Quantification of Opioids in Urine using an Aptamer-based Free-solution Assay

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ABSTRACT: The opioid epidemic continues in the United States. Many have been impacted by this epidemic including neonates who exhibit Neonatal Abstinence Syndrome (NAS). Opioid diagnosis and NAS can be negatively impacted by limited testing options outside the hospital, due to poor assay performance, false-negatives, rapid drug clearance rates, and difficulty in obtaining enough specimen for testing. Here we report a small volume urine assay for oxycodone, hydrocodone, fentanyl, noroxycodone, norhydrocodone, and norfentanyl with excellent LODs and LOQs. The free-solution assay (FSA), coupled with high affinity DNA aptamer probes and a compensated interferometric reader (CIR) represents a potential solution for quantifying opioids rapidly, at high sensitivity, and non-invasively on small sample volumes. The mix-and-read test is 5-to-275-fold and 50-to-1250-fold more sensitive than LC-MS/MS and immunoassays, respectively. Using FSA, oxycodone, hydrocodone, fentanyl and their urinary metabolites were quantified using 10 µL of urine at 28-81 pg/mL, with >95% specificity and excellent accuracy in ~1 hour. The assay sensitivity, small sample size requirement, and speed could enable opioid screening, particularly for neonates and points to the potential for pharmacokinetic tracking.

INTRODUCTION: The United States is experiencing an opioid epidemic of unprecedented proportions, with ~100 Americans dying each day from an overdose, and an economic impact of ~$100B/year.[1] One of the many victims of this epidemic are newborns, as opioid use during pregnancy often resulting in addicted infants, who can then suffer from Neonatal Abstinence Syndrome (NAS).[2] As with adult addiction, NAS can be a serious condition, afflicting ~2% of all neonates born in the U.S. Expectant mothers using opioids have between 2-to-4-fold higher preterm birth rates when compared to the general population.[3] While many NAS patients are diagnosed and treated in the hospital setting, many are birthed at centers and low resource hospitals in poor, rural communities that tend to be the loci of the opioid epidemic. Infants that are sent home untreated can undergo withdrawal, exhibiting continuous crying, difficulty breathing, diarrhea, fever, and an inability to be comforted. As with adults, addiction symptoms can intensify until treatment and, if not diagnosed, severe complications can arise. The general lack of availability and limitations of existing methods, including poor sensitivity, complexity, or long turnaround time points to a need for a non-invasive, rapid, sensitive, and quantitative near-patient assay. The free-solution assay (FSA), performed on a novel benchtop reader could address these limitations. The simplicity, high sensitivity, small urine volumes, and speed of our method can potentially expedite and extend clinical feedback for a wide array of patients.

Urine is a routinely collected and a widely employed clinical matrix,[4] making it an attractive, non-invasive approach to opioid analysis. Yet, sample volume requirements (>100 µL) and time-to-results (24-48 hours, Mayo Clinic)[5] make quantitative testing by LC-MS/MS less than optimal for patient management in the rural or out-patient setting. This is particularly the case for premature babies which produce significantly less urine than the 1 mL/kg/hour produced by term neonate. The main alternatives to MS, the enzyme multiplied immunoassay technique (EMIT II),[6] or the cloned enzyme donor immunoassay (CEDIA),[7] are more rapid (1-2 hrs), but still require 50-100 µL of urine and are not particularly sensitive or quantitiative (Table 1).

Here we capitalized on a recently reported next generation backscattering interferometer, the *compensated* interferometer.[8] Using this optical engine in our new reader affords several advantages over its precursor, Backscattering Interferometry (BSI). The compensated interferometric reader's (CIR) advantages include simultaneous measurement of the test and reference samples, increased throughput, and a unique feature to interferometers, significant immunity to temperature sensitivity. Thermal compensation also enabled the use of capillary tubes for uninterrupted sample introduction and detection in the reader.

The free-solution assay (FSA) used here is unique, providing a universal signal transduction method for the quantification of molecular interactions. As described recently, FSA allows quantification of a target through a probe target interaction and measurement of the intrinsic solution-phase properties resulting from the reaction induced conformation and hydration changes.[9] FSA is unique in that it is assay agnostic and matrix insensitive. Here, by exploiting these properties, the quantification of several opioid targets in urine using aptamers as the probe molecule was demonstrated.

