Analytical Cumulative Examination
16 November 2010
Primary Examiner: T.P. Beebe, Jr.; Secondary Examiner: K. Booksh

The subject of this exam is a recent publication in *Analytical Chemistry*:

"Label-Free Porous Silicon Immunosensor for Broad Detection of Opiates in a Blind Clinical Study and Results Comparison to Commercial Analytical Chemistry Techniques," by Lisa M. Bonanno, Tai C. Kwong, and Lisa A. DeLouise. The article was accepted in October 2010, and is now on-line in *Analytical Chemistry* as an ASAP article. The article is available in PDF format from the UD library, and it is appended here.

Assignment: Write a review and overview paper (see details below). Before 12:00 noon on Tuesday 16 November 2010, please e-mail a PDF version of your paper to Tom Beebe (beebe@udel.edu), and a copy to Karl Booksh (kbooksh@udel.edu). ***Please do not show up in person at the exam room on Saturday 13 November.*** You may work on this paper immediately. If I had sent out this exam two weeks ago, you probably would have spent two weeks on it. I want to see what you can do in one week. You must work alone. You may use any scholarly resources that you care to employ, although your paper must be your original work. You must include and incorporate at least 10 peer-reviewed journal articles or books other than the Bonanno paper in your review and bibliography. UD's rules on plagiarism and academic honesty apply here. Be sure to read these rules first: (http://www.udel.edu/stuguide/10-11/code.html#honesty)

A.) Your review and overview paper should cover *at least* the following subjects, in the order that you deem appropriate, with the headings in **bold**:

A.) **Label-Free Detection.** A general discussion of label-free detection and why it is of interest;
B.) **Porous Silicon as a Detection Platform.** A general discussion of porous silicon, including how it has been used for detection in the past, its strengths and weaknesses;
C.) **Competing Analytical Techniques.** An introduction to the other analytical techniques employed here (and in other related articles);
D.) **Metrics for Comparison.** An overview of the various quantitative and qualitative metrics employed here for comparison to porous silicon;
E.) **Critical Review.** A critical review of the article named above;
F.) Any additional sections that you deem appropriate;
G.) **Bibliography.**

B.) The paper should have the following format, and points will be lost for failure to follow:

- The text of the body of the paper should be no less than 10 pages and no more than 12 pages.
- The pages should each be numbered, with 1-inch margins on all sides.
- The font should be 11 point Arial.
- The line spacing should be 1.5 (*i.e.*, not single-spaced and not double spaced).
- The title page and your bibliography page(s) do not count in the page limits. Number all pages. Start page numbering at "1" on the page following your title page.
- Any figures or tables that you employ should be pasted directly into the body of the text so that text flows neatly around them. These objects count in the page limit.
- Each figure and table should be numbered, and its source should be indicated in a figure caption located immediately below the figure or table.
- Footnotes and references should be indicated in the body of your paper with superscripts, and the reference to which they refer should appear in the bibliography at the end.
- References in your bibliography must have the title of the article indicated, and should use the ACS format (with titles).
- You must include and incorporate at least 10 peer-reviewed journal articles or books other than the Bonanno paper in your review and bibliography.
- A URL (web address) will only count as a valid reference if it is clearly from a peer-reviewed source and is indicated as such. This is usually not the case for a URL, so review them carefully before using them.
- Use the section headings above, as well as others that you deem appropriate.

Label-Free Porous Silicon Immunosensor for Broad Detection of Opiates in a Blind Clinical Study and Results Comparison to Commercial Analytical Chemistry Techniques

Lisa M. Bonanno,[†] Tai C. Kwong,[‡] and Lisa A. DeLouise*[,†,§]

Department of Biomedical Engineering, University of Rochester, Rochester, New York, United States, and Departments of Pathology and Lab Medicine and Dermatology, University of Rochester Medical Center, Rochester, New York, United States

In this work, we evaluate for the first time the performance of a label-free porous silicon (PSi) immunosensor assay in a blind clinical study designed to screen authentic patient urine specimens for a broad range of opiates. The PSi opiate immunosensor achieved 96% concordance with liquid chromatography-mass spectrometry/tandem mass spectrometry (LC-MS/MS) results on samples that underwent standard opiate testing ($n = 50$). In addition, successful detection of a commonly abused opiate, oxycodone, resulted in 100% qualitative agreement between the PSi opiate sensor and LC-MS/MS. In contrast, a commercial broad opiate immunoassay technique (CEDIA) achieved 65% qualitative concordance with LC-MS/MS. Evaluation of important performance attributes including precision, accuracy, and recovery was completed on blank urine specimens spiked with test analytes. Variability of morphine detection as a model opiate target was <9% both within-run and between-day at and above the cutoff limit of 300 ng mL^{-1}. This study validates the analytical screening capability of label-free PSi opiate immunosensors in authentic patient samples and is the first semiquantitative demonstration of the technology's successful clinical use. These results motivate future development of label-free PSi technology to reduce complexity and cost of diagnostic testing particularly in a point-of-care setting.

Advances in nanotechnology and microfabrication techniques have generated an explosion in biosensor research and development, with an estimated $300 US million worldwide investment in 2008.[1,2] In comparison, commercialization of biosensor technology has significantly lagged behind. Slow translation can be attributed to cost considerations and technical barriers such as stability upon sensor storage, reliability, and detection sensitivity of target molecules in authentic patient samples.[1] Design of an ideal biosensor platform should incorporate these features and design for feasibility of system integration for automated high throughput and multianalyte analysis in a clinical laboratory. Overcoming these challenges to develop practical biomolecular detection platforms for rapid and reliable analytical performance in real world environments is a necessary step to improve upon the current system of laboratory-based analytical chemistry methods.[1]

A major focus in diagnostics development is on label-free biosensors that exploit the unique optical and electrical properties of semiconductor nanomaterials and, therefore, have the potential to reduce cost and complexity of analytical techniques, which largely rely on signal generation from secondary enzymatic, radioisotopic, or fluorescent molecules.[3,4] Nanostructured porous silicon (PSi) is one such promising material that satisfies the consensus criteria of an ideal diagnostic device including its inexpensive fabrication, label-free optical analysis,[5−14] and potential for multianalyte analysis with an array of probe molecules and point-of-care colorimetric detection.[6−9]

The response signal of the label-free PSi sensor is generated by effective refractive index changes that arise when targeted biomolecules are selectively captured within the porous sensor with use of surface immobilized probe molecules.[5] Diverse surface functionalization schemes have been used in PSi to detect

* Corresponding author. Address: 601 Elmwood Ave. Box 697, Rochester, NY 14642. Phone: 585.275.1810. Fax: 585 0.273.1643. E-mail: lisa_delouise@urmc.rochester.edu.

