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High-Throughput Sample Preparation and Analysis Using 96-Well Membrane Solid-Phase Extraction and Liquid Chromatography-Tandem Mass Spectrometry for the Determination of Steroids in Human Urine

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A 96-well solid-phase extraction (SPE) system is used to rapidly prepare human urine samples for high-throughput quantitative analysis of two steroids, equilenin and progesterone, by liquid chromatography-tandem mass spectrometry using deuterated estrone as the internal standard. We define high-throughput here as analysis of 384 samples in a 24 h period. A total of 384 samples and standards were extracted by an individual in one day and subsequently analyzed within a 24 h period. The inter- and intratray accuracy and precision obtained over the course of these injections was within 8% coefficient of variation when analyzed by atmospheric pressure chemical ionization mass spectrometry using positive ion detection. A semiautomated sample processing workstation was used to add internal standard and then process 96 samples at a time. The recovery of the analytes from the SPE was approximately 85%. The accuracy and precision obtained was comparable to that ordinarily obtained using manual sample preparation techniques. (J Am Soc Mass Spectrom 1999, 10, 1322–1327) © 1999 American Society for Mass Spectrometry

New techniques in synthetic organic chemistry have created the ability to synthesize large libraries of compounds for biological activity screening. Many high-throughput techniques and screening assays have been developed and automated to expedite lead candidate selection in drug discovery [1]. This capability has led to an even greater need for rapid toxicological and pharmacokinetic evaluation of lead drug candidates and, consequently, for rapid sample preparation and analysis. Two developments that have aided in rapid sample preparation and analysis are the 96-well format for solid-phase extraction (SPE) and atmospheric pressure ionization-tandem mass spectrometry. Although the 96-well format has been utilized for many years for immunoassays and in vitro receptor binding studies, its use for SPE is relatively new [2,3]. The ability to rapidly process large numbers of samples in an automated fashion can shift the analysis bottleneck from sample preparation to analysis itself. High-throughput liquid chromatography-tandem mass spectrometry (LC/MS/MS) analyses can accommodate short chromatographic runs at the expense of good chromatographic separation. Because the quality of analytical results are tantamount to speed, sacrifices in chromatographic separation must also accommodate situations where drug metabolites or sample matrix interfere with the accurate determination of analytes [4]. In many cases, however, after identification of major metabolites, the selectivity of tandem mass spectrometry will allow the use of much shorter chromatographic run times.

To determine what the current limitations are with regard to high-throughput sample preparation and LC/MS/MS analysis, we chose to evaluate semiautomated robotic sample preparation in the 96-well format. One goal was to determine the accuracy and precision values that could be obtained using 96-well SPE when analyses are performed with large numbers of biological samples and over a continuous 24 h period. It was also of interest to determine if other problems might arise when performing hundreds of analyses in a relatively short period of time. To test this, samples were extracted in four 96-well blocks, each containing standards and QC samples. The results were compiled both as individual "runs," or blocks, and as one large run of 384 samples. The samples were prepared and analyzed in a fashion that would comply with commonly accepted good laboratory practices (GLP) criteria used in validation of bioanalytical methods.

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Steroids were chosen which are found in females due to an interest in estrogens and their known influence on breast cancer risk in women [5,6]. An increase in breast cancer risk has been associated with higher lifetime exposure to estrogens [7]. At the same time, estrogen replacement therapy (ERT) has a long history of use in the United States for the treatment of postmenopausal symptoms and is showing additional benefits in reduced incidence of heart disease and osteoporosis [8]. Because there may be an increased need for analyzing large numbers of female urine samples for estrogen monitoring in the future, the described strategy was undertaken to possibly serve this potential need.

Experimental

Chemicals

Steroid standards (progesterone, equilenin, 16 hydroxyestrone) were purchased from Steraloids (Wilton, NH) and 2,4,16,16 d4-estrone from C/D/N Isotopes (Pointe Claire, Canada). Solvents were obtained from Mallinkrodt Baker (Phillipsburg, NJ) and water collected fresh from a Barnstead (Dubuque, IA) Nanopure water purification system. Formic acid, 88% double distilled, was obtained from GFS Chemicals (Columbus, OH).

