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A Microfluidic Technique for Quantification of Steroids in Core Needle Biopsies

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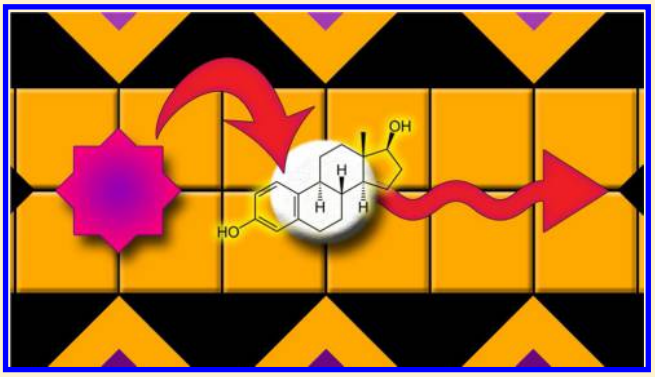
Supporting Information

ABSTRACT: Core needle biopsy (CNB) sampling is known to be inexpensive and minimally invasive relative to traditional tissue resectioning. But CNBs are often not used in analytical settings because of the tiny amount of sample and analyte. To address this challenge, we introduce an analytical method capable of multiplexed steroid quantification in CNB samples—those studied here ranged in mass from 2 to 8 mg. The new method uses digital microfluidics to extract steroids from CNB tissue samples (including a solid-phase extraction cleanup step) followed by analysis by high-performance liquid chromatography tandem mass spectrometry (HPLC–MS/MS). The method has limits of detection of 3.6, 1.6, 5.8, and 8.5 fmol for estradiol, androstendione, testoterone, and progesterone, respectively. We propose that future generations of this method may be useful for regular quantification of steroids in core needle biopsy samples of breast tissue to inform dosage and timing of antihormone or hormone replacement therapies as part of a personalized medicine approach to treating a variety of hormone-sensitive disorders.



Steroid hormones are constitutively involved in a wide range of normal physiological processes, as well as being key players in diseases like breast cancer, prostate cancer, and diabetes.[1,2] In clinical laboratories, there are two principle methods[3,4] used to quantify hormones: immunoassays and liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS). Immunoassays have many advantages, including being simple, inexpensive, and sensitive, but they also suffer from disadvantages such as cross-reactivity, matrix interference, and interlaboratory variation.[4] The biggest drawback for immunoassays is multiplexing—a separate assay must be used for each new analyte that is measured.[5] Methods relying on mass spectrometry, including LC–MS/MS[6] and GC/MS/MS,[7,8] also have advantages (reliability and accuracy) and disadvantages (expense, size, requirement of a trained operator), but they are well-suited for multiplexing—one analysis is often sufficient to quantify many different steroid analytes.[7,8]

Steroid hormones are routinely quantified in blood and serum,[9] but there are many applications for which it is useful to evaluate hormone levels in tissue.[10−12] Traditional methods for this purpose require a surgical biopsy to remove a >500 mg section of tissue,[13] an invasive, costly process that involves a trip to the hospital, anesthesia, and risk of scarring. An attractive alternative to surgical biopsy is the core needle biopsy (CNB), in which a much smaller section (∼1−10 mg) is collected in a physician's office using biopsy needles in few minutes, without general anesthesia and with no risk of scarring.[14−18] Unfortunately, the small size of CNB samples makes them difficult to handle or analyze. As far as we are aware, there have been no previously published reports of the quantification of steroid hormones in CNB samples.

Here we report the first method for quantifying hormones in core needle biopsies. The method relies on digital microfluidics[19] (DMF), a technique in which droplets of samples and reagents are manipulated electrodynamically on an array of electrodes coated with a hydrophobic insulator. The "open" format of DMF makes it particularly well-suited to handling complex samples (for example, dried blood on paper[20−22]) as there are no enclosed channels that might become clogged with

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solids. DMF was recently reported to be useful for extracting steroid hormones from tissue, blood, and serum.[23] Here we report a significant improvement on the previous work, with innovations in sample extraction, cleanup, and derivatization, to enable the first method capable of measuring hormones in CNB samples. To accompany DMF sample processing, a custom HPLC−MS/MS method was developed to allow for the simultaneous quantification of estradiol (E2), androstenedione (AD), testosterone (TS), and progesterone (PG). We propose that this technique and variations thereof may eventually be useful for personalized medicine strategies in which hormone levels are regularly measured to inform dosage and timing of antihormone or hormone replacement therapies. More generally, we propose that these techniques may be useful for a wide range of applications that would benefit from quantification of steroid hormones and other small molecules in CNB samples.

