

Workflow for the detection of fentanyl and norfentanyl down to 0.1 ng/mL in urine after abuse of fentanyl skin patches

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Introduction

Fentanyl, a powerful, synthetic opioid that is 80 times stronger than morphine, is commonly prescribed for pain management. Among opioid users, deaths due to fentanyl overdose have significantly increased in the past few years¹. However, prescribed fentanyl abuse is not the only source of opioid addiction. At the end of a pain treatment regimen or after a surgery, many patients, typically in homes for the elderly and outpatient clinics, are prescribed opioids in the form of a skin patch. These skin patches, discarded after use, still contain 70% of the original concentration of fentanyl². As a result, opioid abusers chew on discarded fentanyl skin patches found in trash cans. Another common practice is to cut up and cook used fentanyl skin patches and intravenously inject the solution³. Drug abuse patients currently in withdrawal therapy often do not disclose their abuse of fentanyl from skin patches, which not only relapses the addiction, but can also result in receiving incorrect treatment. Therefore, there is a pressing need to accurately detect trace levels of fentanyl after abuse of skin patches.



Currently, there are two immunological methods to detect fentanyl: one uses a urine stick to detect fentanyl levels down to 10 ng/mL and the second is a polyclonal antibody test that detects down to 1 ng/mL. Both options are designed to measure only fentanyl. However, of the overall ingested fentanyl, only 30% is excreted in its full form without undergoing any metabolism. In fact, after patch removal, serum levels of fentanyl decline with an average elimination half-life of 17 hours. The majority of fentanyl in the system is metabolized and excreted in urine as norfentanyl⁴.

Given that current tests only detect fentanyl down to 1 ng/mL, they fail to offer the sensitive measurements required for outlining a treatment plan in drug abuse patients. Solely testing for fentanyl levels does not provide withdrawal clinics with the necessary information to examine whether a patient has abused fentanyl skin patches before coming to the hospital. Without these details, incorrect treatments may be administered, resulting in unfavorable outcomes.

The shortcomings of immunological fentanyl testing can be resolved by chromatographic methods that allow the analysis of fentanyl and its metabolite, norfentanyl, down to 0.1 ng/mL in urine samples. Here, we demonstrate a complete method development for the detection of fentanyl and norfentanyl using high-resolution liquid chromatography-mass spectrometry (LC-MS) with the Thermo Scientific™ Orbitrap™ system.

Experimental

The general workflow: from sample to result

As the analytes are excreted in urine, there is no glucuronidation process. Therefore, fentanyl and norfentanyl can be determined without a glucuronidate separation. Since the required limit of quantification (LOQ) and limit of detection (LOD) are very low (0.1 ng/mL), it is necessary to concentrate and clean up the samples by solid phase extraction (SPE). This allows a concentration factor of up to 30 times, depending on the amount of sample used for extraction. Then, the sample is introduced to an ultra-high performance liquid chromatography (UHPLC) system and separation is performed on a high performance liquid chromatography (HPLC) column. For accurate and sensitive detection of the analytes, a high resolution Orbitrap LC-MS system is used.

Method development of SPE

In LC-MS, polymeric SPE is widely used as the contaminants from the resin are not charged and, therefore, not detected. Other alternatives include silica-based materials, but there is a chance that contaminants released from the material are charged and may contribute to a higher background. Additionally, silica materials are only stable at certain pH values and can collapse below a pH of 3. At the start of the development, a Thermo Scientific™ HyperSep™ retain material was used, but the ion exchange capability was not high enough to detect fentanyl and norfentanyl at the level of 0.1 ng/mL.

With the recent introduction of Thermo Scientific™ SOLA™ SPE cartridges (30 mg), there is a new material available that offers high loadability, high ion-exchange capacity, and extremely clear extracts. SOLA SPE products use an organic polymer, such as divinyl-benzyl-styrene, functionalized with a ligand, that is combined with polytetrafluoroethylene to remove frits. Its macro-porous design eliminates issues observed with traditional loosely packed SPE formats by combining the support material and active media components into a solid, uniform sorbent bed. Cleanliness has been tested using gas chromatography (GC)-MS, comparing silica material, that is generally cleaner than polymeric material, to SOLA sorbents (30 mg).

