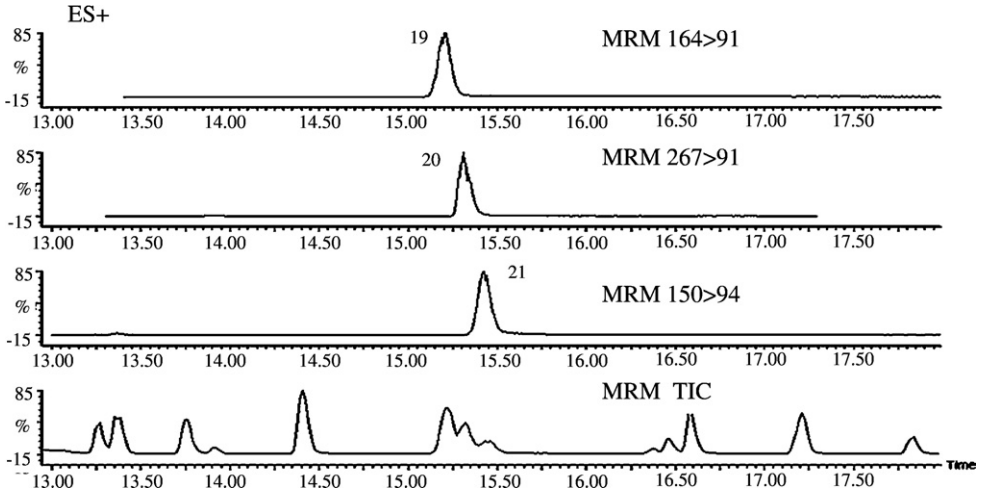

Fig. 3. Selective SRM detection of overlapping fentanyl processing impurities. Individual peak assignments are shown in Fig. 1. See Section 2 for UHPLC–MS/MS conditions.

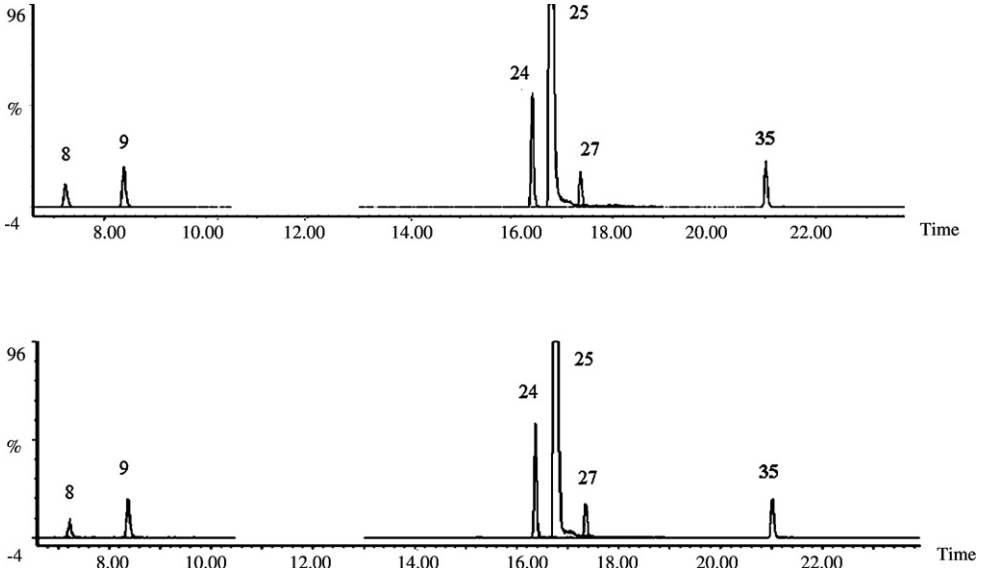

Fig. 4. UHPLC–MS/MS gradient separation representing combined timed programmed SRMs of the product ion of greatest intensity for two seized exhibits A and B. Exhibit A is adulterated with diphenhydramine, while exhibit B is adulterated with heroin, diphenhydramine, acetaminophen, and niacinamide. Individual peak assignments are shown in Fig. 1. See Section 2 for UHPLC–MS/MS conditions.