† Biomedical Engineering Department, University of Rochester.

‡ Department of Pathology and Lab Medicine, University of Rochester Medical Center.

§ Department of Dermatology, University of Rochester Medical Center.

(1) Luong, J. H. T.; Male, K. B.; Glennon, J. D. *Biotech. Adv.* **2008**, *26*, 492–500.

(2) World Markets for Point of Care Diagnostics. Kalorama Information: World Markets for Point of Care Diagnostics; Vol. 13, 2009.

(3) Wang, Y.; Tang, Z.; Kotov, N. A. *Mater. Today* **2005**, *8*, 20–31.

(4) Jain, K. K. *Clin. Chim. Acta* **2005**, *358*, 37–54.

(5) Vinegoni, C.; Cazzanelli, M.; Pavesi, L. Porous Silicon Microcavities. In *Silicon-Based Materials and Devices*; Nalwa, H. S., Ed.; Academic Press: New York, 2001; pp 123−187.

(6) Ressine, A.; Finnskog, D.; Malm, J.; Becker, C.; Lilja, H.; Marko-Varga, G.; Laurell, T. *Nano Biotech.* **2005**, *1*, 93–103.

(7) Cunin, F.; Schmedake, T. A.; Link, J. R.; Li, Y. Y.; Koh, J.; Bhatia, S. N.; Sailor, M. J. *Nat. Mater.* **2002**, *1*, 39–41.

(8) Sailor, M. J. *ACS Nano* **2007**, *1*, 248–252.

(9) Bonanno, L. M.; DeLouise, L. A. *Adv. Funct. Mater.* **2010**, *20*, 573–578.

(10) Pacholski, C.; Martor, M.; Sailor, M. J.; Cunin, F.; Miskelly, G. M. *J. Am. Chem. Soc.* **2005**, *127*, 11636–11645.

(11) Bonanno, L. M.; DeLouise, L. A. *Biosens. Bioelectron.* **2007**, *23*, 444–448.

(12) Gao, L. Z.; Mbonu, N.; Cao, L. L.; Gao, D. *Anal. Chem.* **2008**, *80*, 1468–1473.

(13) Li, Y. Y.; Cunin, F.; Link, J. R.; Gao, T.; Betts, R. E.; Reiver, S. H.; Chin, V.; Bhatia, S. N.; Sailor, M. J. *Science* **2003**, *299*, 2045–2047.

10.1021/ac101804s

proteins,[15,16] oligonucleotides,[17−19] enzymes,[20−22] and small molecules[23,24] in proof-of-principle laboratory studies. However, to our knowledge, there are no published studies adequately addressing the validation of analytical performance of a label-free affinity-based PSi immunosensor in a clinical assay with authentic patient samples, which is a necessary undertaking in developing practical clinical diagnostics.

Recent work by Lowe et al. has successfully combined immunocapture on PSi substrates with laser desorption/ionization mass spectrometry (LDI-MS) techniques to detect drug metabolites in laboratory grade buffer[25] and real-world oral fluid samples.[26] LDI-MS facilitates improved specificity and confirms the identification of captured target molecules; however, in contrast, it also adds cost and complexity to the test. In comparison, the label-free PSi immunoassay format provides straightforward optical sensor read-out desirable for use in point-of-care settings such as clinics and rehabilitation centers. In addition, the photonic properties of PSi can be exploited to facilitate colorimetric readout by the eye to further simplify readout by eliminating the need for external optical instrumentation.[9,22]

Here, we build upon our previous work, in which a proof-of-concept competitive-inhibition immunoassay was developed in a photonic PSi Bragg mirror sensor to achieve improved detection sensitivity of small molecule targets with the capability to tune detection sensitivity and range over ~3 orders of magnitude (18.0 nM−10.8 μM).[24] This assay was designed to have broad cross-reactivity toward opiates through use of an antibody that was selected using enzyme linked immunosorbant assay (ELISA).[24] To move beyond proof-of-concept, in this study, we evaluated the performance of the label-free PSi biosensor assay as a broad screening technology for detecting opiates in authentic patient urine specimens. Results were compared to commercial immunoassays and chemical analysis techniques (gas chromatography/mass spectrometry, GC/MS, and liquid chromatography-mass spectrometry/tandem mass spectrometry, LC-MS/MS).

In addition, special attention has been made to evaluate the capability of the PSi opiate biosensor to detect oxycodone. Oxycodone in a time-release preparation as OxyContin is among the most often-prescribed schedule II controlled substances for pain relief, and its widespread availability and high dependency potential have contributed to its "street" sale and frequent abuse.[27,28] Oxycodone is a semisynthetic opiate chemically related to morphine and codeine that contains a 6-keto substituent group and a hydroxyl group at carbon 14.[24] Most commercial opiate immunoassay kits are designed to detect morphine and codeine and have limitations for oxycodone detection.[25−30] Thus, oxycodone-specific immunoassays have been developed for use in pain clinics and/or as a secondary opiate screening test in clinical laboratories.[27] The use of a broad opiate screening assay that includes sensitive detection of oxycodone and its metabolites would be advantageous in a clinical setting, decreasing false negative screening results and decreasing the cost and time associated with secondary more-specific oxycodone screening techniques.

MATERIALS AND METHODS

Chemicals. Drug standards and chemicals were purchased from Alltech (Deerfield, IL): codeine, hydromorphone (HM), monoacetylmorphine (6-Mam), morphine, and oxycodone; Sigma-Aldrich (St. Louis, MO): morphine-3-glucuronide (M3G), morphine-d_3, codeine-d_3, SKF-525A, and β-glucuronidase from limpets (Patella vulgata); Cerilliant (Round Rock, TX): hydrocodone (HC), codeine, noroxycodone, methadone, benzoylecgonine, dihydrocodeine (DHC), naloxone, buprenorphine, and norbuprenorphine; Pierce (Rockford, IL): *N*-methyl-bis(trifluoroacetamide) (MBTFA). Solid M3G was obtained as a generous gift from the National Institute on Drug Abuse (Bethesda, MD). A monoclonal mouse antimorphine antibody (α-M Ab) was purchased from MyBiosource (MBS318578, San Diego).