Preparation of Standards

Standard solutions were made at a concentration of approximately 1 mg/mL in ethanol. Separate weighings of each compound were used for the quality control standards (QCs) and the calibration standards (CSs). The internal standard, d4-estrone, was then further diluted with water, to a concentration of 625 ng/mL, for addition to each sample. For each block of 96 wells, CSs were placed at the beginning and end by spiking 500 μL of blank male urine with 10 μL of an appropriate ethanol-diluted standard solution. Urine CSs were prepared to give concentration levels of 0, 1, 3, 10, 30, 100, 300, and 1000 ng/mL. QCs were prepared in the same fashion at levels of 8, 50, and 500 ng/mL with six replicates being interspersed among samples, and between each CSs series. The same female pregnancy urines were used as samples for each of the four 96-well blocks.

The 96-well SPE blocks were obtained from 3M Corporation (St. Paul, MN). Polypropylene deep-well blocks were obtained from Chrom Tech. (Apple Valley, MN). The HPLC system used was comprised of two Shimadzu 10AD pumps, an SCL-10A System controller, and a SIL-10A autoinjector upgraded with an MTP-96 ROM chip (Shimadzu Scientific Instruments, Columbia, MD). The latter allows injections to be made directly from a 96-well microtiter plate on the SIL-10A autoinjector. The column used was a Keystone Scientific (Bellefonte, PA) Javelin guard column (2 mm i.d. $\times$ 20 mm) packed with Betasil C8, 5 μm particles. Solvent A was a solution of 10% methanol in water, whereas solvent B was 90% methanol in water. Both solvent mixtures had formic acid added to a final concentration of 0.05% by volume. Isocratic conditions of 65% B, 35% A were used at a flow rate of 400 μL/min.

A Waters (Milford, MA) pneumatically actuated switching valve was used to direct column effluent away from the mass spectrometer during the first 0.65 min of each run and before the analytes of interest eluted. Timing of these events was controlled using the Shimadzu system controller.

Mass Spectrometry

All analyses were performed on a PE Sciex (Concord, ON) API 300 tandem mass spectrometer operated in the atmospheric pressure chemical ionization (APCI) mode with positive ion detection. The heated pneumatic nebulizer was operated at a temperature of 480 °C using a nitrogen gas flow of 2.5 L/min. The mass spectrometer was calibrated using a 10 μM solution of PPG 425 to obtain peak widths of 0.7 u, or less, in both Q1 and Q3 across the mass range of 60 to 800 Da. Additional tuning and optimization of the instrument was performed by infusion of the analytes into the LC mobile phase to obtain optimum settings and sprayer positioning for the selected mass transitions. For all MS/MS experiments the collision gas pressure was set at position "2" on the PE-SCIEX API 300 system while the collision energy was set at 25 V. Data was processed using MacQuan version 1.5 software. For each analyte the protonated molecule served as the precursor ion. A single precursor ion–product ion transition was monitored for each analyte as follows; equilenin, *m/z* 267 to *m/z* 209; d4-estrone, *m/z* 275 to *m/z* 135; progesterone, *m/z* 315 to *m/z* 97. In order to keep scan rates high the internal standard and equilenin were monitored in an initial period using 200 ms dwell times, whereas progesterone was monitored in a second period using a dwell time of 300 ms.