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The UHPLC–MS/MS methodology was tested on two samples of in-house syntheses using the Janssen method and one in-house synthesis using the Siegfried method. As shown in Table 4, all three profiling traces contained route specific markers (4 Siegfried, 4 Janssen, and 2 Janssen, respectively). It should be noted that the two Janssen syntheses were carried out under different conditions, which resulted in vastly different profiles (see Table 4). These results illustrate the ability of the above methodology to classify synthetic route and to differentiate between two exhibits originating from the same synthetic route. For all analyses (including subsequent analysis of exhibits), a processing impurity was only found to be present if the SRM1/SRM2 area ratio for a sample peak was within ±20% of the same ratio for a standard peak. This is equal to or exceeds European Union guidelines[4] for the confirmation of drugs and other contaminants using HPLC–MS/MS [8,9].

Seven in-house prepared, highly adulterated fentanyl samples were analyzed blindly to ascertain which synthetic route was used and to determine whether any of the exhibits were related. UHPLC–MS/MS analysis correctly classified 4 samples as originating from a Janssen synthesis, 2 samples from a Siegfried synthesis, and 1 sample from a mixed route (mixture of sample synthesized by Janssen route and sample synthesized by Siegfried route). The two samples synthesized by the Siegfried route (cut with approximately 100× heroin and diphenhydramine to fentanyl and 5× quinine to fentanyl) were identical and correctly identified by UHPLC–MS/MS normalized chromatographic profiles as originating from a common source. Four adulterated Janssen route samples originating from two different syntheses were also correctly identified using normalized SRM traces as coming from two different sources. These samples were cut with different levels of heroin, quinine, and diphenhydramine (approximately 4–30× relative to fentanyl). Two samples contained caffeine at concentrations of approximately 1 and 12× relative to fentanyl, respectively.

3.3. Analysis of seized drugs containing fentanyl

Seventy-six seized exhibits were analyzed. For 42 of these samples, the presence of the 364S marker indicated a Siegfried synthesis. For two other exhibits, the presence of three and five Janssen markers, respectively, indicated a Janssen synthesis. Thirty-two samples contained both Siegfried and Janssen markers suggesting that the exhibits originated from a mixture of materials prepared from both synthetic routes. These exhibits contained one or two Siegfried markers, and as many as 8 Janssen markers.

For comparative purposes, the exhibits identified as being synthesized by the Siegfried synthesis, the Janssen synthesis, and the mixed routes contained 4–7, 5–7, and 7–19 chemical markers, respectively. The markers found for the various exhibits are listed in Table 4. There were significant differences between most of the exhibits analyzed. As indicated by their very similar UHPLC–MS/MS profiles, a few of the exhibits appeared to be from the same source. For example, as shown in Fig. 4, two alleged Siegfried route exhibits with different combinations of adulterants showed very similar chromatographic profiles. These exhibits were seized in a midwest city within 26 days of each other (Fig. 4A and B). In another example three mixed route exhibits seized in the same northeastern city had the same combination of adulterants and exhibited very similar chromatographic profiles. Two of these exhibits were seized on the same day and contained very similar levels of adulterants (Fig. 5B and C). A third exhibit that had been obtained ten days earlier contained different levels of adulterants.

[4] European Union guidelines specify that SRM ratios for confirmatory purposes are ≥20% depending on the magnitude of the ratio.

Table 3
Relative retention time (RRT)[a] for target analytes, fentanyl and adulterants.

