Determination of Nine Fentanyl Drugs in Hair Samples by GC-MS/MS and LC-MS/MS

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ABSTRACT: We established GC-MS/MS and LC-MS/MS analysis methods for nine fentanyl drugs in hair samples. Human hairs were prepared by soaking in a solution of water-dimethyl sulfoxide with target analytes. The drugs were norfentanyl, acetyl fentanyl, *para*-fluorofentanyl, isobutyryl fentanyl, fentanyl, thio-fentanyl, 4-fluoroisobutyr fentanyl, ocfentanil, and tetrahydrofuran fentanyl. For a single-factor experiment, a Box−Behnken design-response surface was used to optimize the pretreatment conditions of samples. The prepared samples were quantitatively analyzed by GC-MS/MS and LC-MS/MS. The working curve method was used for quantitative analysis with fentanyl-D5 as the internal standard. The concentrations of the nine fentanyl drugs in the samples were 1.488−6.494 ng mg^{-1}, RSDs < 5.0%. For GC-MS/MS, the linear range of the nine fentanyl drugs was 0.5−5.0 ng mg^{-1}, r^2 > 0.999. The detection limits were 0.02−0.05 ng mg^{-1}, and the recovery rates were >86%. For LC-MS/MS, the nine fentanyl drugs had an excellent linear relationship within the concentration range of 3.0−220.0 pg mg^{-1}, r^2 > 0.999. The detection limits were 0.05 pg mg^{-1} and the recovery rates were >84%. The established methods were used for the detection of fentanyl drugs in human hairs, with high sensitivity, accuracy, and specificity. These two methods can be used for the certification of fentanyl certified reference substances (CRMs). In the experiment, the developed hair CRMs, which will continue to be studied in the future, are expected to be used in forensic drug abuse detection.
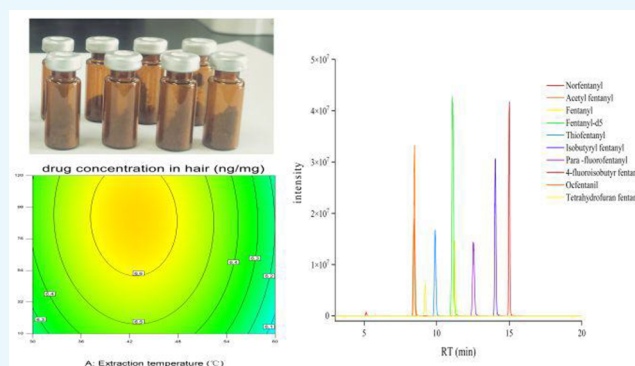
INTRODUCTION

Fentanyl is used as an analgesic and as an adjunct to anesthetic treatment. Since the 1960s, it has been widely used in surgical operations,[1−5] due to its extreme potency, ∼ 100 times that of morphine.[6] Because of its heroin-like effects, fentanyl abuse is rampant in many countries. Fentanyl is also an adulterant in other illicit substances, such as methamphetamine, heroin, and cocaine. Adverse effects of fentanyl and its analogs include hypercapnia, bradycardia, miosis, respiratory depression, reduced consciousness, and coma.[7,8] As early as 1964, the United Nations Office on Drugs and Crime listed fentanyl as an internationally controlled drug. One of the reasons behind the abuse of fentanyl drugs is that the synthesis of fentanyl substances is convenient and straightforward. Many new fentanyl analogs can be derived by modifying the phenylalkyl, propionyl, and especially 4-piperidyl rings in the fentanyl structure. Most of these compounds retain the original potency of fentanyl or are more potent.[9] Since 2013, the number of fentanyl analogs has begun to increase, among which furyl fentanyl, *β*-hydroxythiofentanyl, and valeryl fentanyl are the most commonly used.[10] Consequently, the United States Centers for Disease Control and Prevention reported a significant increase in overdose deaths involving fentanyl drugs. The numbers rose from 5544 deaths in 2014 to 9580 in

2015 and 19 413 in 2016.[11,12] In addition, fentanyl drugs are more effective in potency, cheaper to produce, and easier to transport than heroin, making them ideal for smuggling across borders, as only a tiny amount represents a substantial payout. These characteristics make them a new generation of psychoactive substances.

As a consequence of the increasing prevalence and emergence of new fentanyl analogs, forensic and clinical laboratories worldwide are continuously asked to update their analytical procedures for the identification and quantification of these new drugs in various biological matrices. Few preliminary methods have been published for their detection in conventional matrices such as urine,[13] blood,[14] and hair.[15] Blood and urine analysis can provide short-term information related to drug addiction, whereas long-term medical history needs to be traced through hair sample analysis. Hair sample

analysis is widely applied in forensics to retrospectively document exposure to substances over a prolonged period of up to several months. For the analysis of new psychoactive substances, the hair test is an excellent supplement to the urine test.[16] The commonly used detection methods for fentanyl drugs and their metabolites in biological matrices include immunoassay, GC-MS/MS, and LC-MS/MS.[17−21] Immunoassays are a standard method used to screen biological samples for drugs of abuse. However, many fentanyl analogs or metabolites may not cross-react with immune detecting antibodies, limiting their application. In addition, immunoassays cannot provide structural information about the analytes. The GC-MS/MS method is one of the essential analytical methods for identifying psychotropic drugs in forensic toxicology and doping analysis.[22] The determination of fentanyl in biological samples by GC-MS/MS requires preprocessing such as derivatization, which is cumbersome and not conducive to rapid detection. In addition, the detection limit of the method is relatively high, allowing false negatives in practice. The LC-MS/MS method has the characteristics of high sensitivity, high throughput, fast turnaround time, and a wide detection range. It can simultaneously detect and analyze fentanyl and their metabolites in biological matrices.[23] Before LC-MS/MS, acid hydrolysis, alkali hydrolysis, enzymatic hydrolysis, and other methods are generally used to hydrolyze and release the drugs from the hair samples, and SPE or LLE[24] is used for purification. The sample pretreatment process is complicated. In addition, the lack of fentanyl drug matrix references has led to the failure to guarantee the reliability, accuracy, and traceability of the test results.