EXPERIMENTAL SECTION

Reagents and Materials. Unless otherwise specified, reagents were purchased from Sigma Chemical (Oakville, ON) including 1,3,5-estratriene-3,17β-diol (estradiol, E2) with purity of 98% and 1,3,5-estratriene-3,17β-diol-16,16,17-d_3 (estradiol-d_3, E2d3) with an isotopic purity of 98%. 4-Androsten-3,17-dione (androstenedione, AD) with purity of >98%, 4-androsten-3,17-dione-2,2,4,6,6,16,16-d_7 (deuteroandrostenedione-d_7, ADd7) with an isotopic purity of >98%, 4-androsten-3-17β-ol-3-one (testosterone, TS) with purity of >98%, 4-androsten-3-17β-ol-3-one-16,16,17-d_3 (deuterotestosterone-d_3, TSd3) with an isotopic purity of >98%, 4-pregnen-3,20-dione, (progesterone, PG) with purity of >98%, and 4-pregnen-3,20-dione-2,2,4,6,6,17α,21,21,21-d_9 (deuteroprogesterone-d_9, PGd9) with an isotopic purity of >98% were purchased from Steraloids (Newport, RI). Parylene-C dimer was from Specialty Systems (Indianapolis, IN), and Teflon-AF was obtained from DuPont (Wilmington, DE). All solvents were HPLC grade (>99.9% purity), and all other chemicals used were of the highest grade available (>98% purity).

For each hormone, a stock solution (1 mg/mL) was prepared in methanol. Working solutions of each standard (10 μg/mL) were formed in methanol by serial dilution. Two stock mixtures were prepared from the working solutions in methanol: mixture A contained the four standard hormones (E2, AD, TS, and PG, 200 ng/mL each), and mixture B contained the four deuterated hormones (E2d3, ADd7, TSd3, and PGd9, 200 ng/mL each). All stock and working solutions were stored at −20 °C.

A C12 casting solution was prepared by mixing 279 μL of butyl acrylate (99%), 150 μL of 1,3-butanediol diacrylate (98%), 69 μL of lauryl acrylate (90%), 2.5 mg of 2,2-dimethoxy-2-phenylacetophenone (99%), and 1 mL of porogen comprising a 4:1:1 ratio of acetonitrile, 95% ethanol, and 5 mM phosphate buffer at pH 6.8.

DMF Device Fabrication, Assembly, and Operation. Two-plate digital microfluidic devices were fabricated in the University of Toronto Nanofabrication Centre (TNFC), using microfabrication techniques described previously.[24] Briefly, top plates were formed from indium−tin oxide (ITO)-coated glass substrates (Delta Technologies Ltd., Stillwater, MN) coated with 50 nm Teflon-AF, and bottom plates were formed from chromium-coated glass substrates (Telic, Valencia, CA) coated with 7 μm Parylene-C and 50 nm Teflon-AF. Bottom plates featured an array of 80 actuation electrodes (2.2 mm × 2.2 mm each) connected to eight reservoir electrodes (16.4 mm × 6.7 mm each). The actuation electrodes were roughly square with interdigitated borders (140 μm peak-to-peak sinusoids), with interelectrode gaps of 30−80 μm. Devices were assembled by joining a top and bottom plate with a spacer formed from five pieces of 3 M Scotch double-sided tape (St. Paul, MN) with a total spacer thickness of 450 μm.

The automated open-source DropBot DMF control system (described in detail elsewhere[25]) was used to control droplet movement on chip. Aliquots of reagents were loaded onto a DMF device by pipetting a droplet onto the bottom plate at the edge of the top plate and simultaneously applying sine wave voltages (~100 Vrms, 10 kHz) between the top plate electrode and successive electrodes on the bottom plate via a custom pogo-pin connector (comprising an array of 120 pogo pins on a printed circuit board, each pin wired to solid-state switches in the DropBot system[25]), to draw the fluid into the reservoir and further manipulate droplet movement. Droplet operation was monitored and recorded with a webcam (LifeCam Studio with frame rate of 30 fps, Microsoft Mississauga, ON).

Porous Polymer Monolith Formation. Porous polymer monoliths (PPMs) were prepared by photopolymerization of a C12 casting solution. An aliquot (100 μL) of the casting solution was sandwiched between two pieces of unpatterned glass slides coated with 50 nm Teflon-AF separated by 450 μm spacers. Polymerization was initiated by exposure to UV radiation (100 W, 365 nm, 5 min).[26] The resulting bulk PPM substrates were ~50 mm diameter, 450 μm high, and had masses of ~30 mg. The 2.5 mm diameter PPM discs were separated from the larger substrates using a manual biopsy punch (Miltex, York, U.S.A.) and then manually positioned onto DMF devices as described previously.[27]

Rat Samples. Rat fat samples were obtained from 12 month old rats at the animal facility in the Samuel Lunenfeld Research Institute at Mount Sinai Hospital. Rats were euthanized without anesthesia using CO_2 for ~4 min; tissue specimens were collected from abdominal region with scissors and were stored at −80 °C until use. Tissue specimens were thawed, sectioned, and weighed (samples ranged from 3.6 to 6.3 mg for DMF-scale and 96−113 mg for macroscale sample processing experiments, respectively) prior to placing them in microcentrifuge tubes. Each sample was then spiked with exogenous hormones by pipetting aliquots of dilutions of mixture A in methanol onto the tissue and allowing it to dry at room temperature (~30 min). For experiments evaluating long-term signal stability and extraction time, the spiking solution was a 25 μL aliquot of a dilution of mixture A (5 ng/mL of each analyte). For experiments evaluating extraction efficiency, the spiking solution was a 2.5 μL aliquot (for DMF experiments) or a 20 μL aliquot (for macroscale experiments) of a dilution of mixture A (100 ng/mL of each analyte). As a control, some samples were spiked with identical volumes of neat methanol containing no analytes.