Study Population and Design. Study urine samples were collected for routine drug of abuse testing at the Strong Memorial Hospital Clinical Toxicology Laboratory (CTL, June to July 2009). All opiate results attained by the CTL were kept blind until analysis by the PSi sensor was complete (October, 2009). Current CTL standard opiate testing protocols require that specimens screened positive by the opiate cloned enzyme donor immunoassay (CEDIA, Thermo Scientific, cutoff of 300 ng mL^{-1}) must be validated by chemical analysis. Validation with GC/MS facilitates quantification of total morphine/codeine, and semiquantification with LC-MS/MS identifies the presence of specific opiates and their approximate concentration. Requests by doctors for "pain profile testing" require a second immunoassay test (DRI oxycodone, Thermo Scientific, cutoff 100 ng mL^{-1}) upon a negative CEDIA opiate result. LC-MS/MS analysis was also subsequently performed on specimens that screened positive by the PSi sensor and negative by CEDIA to identify the presence of any specific opiates and approximate concentration.

We analyzed 70 deidentified urine specimens that met federal and University of Rochester criteria for exemption as not qualifying as human subjects research (45 CFR 46.102). Five groups of study samples were selected on the basis of their opiate results attained by the CTL standard drug screening procedures (Groups 1−4) and pain profile testing (Group 5): Group 1, negative by CEDIA

(14) Ouyang, H.; Striemer, C. C.; Fauchet, P. M. *Appl. Phys. Let.* **2006**, *88*, 163108.
(15) Ouyang, H.; DeLouise, L. A.; Miller, B. L.; Fauchet, P. M. *Anal. Chem.* **2007**, *79*, 1502–1506.
(16) DeLouise, L. A.; Miller, B. L. *Mater. Res. Soc. Symp. Proc.* **2004**, *782*, A531.
(17) Rong, G.; Najmaie, A.; Sipe, J. E.; Weiss, S. M. *Biosens. Bioelectron.* **2008**, *23*, 1572–1576.
(18) Di Francia, G.; La Ferrara, V.; Manzo, S.; Chiavarini, S. *Biosens. Bioelectron.* **2005**, *21*, 661–665.
(19) Steinem, C.; Janshoff, A.; Lin, V. S. Y.; Völcker, N. H.; Ghadiri, M. R. *Tetrahedron* **2004**, *60*, 11259–11267.
(20) Kilian, K. A.; Böcking, T.; Gaus, K.; Gal, M. J.; Gooding, J. *ACS Nano* **2007**, *1*, 355–361.
(21) DeLouise, L. A.; Miller, B. L. *Anal. Chem.* **2005**, *77*, 3222–3230.
(22) Orosco, M. M.; Pacholski, C.; Miskelly, G. M.; Sailor, M. J. *Adv. Mater.* **2006**, *18*, 1393–1396.
(23) Lin, V. S. Y.; Motesharei, K.; Dancil, K. P. S.; Sailor, M. J.; Ghadiri, M. R. *Science* **1997**, *278*, 840–843.
(24) Bonanno, L. M.; DeLouise, L. A. *Anal. Chem.* **2010**, *82*, 714–722.
(25) Lowe, R. D.; Guild, G. E.; Harpas, P.; Kirkbride, P.; Hoffmann, P.; Voelcker, N. H.; Kobus, H. *Rapid Commun. Mass Spectrom.* **2009**, *23*, 3543–3548.
(26) Lowe, R. D.; Szili, E. J.; Kirkbride, P.; Thissen, H.; Siuzdak, G.; Voelcker, N. H. *Anal. Chem.* **2010**, *82*, 4201–4208.
(27) Back, R. C.; Monforte, J. R.; Poklis, A. *J. Anal. Toxicol.* **2005**, *29*, 675–677.
(28) Abadie, J. M.; Black, D. A.; Garbin, J.; Saxon, A. J.; Bankson, D. D. *J. Anal. Toxicol.* **2005**, *29*, 825–829.
(29) Heit, H. A.; Gourlay, D. L. *J. Pain Symptom Manage.* **2004**, *27*, 260–267.
(30) Kwong, T. C.; Dasgupta, A.; Garg, U. In *Handbook of Drug Monitoring Methods, Therapeutics and Drugs of Abuse*; Dasgupta, A., Ed.; Humana Press: Totowa, NJ, 2008, pp 297−364, 395−406.

($n = 10$); Group 2, "non-negative negative" by CEDIA (absorbance response less than that of the cutoff calibrator (35−85%), but significantly higher than that of negative control, $n = 10$); Group 3, positive by CEDIA and validated by LC-MS/MS and GC/MS to contain only morphine and its metabolites ($n = 10$); Group 4, positive by CEDIA and indicated by LC-MS/MS to contain the presence of multiple combinations of opiates, including morphine, codeine, M3G, HM, HC, DHC, and 6-Mam ($n = 20$); Group 5, randomly selected samples that underwent pain profile testing ($n = 20$). Analysis with the PSi opiate sensor was repeated on 2 to 3 different days with 3 measurements per run.

Opiate Immunoassay. Urine screening for opiates by immunoassay was performed using Microgenics CEDIA reagents (Thermo Scientific) on a Siemens ADVIA 2400 Chemistry System (Siemens Healthcare Diagnostics) according to manufacturer's protocol. The cutoff used was 300 ng mL^{-1}. Urine screening for oxycodone was performed using DRI Oxycodone (Thermo Scientific) on an Olympus AU 400 Clinical Chemistry Analyzer using the manufacturer's protocol. The cutoff used was 100 ng mL^{-1}.

Opiates Confirmation by Chemical Analysis. The LC-MS/MS and GC/MS chemical analysis techniques completed are production clinical assays that have been reviewed and deemed acceptable by New York State Department of Health and College of American Pathology on-site lab inspections as part of accreditation of the Strong Memorial Hospital Clinical Toxicology Laboratory.

LC-MS/MS Opiate Analysis. Sample (0.5 mL) was transferred into a Spin-X centrifuge filter vial (0.22 μm pore, nylon, Pall Life Sciences) and centrifuged (Eppendorf 5417C microfuge, 12 000 rpm, 3 min). To a glass autosampler vial was added 0.1% formic acid (950 μL), internal standard (SKF 525A, 1 μg mL^{-1} in methanol, 10 μL), and filtered urine (50 μL). The vial was capped and vortex-mixed (10 s) and loaded onto an autosampler tray, and 20 μL was injected into HPLC.