Semiautomated Sample Processing

A Tomtec Quadra 96 Model 320 was used to prepare urine sample extracts in the 96-well format. Deep-well (1 mL/well) microtiter blocks of 96 samples were prepared in the following fashion. First, an accurate 500 μL aliquot of either sample, standard, or QC sample, was placed in appropriate wells of a deep-well block. The block was placed at one station of the instrument and a new set of 96 polypropylene tips was loaded onto the instrument. A new C8 Empore 96-well SPE block was placed on the vacuum manifold and conditioned with 200 μL of methanol followed by 200 μL of water. Immediately after aspiration of the water the vacuum was turned off and the same tips were used to aspirate 400 μL of water containing the d4-estrone internal standard (IS) and dispense it into the block containing the samples. This was followed by a three cycle aspirate

and dispense step to mix IS and sample. Two separate 425 μL aliquots of the resulting solution were applied to the 96-well SPE block and subsequently aspirated through by vacuum. At this point a new set of tips was placed on the 96-well head and three 375 μL aliquots of water were dispensed into each well as a washing step, and aspirated through together. This step was performed twice to give a total water wash volume of over 2 mL. This was followed by a 200 μL wash with 40% methanol in water. The waste collection dish was replaced with a deep-well polypropylene collection block and a single elution with 100 μL of acetonitrile performed. Finally, the 96-well SPE block was removed and 400 μL of 0.2% formic acid in water was added to each well using the same set of tips to give a solution of the analyte in 20% acetonitrile. The collection block was then sealed with a piece of 3M adhesive tape supplied with the SPE blocks, and inserted into the autoinjector.

Results and Discussion

One goal of this work was to develop an analytical strategy for the determination of representative neutral steroidal compounds in urine samples. Our previous work with both corticosteroids [9] and anabolic steroids [10] has highlighted the challenges of identifying and quantifying this class of compounds at trace levels in biological samples. We [11] and others [12] continue to be challenged by the relatively dirty biological extracts when isolating important test articles from biological samples for high-throughput LC/MS/MS applications. Clearly there is a need for improved procedures for characterizing this important group of neutral compounds. Recent studies from van Berkel et al. [13] have employed chemical derivatizations to enhance the detectability of steroidal compounds, while we and others have employed negative ion detection [14] and counterion attachment [15] to enhance the LC/MS sensitivity for neutral chemical entities.

A second goal was to determine how 96-well sample extraction can serve in today's analytical laboratory where high-throughput sample analysis is in demand. There is a growing need to improve sample preparation throughput for analysis of larger numbers of samples in shorter periods of time. Pharmaceutical drug development and drug discovery studies are also requiring more rapid turnaround of analytical results so that subsequent dosing studies can be undertaken. This paper presents a study into the use of multiple 96-well plates, equipped with the Empore disk SPE technology, for parallel sample preparation and subsequent LC/MS/MS analysis of 384 samples in a 24 h period.

Two important steroids were selected for these studies; equilenin, an important component of estrogen replacement therapy, and progesterone, a major steroid component of human pregnancy urine. The product ion mass spectra of these steroids are shown in Figure 1 along with their structures.