Here we describe the development of hair reference materials suitable for quantifying fentanyl analogs and its application to detect fentanyl. We have developed a fast, accurate, and sensitive analytical method based on GC-MS/MS and LC-MS/MS for measuring trace amounts of fentanyl in human hairs. The sample preparation process is quick and straightforward, and convenient to operate. The GC-MS/MS method does not require derivatization and can be directly used for quantitative analysis. The LC-MS/MS analytical method has high sensitivity with the limit of quantification as low as 0.25 pg mg^{-1}. The established methods were used for detecting fentanyl in human hairs with high sensitivity, accuracy, and specificity.

■ MATERIALS AND METHODS

Chemicals and Reagents. LC-MS/MS (TQS, Waters, USA); GC-MS/MS (7890A-7000B, Agilent, USA); norfentanyl (Ministry of Public Security of China, 99.7%); acetyl fentanyl (GBW(E)091075, 99.6%); isobutyryl fentanyl (GBW-(E) 091077, 99.8%); *para*-fluorofentanyl (GBW(E)091074, 99.7%); ocfentanil (GBW(E)091078, 99.7%); thiofentanyl (GBW(E)091073, 99.6%); 4-fluoro-isobutyr fentanyl (GBW-(E)091076, 99.7%); tetrahydrofuran fentanyl (Ministry of Public Security of China, 99.9%); fentanyl (GBW(E)091009, 99.8%).

Hair Sample Preparation. Hair samples were collected from adults without a history of illicit drug use. About 40 g of drug-free hairs were shampooed and washed with a sufficient volume of water followed by methanol three times; they were air-dried and chopped into about 5 cm. Thirty milligrams of acetyl fentanyl, *para*-fluorofentanyl, isobutyryl fentanyl, fentanyl, thiofentanyl, 4-fluoroisobutyr fentanyl, ocfentanil, and tetrahydrofuran fentanyl and 5 mg of norfentanyl were

dissolved in a small volume of distilled water in a 1000 mL glass beaker, respectively. Then, 500 mL of 0.02 M HCl in DMSO were added, followed by 500 mL of distilled water in an ice bath. The drug-free hairs were soaked into the solution and a small portion (about 20 mg) was removed every 2 or 3 days for analysis until the concentrations of fentanyl drugs were plateaued. After 24 days, the soaking liquid was poured out, hairs washed thoroughly with methanol four times, and the fourth washing liquid was reserved for use. The washed hair samples were dried in a vacuum oven for 48 h, ground and crushed by a ball mill, and segmented into about 5 mm. Hairs were then mixed evenly with a mixer for 24 h, aliquoted into 150 vials (ca. 100 mg each), and stored in the dark at room temperature.[25−27] Figure 1 depicts the preparation of the hair samples.
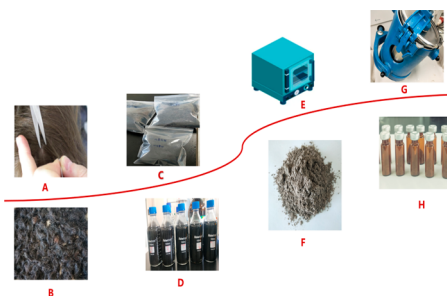

Figure 1. Hair sample preparation: (A) shearing, (B) cleaning, (C) drying, (D) soaking, (E) vacuum drying, (F) crushing, (G) mixing, and (H) bottling.

Sample Preparation for GC-MS/MS Measurement. Approximately 100 mg of sample was accurately weighed into the reservoir, then 25 mL of methanol/5 M HCl (15:1) was added. Fentanyl-D5 (5.0 ng mg^{-1}; 100 μL) was added as a reference and sonicated at 40 °C for 75 min. Hair extracts were dried at 45 °C under N$_2$ gas. The residue was reconstituted with 500 μL of mobile phase and centrifuged at 15 000 × g for 5 min, and the supernatant was filtered through a 0.22 μm microporous membrane before injection for analysis.

Sample Preparation for LC-MS/MS Measurement. Approximately 20 mg of sample was accurately weighed into the reservoir, then 5 mL of methanol/5 M HCl (15:1) was added. Fentanyl-D5 (1.0 ng mg^{-1}; 100 μL) was added as a reference and the samples were then processed as above.