Compound	RRT[a] (min)	Concentration of standard (ng/mL)[b]
Thiamine	0.064	
Niacinimide	0.071	
Dimethylsulfone	0.090	
93	0.108	500
107J	0.159	500
176J	0.159	500
Morphine	0.169	
189J	0.227	500
Acetaminophen	0.256	
191J	0.278	500
121S	0.307	500
Procaine	0.318	
280-1J	0.369	1000
Pseudoephedrine	0.381	
203-1	0.426	500
Codeine	0.449	
205-2	0.489	500
Caffeine	0.516	
O3-monoacetylmorphie	0.557	
Quinine	0.581	
O6-monoacetylmorphine	0.588	
232-2	0.631	500
217	0.636	500
Lidocaine	0.689	
308-1S	0.699	500
203-2	0.739	1000
246	0.756	500
232-1J	0.761	500
247-2	0.784	500
266-2J	0.790	250
247-1J	0.818	500
163J	0.864	1000
266-1J	0.869	250
149	0.881	500
Acetylcodeine	0.884	
Heroin	0.897	
Cocaine	0.919	
252	0.932	250
280-3S	0.938	500[b]
261	0.943	500
Benzocaine	0.966	
Papaverine	0.974	
280-2	0.977	500
211	0.977	250
Noscapine	0.997	
322-2	1.000	250
177S	1.011	100
322-1J	1.034	250
336-2	1.074	250
Fentanyl	1.091	
308-2	1.114	250
Diphenhydramine	1.126	
322-3J	1.159	250
183	1.170	250
Promethazine	1.190	
364-S	1.205	500
336-3S	1.250	250
Carisoprodol	1.260	
Lorazepam	1.270	
Chlorpromazine	1.300	
205-1	1.330	1000
239	1.466	500
233S	1.523	1000
267-2	1.534	500

[a] Relative to 322-2.
[b] Based on GC–FID and GC–MS analysis.

Co-elution of chemical markers with adulterants at levels which could cause ion suppression was not observed in most instances. However, certain solutes and possibly their hydrolysis products were not reliable markers. This included amides such as 177S, 205-1 and 233, which hydrolyzed (in the acidic injection solvent) to amines 121S, 149 and 177S, respectively. The in-house

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Table 4
Chemical markers for in-house synthetic and seized exhibits.

Synthetic route	# of exhibits	Siegfried markers	Janssen markers	Common markers
Siegfried	In-house synthesis	121S, 177S, 336-3S, 364S		149, 205-2, 280-2, 308-2, 322-2
Siegfried[a]	43	364S		149, 203-1[b], 205-2[b], 261[b], 280-2[b], 308-2, 322-2[b]
Janssen	In-house synthesis #1		163J, 232-1J, 247-1J, 322-1J	149, 205-2, 232-2, 246, 261, 280-2, 322-2
Janssen	In-house synthesis #2		232-1J, 322-1J	149, 232-2, 246, 308-2, 322-2
Janssen[a]	2		176J, 191J, 232-1J, 266-1J, 322-1J	205-2[c], 280-2[c]
Mixed	32	121S, 308-1S, 364S	107J, 163J, 176J, 189J, 191J, 232-1J, 247-1J, 266-1J, 322-1J	93, 149, 203-1[d], 205-2[d], 232-2, 247-2, 246, 261[d], 280-2[d], 308-2, 322-2[d]

[a] Apparent synthetic route.
[b] Present in most Siegfried route exhibits.
[c] Present in both Janssen route exhibits.
[d] Present in all mixed route exhibits.

Siegfried synthesis preparation was run as a control during every sample sequence. Over a one year period, %RSDs of 7.4–12.1 were obtained for the normalized SRM1 area of solute,[5] indicating good day-to-day reproducibility of the UHPLC–MS/MS system (which is a necessary requirement for sample comparison).

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[5] For certain solutes, the SRM1 area of a manufacturing impurity was divided by the SRM1 of 322-2. Chemical markers excluded for low signal-to-noise included 121S, 177S (177S hydrolyzes to 121S) and 280-2 (low signal-to-noise).

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