Chromatographic separation was conducted with a Shimadzu HPLC System fitted with Phenomenex Onyx C18 guard cartridge (4.6 × 5 mm) and analytical column (3 × 100 mm) maintained at 40 °C. The flow rate was 1.5 mL min^{-1}, and the mobile phase was composed of solvents A (0.1% formic acid in deionized water) and B (0.1% formic acid in acetonitrile). The gradient program is shown in Table S-1, Supporting Information.

Mass spectrometry was performed using an Applied Biosystem 4000 QTrap operated in positive electrospray ionization mode with source potential (5500 V), temperature (650 °C), and curtain gas (20 mL min^{-1}). Each opiate was identified by two transition ions and the retention time; multiple reaction monitoring (MRM) transitions and operating parameters are listed in Table S-2, Supporting Information. The limits of detection (LOD) are listed in Table S-3, Supporting Information.

The calibration standards M3G, morphine, 6-Mam, codeine, HM, HC, DHC, oxycodone, noroxycodone, and oxymorphone, (1000 ng mL^{-1} each) functioned as a single-point calibrator in the assay, allowing semiquantitative estimation of analyte amounts in control and patient samples. Semiquantitation of opiate concentration was based on the response ratios of the abundances of the opiate precursor ion to that of the internal standard for the calibrator sample and the unknown sample.

GC/MS Opiate Analysis. GC/MS was performed on an Agilent 6890 gas chromatograph and 5973 mass spectrometer fitted with a Agilent Ultra-2 (0.2 mm × 12 m) capillary column. Procedures were performed as described in Broussard et al.[31] Briefly, identification and quantification of urine morphine and codeine concentrations were performed in selected ion monitoring (SIM) mode using the following ions of the derivatives: morphine, 364, 477, 478; morphine-d_3, 367, 480, 481; codeine, 395, 282, 396; codeine-d_3, 398, 285, 399. Quantification values are calculated using the regression equation derived from plotting the mass ion ratios (morphine, 364/367; codeine, 395/398) against calibrator concentrations (400, 1000, 2000, and 4000 ng mL^{-1} each of morphine and codeine). The assay was linear between 150 and 10 000 ng mL^{-1} of morphine and codeine, and between-day assay precision (coefficient of variation, CV) at approximately 125% and 40% of the cutoff (2000 ng mL^{-1}) was 2.2 and 1.6% for morphine and 3.5 and 1.7% for codeine.

PSi Opiate Sensor. The development and optimization of the PSi photonic transducer and immunoassay protocol for detecting opiates in urine (including testing of proper controls for assay specificity and sensor output signal drift) were previously described in Bonanno et al.[24] Briefly, a PSi Bragg reflector was etched into n+ (100) crystalline silicon wafers (Sb-doped, 0.01−0.03 Ω·cm) in electrolyte containing Pluronic L31 (0.1%) and hydrofluoric acid (5%). The Bragg reflector architecture consisted of 16 alternating layers of porosity (78% and 92%) with a total thickness (~2.88 μm) measured by scanning electron microscopy (SEM). The average pore diameters for high- and low-porosity layers (106 and 73 nm, respectively) measured by SEM have previously been shown to allow sufficient infiltration of proteins in an immunoassay.[39−41] The wavelength shift sensitivity (WSS, 231.5 nm RIU^{-1}) was determined by tracking the infiltration of liquids with known refractive index (η) values.

The PSi sensor substrate is functionalized by covalent attachment of an opiate-analogue (M3G) via carbodiimide chemistry to lysine groups present on a bovine serum albumin (BSA) blocked surface. Sensors were rinsed with phosphate buffered saline (PBS, pH 7.4) containing 0.05 wt % Tween 20 surfactant and 1 wt % trehalose to maintain protein folding after drying and rehydration.[35,36] All opiate-analogue functionalized sensors were dried under nitrogen gas and stored at 4 °C until ready for use. These initial experiments did not store sensors for more than 48 h; however, future work could assess stability over longer storage periods. All deidentified urine specimens were received frozen and stored at −20 °C. Urine specimens were brought to room temperature and vortex mixed before adding 15 μL to the sensor directly followed by a fixed aliquot of α-M Ab (15 μL, 275 μg mL^{-1}) and incubated (60 min) in a humidity chamber (Figure 1). Sensors were then rinsed three times (0.05 wt % Tween 20 diluted in PBS, pH 7.4) and dried with nitrogen gas. For optical response

(31) Broussard, L. A.; Presley, L. C.; Pittman, T.; Clouette, R.; Wimbish, G. H. *Clin. Chem.* **1997**, *43*, 1029–1032.
(32) Tinsley-Bown, A. M.; Canham, L. T.; Hollings, M.; Anderson, M. H.; Reeves, C. L.; Cox, T. I.; Nicklin, S.; Squirrell, D. J.; Perkins, E.; Hutchinson; Sailor, M. J.; Wun, A. *Phys. Status Solidi A* **2000**, *182*, 547–553.
(33) DeLouise, L. A.; Miller, B. L. *Anal. Chem.* **2004**, *76*, 6915–6920.
(34) Ouyang, H.; Christophersen, M.; Viard, R.; Miller, B. L.; Fauchet, P. M. *Adv. Funct. Mater.* **2005**, *15*, 1851–1859.
(35) Nan, X.; Shumaker-Parry, J. S.; Zareie, M. H.; Campbell, C. T.; Castner, D. G. *Langmuir* **2004**, *20*, 3710–3716.
(36) Jairo, J.; Michel, P. *Curr. Med. Chem.* **2004**, *11*, 439–446.