GC-MS/MS Measurement. Chromatographic column, DB-5MS (30 m × 0.25 mm × 0.25 μm); column temperature, 180 °C (1 min)−10 °C/min−300 °C (8 min); carrier gas, helium with a flow rate of 1.0 mL/min; inlet temperature, 280 °C; injection volume, 1 μL; split injection with a split ratio of 5:1; solvent delay time, 4 min; electron impact ionization source (EI), electron energy 70 eV; ion source temperature, 230 °C; interface temperature, 250 °C. The mass detector was operated in electron ionization at 70 eV in SIM/SCAN mode. The full scan acquisition range was m/z 50−450. The selected ion monitoring mode (SIM) was used for quantitative analysis. The first monitoring group was at 5.5−7.0 min, monitoring m/z 120, 159, 175, and 83. The second monitoring group was at 11.5−14.0 min, monitoring m/z 231, 146, 188, 279, 176, 280, 245, 146, 93, 189, 280, 263, 164, 220, 259, 189, 277, 207, 250, 151, 194; Group 3 monitoring was at 14.5−16.0 min, monitoring m/z 287, 189, 146, and 158. The diagnostic ions

Table 1. Mass Spectrometric Parameters of Fentanyl Drugs

drug name	SIM ions	mol wt	parent ion (m/z)	daughter ions(m/z)	cone (V)	collision energy (V)	internal standard, IS
norfentanyl	120, 159, 175, 83[a]	232.32	233.25	84.25[a]	82	18	fentanyl-D5
acetyl fentanyl	231,[a] 146, 188	322.45	323.31	188.29[a]	10	20	
				105.23	10	32	
ocfentanil	279,[a] 176, 280	370.47	371.33	188.29[a]	10	24	
				105.23	10	34	
thiofentanyl	245,[a] 146, 93	342.18	343.23	194.27[a]	16	20	
				111.21	16	46	
fentanyl	245,[a] 146, 189, 280	336.48	337.34	188.29[a]	4	20	
				105.23	4	34	
para-fluorofentanyl	263[a],164,220	354.47	355.33	188.29[a]	8	26	
				105.23	8	34	
isobutyryl fentanyl	259,[a] 146, 189	350.51	351.37	188.29[a]	16	24	
				105.23	16	34	
4-fluoroisobutyrfentanyl	277, 164,[a] 207	368.50	369.36	188.29[a]	6	24	
				105.23	6	36	
tetrahydrofuran fentanyl	287,[a] 189, 146, 158	378.52	379.29	188.29[a]	94	38	
				105.23	94	22	
fentanyl-D5	250, 151,[a] 194	341.48	342.36	105.17	62	38	
				188.29[a]	62	22	

[a]Quantitative ion.

monitored for each substance in SIM mode are listed in Table 1.

LC-MS/MS Measurement. Measurements were performed on a Waters TQS LC-MS/MS with ESI in the positive ion mode using MRM monitoring. Nine fentanyl drugs were separated by LC on an ACQUITY UPLC HSS T3 column (100 mm × 2.1 mm, 1.8 μm), maintained at 30 °C in a column oven. The injection volume was 2 μL. For hair samples, the analytes were separated with a gradient mobile phase consisting of 0.1% formic acid in 10 mmol/L ammonium acetate aqueous solution (A):acetonitrile (B), at a constant flow rate setting of 0.20 mL/min. A gradient elution was used with the following pump program: 15% B increased to 28% over 4 min, 28% B maintained for 1 min and then increased to 30% in 5 min using a linear gradient, 30% B increased to 45% in 3 min and then increased to 95% over 0.5 min, and then decreased to 15% over 1 min, and maintained for 1 min. The total runtime was 20 min. Ion source, (ESI⁺); temperature, 150 °C; capillary voltage, 1.52 kV; desolventizing gas temperature, 600 °C; desolventizing gas flow rate, 800 L/h; cone gas flow rate, 150 L/h; mass spectrometry parameters are shown in Table 1.

■ RESULTS

Selection of Soaking Time for Hair Sample Preparation. Figure 2 shows the relationship between the amounts of fentanyl drugs incorporated in hairs and the time of soaking in the DMSO solution. Within 7−22 days, drugs in hairs increased with soaking time; they peaked at 24 days. On the 25th day, the decrease in the concentration of the drug entering the hair may be due to some drugs being released from the hair during the soaking process, causing the concentration to drop. During the soaking process, the concentration of the drug in the hair may reach saturation, resulting in the precipitation of part of the drug and a decrease in the concentration.

Optimization of Sample Preparation Conditions. In hair analysis, to hydrolyze and release drugs from the hair, hydrolysis methods such as acid hydrolysis, alkali hydrolysis,
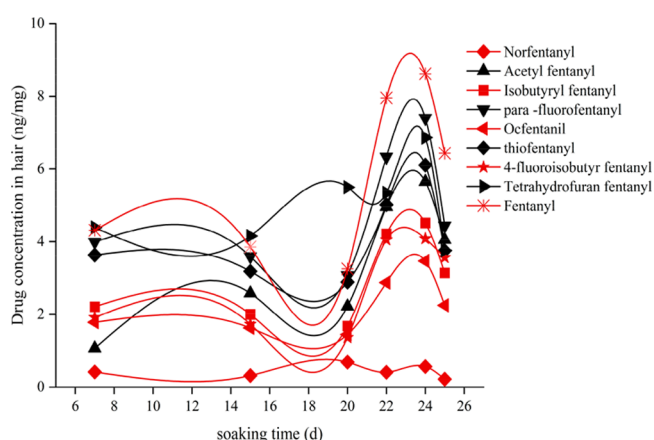