Human Samples. Ethical approval for the study was granted by the Research Ethics Board of Mount Sinai Hospital (reference no. 07-0015A). Written informed consent was obtained from each participant before enrollment. Breast tissue samples were collected from four different groups of patients: (1) premenopausal women, (2) postmenopausal women [at least 12 months since the last menstrual period that have never taken menopausal hormone therapy (HT)], (3) postmenopausal women on HT (1.0 mg/day 17ß-estradiol, Shire Canada Inc., Saint-Laurent, QC), and (4) postmenopausal women on

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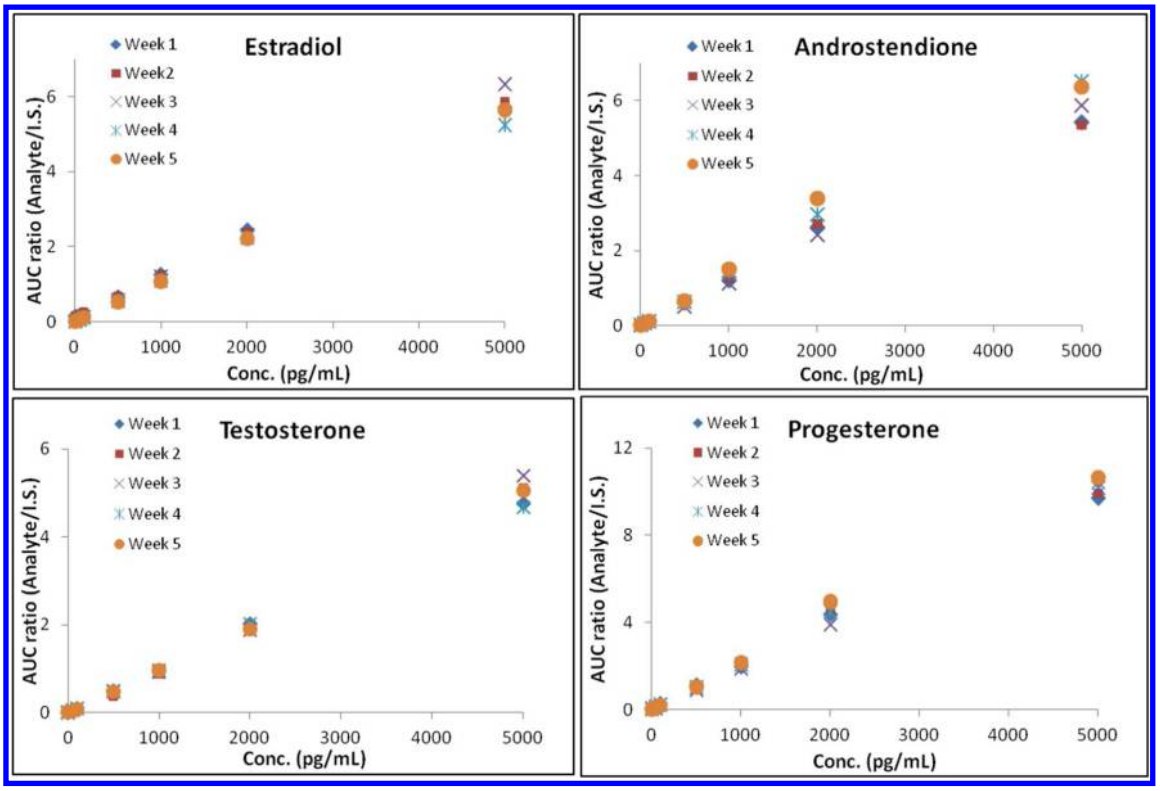

Figure 1. HPLC–MS/MS calibration plots for derivatized estradiol, androstenediol, testosterone, and progesterone obtained in week 1 (blue diamonds), 2 (red squares), 3 (purple X's), 4 (blue asterisks), and 5 (orange circles).

HT plus aromatase inhibitor (AI) therapy (2.5 mg/day letrozole, Novartis Canada, Dorval, Quebec). The samples were collected using Quick-Core Biopsy Needles (QC-16-6.0-20T, Cook Medical, U.S.A.) after local intradermal injection of 0.5 mL of 2% Xylocaine (AstraZeneca, Wilmington, DE). All samples were taken from the right breast at the 10 o'clock position, 5 cm from the border of the areola. The samples were stored in centrifuge tubes at −80 °C until use.

