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ARTICLE

Detection of codeine and fentanyl in saliva, blood plasma and whole blood in 5-minutes using a SERS flow-separation strip

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A simple-to-use device to measure drugs in saliva, blood plasma, and whole blood for point-of-care analysis and treatment of overdose patients has been investigated. A rudimentary flow strip has been developed to separate opioids from these biofluids for analysis by surface-enhanced Raman spectroscopy (SERS). The strips are based on lateral flow assays, in which the antibodies have been substituted by SERS-active pads for detection. Samples of codeine and fentanyl, artificially added to these biofluids, were measured using the strips by a field-usable Raman spectrometer. We report measurement of these drugs in these biofluids from 0.5 to 5 µg/mL in 5 minutes. Calculated limits of detection for the spectra suggest that these drugs could be measured at 5 to 20 ng/mL with improvements in the strips' separation capability.

Introduction

Opioids are unequalled for their ability to treat pain. They also stimulate the reward (pleasure) pathway in the brain, which, unfortunately, has contributed to the current opioid crisis involving the overuse of prescription opioids and the use of powerful synthetic opioids mixed with recreational drugs. In 2017, the former contributed to ~17,000 US overdose fatalities,[1] while in 2016 fentanyl added to cocaine and heroin contributed to ~20,000 deaths.[1,2] According to US agency statistics, most of these fatalities occurred while driving, in the home, enroute to or within hospitals. The Department of Transportation reported 15,600 driving fatalities involving drugs in 2016,[3,4] the National Safety Council reported 45,800 in home fatalities due to poisoning (mostly prescribed and illicit drugs) in 2017,[5] and the Center for Disease Control reported over 365,000 emergency department visits related to opioid misuse between July 2016 to September 2017.[6-8] Most of the opioid users entering the emergency departments survived, because the hospitals have laboratory equipment, such as chromatography coupled mass spectrometers, to determine the drug type and concentration in biofluids, such as saliva, blood plasma or urine. The lab analyses allow the medical staff to administer the appropriate medication, such as naloxone for fentanyl overdose.[9,10] In contrast, emergency responders, such as police and ambulance personnel do not have such lab facilities. And, although portable drug test kits are easy to use, they are not reproducible or quantitative. We believe that a point-of-care device, based on Raman spectroscopy, could be developed so that emergency responders could rapidly determine if a person is suffering from opioid overdose, abuse or use; and then administer treatment that saves lives.

A decade ago, portable Raman spectrometers were investigated for their ability to identify powders suspected of being explosives or drugs.[11-15] Today, they are a relatively common tool used by a number of government agencies for border security and drug seizers.[14,16,17] However, the sensitivity of Raman spectroscopy is insufficient to detect drugs in biofluids. Fortunately, the Raman signal of a drug can be amplified by as much as 6 orders-of-magnitude,[18] when it is in the plasmon field at the surface of gold or silver nanoparticles generated by a Raman excitation laser.[19] This "surface-enhanced" Raman spectroscopy (SERS) allows measuring ng/mL samples, while the detailed spectra allow drug identification. We have been investigating the ability of SERS to measure drugs in saliva for almost 15 years,[20-24] including recent measurements of opioids and opioid treatment drugs in the saliva of US military veterans.[25-27] However, these previous measurements required the use of liquid or solid extraction methods, limiting the ability to perform the analysis at the point-of-care.

Recently, we developed a pad suitable for swabbing surfaces suspected of having trace quantities of illicit drugs.[28] The pads successfully detected 500 pg of fentanyl on glass surfaces. Here we describe the development of a rudimentary SERS flow-separation strip (SERS strip) that rapidly separates drugs from biofluids, and we report SERS measurements of codeine and fentanyl artificially added to saliva, blood plasma and whole blood. Codeine and fentanyl were chosen, as they represent overdose from prescription medications and illicit drugs, and their structures represent traditional opioids and the newer synthetic opioids, respectively. The toxic concentrations of codeine and fentanyl in plasma are reported as 200 to 500

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ng/mL and 5 to 10 ng/mL,[29,30] respectively, which represent the required sensitivities for the desired point-of-care device.