Figure 2. Amounts of nine fentanyl drugs incorporated into hair with time.
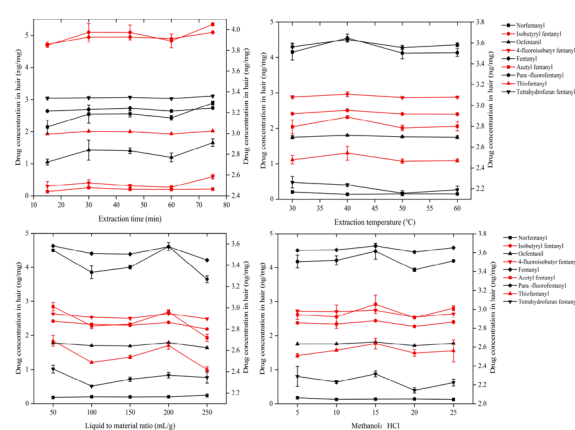

Figure 3. Effect of different extraction temperatures, extraction times, material-to-liquid ratios, and hydrochloric acid acidity on drug concentration.

enzymatic hydrolysis and organic solvent ultrasonic hydrolysis are generally used.[28,29] The single-factor variable method was
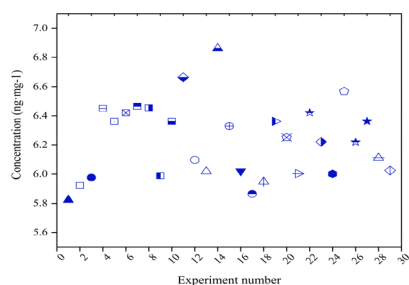
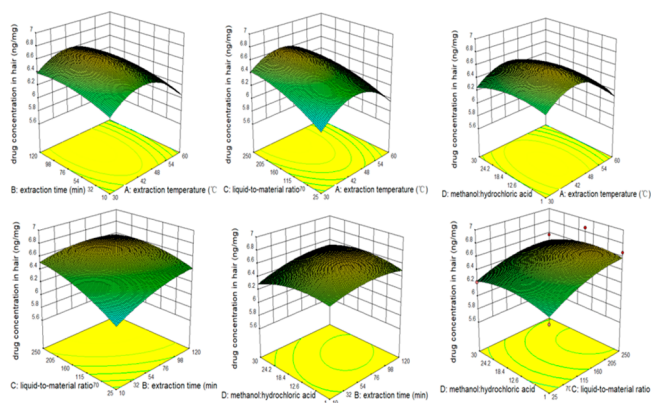**Figure 4.** Response surface experiment design and results.

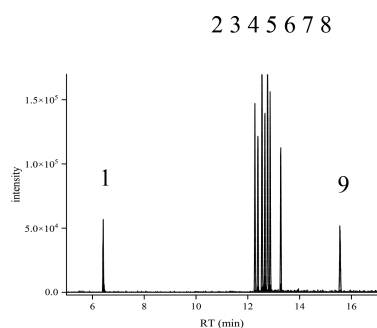**Figure 5.** Fentanyl drug response surface map.

Figure 6. Total ion current diagram of fentanyl compounds in scan mode (1, norfentanyl; 2, acetyl fentanyl; 3, *para*-fluorofentanyl; 4, isobutyryl fentanyl; 5, fentanyl; 6, thiofentanyl; 7, 4-fluoroisobutyr fentanyl; 8, ocfentanil; 9, tetrahydrofuran fentanyl).
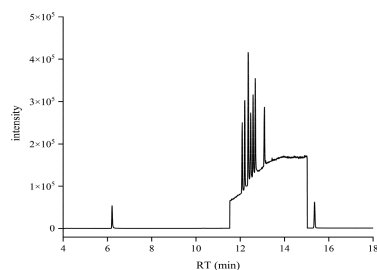

Figure 7. SIM chromatogram of fentanyl drugs in hair samples.

used to investigate the influence of different extraction solvents (methanol, acetonitrile, methanol: hydrochloric acid) on drug concentration. The results show that the ultrasonic extraction of methanol:HCl has the largest amount of drug extraction. The single-factor experiments included drug concentration, extraction temperature, extraction time, liquid-to-material ratio, and hydrochloric acid concentration. Figure 3 depicts
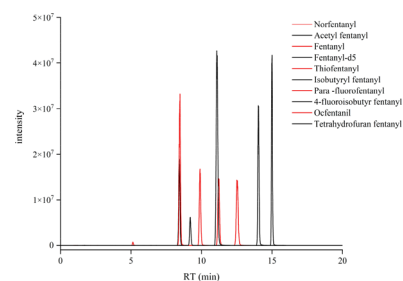

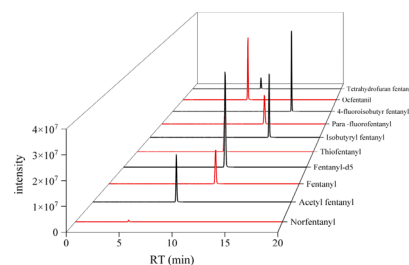**Figure 8.** Total ion chromatogram of fentanyl drugs in hair samples.

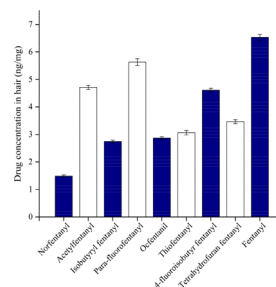**Figure 9.** MRM chromatogram of fentanyl drugs in hair samples.