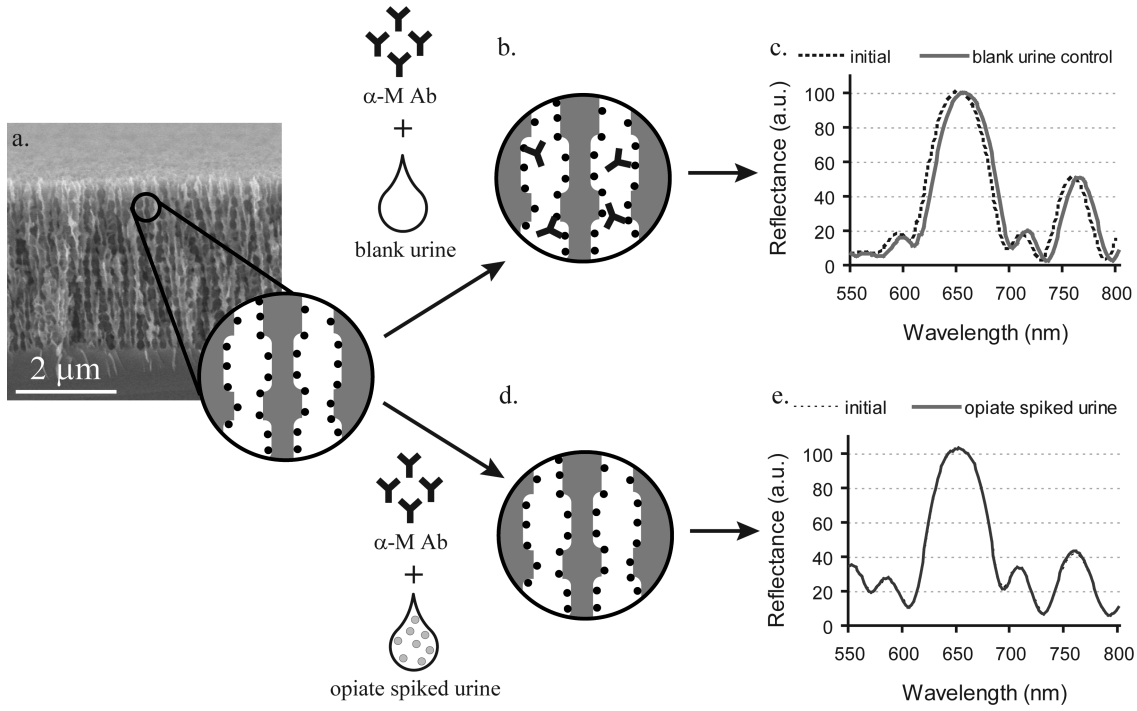

Figure 1. Principle of the PSi sensor technique. (a) Cross-sectional SEM of PSi Bragg mirror architecture. Inset depicts schematic of opiate-analogue functionalized PSi. (b) In the absence of free drug in urine, maximum α-M Ab binds to surface-attached opiate analogue resulting in (c) a maximum wavelength shift response (6.0 ± 0.7 nm). (d) Opiates in urine compete with surface attached opiate-analogue for α-M Ab binding sites. α-M Ab bound to free drug is washed away, resulting in a proportional decrease in the wavelength shift response. (e) Negligible wavelength shift results when a high concentration of oxycodone (3000 ng mL^{-1}) is spiked into urine.

measurement, the PSi sensors were held within a custom fixture and exposed to a normal incident beam of white light (spot size, ~1.3 mm^2). Reflectance spectra normal to the surface were measured using an Avantes 3648-USB2 spectrophotometer (Broomfield, CO) with optical resolution of 0.06 nm pixel^{-1} at three different locations on each sensor. The largest increase in refractive index is attained for blank urine specimens; where maximum Ab binds to the opiate-analogue covered PSi surface and results in maximum wavelength shift of the reflectance spectrum. Free opiates in the urine specimen compete for binding sites on the Ab and a proportional decrease in Ab binding to the opiate-analogue functionalized PSi, producing a corresponding decrease in the wavelength shift.

Dose response calibration curves were created for various drug standards diluted (1−10 000 ng mL^{-1}) in blank urine from healthy volunteers (between day $n \geq 2$, within assay $n = 3$). All data is normalized to the maximum wavelength shift attained for the blank urine control that is concurrently run with the test specimens to improve run-to-run variability. These data were used to create a calibration curve for the PSi opiate sensor and to quantify assay cross-reactivity.

Analytical Methods and Statistical Analysis. PSi sensor wavelength shift data for all reactive opiates were imported into OriginLab 7.1 Data Analysis and Graphing Software (OriginLab Corporation) where nonlinear regression models (95% confidence intervals) were used to fit the data to a nonlinear 4-parameter logistic model[37,38] expressed as:

$$y = \frac{\mathrm{A1} - \mathrm{A2}}{1 + ({}^{x}/_{x_0})^{p}} + \mathrm{A2} \quad (1)$$

where y is the sensor optical response, x is the concentration of total opiates in urine, A1 is the upper asymptote, A2 is the lower asymptote, x_0 is the half maximal effective concentration (EC50), and p is the slope factor. The resulting logistic curve was utilized as a calibration curve from which semiquantitative results are determined (between day $n \geq 2$, within assay $n = 3$).[37,38] The upper limit of the analytical measurement range was determined by the linear portion of the curve, calculated by bend point analysis in MATLAB software (Mathworks Inc., Natick, MA).[39] The lower limit of quantification was determined by the concentration at which the CV and deviation from spiked target concentration were both not more than 20%.[37]

Computations were performed using Microsoft Excel 2008 (Microsoft Corporation) software. Cross-reactivity was evaluated at the cutoff (300 ng mL^{-1}) by dividing the recovered concentration of opiates by the spiked concentration of each compound into blank urine. It is noted that various methods exist for measuring cross-reactivity across the assay range;[40] however, this method was utilized so that a direct comparison could be made with manufacturer's published values for the CEDIA opiates and DRI oxycodone techniques at the cutoff.[41−43]

(37) Linnet, K.; Boyd, J. C. In *Tietz Fundamentals of Clinical Chemistry: Selection and analytical evaluation of methods with statistical techniques*; Burtis, C. A., Ashwood, E. R., Bruns, D. E., Eds.; Elsevier Saunders: St. Louis, 2008; pp 201−228.

(38) Meddings, J. B.; Scott, R. B.; Fick, G. H. *Am. J. Gastrointest. Liver Phys.* **1989**, *257*, G982–989.

(39) Sebaugh, J. L.; McCray, P. D. *Pharm. Stat.* **2003**, *2*, 167–174.

(40) Miller, J. J.; Valdes, R., Jr. *J. Clin. Immunoassay* **1992**, *15*, 97–107.