Aptamers are DNA- or RNA-based ligands capable of binding practically any molecular target and are commonly identified by

an *in-vitro* method of selection referred to as Systematic Evolution of Ligands by Exponential enrichment or "SELEX."[10] Aptamers with properties rivaling antibodies in both affinity and specificity have been developed as ligands to a wide range of targets including peptides, proteins, small organic molecules, cellular toxins, viruses, and even heavy metal ions. The advantages of aptamers have been well chronicled.[11] Relative to FSA, aptamers have benefits because they can be designed to undergo large conformation and hydration changes upon binding.

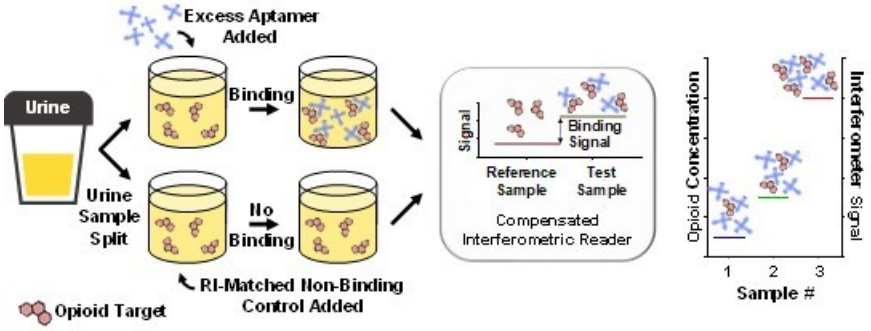

Figure 1. Schematic of the free-solution assay (FSA) workflow. A urine sample is split into two aliquots. One aliquot is combined with the probe aptamer to form the test sample, and the other aliquot is combined with an RI-Matched non-binding control solution. The difference in interference pattern is measured using the compensated interferometric reader,

Reported here is specific opioid quantification in 5 µL of urine, at the pg/mL level, by capitalizing on the marriage of these three synergistic technologies; the unique label-free, solution-phase assay (FSA),[9] high-affinity, high-selectivity DNA aptamer probes,[11b] and a recently developed compensated interferometric reader. Once calibrated, 24 sample determinations can be performed in a total analysis time of ~1 hour. Depending on the target, FSA-CIR provides opioid limits of quantification (LOQs) 5-to-275-fold better than LC/MS-MS and ~50-to-1000-fold better than commercially available enzyme multiplied immunoassay techniques (EMIT)[6] or cloned enzyme donor immunoassays (CEDIA).[7]

EXPERIMENTAL: The FSA method, shown in **Figure 1**, is described in detail elsewhere and in the supporting information.[9, 12] Briefly, the mix-and-read approach is based on splitting a urine sample into two parts, adding the probe to one part and a refractive-index matched non-binding control solution to the other, then analysis by the interferometric reader.

Solution preparation, detailed in the SI, consisted of making stock aptamer and opioid solutions, either for K_D determination or for target quantification. Aptamer selection for the six opioid targets (**Figure 2**) was performed using a modified version of SELEX,[13] and aptamer candidates were characterized using next-generation sequencing methods, and are available for procurement from Base Pair Bio.[14] Once synthesized, aptamers were reconstituted from a lyophilized pellet to prepare a 100 µM stock solution in phosphate buffered saline (PBS) modified to include 1 mM $MgCl_2$. The stock solution was subsequently diluted to the working concentrations (**Table S1**). To ensure the aptamers were in the desired conformation, they were refolded by heating the solution to 90°C for 5 minutes in a water bath, then cooled to room temperature for 15 minutes.

Opiate target solution preparation followed normal analytical procedures described in detail in the SI. Briefly, the six opioid targets and cortisol were obtained at 1 mg/ml (2.7 – 3.3 mM) in methanol from Sigma Aldrich. Working solutions of 27 - 32 µM were prepared by diluting 10 µL of the opioid standard solution with 990 µL of PBS. The appropriate volume of the working solutions was further diluted to give 200 nM opioid solutions. A list of precise volumes is included in **Table S1**. Care was taken to ensure the 1% methanol in PBS solution was kept constant across all dilutions so that sample and reference solutions were index matched. The concentration of the target samples stayed well below the 1 mM aqueous solubility limit. Preparation of solutions used for aptamer affinity measurements, LOQ determinations for the opioid targets, and the unknowns was performed in 50% urine.