Figure 10. Quantification of fentanyls in hair (GC-MS/MS).
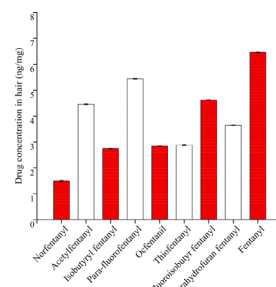

Figure 11. Quantification of fentanyls in hair (LC-MS/MS).

the optimization of the sample preparation process by a univariate approach. A response surface experiment based on the single-factor test was used to optimize the process. Using the *Design-Expert 10.0* software, a four-factor, three-level experiment was designed. We used the drug concentration in the hair, with the extraction temperature (30−60 °C), the extraction time (10−120 min), the liquid-to-material ratio (25−250 mL g^{-1}), and the methanol:hydrochloric acid ratio (methanol:HCl = 1:1−30:1) as independent variables, to explore the best extraction conditions of nine fentanyl drugs in hair. Figure 4 depicts the response surface experimental design and results, and Figure 5 depicts the results of the fentanyl response surface experiment. The second-order polynomial regression equation between the drug concentration (ng mg^{-1}) and extraction temperature (*a*), extraction time (*b*), liquid-to-

Table 2. Comparison of GC-MS/MS and LC-MS/MS Results (ng mg^{-1})^a

| | LC-MS/MS | | GC-MS/MS | | | | | |
drug name	mean	RSD (%)	mean	RSD (%)	difference (%)	results mean	F_{calcd}	t_{calcd}
norfentanyl	1.491	1.8	1.486	2.8	0.4	1.489	0.15	0.56
acetyl fentanyl	4.451	0.4	4.709	1.5	−5.6	4.580	0.01	0.01
isobutyryl fentanyl	2.738	0.7	2.739	1.7	0.1	2.739	0.02	0.91
para -fluorofentanyl	5.435	0.3	5.629	2.2	−3.5	5.532	0.01	0.01
ocfentanil	2.840	0.3	2.863	1.8	−0.8	2.852	0.01	0.06
thiofentanyl	2.876	0.7	3.064	2.8	−6.3	2.970	0.01	0.01
4-fluoroisobutyr fentanyl	4.616	0.3	4.610	1.4	0.1	4.613	0.01	0.49
tetrahydrofuran fentanyl	3.643	0.3	3.468	2.0	4.9	3.556	0.01	0.01
fentanyl	6.459	0.2	6.529	1.6	1.1	6.494	0.01	0.01

$^a F_{calcd}$, calculated F-value; F_{crit}, critical F-value of $\alpha = 5\%$; t_{calcd}, calculated t-value; t_{crit}, critical t-value of $\alpha = 5\%$;

material ratio (c), and methanol:hydrochloric acid (d) obtained by fitting is as follows: drug concentration (ng mg^{-1}) = $6.56 - 0.08a + 0.11b + 0.15c - 0.02d - 0.013ab - 0.045ac - 0.076bc + 0.042bd - 0.031cd - 0.33a^2 - 0.08b^2 - 0.088c^2 - 0.096d^2$. In the established model, $F = 6.97$, $p = 0.0004 < 0.05$, indicating that the model has significant differences and is accurate and reliable. It can be seen from the F test that the main factors affecting the drug concentration are extraction time > liquid-to-material ratio > extraction temperature > methanol:HCl. The calculation using the *Design Expert 10.0* software shows that the extraction time is 75 min, the liquid-to-material ratio is 250:1 (mL g^{-1}), the extraction temperature is 40 °C, and the drug concentration is highest when methanol:HCl = 15:1 (v/v). Under this condition, the maximum predicted value of the concentration of fentanyl in the hair is 6.651 ng mg^{-1}, close to the actual content of fentanyl in the hair (6.494 ng mg^{-1}). Thus, it is reasonable and feasible to adopt the response surface method to optimize the pretreatment conditions of hair samples.

Method Validation. The LOD value was considered the concentration value giving $S/N > 3$ for at least three diagnostic ions for each substance, whereas the LOQ was the minimum concentrations giving $S/N > 10$ for at least three diagnostic ions. In the GC-MS/MS method, when the concentration of nine fentanyl drugs was 0.02−0.05 ng mg^{-1}, the S/N was >3, and the characteristic ion peak of each compound was evident. When the concentration of nine fentanyl compounds was 0.08−0.20 ng mg^{-1}, S/N was >10. In LC-MS/MS, the detection limit was 0.05−0.10 pg mg^{-1}, and the quantification limit was 0.25−0.50 pg mg^{-1}. The sensitivity of these two methods is high and meets the detection requirements of drugs in hairs. The internal standard working curve method was used for quantitative analysis. Calibration curves were built by linear regression of the area ratio of each substance with the corresponding IS versus the concentration of analyte. Take the peak area ratio (y) of the target substance and the internal standard quantitative ion pair as the ordinate and the mass concentration of the target substance (x) as the abscissa to perform linear regression, and draw the standard curve. For GC-MS/MS, the linear range of nine fentanyl drugs was 0.5 to 5.0 ng mg^{-1}, $r^2 \geq 0.999$. For LC-MS/MS, the nine fentanyl drugs had a good linear relationship within the concentration range of 3.0−220.0 pg mg^{-1}, $r^2 \geq 0.999$. The hair extracts were used to investigate the intraday and interday deviations. The results showed that the intraday precision of the GC-MS/MS method was RSDs < 2.0% ($n = 6$). Within 5 days, the interday precision was RSDs < 3.0%. For LC-MS/MS, The intraday precision is RSDs < 5.0%, and the interday precision is RSDs <