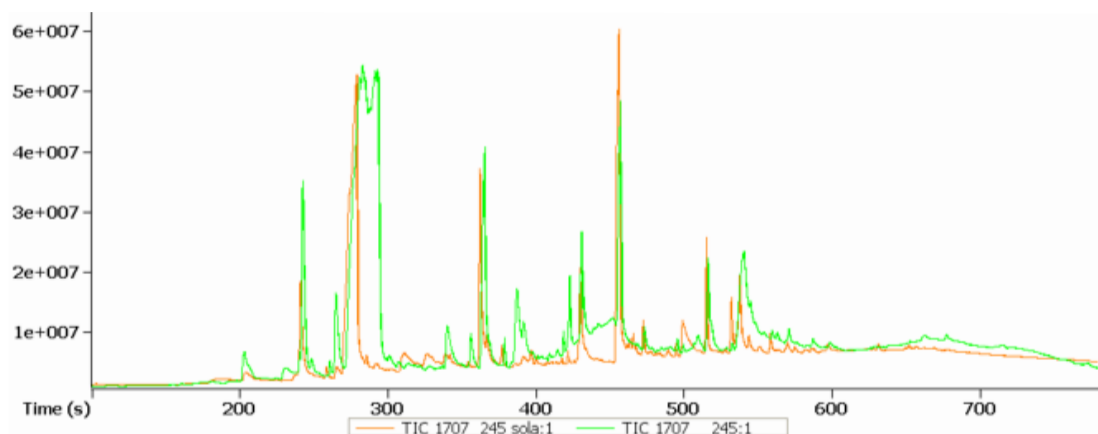


Figure 1. Using GC-MS to compare the cleanliness of SOLA SPE (30 mg) cartridges and plates (orange) with a silica-based cation exchanger (green)

The orange chromatogram in Figure 1 shows that SOLA sorbent (30 mg) is cleaner than the silica material. In this case, a general unknown drug screening was used to make the comparison. It is worth mentioning that, due to its cleanliness, the same extract gained from the SOLA (30 mg) SPE cartridges could be injected to a GC-MS workflow, eliminating the need for another sample preparation step. The advantage of using a polymer, such as SOLA material, is that even low pH settings can be used. Different SPE cleanup steps were tested to determine fentanyl and norfentanyl levels. Table 1 shows the different loading and washing conditions for SOLA strong cations exchange (SCX) cartridges at 3 mL/30 mg.

The washing steps were modified to get a clean extract. As every human matrix is different, over 100 urine samples, spiked with 1 ng/mL fentanyl, fentanyl-d₅, and norfentanyl, were tested for ion suppression. In general, urine samples containing many other drugs—around 5% of all measured samples—showed ion suppression.

The chromatogram overlay (Figure 2) shows the different washing steps performed to remove fats and lipids from samples and their respective influence on norfentanyl peaks. As shown, hexane cannot be used in washing as it causes the resin to swell.

Table 1. Different SPE cleanup steps performed using SOLA SCX cartridges at 3 mL/30 mg

| Conditioning | pH in sample | Washing step | Elution step |
|--------------|---|---|--|
| 3 mL MeOH | pH 2 0.55% formic acid 1.5 mL sample + 3 mL 0.55% 1:2 dilution Best pH to charge the analytes | 0.5 mL 0.55% formic acid 2 X 0.5 mL hexane, not working Bad interaction with resin | 2 times 0.5 mL 5% NH ₃ in MeOH |
| X | pH 6 phosphate buffer -NaOH 1.5 mL sample + 3 mL buffer 1:2 dilution | 0.5 mL 0.55% formic acid 2 X 0.5 mL MeOH Extract not clean enough | X |
| X | | 0.5 mL 0.55% formic acid 2 X 0.5 mL acetonitrile Extract not clean enough | X |
| X | | 0.5 mL 0.55% formic acid 2 X 0.5 mL dichloromethane Clean extract | X |
| X | | 1 mL MeOH/H ₂ O 60:40 With 0.55% formic acid 2 X 0.5 mL dichloromethane Cleanest extract | X |

