CAPSULES
preliminary notes and applications from Bioanalytical Systems, Inc.

Urinary Estriol By LCEC

Estriol is synthesized in the placenta from the fetal adrenal cortical precursor, DHEA[1]. Urinary and serum levels rise progressively during normal pregnancy, and any decline in estriol (40% from previous determinations[1]) is considered a warning of placental malfunction.

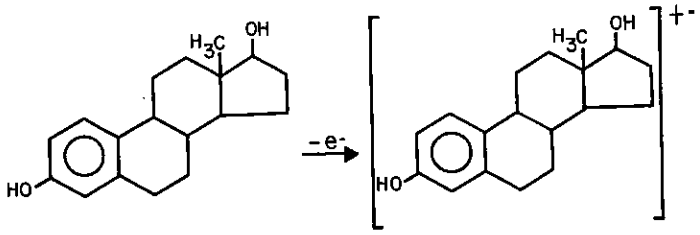

Figure 1. Electrochemical reaction of estriol.

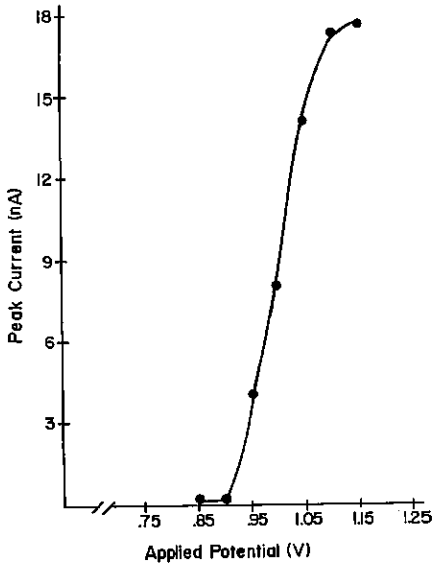

Figure 2. Hydrodynamic voltammogram of repetitive 25 ng injections of estriol.

Estriol in serum is in its unconjugated form, and is found in low ng/mL concentrations. In urine, however, estriol is largly conjugated as the glucuronide and/or sulfate, and excreted levels reach mg/day after the 16th week of pregnancy.

Clinically, urinary estriol levels are of considerable value in the management of "high risk" patients facing complicatons such as diabetes, RH immunizatlon, and fetal growth retardation.[1] Estriol levels (both urinary and serum) are subject to much individual variation. Therefore inter-patient comparisons are of limited utility. One can only compare estriol levels to a "wide range"[1] of values calculated for a particular week of pregnancy (weeks 30-40). It is necessary to perform at least two daily measurements and compare these to previous determinations. Because of this problem, there is a need for an assay that is quick, relatively simple, and reliable for the measurement of urinary estriol.

Liquid chromatography with fluorescence or ultraviolet detection [3-5] and radioimmunoassay (RIA) have been used for estriol determinations. The use of LC with UV detection employs a liquid-liquid extraction technique for the isolation of estriol. However, the sensitivity is poor, necessitating the use of large urine volumes and therefore large volumes of extracting solvents (which can be costly if running many samples). Other LC assays have been reported, but these demonstrated poor extracton efficiencies[4] or significant differences is extraction efficiency.[5] RIA has been quite popular, but the cost per sample using the commercially available kits is high, and disposal of the treated samples is a problem. Others[2] have described the use of LC with electrochemical detection for the assay of urinary estriol.

Electrochemistry Of Estriol

Estriol is electrochemically oxidized at a carbon electrode according to the reaction of F1. One electron is released to form the initial free-radical intermediate. F2 is a hydrodynamic voltammogram of repetitive 25 ng injections of estriol. In order to

reach the limiting current plateau, a high potential (+1.1 V vs Ag/AgCl) must be applied. The Figure does not demonstrate this plateau well because with high applied potentials, oxidation of the mobile phase occurs and high background currents result. The optimum operating potential for any compound is a tradeoff between minimizing the background current and maximizing the limiting current. For the oxidation of the phenolic ring on estriol, an applied potential of +1.15 V was chosen to be the optimum.