The CIR (**Figure S1**) described in the SI, consisted of a droplet generator, the compensated interferometer, and a syringe pump. The interferometer is based on a laser, a fused silica capillary, and a CCD camera. The laser – capillary interaction produces fringes that are directed onto the camera and proper windowing of the captured image using an in-house Labview™ program facilitates interrogation of adjacent regions of the capillary for differential comparison of sample and reference droplets. The positional shifts in the fringes within each of these two windows is proportional to molecular binding and is quantified by a fast Fourier transform (FFT)[15] (SI). Droplet trains were generated directly in the capillary tube by a Mitos Dropix (SI). A single section of capillary served both as the reader cell and transfer line. The sample-reference pairs were separated by a 40nL droplet of oil (Flourinert FC-40, Sigma-Aldrich). Prior to droplet train generation, the capillary was filled with PBS and the syringe pump operated at a flow rate of 10 µL/minute for 10 minutes to establish stable flow. Then the assay was run by introducing 0.75 µL of each sample/reference solution pair 5 times, followed by two PBS rinses of 4 µL. This process was repeated for each concentration, or unknown, completing a full dropix sample tray. Analysis of a fully loaded sample-well tray (6 sample-reference pairs) requires about 14 minutes. Prior to the analysis of the next dropix sample tray, the capillary tube was rinsed thoroughly with PBS. Glycerol calibration confirmed CIR performance with the desired values of: a) response ~0.110 radians/mM glycerol, b) standard deviation of replicate determinations of ~0.012 radians c) an LOQ of ~0.33 mM glycerol calculated as 3×(σ of 5 replicate measurements) / (slope).

Isothermal end-point binding affinity assays were performed using a 7-point serial dilution series of the opioids ranging from 50 – 0.780 nM in 50% Urine / 50% PBS. The binding samples were prepared by incubating each concentration of opioid with 1 nM of the aptamer selected for that opioid. The reference solution consisted of the same concentration of opioid (50 - 0.780 nM) in 50 % Urine / 50% PBS solution, devoid of aptamer. Dissociation constants were quantified by analysis using CIR on 5 replicates at each opioid concentration and fitting the data to a single-site saturation isotherm using Graphpad Prism™, using the equation:

$$Y = \frac{B_{MAX} \cdot X}{K_D + X}$$

Calibration curves for the opioid assays were obtained by creating a dilution series of the target ranging from 100-0 nM in 50% Urine / 49.5% PBS / 0.5% Methanol with 1000 nM aptamer and an RI matched reference, incubating for 1 hour, then performing FSA-CIR analysis (SI). The phase shift between binding and reference sample was measured using the CIR, and the response was fit with a saturation isotherm. The slope in the linear region was used to calculate the LOD (3×σ instrument baseline noise/slope) and the LOQ (3×σ (of replicate determinations)/slope).

Test "unknowns" were prepared by spiking blank human urine with the opioid target and performing the FSA measurement as described above and in the SI. Unknowns were prepared so that the operator remained blinded to the sample's true concentration until after the determination was completed.

Aptamer specificity (cross-reactivity) was tested between the target drug and most common metabolite, as well as cortisol (SI). Briefly, high concentration solutions (2000 nM) of the target opioid, the metabolite, and cortisol were prepared in 50% Urine / 49%PBS / 1% methanol, allowing subsequent preparation of 1000 nM, 500 nM, and 0 nM solutions. These solutions were incubated with the target aptamer at 1000 nM in 50% Urine / 50% PBS and then assayed by CIR in an endpoint format.

For brevity, the pharmacokinetic modelling efforts undertaken here are described in significant detail in the SI.