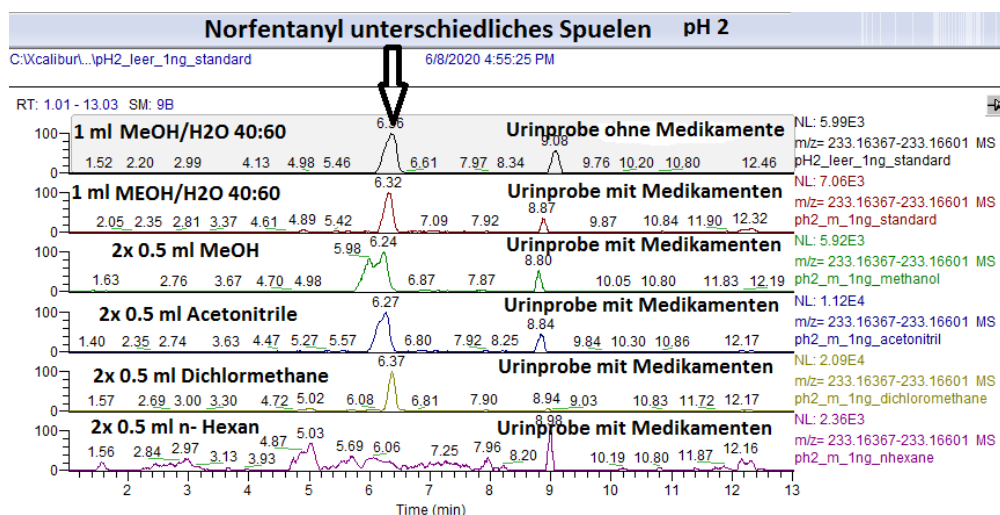


Figure 2. Different washing steps and their influence on norfentanyl peaks

Another parameter tested was the influence of the loading pH—the sample and sorbent pH. Two different loading steps were tested since the pKa of fentanyl is 8.3 and norfentanyl is 10.3. At a loading pH of 6, typically 2 units below pKa, both analytes were retained due to the hydrophobicity of the sorbent. However, at a pH of 6, the washing steps are limited, and norfentanyl detection limits were not low enough. The polymer used in SOLA (30 mg) products utilizes the cation exchanger to its fullest capability, allowing loading conditions at a pH of 2, and yielding the best LODs and LOQs. The final SPE method is shown in Figure 3.

Method development of HPLC

Beyond the clean-up offered by SPE, the phase of the HPLC column is also an important factor contributing to ion suppression. As mentioned above, 5% of the urine samples measured still show ion suppression. Therefore, the influence of HPLC column choice on eliminating ion suppression was tested.

Different HPLC materials were considered for this method development. Both spherical silica and core shell materials, in different pore sizes and different chemistries, were tested for their ability to move the analytes out of the ion suppression front. Figure 4 shows the Tanaka plots for the HPLC columns that were tested. These columns had a length of 100 mm and an inner diameter of 2.1 mm.

Thermo Scientific™ Hypersil GOLD™ (175 Å pore) and Acclaim™ columns (200 Å pore) are made of spherical silica, while Thermo Scientific™ Accucore™ columns (80 Ångstrom pore) are made of core shell materials. A pre-column was used to protect the analytical column and was changed every 400 injections. The Hypersil GOLD column separates by hydrophobicity and is slightly more polar compared to standard C18 columns. The Accucore Biphenyl column separates using hydrophobic and pi-pi interactions. C30 materials show more separation by hydrophobicity. The Tanaka plots (Figure 4) for the Accucore C30 column and the Acclaim C30 column look very similar.