DMF Sample Processing. For each experiment, a tissue sample and a PPM disc were positioned on a DMF bottom plate. An amount of 25 μL of lysing solvent (dichloromethane/acetone 80:20) was pipetted onto the sample and allowed to evaporate (~30 s), and then the device was assembled with a top plate. An amount of 25 μL of a dilution of mixture B (E2d3, ADd7, TSd3, and PGd9, 2 ng/mL each) was loaded into a reservoir and driven onto the tissue sample. The droplet was moved across the tissue sample in a clock-wise circular motion for 5 min, incubated statically for 10 min, and the sample-extract droplet was driven away from the tissue sample and delivered to the PPM disc for cleanup. Prior to this step, the PPM disc was activated by dispensing a droplet of methanol, delivering it to the PPM disc, incubating for 5 min, and driving the remaining methanol to waste. The sample-extract droplet was actuated back and forth across the PPM disc for 2 min before it was driven away to a collection reservoir.

The process described above was repeated for four times, generating four separate extract-droplets, which were typically pooled and allowed to dry at room temperature for ~30 min. In some experiments, each extract droplet was collected and analyzed individually. The top plate was removed, and the dried samples were reconstituted in a 25 μL aliquot of a 1:1 mixture of 1 mg/mL dansyl chloride in acetone and 100 mM sodium bicarbonate (pH 10.7). This solution was transferred to a capped vial and incubated in a 60 °C water bath for 5 min, then mixed with 25 μL of 50:50 methanol/DI water and transferred into a 96-well plate for analysis.

Macroscale Sample Processing. Macroscale tissue samples (spiked and unspiked with exogeneous analytes, as described above) were processed according to an adaptation of a previously reported method.[28] Briefly, each sample was ground and suspended in a 20 μL aliquot of a dilution of mixture B (20 ng/mL of each deuterated hormone). A 4 mL aliquot of methanol was added, and the resulting suspension was mechanically homogenized and centrifuged (1200*g*) for 5 min. The supernatant was transferred to a new tube and evaporated to dryness under a stream of nitrogen at 40 °C. Each dried sample was reconstituted in a 200 μL aliquot of a 1:1 mixture of 1 mg/mL dansyl chloride in acetone and 100 mM sodium bicarbonate (pH 10.7). This solution was incubated in a 60 °C water bath for 5 min in a capped vial, then mixed with 200 μL of 50:50 methanol/DI water and finally transferred into a HPLC vial for analysis.

HPLC–MS/MS. A QuattroMicro triple quadrupole mass spectrometer (Waters, Milford, MA) was operated in multiple reaction monitoring (MRM) mode using MassLynxv4.0 (Waters). Electrospray ionization (ESI) was used in positive ion mode. The capillary voltage was 3.5 kV, the extractor cone voltage was 3 V, and the detector voltage was 650 V. The cone voltage ranged from 30 to 50 V (Table S1 in the Supporting Information). Cone gas flow was 30 L/h, desolvation gas flow was 600 L/h, and the source temperature and desolvation temperatures were 100 and 400 °C, respectively.

Chromatographic separations were performed using an Agilent Technologies 1200 series HPLC system (Santa Clara, California). The column was an Agilent Zorbax Eclipse Plus C18 column (2.1 mm i.d. × 100 mm long, 1.8 μm particle

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diameter) protected by a C18 Zorbax guard column (2.1 mm i.d. × 12.5 mm long, 5 μm particle diameter, Agilent) and an in-line filter (2.1 mm diameter, 0.2 μm pore diameter, Agilent). The HPLC was operated at ambient temperature in gradient elution mode at a flow rate of 0.1 mL/min. The autosampler injection volume was 20 μL. The gradient started with 50% mobile phase A (0.1% formic acid in DI water) and 50% mobile phase B (0.1% formic acid in methanol), changed linearly to 80% mobile phase B over 2 min, and then changed linearly again to 100% mobile phase B over the next 2 min. Mobile phase B was then held at 100% for 5 min before linearly decreasing back to 50% over 2 min and holding at 50% for 13 min for a total run time of 22 min.

Calibration Curves and Quantitative Analysis. HPLC–MS/MS conditions were optimized using dilutions of hormone mixtures A and B in 50:50 methanol/DI water. Briefly, dilutions of nondeuterated standards at 20, 50, 100, 500, 2000, and 5000 pg/mL were formed from mixture A containing 1000 pg/mL of deuterated standards (from mixture B). MRM transitions of each analyte and labeled internal standard, as well as ionization conditions, collision energies, and retention time windows are listed in Table S1 in the Supporting Information.

Calibration curves were constructed by plotting the ratio of the area under the curve (AUC) for analyte product ions relative to those of their internal standards as a function of concentration. Regression lines were fit to the data, and limits of detection (LODs) were determined as the analyte concentrations corresponding to the signal of the sample blank plus three standard deviation of the blank value. A new calibration curve was generated before and after each tissue analysis (rat or human) for quality control (QC) to evaluate instrument performance and to assess errors related to analytical variation. The average calibration data from the two QC plots (collected before and after the sample) were used to quantify the extracted hormones in each tissue sample.