RESULTS and DISCUSSION The mix-and-read, urine, opioid assay reported here is enabled by comparing the signal of 'matched' sample-reference pairs from the CIR.[9] As shown in **Figure 1**, we split a small volume of a urine sample into two aliquots and then process them to provide 'binding' and 'reference' solutions. To quantify a target, we add an excess of probe (DNA aptamer) to one of the aliquots, giving the "binding/test" sample, and to the other we add an RI matching solution (buffer) or "reference/control." Assay/instrument calibration solutions are prepared by first spiking blank urine with increasing concentrations of the opiate target, then splitting this sample into two aliquots and proceeding as described here and in the Supporting Information (SI). These solutions are allowed to equilibrate and then introduced into adjacent wells of a droplet generator (SI) for analysis by the reader as pairs separated by an oil droplet. The difference in interferometric signal between the sample–reference pairs provides a quantitative measure of target by reporting the concentration of aptamer-target complex, while allowing the matrix signal to be nullified. Since the aptamer probe is used in large excess relative to the target (μM vs. nM) for target quantification, the signal due to the aptamer is essentially constant so it can be ignored.

The CIR, described in detail in the SI, is relatively simple, consisting of a commercial droplet generator (Mitos Dropix, Dolomite Microfluidics, UK), an interferometer, and syringe pump (**Figure S1**).[8] The interferometer is based on a diode laser (Lasermate, USA), a capillary flow cell (Polymicro, Molex, USA), and a camera (Basler avA2300 2D CCD array, USA) and has been shown to provide numerous improvements over previous scattering interferometers.[8, 16] In the CIR, an expanded laser beam illuminates the long axis of the capillary, producing 'elongated' fringes that shift in proportion to the analyte concentration. The simultaneous analysis of fringe shift for sample-reference pairs of 1.0 μL droplets separated by a 40 nL oil droplet in an uninterrupted fluid-droplet-train provides efficient, sample-conserving FSA analysis. The use of a continuous section of fused silica capillary (250 μM ID × 350 μM OD) as both the transfer line and the detector cell for the interferometer, and the appropriate settings for the droplet generator and syringe pump (SI), enables semi-automated analysis of up to 24 sample pairs in less than 1 hour.

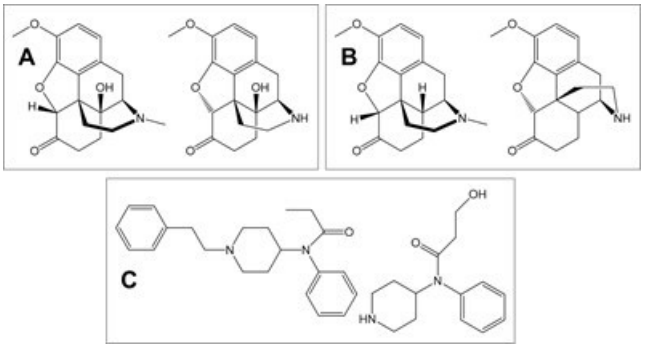

Figure 2. Structures for target molecules: A) Oxycodone (left) and its major urinary metabolite, noroxycodone (right). B) Hydrocodone (left) and major urinary metabolite, norhydrocodone (right). C) Fentanyl (left) and its major urinary metabolite, norfentanyl (right).

Avoiding many of the issues associated with detecting small molecules with antibodies was facilitated by using DNA-aptamers as highly selective opioid probes.[11b] DNA aptamers are advantageous because they exhibit high selectively to small molecule targets, don't require a cold chain for storage/transport, are readily synthesized, and can be selected in the matrix of interest (urine in this case).[13] The aptamers employed here were obtained for the six opioid targets displayed in **Figure 2**, and were prepared using a multiplex version of "structure-switching" systematic evolution of ligands by exponential enrichment