9.0%. It shows that the established method has good repeatability. The stability of the substances in hairs was determined by analyzing the reference hair spiked with all the substances included in the study once a day for 5 days. The stability of extracted samples was evaluated by storing in the autosampler at room temperature and injecting them at different times (1, 24, 48, 72, and 96 h). The eventual appearance of unexpected interfering peaks was evaluated, along with whether there were significant differences in the quantitative results (established at ±15% of theoretical concentration).[17] The results show that the fentanyl hair extracts had good stability within 96 h at room temperature. Recoveries were determined using LOCTRL, MEDCTRL, and HICTRL ($n = 3$). The average recoveries for LOCTRL, MEDCTRL, and HICTRL in GC-MS/MS were 105.3− 121.8%, 98.74−117.6%, 86.49−96.16%, RSDs ≤ 2.5%. For LC-MS/MS, the average recoveries for LOCTRL, MEDCTRL, and HICTRL were 86.30−111.6%, 86.29−112.2%, 84.02− 108.8%, RSDs ≤ 2.4% (Tables S1 and S2). These results demonstrate that the GC-MS/MS and LC-MS/MS methods are accurate, reliable, and suitable for analyzing nine fentanyl drugs in hair samples. The matrix effect was defined as the ratio of the mean peak area obtained by analyzing six different blank hair matrices spiked after extraction with nine fentanyl drugs at two concentrations to the mean peak area obtained in an methanol solution at the same concentrations (ME (%) = ($A/B - 1$)100, where A represents the area of the samples in hairs and B represents the area of the samples in methanol solution; MEIS (%) = ($C/D - 1$)100, where C represent the area of the fentanyl-D5 in hair and D represents the area of the fentanyl-D5 in methanol solution). The acceptance criteria for ME and MEIS was a matrix effect lower than 25% and an RSD < 15%.[30] Negative values indicate ion suppression occurred, and positive values indicate enhancement. Positive ESI polarity could be caused by proteins, peptides, amino acids, and other substances in the matrix, which form positively charged ions and result in a higher degree of ion suppression. In addition, the use of acidic mobile phases in reversed-phase chromatography may increase the number of positively charged ions and cause ion suppression.[31] The matrix effect in the GC-MS/MS and LC-MS/MS methods was in an acceptable range.

Qualitative Analysis. Qualitative analysis was carried out by the GC-MS/MS method. The scan method was used to combine the characteristic ions, and the qualitative analysis was performed according to a combination of retention time and a NIST mass spectral library search. Figure 6 depicts the total ion chromatograms of nine fentanyls under GC-MS/MS conditions. Beyond tetrahydrofuran fentanyl, the mass spectra

of the other eight fentanyls were matched to the NIST standard library. The mass spectrum of tetrahydrofuran fentanyl was not included in the NIST standard library. However, using the characteristic ion peaks m/z 287, 189, and 146 in the mass spectrum, combined with the retention time, qualitative analysis can be performed. The mass spectra of nine fentanyl drugs are shown in Figure S1.

Quantitative Analysis. *GC-MS/MS.* Eleven accurately weighed hair samples (approximately 100 mg) were used in GC-MS/MS for quantitative analysis. Figure 7 depicts the SIM chromatograms of nine fentanyls under GC-MS/MS conditions. The concentrations of the nine fentanyl drugs in the prepared hair samples were 1.486−6.529 ng mg^{-1}, RSDs ≤ 5.0% (n = 11): norfentanyl, 1.486 ± 0.04 ng mg^{-1}; acetyl fentanyl, 4.709 ± 0.07 ng mg^{-1}; isobutyryl fentanyl, 2.739 ± 0.05 ng mg^{-1}; *para*-fluorofentanyl, 5.629 ± 0.12 ng mg^{-1}; ocfentanil, 2.863 ± 0.05 ng mg^{-1}; thiofentanyl, 3.064 ± 0.09 ng mg^{-1}; 4-fluoroisobutyr fentanyl, 4.610 ± 0.06 ng mg^{-1}; tetrahydrofuran fentanyl, 3.468 ± 0.07 ng mg^{-1}; fentanyl, 6.529 ± 0.11 ng mg^{-1}.

LC-MS/MS. Multireaction monitoring mode (MRM) was used for quantitative analysis. Only fentanyl-D5 was detected in the blank hair sample. Figure 8 depicts the TIC chromatogram of fentanyls under LC-MS/MS conditions, and Figure 9 shows the MRM chromatograms of fentanyls under LC-MS/MS conditions. The concentrations of the nine fentanyl drugs in the prepared hair samples were 1.491−6.459 ng mg^{-1}, RSDs ≤ 2.3% (n = 11): norfentanyl, 1.491 ± 0.03 ng mg^{-1}; acetyl fentanyl, 4.451 ± 0.02 ng mg^{-1}; isobutyryl fentanyl, 2.738 ± 0.02 ng mg^{-1}; *para*-fluorofentanyl, 5.435 ± 0.02 ng mg^{-1}; ocfentanil, 2.840 ± 0.01 ng mg^{-1}; thiofentanyl, 2.876 ± 0.02 ng mg^{-1}; 4-fluoroisobutyr fentanyl, 4.616 ± 0.01 ng mg^{-1}; tetrahydrofuran fentanyl, 3.643 ± 0.01 ng mg^{-1}; fentanyl, 6.459 ± 0.01 ng mg^{-1}. The quantitative results of GC-MS/MS and LC-MS/MS are shown in Figures 10 and 11. Table 2 shows that F_{calcd} < F_{crit} = 3.982, there was no significant difference in the precision of the two sets of data. A t test was performed on the average of the two methods, t_{calcd} < t_{crit} = 2.086, and there was no significant difference between the average values of the two analytical methods. The concentrations of the nine fentanyl drugs in the prepared hair samples were 1.489−6.494 ng mg^{-1}, RSDs < 4.0%.