Experimental

Analytical grade phosphate buffered saline (PBS), sodium citrate, chloroauric acid, cetyltrimethyl-ammonium bromide, and HPLC-grade water were all purchased from Sigma Aldrich (St Louis, MO). Forensic grade codeine and fentanyl, 1 mg/mL methanol and acetonitrile, respectively, were purchased from Cerilliant Corp (Round Rock, TX). Eppendorf plastic centrifuge tubes, used to hold samples, were purchased from VWR International (Radnor, PA). Glass fiber sheets were purchased from Sigma Millipore (Burlington, MA) to produce the SERS-active pads. The gold nanoparticles and the SERS-active pads were synthesized in-house according to previously published procedures (Figure 1A).[28] De-identified pooled saliva (liquid), pooled blood plasma (lyophilized), and pooled whole blood (liquid) were purchased from Lee BioSolutions (Maryland Heights, MO). Samples were prepared by adding 10 µL of the forensic drug to 90 µL saliva, reconstituted plasma or blood to produce 100 µg/mL samples. These samples were further diluted with the corresponding biofluid for the lower 10 to 0.5 µL/mL measurements. All used samples were autoclaved and packed in biohazard bags before disposing.

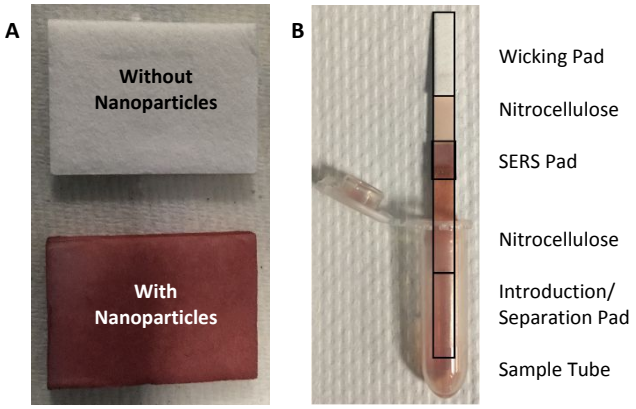

Figure 1. A) Collection pad before and after impregnation with gold nanoparticles. The pads are ~5 mm by 7 mm. B) SERS flow-separation strip used to measure 100 µL blood contained in a 2 mL Eppendorf tube.

SERS measurements were performed using a 5-lb field-usable Raman spectrometer of in-house design. It employed a 785 nm diode laser and a room temperature 512 channel Si array detector. The strips were inserted into a simple enclosure attached to the spectrometer that aligned the focal point of the laser on the SERS-pad. All measurements used 40 mW laser power and a 3-sec acquisition time. All experiments using these samples were performed in a Biosafety Level 2 cabinet following standard safety precautions.

Results and discussion

The design of the SERS flow-separation strips are based on the components of lateral flow assays: 1) the sample introduction/separation pad, 2) the nitrocellulose flow membrane, 3) test and control lines composed of antibodies, and 4) an absorbent/wicking pad, all on a thin plastic support. Here, the antibody test lines were replaced with an in-house pad consisting of a glass-fiber pad impregnated with gold nanoparticles. The SERS-pad details have been previously published.[28] Rudimentary SERS strips were prepared by sticking 5 mm by 1-3 cm introduction/separation pads, 5 mm by 4 cm nitrocellulose flow membranes, 5 mm by 7 mm SERS pads, and 1-3 cm absorbent/wicking pads to 5 mm wide by 8-10 cm long plastic supports with an adhesive surface. The SERS pad was dried in-place on the nitrocellulose section, between the introduction and wicking pads as done for antibodies. The strips were placed in 2 mL plastic tubes containing the various samples, which were allowed to flow up the strip, driven by capillary action, prior to removal for measurement (Figure 1B).