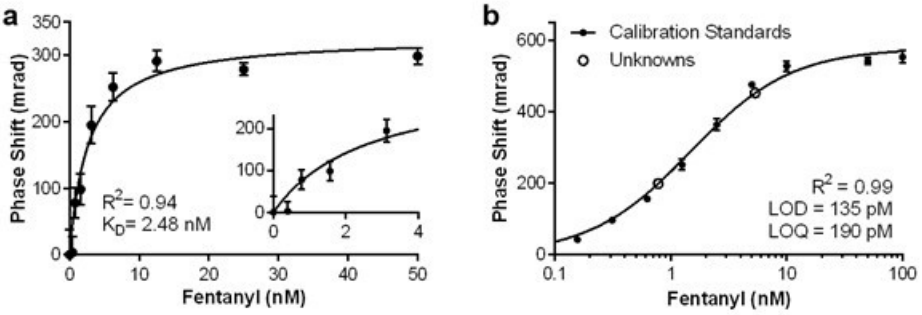

Figure 3: A) Fentanyl aptamer K_D determination giving an affinity of 2.44 nM and $R^2 = 0.94$. Error bars show standard deviation of 7 replicates. B) Calibration curve for fentanyl.

(SELEX).[14, 17] Once identified and prior to FSA-CIR testing, candidate aptamers were sequenced to provide a preliminary indication of how they might perform in the assay.

With aptamer candidates in hand, FSA-CIR was used to quantify the binding affinity (K_D) to their cognate targets, providing insight into opioid assay performance. These K_D determinations were performed in 50% urine/50% phosphate buffered saline (PBS) using the end-point saturation isotherm assay described previously[9, 11b] and in the SI. In short, urine binding samples were prepared with 1 nM aptamer (e.g. receptor) and increasing concentrations of the target opioid (0-50 nM, e.g. ligand). The reference solutions were made from the individual opioid-containing urine solutions that are refractive index (RI) matched with PBS devoid of aptamer (SI). **Figure 3A** displays the saturation isotherm for the fentanyl aptamer based on seven replicate determinations by FSA-CIR for each sample-reference pair and is typical of our K_D experiments (SI). K_D's for all aptamer-opioid interaction were calculated by fitting a single-site saturation isotherm to the FSA signal. The results for K_D determinations for all aptamer/opioid pairs are displayed in **Figure S2**. The aptamer probes exhibited affinity in the hundreds of picomolar to low-nanomolar range (**Table S2**), with the K_D's for fentanyl = 2.48 nM, norfentanyl = 0.93 nM, oxycodone = 0.66 nM, noroxycodone = 1.33 nM, hydrocodone = 4.49 nM and norhydrocodone = 0.72 nM. As shown below, K_D values in this range enabled a single probe, mix-and-read approach to provide pg/mL sensitivity and excellent specificity for the target in urine. **Figure 3A** and **S2** also illustrates that the aptamer binding affinity measurements are robust and reproducible. So as to match the final quantification assay conditions, KD determinations where performed on solutions containing a final concentration of 50% urine. The urine samples start out at 100%, but when combined with a probe solution or control solution, the result is a 50% urine solution at the time of measurement.

Next, we determined the limits of detection (LODs) and limits of quantification (LOQs) for the 6 opioid-aptamer assays. Solutions were prepared to contain an opioid concentration ranging from 0–100 nM in 50% urine and an excess of the aptamer probe. The reference solutions contained the respective opioids at a concentration from 0–100 nM and the appropriate volume of RI matching control solution (SI). **Figure 3B** and **Figure S3** illustrate the opioid calibration curves obtained by our assay in 50% urine. Assay performance for fentanyl (**Figure 3B**) was an LOD of 45 pg/mL (13 pM) (LOD = 3×σ (5 sec. of baseline noise)/(calibration curve slope)), and an LOQ of 63 pg/mL (190 pM) (LOQ = 3×(σ for all replicates)/slope). All assays reported a dynamic operating range of ~2.5 orders of magnitude in concentration and a correlation coefficient, R^2 = 0.99 (Table S2). The opioid assays performance (**Table S2**) gave LODs ranging from ~28-81 pg/mL (90-245 pM) and LOQ's of 44-183 pg/ml (141-611 pM). The fentanyl assay, particularly interesting due to its recent explosion in illicit use, has an LOQ at least 5-fold better than the best value reported for the considerably more time-consuming and complex LC-MS/MS assay.[18]

Assay performance, regardless of methodology, can be impacted by undesirable off-target response.[19] Aptamer-based assays are no exception, with cross-reactivity to non-target species and matrix components a potential problem. Specificity