RESULTS AND DISCUSSION

HPLC–MS/MS Analysis of Steroid Hormones. The primary goal of this work was to develop means to quantify steroid hormones in CNB tissue samples. Four hormones, estradiol, androstenedione, testosterone, and progesterone, were chosen as test candidates, given the importance of these analytes in breast cancer.[29,30] AD, TS, and PG are readily ionizable by ESI and thus are compatible with HPLC–MS/MS analysis of trace amounts of analyte.[31] In contrast, the ionization efficiency of E2 (even in negative ESI mode) is relatively low because of its phenolic functional group.[32,33] To improve ionization efficiency of E2, we developed a method relying on derivatization with dansyl chloride following sample extraction, where the phenolic group of E2 is modified with a dansyl moiety bearing a readily ionizable secondary amine. Figure S1 in the Supporting Information demonstrates the difference in signal intensity observed for (1) dansylated E2 in positive mode and (2) underivatized E2 in negative mode. As shown, the signal intensity of dansylated E2 is ∼30 times higher than that for underivatized E2. The strong signal of dansylated E2 allowed for the development of a sensitive method with low limit of detection (described below).

While dansylation of estradiol has been reported previously,[34−37] it has only been used in applications in which the analytes to be evaluated were all estrogens (i.e., E2, estrone, and estriol), not a mixture of estrogens, progestogens, and androgens (as desired for this work). Importantly, the dansylation derivatization procedure was found to not affect the signals for AD, TS, and PG. In the work reported here, the E2 derivatization was implemented off-chip (to avoid problems associated with evaporation of acetone at elevated temperature), but in the future, we propose to implement this step on-chip in a miniature sealed chamber (as described previously[38]) for a completely automated method.

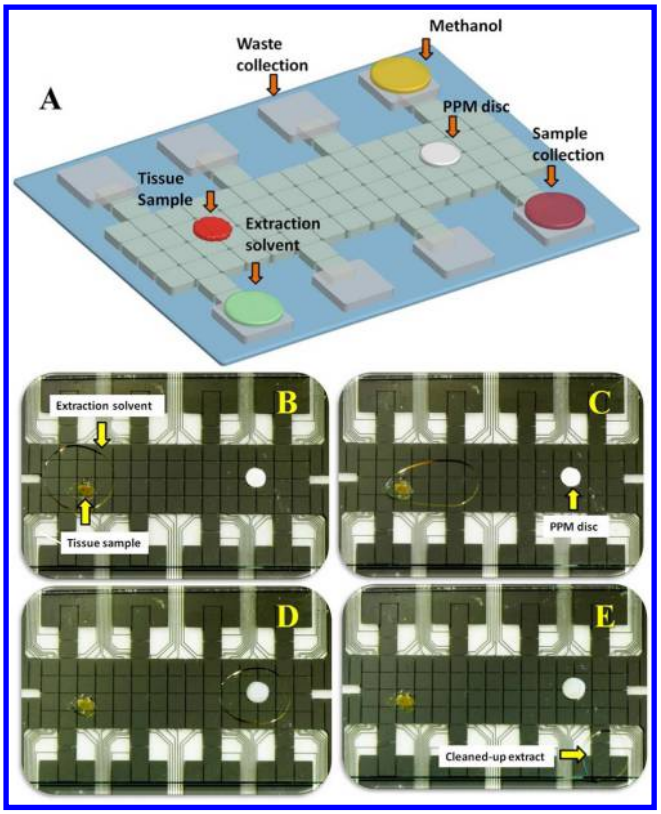

Figure 2. Digital microfluidic extraction/cleanup from tissue samples. Schematic of the device (A) and frames from a movie depicting extraction from tissue sample (B and C) and cleanup on a porous polymer (PPM) disc for solid-phase extraction (SPE) (D and E).

Armed with a method for selective derivatization of E2, an HPLC–MS/MS method was developed for simultaneous quantification of the four analytes (Table S1 in the Supporting Information). The method was validated by running a series of dilutions of a mixture of native hormones (mixture A) containing deuterated internal standards at 1000 pg/mL (mixture B) as described in the methods section. The ratio of AUC for each standard relative to its deuterated IS was plotted as a function of standard concentration, and the results are described in Table S2 in the Supporting Information. Good linearity was observed for all four analytes; linear regression equations were generated with R^2 values ranging from 0.9967 to 0.9997 for all of the hormones. The LODs/LOQs were 3.6/13.2, 1.6/5.4, 5.8/20.2, and 8.5/27.0 fmol for E2, AD, TS, and PG, respectively.

Calibration plots were repeatedly generated on multiple days (and with multiple operators), and the mean interday and intraday precision was 4.6% and 5.0% RSD. Five such plots collected over the span of 5 weeks (during which tissue samples were evaluated, as described below) are shown in Figure 1. The bias in slope was less than 10% for all analyses, with the highest agreement for the (most relevant) lower concentration range (as is often the case in such analyses[39]). In practice, for each