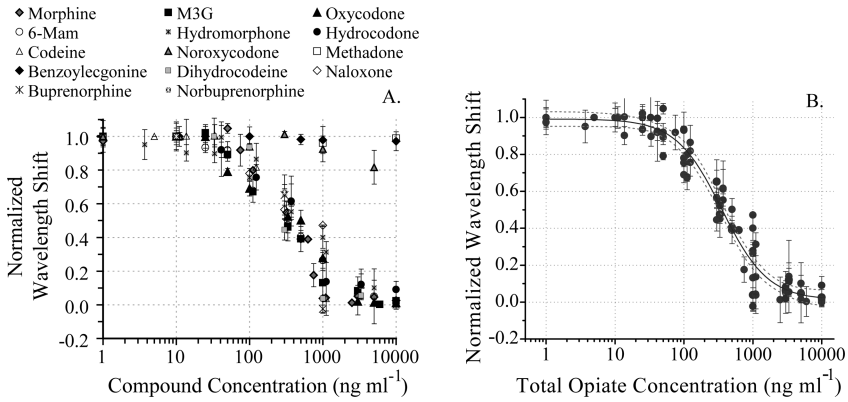

Figure 2. (A) Dose response of PSi opiate sensor to various compounds. (B) PSi opiate sensor calibration curve; 4-parameter logistic fit to dose response of compounds with cross-reactivity >75%: morphine, codeine, M3G, 6-Mam, dihydrocodeine, hydrocodone, hydromorphone, oxycodone, oxymorphone, noroxymorphone, buprenorphine, and naloxone. Solid black line, $y = 0.011 + (0.992 - 0.011)/(1 + (x/0.356)^{1.308}$, $R^2 = 0.956$; dotted black line and all error bars, 95% confidence intervals (between day $n = 3$, within assay $n = 3$).

The 95% confidence intervals of the extrapolated mean concentration of each blind test urine specimen were calculated (between-day $n \geq 2$, within-assay $n = 3$). Both clinical sensitivity (probability of classifying a result as positive) and clinical specificity (a measure of the probability that a test result is negative if the condition investigated is not present) is evaluated in comparison to commonly used CEDIA opiate and verified by LC-MS/MS or GC/MS.

RESULTS

Optical Sensor Response to Morphine as a Model Opiate. The PSi Bragg mirror architecture used for this study (Figure 1a) displays macroscopic pore diameters (106 and 73 nm) and low contrast between alternating high and low porosity layers facilitating sufficient mass transport characteristics for antibody−antigen competitive binding interactions.[32−34] An average maximum wavelength shift of 6.0 ± 0.7 nm (between-day $n = 35$) was attained for control blank urine specimens where maximum α-M Ab bound to the opiate-analogue functionalized PSi sensor (Figure 1b,c). This can be observed in Figure 1c where representative reflectance spectra experimentally attained illustrate a red wavelength shift in the Bragg mirror peak from 650.1 to 656.3 nm. Negligible wavelength shift is observed when high concentrations of reactive opiates are spiked into urine due to competitive binding of the free opiates to α-M Ab binding sites and subsequent washing away of α-M Ab−opiate complexes (Figure 1d). This can be observed in Figure 1e as no shift in the Bragg mirror reflectance peak is observed, with addition of 3000 ng mL^{-1} oxycodone and subsequent washing with PBS (0.05 wt % Tween 20). Initial experiments demonstrated that the optical dose−response behavior was consistent in PBS and blank urine samples with varying pH (5−8; Figure S-1, Supporting Information). All data in the remainder of this study was normalized using the formula (mean specimen wavelength shift/mean blank urine control wavelength shift, with an intra-assay $n = 3$ measurements per PSi sensor to attain mean values) to improve between-day assay variability. A single blank urine control sample was tested daily for normalization.

PSi Opiate Sensor Broad Specificity. Broad specificity of the PSi opiate sensor toward numerous opiates, including oxycodone, was attained (Figure 2A). Calibration curves illustrate similar dose response behavior to morphine, codeine, M3G, 6-Mam, DHC, HC, HM, oxycodone, oxymorphone, noroxymorphone, buprenorphine, norbuprenorphine, and naloxone while diminished response to noroxycodone was found (Figure 2A). Dissimilar chemical structures (benzoylecgonine and methadone) demonstrated minimal dose response in the opiate assay. Table 1 quantifies the cross-reactivity for each spiked compound in urine and compares results to commercial CEDIA opiate and DRI oxycodone assays.[41−43]

Calibration Curve. The dose response of all opiates that displayed ≥75% cross-reactivity (morphine, codeine, M3G, 6-Mam, DHC, HC, HM, oxycodone, oxymorphone, noroxymorphone, buprenorphine, and naloxone; Table 1) were plotted together and fit to a 4-parameter logistic model to create an opiate calibration curve for the assay (goodness of fit, $R^2 = 0.9556$, standard deviations of the residuals, $\chi^2/\mathrm{DoF} = 0.0069$, Figure 2B).[37,38] The linear range of the calibration curve (109−1161 ng mL^{-1}, Figure 2B) included the cutoff limit (300 ng mL^{-1}). For quantitative values of the dose response fit to each individual opiate refer to Table S-4, Supporting Information.

Precision and Accuracy. Within-run and between-day precision was quantified using morphine as a model opiate spiked in urine at three levels. At the cutoff limit (300 ng mL^{-1}) and at 117% the cutoff limit (350 ng mL^{-1}), a between-day CV of <9% and apparent recovery[44] of ≥95% was observed (Table 2). For low morphine concentration (47% cutoff limit, 140 ng mL^{-1}), between-day CV remained below 13% and apparent recovery of 105% was attained. The semiquantitative analytical detection range of morphine was calculated to be 114−1161 ng mL^{-1}.

Method Comparison of Opiate Detection in Authentic Patient Samples. Agreement between results of the CEDIA and the PSi sensor was compared on 70 patient urine samples collected

(41) ThermoFisher Scientific Specialty Diagnostic Groups. CEDIA Opiate Assay Specification Sheet. http://www.thermo.com/eThermo/CMA/PDFs/Articles/articlesFile_10171.pdf (Accessed February 2010).

(42) ThermoFisher Scientific CEDIA Opiate 300 Cross-Reactivity Table. http://www.thermo.com/eThermo/CMA/PDFs/Articles/articlesFile_8379.pdf (Accessed February 2010).

(43) ThermoFisher Scientific Specialty Diagnostic Groups. DRI Oxycodone Assay Specification Sheet. http://www.thermo.com/eThermo/CMA/PDFs/Product/productPDF_10133.pdf (Accessed February 2010).

(44) Burns, D. T.; Danzer, K.; Townshend, A. *Pure Appl. Chem.* **2002**, *74*, 2201–2205.