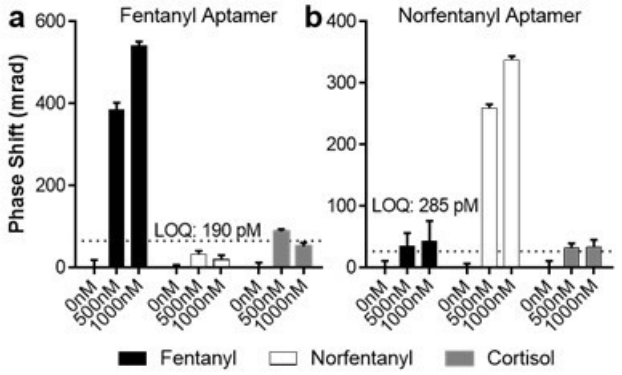

Figure 4. A) Fentanyl and B) norfentanyl cross reactivity results. High specificity aptamers result in signals near or below the assay LOQ for the off-target species.

investigations we done by measuring the magnitude of cross-reactivity of the aptamer to the primary metabolite (e.g. fentanyl vs. norfentanyl), and to the common biological signaling molecule, cortisol. Cortisol was chosen because it is a known marker of stress, widely found in urine, with levels often being correlated with opiate exposure.[20] FSA was used to screen (SI) and quantify the signal for aptamer binding to 0 nM, 500 nM, and 1000 nM of target, the structurally similar urinary metabolite and solutions spiked with cortisol, all in 50% urine samples. All assays contained 1000 nM of the aptamer probe. Here, the large concentration of the aptamer contributes measurably to the signal (bulk RI of the solution), therefore we subtract this aptamer background signal to zero the instrument readout. **Figure 4** illustrates the results of these target and off-target binding experiments for the fentanyl and norfentanyl aptamers. Norfentanyl exhibited minimal cross-reactivity to the fentanyl aptamer (**Figure 4a**), producing a signal below the LOQ of the measurement. Cortisol did produce a response to the fentanyl aptamer with a binding signal slightly larger than the fentanyl LOQ. Being near the LOQ is likely the reason for the discrepancy between the 500 and 1000 nM responses observed for cortisol with fentanyl aptamer. The norfentanyl aptamer exhibited a small amount of cross-reactivity for fentanyl above the LOQ (5%) (**Figure 4b**), and a cortisol signal which is slightly above the norfentanyl LOQ. In general, the aptamer assays performed well, with off-target binding signal magnitudes for the cognate species near or below the LOQ (**Table S2**). While not a statistically significant approach of using phase values below the LOQ, the relative phase values yield off target binding of 0% to ~13% (**Figure S4**, **Table S2)**. Putting these results in perspective, to introduce a quantifiable inaccuracy into the target measurement of just a few percent, the off-target species concentrations would need to be 4,000-10,000 times higher than the target species. For example, typical cortisol levels in the blood range from 20-300 nM, which is only 100-1500 times higher than the fentanyl LOQ.[21] Therefore, typical cortisol levels are still 2-to-10 fold too low to introduce a 5% quantification inaccuracy into a fentanyl measurement at a concentration near the LOQ. While we acknowledge that clinical translation and false positives/negatives prediction will require more rigorous off-target binding determinations, our results performed in the

Table 1: Opioid assay method comparison.

	FSA-CIR	LC-MS/MS	Mayo Clinic[i]	Immuno-assays[ii]
Volume Required	10 µL	100 µL – 30 mL	3 – 20 mL	50 µL
Analysis Time	60 min	30-80 min	24-48 hr	60 min
Sensitivity:	LOQ (pg/mL)	LOQ (pg/mL)	Cutoff	Cutoff
Fentanyl	60	300	100	2,000
Norfentanyl	90	1,400	2,000	2,000
Oxycodone	40	10,000	25,000	50,000
Noroxycodone	180	50,000	25,000	50,000
Hydrocodone	160	10,000	25,000	50,000
Norhydrocodone	80	50,000	25,000	50,000

Values in pg/mL. [i]Two LC-MS/MS tests offered: FENTU for Fen/Norfen. OPATU for Oxy/Noroxy/Hyd/Norhyd. [ii]CEDIA, ELISA, FPIA, DRI, and EMIT II report positive or negative results relative to defined cutoff

urine matrix indicate the reported aptamer-urine assays exhibit excellent specificity.