The capabilities of the SERS strips were initially tested using 10 µg/mL samples of forensic codeine and fentanyl diluted in PBS. For each measurement, a 100 µL sample was added to a plastic tube, a SERS strip was inserted into the tube until the sample reached the wicking pad. The SERS strip was then air dried for 5 min, placed in the Raman spectrometer and measured. For these first measurements, the sample flowed to the wicking pad in ~1 min. Spectra with very good signal-to-noise ratios (S/N) were obtained with only a 3-sec acquisition, matching previously reported SERS for these drugs using gold nanoparticles (Figure 2).[28]

Codeine and fentanyl are two major contributors to the opioid epidemic, as codeine, a natural drug extracted from opium, represents the traditional structure of opioids, while fentanyl, a fully synthetic opioid, represents the latest in abused drugs that are far more potent in affecting the opioid receptors. Codeine, a Schedule II drug in the US, is often prescribed in combination with nonsteroidal anti-inflammatory drugs, such as acetaminophen, aspirin, and ibuprofen, to treat varying degrees of pain. In 2013, it was the most common opiate used in the world.[31] Fentanyl, also a Schedule II drug, is not a prescription drug, but is often used as an anaesthetic during surgery. Unfortunately, it is currently mixed with the recreational drugs cocaine or heroin for illicit use.[32] Due to its potency, ~100 times as strong as morphine,[33] only a small amount is required for a "high", making it ideal for smuggling across borders. In January 2019, 250 pounds were seized at the US-Mexican border.[34]

The codeine spectrum is dominated by six peaks. The 535 cm^{-1} peak is due to out-of-plane CH bending of the rings, the 630 cm^{-1} peak is due to ring breathing, the 1250 to 1280 cm^{-1} doublet is due to combinations of the ring bending modes, the 1435 cm^{-1} peak is due to CH_3 bending, and the 1595 cm^{-1} peak is due to ring CC stretching. (Figure 2A).[35,36] The fentanyl spectrum is dominated by the symmetric and asymmetric phenyl ring breathing modes at 1000 and 1030 cm^{-1}. It also contains several modest intensity peaks. The 830 cm^{-1} peak is due to out-of-

plane phenyl CH bending, the 1180 cm^{-1} peak is due to phenyl ring CC stretching, the 1200 cm^{-1} peak is due to phenyl-C symmetric stretching, the 1330 cm^{-1} peak is due to piperidine CH wagging, and a doublet at 1585 and 1600 cm^{-1} is due to CC stretching of the two phenyl rings (Figure 2B).[37,38]

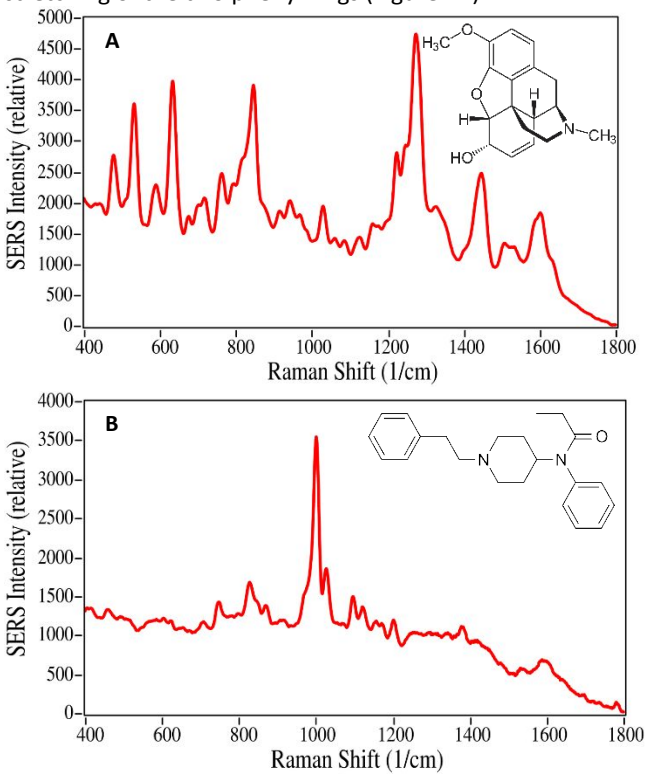

Figure 2. Structures and SERS of A) codeine and B) fentanyl at 10 µg/mL in PBS in glass vials. Spectral conditions: 40 mW at 785 nm, 3-sec scan.