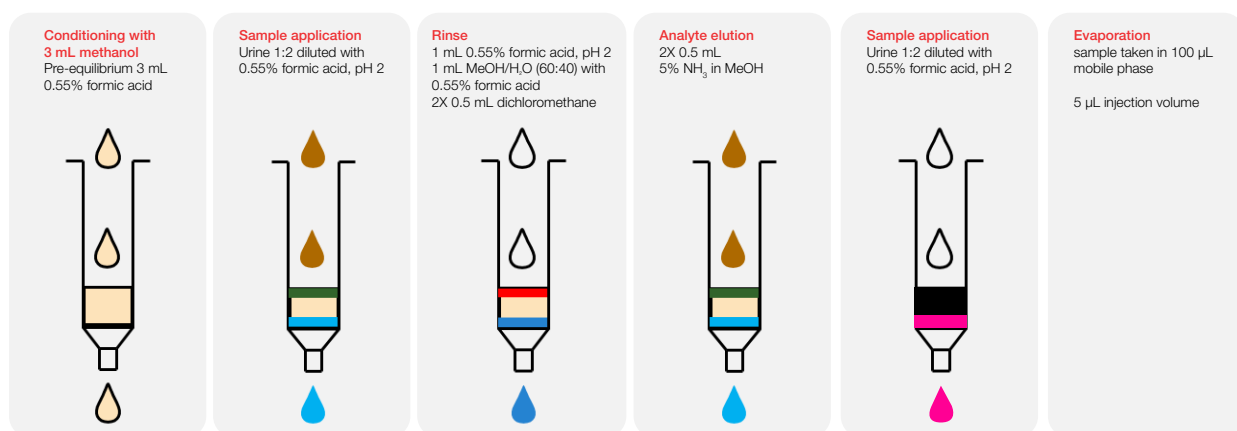


Figure 3. Final SPE workflow for fentanyl and norfentanyl using SOLA SCX cartridges at 3 mL/30 mg with mixed mode for retention

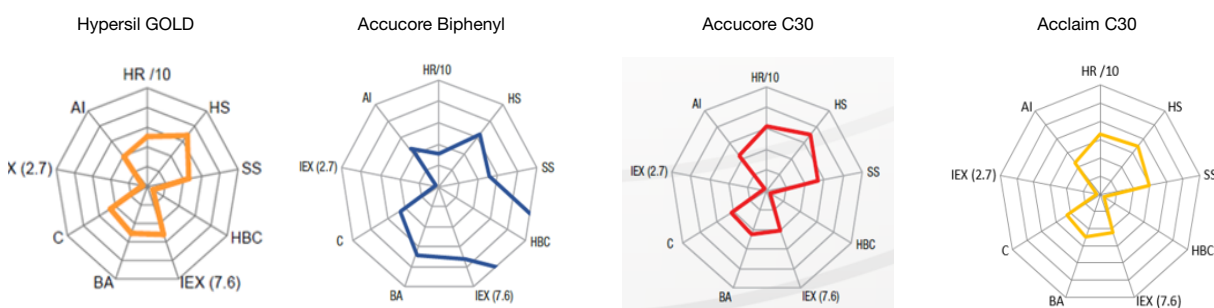


Figure 4. Tanaka plots for the different HPLC columns tested

However, the Accucore C30 column has a 5% carbon load and the Acclaim C30 column has a 13% carbon load. This is a very important factor as seen in the comparison performed during the method development (Figure 5).

As the comparison shows, the carbon load on the Acclaim C30 column impacts analyte separation. Norfentanyl, as seen in the chromatograms, is still affected by ion suppression. Based on the performance of the Acclaim C30 column, it was shown that a spherical silica in

combination with the highest carbon load and a 200 Å porosity could move the analyte peaks out of the ion suppression front.

The Hypersil GOLD and Accucore Biphenyl columns were then tested for ion suppression effects. As seen in the chromatograms in Figure 6, neither had enough hydrophobic retention and norfentanyl ended up in the ion suppression front.