At the time of this research true clinical samples were not available, therefore we prepared spiked urine samples to serve as "unknowns." Upon performing the appropriate calibration curve for each target, a blinded operator quantified the opioid concentration in the "unknown" samples using the FSA-CIR measurement. For fentanyl, these unknowns contained 250 pg/mL and 4 ng/mL of opioid target in pooled human urine. The quantification of these "unknowns" is demonstrated in **Figure 3B** as the open circles overlaid upon the calibration curve. . Results for all "unknown" measurements are summarized in **Table S3**. Plotting the experimental versus the actual 'spiked' concentrations for all assays results gives a linear plot (R^2 = 0.996, **Figure S5**). In all cases the assay provided quantification of the unknown target concentration with less than 6% error (**Tables S2 and S3**). All unknown concentrations evaluated here were 75-to-1000-fold below the standard clinical cut-off of 300 ng/ml,[22] or 500-to-8000-fold below the Substance Abuse and Mental Health Services Administration (SAMHSA) drug-testing cut-off of 2000 ng/ml.[23]

Table 1 summarizes the performance of the aptamer-based FSA-CIR opioid assay compared to other methods. We have excluded gas chromatography, which has mostly given way to immunoassays for screening and LC-MS/MS for analyte confirmation/quantification. Although differences exist, FSA-CIR screening is most akin to immunoassay. Besides free-solution, label-free operation, a major distinction is that FSA-CIR is quantitative, whereas EMIT® II Plus (Siemens) is qualitative, or semi-quantitative (+/- 1.5 ng/mL) within the opioid determination operating range of 5-20 ng/mL[6], while FSA is quantitative over about 2.5 decades (**Figure 3B and S3**). CEDIA® (Thermo Scientific) has similar performance to the EMIT II Plus.[7] **Table 1** further illustrates that FSA-CIR is 50-1000-fold more sensitive than commercial immunoassays. LC-MS/MS methods are currently reported to provide LOQs of 10-50 ng/ml for hydrocodone, oxycodone, noroxycodone and norhydrocodone in urine.[24] With pg/mL LOQs attainable, FSA can be used to provide a sensitivity improvement of 10-to-1000-fold over typical LC-MS/MS (**Table 1**). FSA was found to be 5-fold and 20-fold better than the standard Mayo Clinic test for fentanyl and norfentanyl, respectively, yet it requires 20 times less sample and was performed in a mix-and-read format on relatively simple instrumentation. To our knowledge, our aptamer-based FSA is 2-fold and 15-fold more sensitive for fentanyl and norfentanyl respectively compared to best reported MS-based assay.[25] While MS methods do allow simultaneous measurement of multiple analytes, specificity does depend on spectrometer resolution. Furthermore, MS instrumentation is complicated, costly, and impractical for the near-patient setting, with results presented here indicating that the aptamer-based FSA represents an attractive alternative.

In addition to the advantages of small volume and instrument simplicity, the enhanced sensitivity of FSA has the potential to speed time-to-result and extend analyte detection time, allowing previously unavailable pharmacokinetic analysis. Neonate opiate pharmacokinetics represents an interesting case to consider, in part because it varies considerably.[18] Using observations by Valitalo[26] allows us to place reasonable bounds on expected neonate opiate circulation lifetimes. Taking these

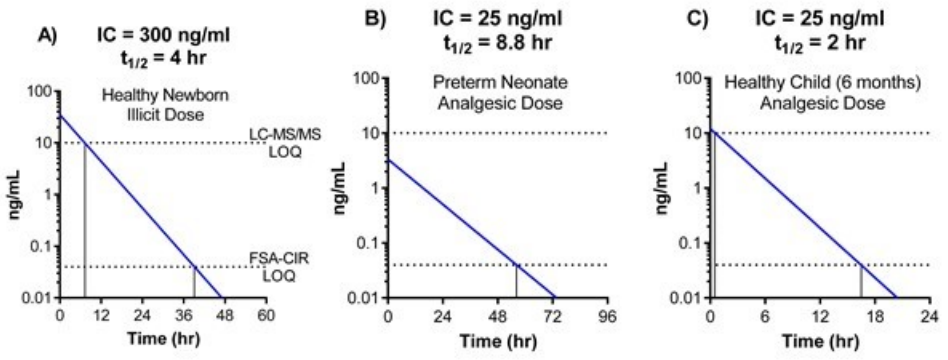

Figure 5. Neonate oxycodone concentration vs. time for several representative cases, illustrating how lower LOQs by FSA-CIR could provide increased time for detection relative to LC-MS/MS.