Saliva was used to prepare the next samples, representing a moderate separation challenge for the SERS strips, as it is composed of ~90% water. Samples were prepared at 10, 5, 1 and 0.5 µg/mL in de-identified, pooled saliva. Sample flow up the SERS strip took 1-2 min. For both drug spectra, the signal intensity decreased by a factor of ~2 compared to PBS, and the noise level increased (Figure 3A). In the case of codeine, the relative peak intensities also changed compared to the pH 7.0 buffer. This is likely due to the saliva pH, which may have been slightly basic, e.g. 8.0 or higher, resulting in at least some of the codeine being in the undissociated form, since its pKa is 8.2.[39] Nevertheless, the primary peaks, specifically the 625 and 1280 cm^{-1} peaks, were readily measured at 1 µg/mL in saliva, but not at 0.5 µg/mL, due to the increased noise. In contrast, fentanyl was easily measured at 0.5 µg/mL, and the spectrum is virtually identical to that in PBS (Figure 3B).

Blood plasma was used to prepare the next samples, representing a potentially greater separation challenge for the SERS strips. While plasma is also ~90% water, it contains a number of much larger biomolecules that can impede separation, such as fibrinogen and globulins at 340 and 1200 kD,[40] compared to glycans and amylase in saliva at 20 and 80 kDa.[41] The samples were prepared as before at 10, 5, 1 and 0.5 µg/mL, but in de-identified, pooled blood plasma. Sample flow up the SERS strip took ~1 min. The codeine spectrum was once again dominated by the 1280 cm^{-1}, while the 625 cm^{-1} peak, due to ring breathing, became more prominent (Figure 4A).

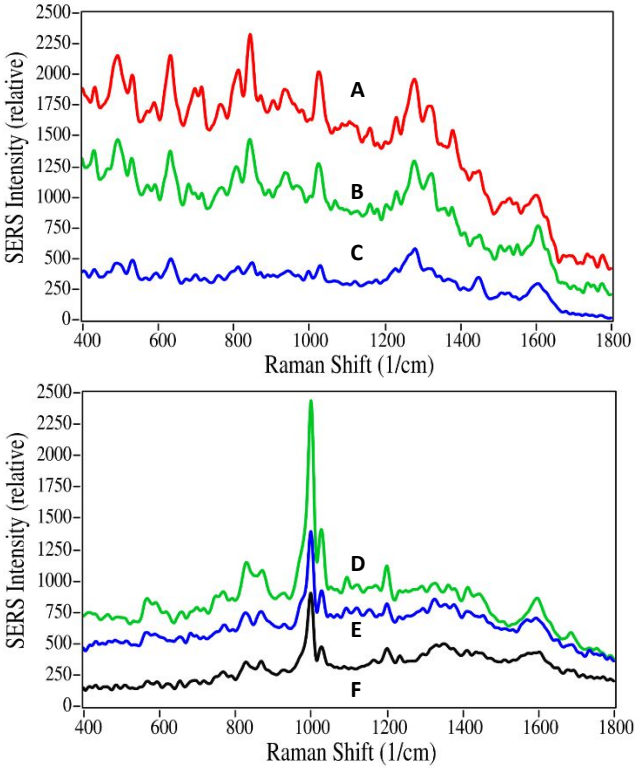

Figure 3. SERS of codeine measured at A) 10, B) 5 and C) 1 µg/mL; and fentanyl measured at D) 5, E) 1 and F) 0.5 µg/mL in saliva on SERS strips. Sample conditions: strips placed in samples diluted with saliva. Spectral conditions as in Figure 2.