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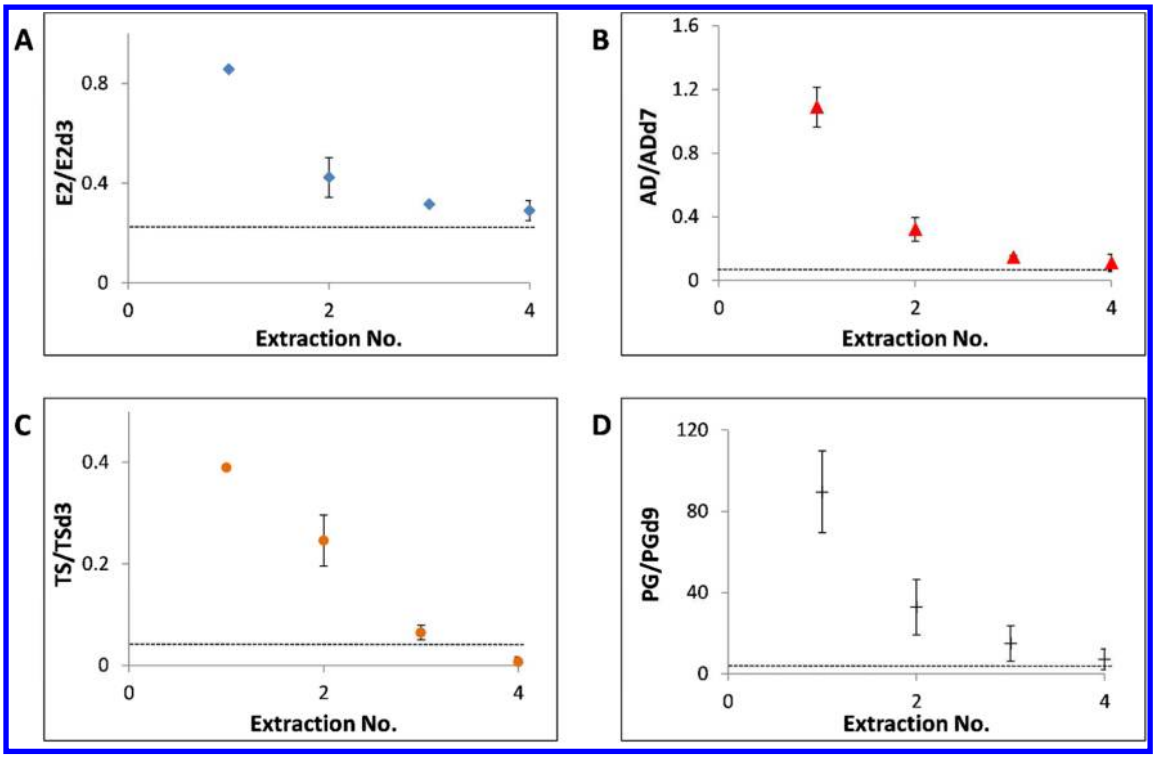

Figure 3. Extraction time. HPLC−MS/MS MRM chromatogram area under curve (AUC) ratios of standard (spiked in ∼5 mg rat tissue samples spiked with 25 μL of 5 ng/mL of each hormone) to deutrated IS (dissolved in extraction solvent) for (A) dansylated E2 (blue diamonds), (B) AD (red triangles), (C) TS (orange circles), and (D) PG (black crosses) after extraction from rat tissue samples by DMF. Data are plotted as a function of extraction number, where each number represents one of four sequential droplets (i.e., droplets 1, 2, 3, and 4) used to extract analytes from a given sample in succession. Each condition was repeated three times, and error bars represent ±1 SD. The black dotted lines represent the AUC ratios of blanks.

tissue sample analyzed, a separate calibration plot was collected before and after analysis as a measure of quality control.

There have been several methods reported previously[28,40−50] for the analysis of steroid hormones by HPLC−MS. There are only four previous reports (those of Guo et al.,[40] Koal et al.,[41] Kinoshita et al.,[50] and Gaikwad[28]) of methods capable of quantifying the four analytes described here (E2, AD, TS, and PG) simultaneously. These techniques are useful, but they require large sample volumes (200 μL,[40] 500 μL,[41] 1 g,[50] and 200 mg[28]), making them a poor match for analyzing very small samples. The new method reported here was specifically designed to be compatible with microfluidic-processed ∼5 mg core needle biopsy tissue samples, as described below.

On-Chip Extraction and Cleanup. A digital microfluidic device (Figure 2A) was designed for extraction of hormones from approximately milligram size tissue samples and in-line sample cleanup. In a typical experiment, a tissue sample is placed onto a device, and a droplet of extraction solvent (neat methanol containing internal standards) is driven onto the tissue sample and then incubated to extract the exogenous analytes (Figure 2B−C). After extraction, the droplet is driven to a PPM disc to remove unwanted nonpolar constituents by solid phase extraction (SPE) (Figure 2D−E), and then the purified sample is driven to a collection reservoir. This extraction and cleanup process is repeated multiple times, and the pooled, processed sample is collected for off-line dansylation and analysis by HPLC−MS/MS. In practice, the process requires <2 h from sample to analysis; in the future, the extraction and purification might be implemented in parallel (as has been reported for DMF dried blood spot analysis[22]) to improve throughput.