Table 1. Comparison of Immunoassay Specificity to Various Opiates

	PSi sensor opiates		CEDIA opiates[a]		DRI oxycodone[a]	
compound	spiked conc. (ng mL^{-1})	% cross reactivity	tested conc. (ng mL^{-1})	% cross reactivity	tested conc. (ng mL^{-1})	% cross reactivity
oxycodone	300	111	10 000	3.1	300	100
oxymorphone	300	101	20 000	1.9	300	103
hydrocodone	300	84	300	48	75 000	N[b]
hydromorphone	300	76	300	57	30 000	N[b]
noroxymorphone	300	100	100 000	N[b]	500 000	<0.1
noroxycodone	5000	2	*c*	*c*	50 000	<0.1
morphine	300	99	300	100	350 000	N[b]
codeine	300	98	300	125	500 000	N[b]
M3G	300	131	300	81	900 000	N[b]
6-Mam	300	116	300	81	50 000	N[b]
dihydrocodeine	300	142	300	50	100 000	N[b]
buprenorphine	300	75	100 000	P[d]	40	<0.1[21]
norbuprenorphine	300	69	*c*	*c*	*c*	*c*
naloxone	300	98	6000	N[d]	200 000	N[b]
benzoylecgonine	10 000	0.1	500 000	N[d]	2 000 000	<0.1
methadone	10 000	<0.1	500 000	N[d]	1 000 000	<0.1

[a] Values attained from Microgenics product package insert [29−31] unless otherwise noted. [b] DRI qualitative response is positive (P) or negative (N) at a cutoff of 100 ng mL^{-1} oxycodone. [c] Cross-reactivity data is not available. [d] CEDIA qualitative response is positive (P) or negative (N) at a cutoff of 300 ng mL^{-1} opiates.

Table 2. Precision of the PSi Opiate Sensor Semiquantification of Morphine as a Model Opiate in Urine

	within-assay			between-day		
spiked concentration (ng mL^{-1})	recovery (ng mL^{-1})	CV (%)	*N*	recovery (ng mL^{-1})	CV (%)	*N*
140	148.9	11.2	14	147.0	12.8	7
300	308.9	6.5	14	297.5	8.2	7
350	339.0	5.32	14	332.0	7.6	7

Table 3. Qualitative Result Comparison of CEDIA Opiate Assay, PSi Opiate Sensor, and LC-MS/MS (at 300 ng mL^{-1} cutoff) in Authentic Patient Urine Samples that Underwent Standard Opiate Screening Procedures at Strong Memorial Hospital CTL

CEDIA	PSi Sensor +	PSi Sensor −
+	29	1[a]
−	3[b,c]	17

LC-MS/MS	PSi Sensor +	PSi Sensor −
+	30	0
−	2[c]	1

[a] LC-MS analysis reported 156 ng mL^{-1} morphine in sample # 22. [b] LC-MS analysis reported 552 ng mL^{-1} oxycodone and 320 ng mL^{-1} oxymorphone in sample # 18. [c] LC-MS analysis reported ~50 ng mL^{-1} norbuprenorphine and ~120 ng mL^{-1} naloxone in sample # 11 and <100 ng mL^{-1} naloxone in sample # 12; however, these drugs are not validated procedures and cannot be confirmed.

Table 4. Qualitative Result Comparison of CEDIA Opiate (300 ng mL^{-1} cutoff), DRI Oxycodone (100 ng mL^{-1} cutoff), PSi Opiate Sensor (300 ng mL^{-1} cutoff), and LC-MS/MS (300 ng mL^{-1} cutoff) on Randomly Selected Samples (Group 5) that Underwent Pain Profile Testing at Strong Memorial Hospital CTL

CEDIA	PSi Sensor +	PSi Sensor −
+	10	0
−	7[a]	3

DRI	PSi Sensor +	PSi Sensor −
+	7	3[b]
−	0	0

LC-MS/MS	PSi Sensor +	PSi Sensor −
+	17	0
−	0	3

[a] All of these samples (IDs: 53, 54, 64, 65, 66, 67, and 68; refer to Table S-6, Supporting Information) were screened positive for oxycodone by the DRI oxycodone assay. [b] DRI oxycodone and LC-MS semiquantitative results report an oxycodone concentration of <300 ng mL^{-1} for all three samples (sample IDs 60, 69, and 70 in Table S-6, Supporting Information) so the lower cutoff of 100 ng mL^{-1} of the DRI oxycodone assay compared to the higher cutoff of 300 ng mL^{-1} of the PSi opiate sensor resulted in a discrepancy of qualitative results.

and tested for opiates at the Strong Memorial Hospital CTL (Tables 3 and 4). Analysis of samples 1−50 that underwent normal opiate testing at the CTL (Groups 1−4, Table 3) resulted in 92% qualitative agreement between the CEDIA and PSi sensor. Qualitative comparison with LC-MS/MS results showed 94% concordance with both CEDIA and PSi opiate screening results (Table 3).

Three discrepancies occurred for specimens that were screened as negative by CEDIA and positive by the PSi sensor (Table 3). All three of these samples were considered to be "non-negative negatives" by CEDIA, indicating the presence of opiates above the negative control calibrator (specimens 11, 12, and 18 in Table S-5, Supporting Information). LC-MS/MS confirmed the presence of oxycodone (552 ng mL^{-1}) and oxymorphone (320 ng mL^{-1}) in one of these samples. Higher cross-reactivity to these compounds in the PSi sensor (Table 1) facilitated a positive result (674.9 ng mL^{-1} calibrator equivalent of total opiates). LC-MS/MS analysis of the other two remaining mismatched samples detected levels of buprenorphine (~50 ng mL^{-1}) and norbuprenorphine (~120 ng mL^{-1}, LOD = 30 ng mL^{-1}) in the first and naloxone (<LOD of 100 ng mL^{-1}) in the second. Quantitative analysis of these opiates is not validated on our LC-MS/MS; however, the PSi sensor displays higher cross-reactivity for each of these compounds (buprenorphine 75%, norburprenorphine 69%, and naloxone 98%; Table 1). The final disagreement in which CEDIA was positive and PSi was negative was confirmed by GC/MS to be negative with 268 ng mL^{-1} total opiates.

Qualitative results of Group 5 (samples 51−70), which underwent pain profile testing at the Strong Memorial Hospital CTL, are compared in Table 4 for the CEDIA opiate, DRI oxycodone, and PSi opiate sensor immunoassays and chemical analysis by LC-MS/MS. Chemical analysis with LC-MS/MS indicated variable presence of oxycodone, oxymorphone, noroxycodone, and noroxymorphone (Table S-6, Supporting Information). CEDIA produced ten negative results, of which the subsequent DRI oxycodone assay screened all ten of these samples to be positive for oxycodone (sample IDs: 53, 54, 60, and 64−70, Table S-6, Supporting Information). PSi sensor results were in 85% concordance with the pain profile test results. Discrepancies of qualitative results by DRI oxycodone and the PSi opiate sensor occurred on 3 samples, which were verified by the DRI oxycodone assay and LC-MS/MS to contain <300 ng mL^{-1} total opiates. The lower cutoff used for the DRI oxycodone assay (100 ng mL^{-1}) causes them to screen positive in pain profile test procedures.