DISCUSSION

We have developed a fast, accurate, and sensitive analytical method for fentanyl drugs in hairs based on GC-MS/MS and LC-MS/MS. The sample preparation process of these two methods is simple and easy to implement. After adding the reference fentanyl-D5 to the hair samples, ultrasonication is performed and the extracts are dried with nitrogen. Samples are reconstituted with mobile phase, ultrahigh-speed centrifugation, and membrane filtration and subsequently directly injected for analysis. The LOD, LOQ, linearity, repeatability, stability, recovery rate, and matrix effect of the method are verified. According to the results of this study, we believe that the two established methods can be used for the certification of fentanyl certified reference substances (CRMs). In the experiment, the developed hair CRMs, which will continue to be studied in the future, are expected to be used in forensic drug abuse detection.

ASSOCIATED CONTENT

ⓈI Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.2c00087.

> Correlation coefficients, LODs, LOQs, analytical bias, stability, matrix effect, recoveries and RSDs of nine drugs using GC-MS/MS (n = 6) and LC-MS/MS (n = 6); GC-MS/MS mass spectra of nine fentanyl drugs; Tables S1 and S2 and Figure S1 (PDF)

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Both authors contributed equally. The manuscript was written by all authors. All authors have approved the final version of the manuscript.

Notes

The authors declare no competing financial interest.

REFERENCES

(1) Lust, E. B.; Barthold, C.; Malesker, M. A.; Wichman, T. O. Human Health Hazards of Veterinary Medications: Information for Emergency Departments. *J. Emerg. Med.* **2011**, *40* (2), 198−207.

(2) Mounteney, J.; Giraudon, I.; Denissov, G.; Griffiths, P. Fentanyls: Are we missing the signs Highly potent and on the rise in Europe. *Int. J. Drug Policy.* **2015**, *26* (7), 626−631.

(3) D'Errico, S. Commentary. Fentanyl-related death and the underreporting risk. *J. Forensic Leg Med.* **2018**, *60*, 35−37.

(4) Misailidi, N.; Papoutsis, I.; Nikolaou, P.; Dona, A.; Spiliopoulou, C.; Athanaselis, S. Fentanyls continue to replace heroin in the drug arena: the cases of ocfentanil and carfentanil. *Forensic Toxicol.* **2018**, *36* (1), 12−32.

(5) Uvaraj, U.; Colin, L.; Curtis, O.; Gregg, E.; E, A. R. The Pharmacokinetics and Pharmacodynamics of Carfentanil After Recreational Exposure: A Case Report. *Pharmacotherapy.* **2018**, *38* (6), 41−45.

(6) Suzuki, J.; El-Haddad, S. A review: Fentanyl and non-pharmaceutical fentanyls. *Drug Alcohol Depend.* **2017**, *171*, 107−116.

(7) Backberg, M.; Beck, O.; Jonsson, K.-H.; Helander, A. Opioid intoxications involving butyrfentanyl, 4-fluorobutyrfentanyl, and fentanyl from the Swedish STRIDA project. *Clin. Toxicol.* **2015**, *53* (7), 609−617.

(8) Muller, D.; Neurath, H.; Neukamm, M. A.; Wilde, M.; Despicht, C.; Blaschke, S.; Grapp, M. New synthetic opioid cyclopropylfentanyl together with other novel synthetic opioids in respiratory insufficient

comatose patients detected by toxicological analysis. *Clin. Toxicol.* **2019**, *57* (9), 806−812.

(9) Burns, S. M.; Cunningham, C. W.; Mercer, S. L. DARK Classics in Chemical Neuroscience: Fentanyl. *ACS Chem. Neurosci.* **2018**, *9* (10), 2428−2437.

(10) Vardanyan, R. S; Hruby, V. J Fentanyl-related compounds and derivatives: current status and future prospects for pharmaceutical applications. *Future medicinal chemistry.* **2014**, *6* (4), 385−412.

(11) Centers for Disease Control and Prevention. National Vital Statistics System, Mortality. *CDC WONDER.* https://wonder.cdc. gov/ (accessed 2018-08-15).

(12) Rudd, R. A.; Seth, P.; David, F.; Scholl, L. Increases in Drug and Opioid-Involved Overdose Deaths - United States, 2010−2015. *MMWR. Morb. Mortal. Wkly. Rep.* **2016**, *65* (5051), 1445−1452.

(13) Palamar, J. J.; Le, A.; Guarino, H.; Mateu-Gelabert, P. A comparison of the utility of urine- and hair testing in detecting self-reported drug use among young adult opioid users. *Drug Alcohol Depend.* **2019**, *200*, 161−167.

(14) Strayer, K. E.; Antonides, H. M.; Juhascik, M. P.; Daniulaityte, R.; Sizemore, I. E. LC-MS/MS-Based Method for the Multiplex Detection of 24 Fentanyl Analogues and Metabolites in Whole Blood at Sub ng mL^{-1} Concentrations. *ACS omega.* **2018**, *3* (1), 514−523.