parameters for oxycodone half-life as an example (SI), we calculated opioid concentration in urine over time for preterm and term neonates.

Figure 5 shows how improved LOQs can extend the period of accurate urine quantification for exposure and potentially aid in pain management for a term neonate and a healthy child. In a term neonate exposed to an illicit level of oxycodone (300 ng/ml, $t_{1/2}$ = 4 hrs), the quantification window provided by FSA is about 40 hours (**Figure 5A**), as compared to just 8 hours by MSMS.

The calculation for a typical analgesic dose of 25 ng/ml in a preterm neonate ($t_{1/2}$ = 8.8 hrs) yields **Figure 5B**, which illustrates that the oxycodone concentration in urine *never reaches* the LOQ for LC-MS/MS. However, the aptamer-based FSA method makes oxycodone urine analysis viable immediately, and for 56 hours after administration. In a healthy child (age = 6 months, $t_{1/2}$ = 2 hrs) (**Figure 5C**) receiving an analgesic dose of oxycodone (25 ng/ml), the drug is only quantifiable for about half an hour by MS/MS, whereas with FSA the quantification window is extended by an additional 16 hours. These examples show how improved LOQs could allow quantification of opioids in patients, at earlier time-points and for considerably longer time periods, potentially improving the clinical management of these patients.

In conclusion, we have demonstrated a small volume urine assay for oxycodone, hydrocodone, fentanyl, noroxycodone, norhydrocodone, and norfentanyl with excellent LODs, LOQs and specificity. The aptamer-based FSA-CIR approach, coupled with high affinity DNA aptamer probes represents a potential solution for quantifying opioids at high sensitivity and non-invasively on small sample volumes. The method reported here is rapid when compared to currently available methods and exhibits accuracy of better than 95% across a wide range of opioid concentrations. Even with the use of a commercial droplet generator, a relatively low-cost system can be configured in a bench-top format, making it compatible with the near-patient setting. Off-the-shelf components can be used to construct the device on a small optical bread board, cost about $5000 for the optical engine, (CCD, laser diode, and miscellaneous mounting equipment). In addition, a commercial droplet generator (ca. $16,000), syringe pump ($2000), and PC ($1000) are required to construct a CIR. By all accounts constructing a CIR is considerably less expensive than purchasing an LC-MS/MS system. Including all reagents, we estimate an assay costs about ~ $15 (capillary, buffers, standards, aptamers, etc.), as compared to cost of maintaining an LC-MS/MS, purchasing columns, LC reagents and the like, which can run into the thousands of dollars. The simplicity of the reader and recent demonstration of temperature controller-independent operation,[8b] point to the potential for developing a hand-held reader. Enabled by highly selective aptamers and a recent description of the signal transduction mechanism for FSA,[9] our technology has the promise to revolutionize near-patient screening in a variety of contexts, particularly for neonatal opioid quantification.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

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Author Contributions

M.N.K., A.K.K., G.W.J. and D.J.B. wrote the manuscript; M.N.K., A.K.K., G.W.J. and D.J.B designed the research; M.N.K., A.K.K, I.G., and R.D. performed the research; M.N.K., A.K.K., G.W.J. and D.J.B analyzed the data; M.N.K., A.K.K., G.W.J. and D.J.B contributed new reagents/analytical tools.

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Conflict of Interest

R.D., R.H.B., and G.W.J. are employees of Base Pair Biotechnologies, Inc., as such have a financial interest in commercializing the aptamers used in the assay methodology. M.K., A.K., and D.J.B. declare no conflict of interests.

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