DISCUSSION

Label-free PSi optical sensors can simplify and provide cost advantage to current clinical screening methodologies due to their inexpensive fabrication and straightforward optical readout. Stability concerns associated with labels or enzymes are also minimized. Additionally, sensor design was selected so that functionalized (drug analogue bound) PSi sensors do not have surface-bound antibody allowing for more robust storage prior to testing of patient specimens. This is the first demonstration of the use of label-free PSi optical sensors in a blind clinical study and highlights the potential for further technology development and automation.

The broad cross-reactivity of the PSi opiate sensor allowed for improved clinical sensitivity and a reduction in false negative results in screening authentic patient urine samples for opiates when compared with the commercial CEDIA opiate immunoassay. The high cross-reactivity to oxycodone and oxymorphone (Table 1) facilitated improved detection of oxycodone in 5 of the total 70 urine samples (Table 3, 4). However, due to the fixed cutoff of 300 ng mL^{-1} total opiates, the broad PSi opiate assay was less effective in screening for oxycodone than the DRI oxycodone-specific assay that uses a lower cutoff of 100 ng mL^{-1}.

Although more specific assays are better suited for quantification or screening in particular settings, use of a single broad assay is advantageous for screening in a high volume clinical laboratory.[45] Screening for multiple drugs within a class is less expensive than using separate assays (e.g., assay for oxycodone or buprenorphine) or different antibodies in the case of a multiplexed assay for each individual drug.[46] Additionally, in the case of clinical screening for opiates in an emergency setting, identification of a specific opiate is not absolutely necessary for the treatment of opiate overdoses.[45] It is for these reasons that the α-M Ab with broad reactivity to opiates (including oxycodone) was chosen to demonstrate the clinical screening ability of PSi sensor technology. The broad specificity of the α-M Ab was validated in ELISA in our previous work.[24] As is true with any immunoassay technique, higher specificity would be dictated by the choice of a more specific antibody.[46] Therefore, translation of PSi sensor technology in various clinical screening applications would require optimization of immunoassay parameters for the desired antibody.

Future work to extend the dynamic range of the PSi sensor is underway by amplifying the optical response of the sensor upon target binding with integration of responsive polymers. Practically, however, semiquantification with such a broad screening assay would be unfeasible as one expects to trade-off broad specificity with meaningful semiquantification. Quantification is lost as a result of the variable cross-reactivity with different drugs within a drug class. (Refer to Figure S-2, Supporting Information.)[45] Cross-reactivity to various opiate molecules at the cutoff are listed in Table 1, and variability in the dose response of each tested opiate in the PSi sensor can be qualitatively observed in Figure 2A.

An advantage in versatility of the PSi opiate sensor design was demonstrated in our previous work.[24] Capability to tune the opiate detection range was systematically shifted over ∼3 orders of magnitude (5−3100 ng mL^{-1}) by varying the applied urine specimen volume (100−5 μL). This would facilitate the same opiate sensor to screen at multiple cutoffs (e.g., 2000 ng mL^{-1} for forensic screening) with only changing the volume of urine specimen applied and referencing the according calibration curve for the tested specimen volume.

CONCLUSION

This study has shown initial demonstration that the label-free PSi immunosensor technique is suitable to screen for common opiates in real patient urine samples and supports its potential for future development in various clinical assays. In addition to being label-free, the observed high cross-reactivity to oxycodone identifies an advantage of PSi assay over current labeled opiate immunoassay methods.[27,29,30] Although low cross-reactivity to two common non-opiate control analytes in urine (benzoylecgonine, methadone) was demonstrated here, clinical translation would require a more detailed cross-reactivity study for nonopiate analyte interfering agents. Future optimization would also need to reduce assay time and automate sensor response data collection for clinical use. Current work is in progress to achieve label-free colorimetric readout by the naked eye for opiate detection in these photonic PSi sensors for application in point-of-care diagnostics where no optical reader would be required.

ACKNOWLEDGMENT

The authors thank the NIH/NIAID (5K25AI060884) and NIH/NIDA (T32DA007232, F31DA025398) for financial support, NIH/NIDA for their generous gift of M3G compound, Prof. Jean Bidlack and Prof. Benjamin Miller for helpful guidance, and Jennifer Wojakowski and Jeffrey Varley for help in facilitating the blind study at the Strong Memorial Hospital CTL.

SUPPORTING INFORMATION AVAILABLE

The following listed figures and tables: I. Table S-1, HPLC gradient program used in GC/MS; II. Table S-2, operating parameters for tested opiate confirmation by LC-MS/MS; III. Table S-3, limits of detection for various tested opiates by LC-MS/MS procedures. In addition, further quantitative details of the PSi opiate sensor assay results: IV. Table S-4, Logistic fit parameters for various tested opiates response in PSi opiate sensor; V. Table

(45) Hammett-Stabler, C. A.; Pesce, A. J.; Cannon, D. J. *Clin. Chim. Acta* **2002**, *315*, 125–135.

(46) Ellington, A. A.; Kullo, I. F.; Bailey, K. R.; Klee, G. G. *Clin. Chem.* **2010**, *56*, 186–193.

S-5, result comparison of standard opiate testing procedure results (CEDIA opiate immunoassay, GC/MS, LC-MS/MS) at Strong Memorial Hospital CTL to total opiates detected by PSi opiate sensor; VI. Table S-6, result comparison of patient samples that underwent pain profile testing (CEDIA opiate and DRI oxycodone immunoassays and LC-MS/MS) at Strong Memorial Hospital CTL in comparison to total opiates detected by PSi opiate sensor; VII. Figure S-1, Dose-dependent wavelength shift response of PSi opiate sensor to morphine as a model opiate in urine at various pH and PBS; VIII. Figure S-2, Quantitative Comparison of LC-MS/MS and PSi sensor total detection of opiates in urine specimens. This material is available free of charge via the Internet at http://pubs.acs.org.

Received for review July 7, 2010. Accepted October 21, 2010.

AC101804S