In preliminary work it was determined that the best

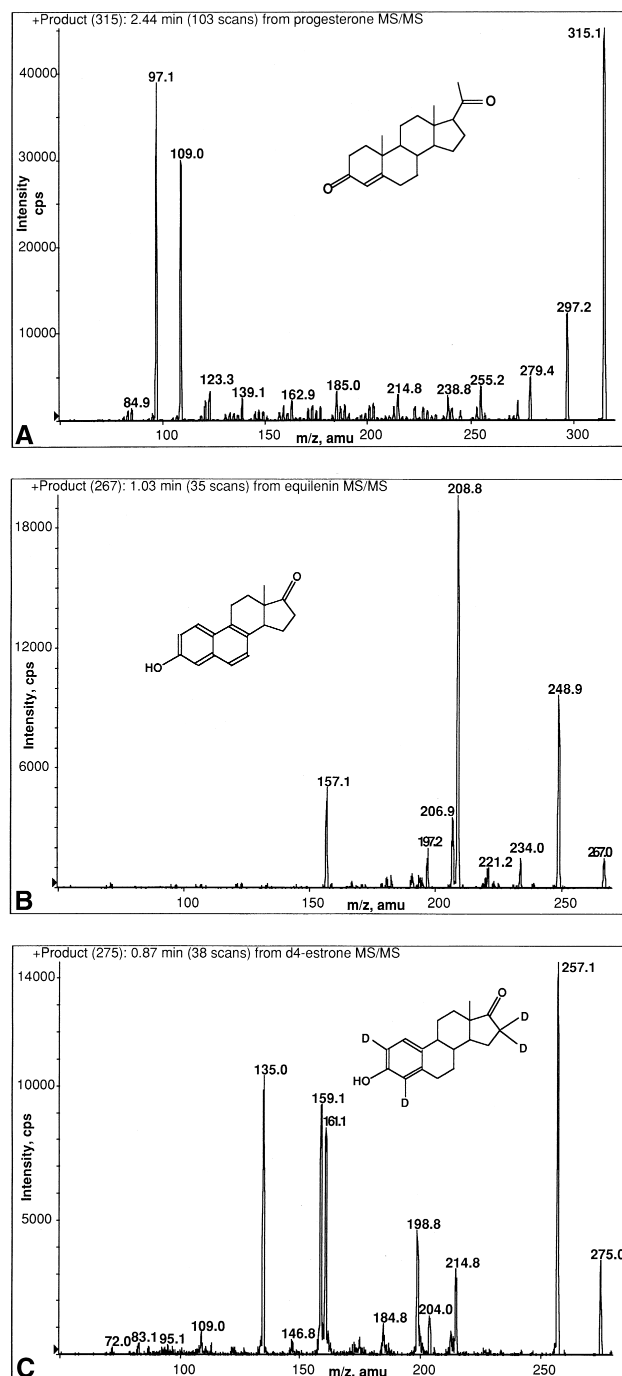

Figure 1. Collision-induced dissociation product ion mass spectra and structures of progesterone, equilenin, and d4-estrone.

APCI sensitivity was obtained using 0.1% formic acid in the mobile phase, with the protonated molecule ion selected as the precursor ion. An optimized SPE extraction method for several steroids was reported previously [16]. Essentially, extraction optimization showed that (1) 200 μL of 30% methanol in water could be used as a washing step following a water wash, (2) a single 100 μL elution with acetonitrile was sufficient to recover 85% of each analyte, and (3) while urine pH had

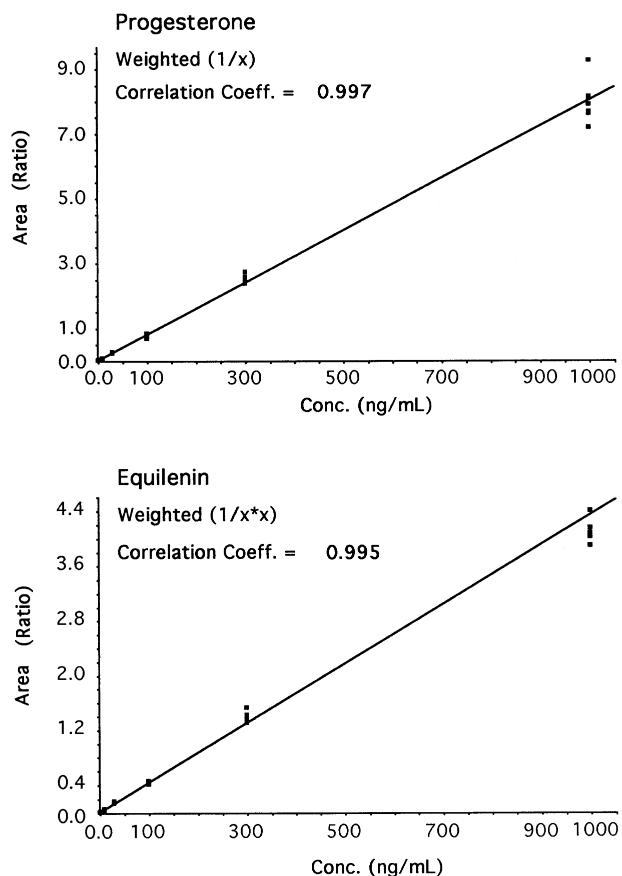

Figure 2. Calibration curves created for equilenin and progesterone extracted from urine using eight replicates at each concentration level and analyzed over a 23.5 h period.