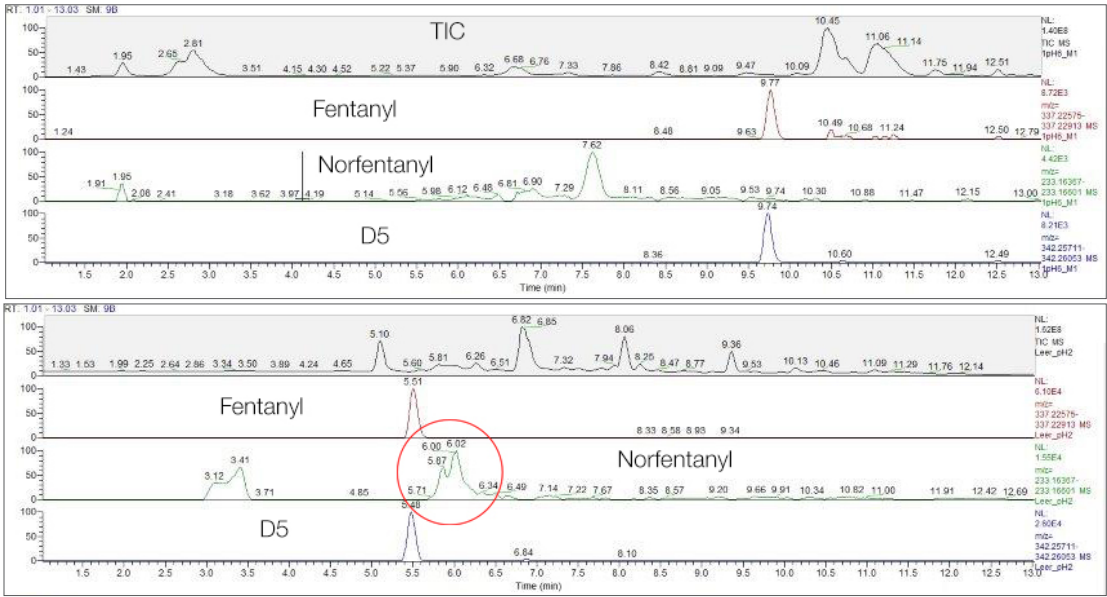


Figure 5. Ion suppression effects with the Acclaim C30 column (upper chromatogram) and Accucore C30 columns (lower chromatogram)

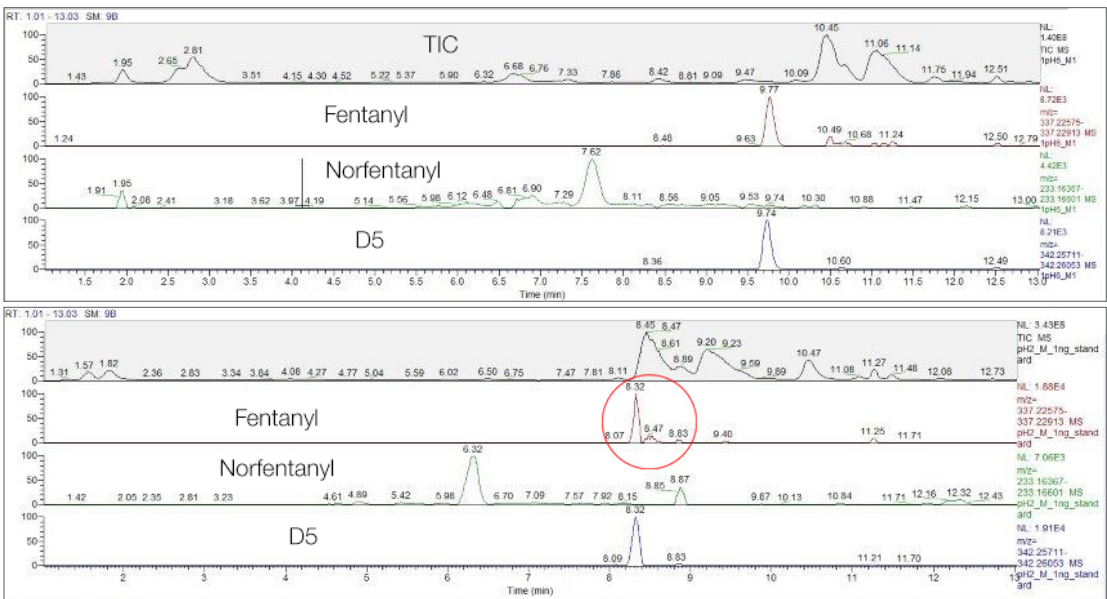


Figure 6. Ion suppression effects with Accucore Biphenyl column (lower chromatogram) compared to Acclaim C30 column (upper chromatogram)

As demonstrated in this method development, it is important to not only consider SPE, but also take HPLC column choice into account. In the workflow for fentanyl and norfentanyl analysis, column selectivity makes a big difference. Ion suppression is an issue even during accurate mass measurements in the Orbitrap mass spectrometer. Interestingly, 95% of the urine samples performed well on the Accucore Biphenyl column. However, as the composition of a urine sample is not known in advance, it is necessary to find a solution for all possible varieties of urine samples.