A series of ∼5 mg samples of abdominal rat fat with spiked with exogenous E2, AD, TS, and PG (designed to mimic CNB samples from human breast tissue) were used to optimize the method shown in Figure 2. Three key parameters were evaluated: long-term signal stability, extraction time, and extraction efficiency. For the first parameter (long-term signal stability), in initial tests with rat fat samples, it was observed that analyte peak intensities decreased steadily over time (on the scale of days−weeks) if samples were evaluated directly after extraction (with no SPE purification step). We hypothesized that this was caused by ion suppression and instrument fouling from tissue constituents other than the analytes. To circumvent this problem, a SPE process was developed (Figure 2, parts D and E), building on recent reports of the use of PPM discs[26,27] for DMF-SPE. Note that the method used here is different from "standard" SPE techniques (and those reported previously[24,25,51]) in that the analytes remain in solution, and the putative interfering agents (e.g., endogenous amines, carbohydrates, or lipids) are retained by the solid matrix, which bears C12 moieties. Mass spectra demonstrating the effect of the cleanup procedure are shown in Figure S2 in the Supporting Information. The addition of the SPE step has modest effect on analyte peak heights (labeled 1, 2, and 3), but significantly reduces the peak heights of a series of unidentified interferences (m/z 400−700). Most importantly, upon inclusion of the SPE step into the standard analysis method, mass spectral signal intensities were observed to be stable for several weeks of replicate analyses.

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Table 1. Measured Hormone Amounts in Core Needle Biopsy Breast Tissue Samples from Human Subjects Normalized by Sample Mass[a]

	E2 (fmol/mg)		AD (fmol/mg)		TS (fmol/mg)		PG (fmol/mg)
CNB sample no.	raw	corrected	raw	corrected	raw	corrected	raw
1	10.0	15.4	12.5	15.5	10.7	12.0	6.5
2	4.8	7.4	17.1	21.3	15.0	16.9	8.5
3	4.6	7.0	5.0	6.2	12.6	14.2	6.6
4	5.4	8.3	8.0	10.0	3.7	4.1	44.0
5	6.4	9.9	11.3	14.1	10.8	12.1	4.4
6	21.5	33.0	5.9	7.4	5.3	6.0	52.8
7	22.9	35.1	8.5	10.6	32.4	36.3	11.7
8	11.6	17.8	5.6	6.9	20.9	23.5	11.2
9	7.8	12.0	13.4	16.7	4.2	4.7	6.7
10	6.4	9.9	2.5	3.1	22.2	24.9	2.2
11	3.9	6.0	4.5	5.5	15.2	17.0	3.6
12	25.5	39.0	4.0	5.0	15.1	17.0	3.6
13	7.0	10.8	1.6	2.0	10.2	11.5	0.9
14	26.7	40.9	8.2	10.2	40.4	45.3	5.0
15	4.6	7.1	2.8	3.5	7.6	8.5	2.8
16	19.4	29.7	4.5	5.6	22.9	25.7	1.8
17	56.6	86.8	1.8	2.3	7.5	8.4	13.8
18	47.0	72.1	4.2	5.2	9.6	10.8	14.4
19	15.9	24.3	1.0	1.3	3.1	3.5	4.6
20	12.0	18.4	<LOD	<LOD	2.3	2.5	1.1
21	1.1	1.7	0.9	1.2	0.8	0.9	0.8
22	11.0	16.9	2.3	2.9	0.7	0.7	1.1
23	18.1	27.8	9.6	12.0	7.2	8.0	7.4
24	4.8	7.4	2.9	3.6	2.3	2.5	32.5
25	10.5	16.1	7.3	9.1	5.4	6.1	47.1
26	6.0	9.2	9.2	11.5	2.5	2.8	3.6
27	5.3	8.2	4.7	5.8	2.0	2.3	6.1
28	9.5	14.5	6.4	7.9	7.9	8.8	17.3
29	7.4	11.4	5.1	6.3	3.8	4.2	5.6
30	10.7	16.5	2.4	3.0	2.3	2.6	4.7
31	13.1	20.1	2.2	2.7	1.5	1.7	8.1
32	24.9	38.2	2.4	3.0	8.9	10.0	27.1

[a]Raw values are the measured amounts; corrected values are extrapolated from the extraction efficiencies (Supporting Information Figure S3) for E2, AD, and TS, respectively. The subjects were from four groups premenopausal with no treatment (group 1: 1−4), postmenopausal with no treatment (group 2: 5−16), postmenopausal with HT/AI (group 3: 17−23), and postmenopausal with HT only (group 4: 24−32).

For the second parameter (extraction time), samples spiked with exogenous analytes were sequentially extracted in one, two, three, and four droplets of extraction solvent (each with 15 min incubation, as described in the Experimental Section). As shown in Figure 3, for each of the analytes, the amount of signal (measured as the ratio of the AUC for the standard relative to that of the deuterated IS) decreased for each successive droplet, approaching the level of the blank in the fourth extraction. Thus, in all subsequent experiments, four successive extractions (representing a total of 1 h of incubation) were performed and pooled prior to analysis.