no influence on recovery of analyte it did have a significant influence on retention of unwanted urine components as determined by HPLC with UV detection (220 nm). Decreasing the urine pH from 8 to 2, by adjustment with dilute formic acid prior to SPE, showed increasing amounts of early eluting urine contaminants. In separate experiments the influence of the percentage of organic solvent in the sample solution was evaluated with regard to peak shape. Up to 30% acetonitrile in water gave reasonable chromatographic peak shape while at 40% acetonitrile the peak heights were sharply reduced and peaks broadened. To eliminate a sample blowdown step larger injection volumes (200 μL) of sample were used with a conservative percentage of organic solvent (20% acetonitrile in water) which affected sample focusing on the head of the reversed-phase HPLC column.

A calibration curve for equilenin and progesterone, generated over the 23.5 h analysis time period, is shown in Figure 2. Including each of the eight calibration data points in the fit, correlation coefficients of 0.995, or better, were obtained for the two analytes. For equilenin, only three points out of 56 were excluded from the calibration curve while another one deviated by more than 20% from the expected concentration. For progesterone, three points were excluded with one additional point deviating by more than 20%. Using the calibration standards at the beginning and end of the analysis, e.g., standard sets one and eight separated by 23 h in time, also provided correlation coefficients of sufficiently good fit (0.993 or better) to quantify the samples and QCs. In this case, two points were excluded from the regression (out of 14 total) for equilenin with another two points having deviations of greater than 15%. For progesterone, just one point was excluded with another single point having a deviation of more than 10%. In no case were both points at a single concentration excluded. The agreement seen between CSs analyzed at the beginning and end of the 384 sample run demonstrates the feasibility of performing large analytical runs with standards placed at the beginning and end of the run only. However, in those instances where the developed method is not sufficiently robust to provide reliable performance, it may be recommended to use two sets of standard samples in each 96-well plate. In these latter situations if a subsequent plate fails to meet acceptance criteria, it may be that the analytical determinations from the earlier samples may not be lost.

Table 1. Accuracy of QC determinations using either two or eight replicates at each standard concentration[a]

	QC1 (8 ng/mL)	QC2 (50 ng/mL)	QC3 (500 ng/mL)
(A) Equilenin			
Two standard series	92.2	95.6	94.5
Eight standard series	93.4	98.3	97.3
(B) Progesterone			
Two standard series	93.2	95.4	102.5
Eight standard series	94.7	95.4	102.3

[a]Accuracy is calculated as [1 − (calculated mean/theoretical value)] × 100.

Accuracy values for equilenin and progesterone QC samples are shown in Table 1 for results generated by use of the two extreme calibration standard series and with all eight calibration series. One result of using only two calibration curves is a slight increase in the deviation of the QCs for equilenin. Upon examination it was observed that the first calibration series was somewhat higher in value than the other points of the curve. By inclusion of all eight points the regression is placed more accurately across the points and this is reflected in the lower deviation of the QCs. For progesterone, the deviations had only minor differences between the use of two and eight calibration series.

The results of an analysis of variance (ANOVA) [17] for each analyte, from calculations generated by use of two calibration series, is shown in Table 2. For all QC levels, and for both analytes, there was found to be a significant contribution to the overall variance, at a confidence level of $\propto = 0.05$, as a result of performing the extraction in separate blocks of 96. Thus, despite the fact that all four blocks were prepared by a single

Table 2. Results from one-way analysis of variance (ANOVA)[a]

	Intra-assay precision	Inter-assay precision	*p* value	F_{calc}
Equilenin				
QC1 (8 ng/mL)	6.02%	3.94%	0.0323	4.26
QC2 (50 ng/mL)	3.43%	4.28%	0.0003	3.41
QC3 (500 ng/mL)	2.81%	3.69%	0.0001	7.37
Progesterone				
QC1 (8 ng/mL)	7.98%	5.88%	0.0176	3.57
QC2 (50 ng/mL)	6.24%	3.95%	0.0375	10.36
QC3 (500 ng/mL)	6.33%	6.52%	0.0016	11.34

[a]$F\ critical_{0.05,3,20} = 3.1$.

analyst and analyzed within 24 h, there are additional influences that could be examined in an attempt to achieve lower interassay variability. However, the coefficient of variation (both inter- and intra-assay precision) is seen to be less than 8% in all cases.