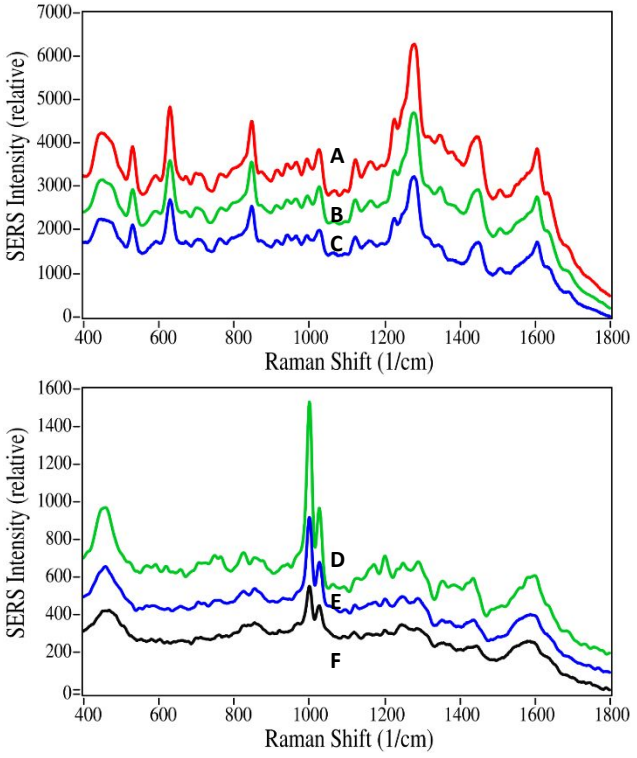

Figure 4. SERS of codeine measured at A) 10, B) 5 and C) 1 µg/mL; and fentanyl measured at D) 5, E) 1 and F) 0.5 µg/mL in blood plasma on SERS strips. Sample conditions: strips placed in samples diluted with plasma. Spectral conditions as in Figure 2.

Again, however, the 0.5 µg/mL codeine spectrum was dominated by noise, while the fentanyl spectrum at this concentration was easily measured (Figure 4B). The better spectral S/N for both drugs suggest that the SERS strips effectively removed the larger biomolecules, which appeared to limit previous researcher's measurements of codeine in plasma to ~3 µg/mL, even when centrifugation and filtering were used.[36]

Whole blood represents the greatest separation challenge for the SERS strips. Whole blood is only ~50% water, with the other half composed of almost all red blood cells.[42] Once again, the samples were prepared at 10, 5, 1 and 0.5 µg/mL, this time in de-identified, pooled whole blood. The separation was less successful, and spectra were only obtained for the 10 and 5 µg/mL samples. Sample flow up the SERS strip was somewhat slower at ~2 min. The codeine peaks at 625 and 1280 cm^{-1} were readily observed for both concentrations, as was the dominant fentanyl doublet at 1000/1030 cm^{-1} (Figure 5).