The Acclaim C30 column ultimately showed the best results for fentanyl and norfentanyl measurements, using a gradient of formic acid (0.1% formic acid in water, buffer A) and MeOH (with 0.1% formic acid, buffer B) going from 10% → 80% → 90% organic. The final gradient appears in Figure 7.

Glass vial choice

Another important variable is the glass vial material. If analytes stick to the surface of the glass, they cannot be injected into the HPLC and their detection limits will be further decreased. Chapter 3.2 of the European Pharmacopoeia (Ph. Eur.) recommends using first hydrolytic class vials⁵. Therefore, to gain full sensitivity of fentanyl and norfentanyl detection, choosing the right glass quality is important. Thermo Scientific™ Chromacol™ GOLD-Grade Inert Vials have only 29% of free silanol groups on the surface. This makes them ideal for the analysis of compounds containing steric hindered nitrogen, halogenated substances, and sulfur- or phosphor-containing species⁶.

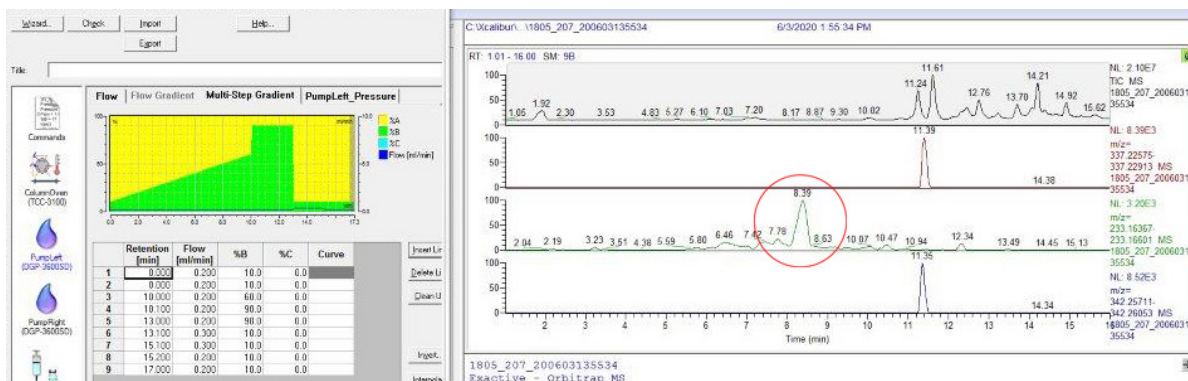


Figure 7. Final gradient using the Acclaim C30 column performed on a high-resolution Orbitrap LC-MS system

Conclusion

There is a need to detect fentanyl and norfentanyl levels in urine samples following the abuse of fentanyl skin patches; however, current immunological methods do not offer the sensitivity required to measure lower levels of fentanyl or its metabolites. Here, a method development for analytical detection of fentanyl and norfentanyl was performed using LC-MS.

SOLA SCX (30 mg) sorbents are suggested when the cleanest extracts are required. The characteristics of this material provided clean samples that could directly be used for GC-MS. Laboratories can use the SOLA SCX cartridges not only for fentanyl analysis, but also for general unknown drug screening using GC-MS.

Ion suppression is an issue even with MS technology. By choosing the right HPLC column, the analyte peaks of interest can be moved outside the ion suppression front. The Acclaim C30 column made of spherical silica and having 200 Å porosity and high carbon load performed the best for fentanyl and norfentanyl analysis, eliminating ion suppression effects on the analytes.

Together with highly inert glass vials, it was possible to achieve an LOD of 0.5 ng/mL and an LOQ of 0.1 ng/mL. Both these limits are a factor of 10 below the actual detection limits offered by immunological tests. Compared to current methods for fentanyl detection, this analytical method is highly sensitive for not only fentanyl, but also its metabolite, norfentanyl, thus providing a more detailed readout. Fast, accurate fentanyl analysis allows withdrawal clinics to perform timely tests, even if a small concentration of fentanyl was abused, and subsequently plan appropriate treatments for drug abuse patients.

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