(15) Salomone, A.; Palamar, J. J; Bigiarini, R.; Gerace, E.; Di Corcia, D.; Vincenti, M. Detection of Fentanyl Analogs and Synthetic Opioids in Real Hair Samples. *J. Anal. Toxicol.* **2019**, *43* (4), 259−265.

(16) Kintz, P. Evidence of 2 Populations of Mephedrone Abusers by Hair Testing. Application to 4 Forensic Expertises. *Curr. Neuropharmacol.* **2017**, *15* (5), 658−662.

(17) Fernández, M. d. M. R.; Wille, S. M. R.; Jankowski, D.; Hill, V.; Samyn, N. Development of an UPLC−MS/MS method for the analysis of 16 synthetic opioids in segmented hair, and evaluation of the polydrug history in fentanyl analogue users. *Forensic Sci. Int.* **2020**, *307* (C), 110−137.

(18) Busardò, F. P.; Carlier, J.; Giorgetti, R.; Tagliabracci, A.; Pacifici, R.; Gottardi, M.; Pichini, S. Ultra-High-Performance Liquid Chromatography-Tandem Mass Spectrometry Assay for Quantifying Fentanyl and 22 Analogs and Metabolites in Whole Blood, Urine, and Hair. *Front. Chem.* **2019**, *7*, 184.

(19) Geile, J.; Maas, A.; Kraemer, M.; Doberentz, E.; Madea, B. Fatal misuse of transdermal fentanyl patches. *Forensic Sci. Int.* **2019**, *302*, 109858.

(20) Larabi, I. A.; Martin, M.; Etting, I.; Pfau, G.; Edel, Y.; Alvarez, J. C. Development and validation of liquid chromatography-tandem mass spectrometry targeted screening of 16 fentanyl analogs and U-47700 in hair: Application to 137 authentic samples. *Drug Test. Anal.* **2020**, *12* (9), 1298−1308.

(21) Wang, G.; Huynh, K.; Barhate, R.; Rodrigues, W.; Moore, C.; Coulter, C.; Vincent, M.; Soares, J. Development of a homo-geneous immunoassay for the detection of fentanyl in urine. *Forensic Sci. Int.* **2011**, *206* (1−3), 127−131.

(22) Strano-Rossi, S.; Bermejo, A. M.; de la Torre, X.; Botre, F. Fast GC-MS method for the simultaneous screening of THC-COOH, cocaine, opiates and analogues including buprenorphine and fentanyl, and their metabolites in urine. *Anal Bioanal Chem.* **2011**, *399* (4), 1623−1630.

(23) Qin, N.; Xiang, P.; Shen, B.; Zhuo, X.; Shi, Y.; Song, F. Application of a validated UHPLC-MS/MS method for 28 fentanyl-analogue and novel synthetic opioids in whole blood in au-thentic forensic cases. *J. Chromatogr B Analyt Technol. Biomed Life Sci.* **2019**, *1124*, 82−99.

(24) Larabi, I. A.; Martin, M.; Fabresse, N.; Etting, I.; Edel, Y.; Pfau, G.; Alvarez, J. C. Hair testing for 3-fluorofentanyl, furanylfentanyl, methoxyacetylfentanyl, carfentanil, acetylfentanyl and fentanyl by LC−MS/MS after unintentional overdose. *Forensic Toxicol.* **2020**, *38* (4), 277−286.

(25) Lee, S.; Park, Y.; Yang, W.; Han, E.; Choe, S.; In, S.; Lim, M.; Chung, H. Development of a reference material using methamphet-amine abusers' hair samples for the determination of methamphet-amine and amphetamine in hair. *J. Chromatogr. B* **2008**, *865* (1), 33−39.

(26) Welch, M. J.; Sniegoski, L. T.; Tai, S. Two new standard reference materials for the determination of drugs of abuse in human hair. *Anal. Bioanal. Chem.* **2003**, *376* (8), 1205−1211.

(27) Lee, S.; Park, Y.; Han, E.; Choe, S.; Lim, M.; Chung, H. Preparation and application of a fortified hair reference material for the determination of methamphetamine and amphetamine. *Forensic Sci. Int.* **2008**, *178* (2), 207−212.

(28) Khajuria, H.; Nayak, B. P.; Badiye, A. Toxicological hair analysis: Pre-analytical, analytical and interpretive aspects. *Med., Sci. Law.* **2018**, *58* (3), 137−146.

(29) Sachs, H.; Arnold, W. Results of Comparative Determination of Morphine in Human Hair Using RIA and GC/MS. *Clin. Chem. Lab. Med.* **2009**, *27* (11), 873−878.

(30) Húšková, R.; Matisová, E.; Hrouzková, S.; Švorc, L. Analysis of pesticide residues by fast gas chromatography in combination with negative chemical ionization mass spectrometry. *J. Chromatogr. A* **2009**, *1216* (35), 6326−6334.

(31) Sofalvi, S.; Schueler, H. E.; Lavins, E. S.; Kaspar, C. K.; Brooker, I. T.; Mazzola, C. D.; Dolinak, D.; Gilson, T. P.; Perch, S. An LC-MS-MS Method for the Analysis of Carfentanil, 3-Methylfentanyl, 2-Furanyl Fentanyl, Acetyl Fentanyl, Fentanyl and Norfentanyl in Postmortem and Impaired-Driving Cases. *J. Anal. Toxicol.* **2017**, *41* (6), 473−483.