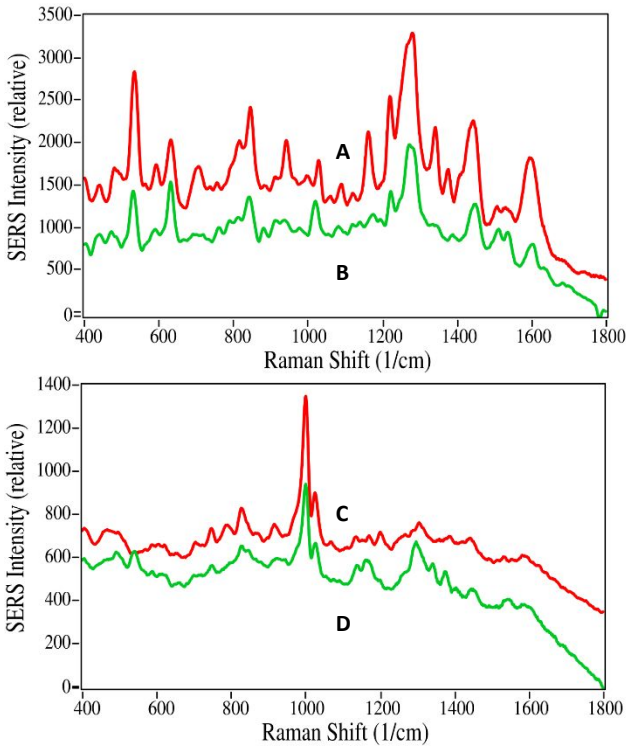

Figure 5. SERS of codeine measured at A) 10 and B) 5 µg/mL; and fentanyl measured at C) 10 and D) 5 µg/mL in whole blood on SERS strips. Sample conditions: strips placed in samples diluted with whole blood (see Figure 1B). Spectral conditions as in Figure 2.

From a spectral standpoint, the S/N indicates that these drugs could be measured at much lower concentrations. The limits of detection (LOD), based on a S/N of 3,[43] were calculated by measuring the codeine and fentanyl baseline corrected peak heights at 1280 and 1000 cm^{-1}, respectively, and measuring the standard deviation noise between 1900 and 2000 cm^{-1}, where the spectrum is devoid of drug contributions. The LODs for codeine and fentanyl were 70 and 22 ng/mL, 18 and 13 ng/mL, and 244 and 152 ng/mL for saliva, blood plasma, and whole blood, respectively. These LODs for codeine in saliva and plasma are sufficient to detect toxic levels, which are reported as ~0.5 to 1.2 µg/mL and ~0.2 to 0.5 µg/mL, respectively.[44,29] In contrast, the LOD for detecting fentanyl in plasma is not sufficient to detect toxic levels, which is reported as ~5 ng/mL.[30] No literature data were found indicating fentanyl toxicity levels in saliva.

Conclusions

A novel SERS flow-separation strip was developed that successfully separated codeine and fentanyl from saliva, blood plasma and whole blood, so that drug concentrations of 1, 1, 5 and 0.5, 1, and 5 µg/mL, respectively, could be measured using a small Raman spectrometer. In a preliminary study of these pads, codeine and fentanyl were measured in PBS at 100 and 50 ng/mL, respectively, with both LODs calculated at 6 ng/mL.[28] These results suggest that a more efficient separation system could measure similar concentrations, and detect toxic levels in biofluids. It was clear from these measurements, that sensitivity was hampered by the fact that the samples spread across the entire strip. Future work will test methods to concentrate the drugs on the SERS pad to increase sensitivity. Future work will also expand the analysis to a growing number of synthetic drugs of concern, such as cannabinoids and cathinones;[45,46] exploiting the one to one chemical structure to Raman spectrum relationship. This is an important advantage when compared to assay kits. Besides the kits false-positive rate of 25%,[47] they often require years to develop a unique antibody for a new drug. Consider that that the first fentanyl specific assay wasn't introduced until 2017.[48] In the case of Raman and SERS, it requires little more than measuring the new drug for identification and a concentration series in the biofluid for quantitation.

We believe that these measurements of opioids, using the rudimentary SERS strips with a portable Raman spectrometer, lay the foundation for a simple-to-use, point-of-care analyser that could be used by police, emergency responders, and medical practitioners to rapidly identify the drug(s) involved in an overdose, and thereby provide appropriate treatment that could potentially save lives. Furthermore, such a point-of-care analyser, would also be valuable to homeland security and NATO military countries, as fentanyl and its analogues, such as carfentanil, are considered potential agents of terrorism and war.[49-51]

Conflicts of interest

All authors were employed at Real-Time Analyzers, Inc. when this work was performed.

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A rudimentary flow strip, based on surface-enhanced Raman spectroscopy, was developed and used to measure drugs, such as fentanyl, in saliva, blood plasma, and whole blood. When fully developed, the strip could be used with hand-held Raman spectrometers as a simple-to-use point-of-care drug analyzer.