For the third parameter (extraction efficiency), in an initial experiment, extracts of nonspiked rat fat samples were generated on chip (as above) and were evaluated using the calibration data (Table S2 in the Supporting Information). The extracts were found to contain undetectable endogenous levels of E2, AD, and TS, but high concentrations (above the calibration range) of endogenous PG. Thus, the samples were deemed to be a good model for evaluating extraction efficiency of E2, AD, and TS, but not PG (in the future, a different type of tissue should be used to extract and quantify PG). A series of tissue samples was then spiked with exogenous E2, AD, and TS, and the recovered concentrations were determined using the calibration data (Table S2 in the Supporting Information). The ratios of recovered/extracted amounts to spiked amounts (i.e., extraction efficiency) were determined after subtracting background levels. For comparison, large sections (∼100 mg each) of identical tissue samples were processed off-chip using conventional techniques.[28] As shown in Figure S3 in the Supporting Information, the extraction efficiencies of the DMF method were 65.3%, 80.3%, and 89.1% (with CVs of 22.5, 21.4, and 11.2) for E2, AD, and TS, respectively. These values were comparable to those of the macroscale technique which had recoveries of 101.7, 79.8, and 102.9 and CVs of 3.7, 27.6, and 18.9 for E2, AD, and TS, respectively. The similarities are remarkable given the differences in size and technique (e.g., microscale samples were not homogenized or centrifuged while macroscale samplers were extensively homogenized and then centrifuged) for the two methods.

The extraction efficiencies described above for the DMF method were deemed acceptable for E2, AD, and TS and were used to correct values obtained for human CNB samples (below). Remaining (unextracted) analytes are likely retained in adipose cells and tissue debris. In the future (if necessary),

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more aggressive cell lysis techniques might be adopted in the DMF workflow, perhaps employing enzymatic digestion.[10]

Analysis of Human CNB Samples. The DMF−HPLC−MS/MS method described above was applied to 32 CNB breast tissue samples. These samples were the first evaluated from a large (and ongoing) study designed to evaluate the effects of aromatase inhibitor (AI) therapy and menopause hormone therapy (HT) on local and systemic hormone concentration in pre- and postmenopausal women. The samples tested here were obtained from four different groups of subjects, including (1) 4 samples from premenopausal subjects not undergoing therapy, (2) 12 samples from postmenopausal subjects not undergoing therapy, (3) 7 samples from postmenopausal subjects undergoing a combination of AI/HT, and (4) 9 samples from postmenopausal subjects undergoing HT. The results were normalized to the mass of the samples and are listed in Table 1.

As shown in Table 1, a "raw" result (i.e., the amount measured) for each analyte is listed for each sample. In an ideal case, standard addition analysis (comprising splitting each sample and spiking different amounts of analyte) would be used to convert the raw value to an actual concentration in the sample; unfortunately, CNB samples are too small to split and analyze by standard addition. Thus, in the work described here, "corrected" values for E2, AD, and TS were extrapolated using the extraction efficiencies determined from rat tissue samples as a proxy (Figure S3 in the Supporting Information). For PG (the only analyte for which an extraction efficiency could not be determined in rat tissue samples), only the raw result is listed. In future work, human breast tissue samples will be obtained to measure extraction efficiencies in samples that more closely match the matrix of CNB samples. But we propose that the technique used here (with correction from rat tissue measurements) is sufficient to confirm that this type of analysis is possible.

A one-way analysis of variance (ANOVA) was conducted to compare the corrected values of E2, AD, and TS (and the raw values of PG) for the four experimental groups. For AD and TS, the ANOVA differences were significant, and individual t tests were performed to identify the affected groups. For AD, the average value for group 1, 13.3 ± 6.6, was significantly greater than the average value for group 2, 7.5 ± 4.5 ($p < 0.05$). For TS, the average value for group 2, 19.4 ± 12.4, was significantly greater than the average value for group 3, 4.5 ± 4.0 ($p < 0.01$), and group 4, 4.6 ± 3.05 ($p < 0.01$). No statistical differences were observed for E2 or PG. In general, the sample size numbers for each of the groups described here is small, so any potential conclusions that might be drawn are tenuous. Regardless, these data prove the principle that measuring hormone levels in CNB samples is possible, and opens the door for future studies with larger sample sets.

When evaluating the entire group of samples tested here (n = 32), the ratios between the average raw measured levels of hormones, roughly 2:3:4:5 for AD/TS/PG/E2 (all in the same order of magnitude), are quite different than the relative levels reported[52,53] for the same hormones in circulation (serum) in similar patient groups. This suggests that the assumption that circulating hormone levels (which are routinely measured) are replicated in tissue (which is not routinely measured) is a poor one. The hormone levels in breast tissue are particularly important for patients undergoing AI therapy and/or HT; thus, we propose that in the future, variations on the method described here may be useful for a personalized medicine approach to dosing and timing of these therapies.

CONCLUSION

We report the first technique capable of quantitation of steroid hormones in core needle biopsy samples, relying on digital microfluidics, solid phase extraction, analyte derivatization, and HPLC−MS/MS. We propose that this type of method will be useful in the future for a wide range of applications, including personalized approaches to diagnosing and treating hormone-sensitive cancers.

ASSOCIATED CONTENT

Supporting Information

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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