Representative chromatograms for a control blank urine followed by QC samples spiked with (A) equilenin and (B) progesterone, at the beginning, middle, and end of the entire analytical run (384 samples) are shown in Figure 3. Retention time, peak intensity, and peak shape reproducibility are seen to be excellent over the course of the entire run. Although some increase in the column pressure was noted over the course of the analytical run, it was not sufficient to cause system failure and could be alleviated by replacement of the frit filter at the beginning of each day or by replacement of the relatively inexpensive analytical, "guard" column altogether.

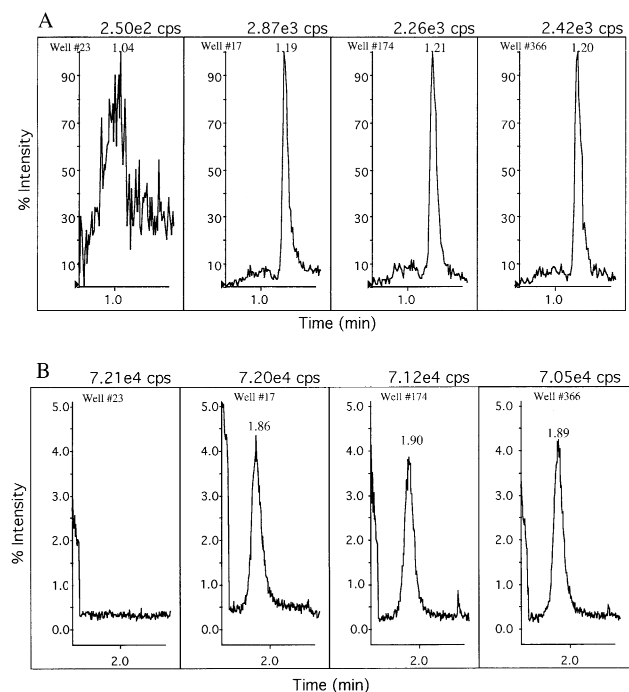

Figure 3. Representative chromatograms of a control blank (Well #23) and of the low QC for (**A**) equilenin and (**B**) progesterone at the beginning (Well #17), middle (Well #174), and end (Well #366) of the 384 sample run.

Conclusions

The purpose of this work was to determine what the current practical limitations are to performing high-throughput sample preparation and LC/MS/MS analysis. For the purposes of this work we define high-throughput as 384 samples prepared and then analyzed within a 24 h period. To evaluate high-throughput sample processing capabilities the 96-well SPE blocks were used in a semiautomated instrument designed to aspirate and dispense solutions using 96 tips at a time. This is in contrast to other types of automated sample processors which prepare four, or eight, samples at a time. Although there are several robotic instruments which provide excellent liquid handling, and which perform extractions without operator attendance, the system used in this work does require operator attendance to control vacuum application to the SPE manifold.

Although there are advantages to increasing the analytical run size there are also risks. For example, in some percentage of analytical runs we might find that the last series of CSs may not produce a sufficiently good fit to quantify the data. In such a case it is of benefit to have multiple calibration curves in each 96-well plate and to use all those which are acceptable up to the point that a failure occurs. The authors are currently evaluating options for decreasing the number of calibration series used in large analytical runs. The benefits of reducing the number of curves per analytical run must be carefully balanced with the risk of the run failing and the consequence of having to repeat the entire run.

An approach has been described for significantly increasing sample preparation and analysis throughput by LC/MS/MS using an APCI source. The results shown demonstrate the level of throughput achievable when chromatographic conditions allow the use of short analysis times.

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