Conditions

Liquid Chromatograph: LC-154T or BAS 400 (Bioanalytical Systems Inc.)
Mobile Phase: 50 mM $NaClO_4$, 0.5% $HClO_4$,in 60% MeOH
Flow Rate: 1 mL/min.
Stationary Phase: Biophase OCTYL 5µm (p/n 6032, (Bioanalytical Systems)
Temperature: 30°C
Amount Injected: 10 µL
Electrode: glassy carbon working electrode
Detector Potential: +1.15 volts (vs. Ag/AgCl)
Estriol Stock Solution. Dissolve 10 mg estriol (Sigma) in 20 mL n-propanol.

Procedure

1. To 1 mL of urine, add 180 µL of conc. HCl. Heat for 30 minutes at 100°C in a water bath. Cool.

2. Extract each hydrolyzed urine sample three times with 2 mL aliquots of diethyl ether.

3. Evaporate the combined ether layers under a nitrogen steam at 40-45°C to dryness.

4. Dissolve the residue in 1 mL mobile phase.

5. Inject 10 µL into the LC.

For calibration purposes, synthetic samples consisting of spiked male urine are prepared along with the real samples.

Sample Calculations

A calibration curve using spiked male urine is run. The concentration of an unknown sample is determined from the calibration curve and the unknown's peak height. To calculate mg estriol/day, the volume of the specimen and the total collection time of the specimen must be taken into account.

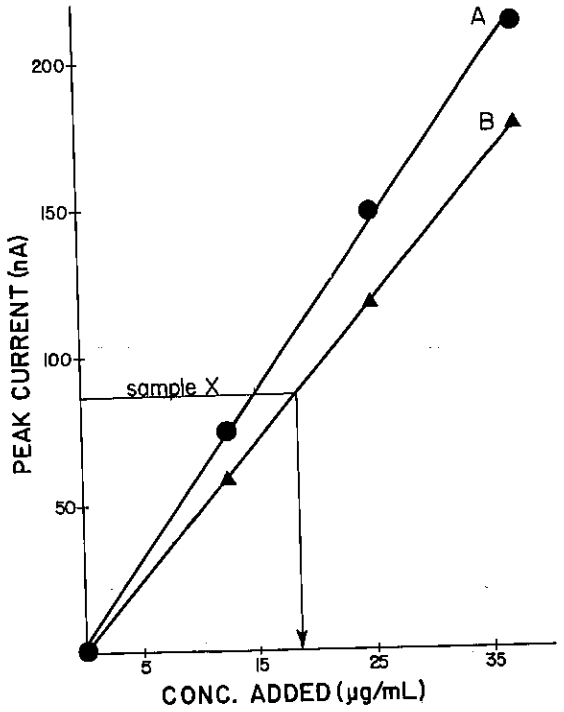

Figure 3. Linearity of LCEC for estriol by the standard addition method. Legend: A = estriol standards, B = male urine.

For example, an unknown urinary sample demonstrated a peak height of 87.2 nA for estriol (refer to F3). This peak corresponds to a concentration of 18.5 µg/mL. Since the specimen was collected over a 103 minute interval and the total volume was 210 mL, the amount of estriol excreted on a mg/day basis is 54.3 mg/day (see T2). The slope and intercept of such a calibration curve must be established by the chemist. The curve given in this note refers only to work done by BAS with a specific LC, samples and methodology.

Results And Discussion

F3 describes the linearity of the system for injections of both standard estriol and spiked urinary samples over the range of 125-400 ng estriol. The extraction efficiency was found to be 80.0 ± 4.1% (n=3). The minimum detectable concentration was 0.4 µg/mL in urine, corresponding to a signal-to-noise ratio of 3.8

for 4 ng injected. Since the volume of the extract equals the original sample volume, no preconcentration is effected. The detection limits in terms of original sample concentration could be lowered dramatically by redissolving the residue in a volume smaller than 1 mL. However, for the purposes of this urinary assay, it was deemed unnecessary. Only 1% of the total estriol extract is injected.

F4A is a chromatogram obtained from a 1 mL urine sample extract, without hydrolysis, and is representative of the amount of unconjugated estriol excreted. F4B was obtained from the same specimen after hydrolysis, representing the amount of total (unconjugated and conjugated) estriol excreted. The large proportion of conjugated estriol is consistent with previous reports.

T1 shows urinary estriol values obtained from two patients, demonstrating the progressive rise in estriol excreted during pregnancy. Assay time for 9 samples requires 1 1/2 hours for batch preparation including hydrolysis, followed by 8-10 minutes of chromatography per sample.

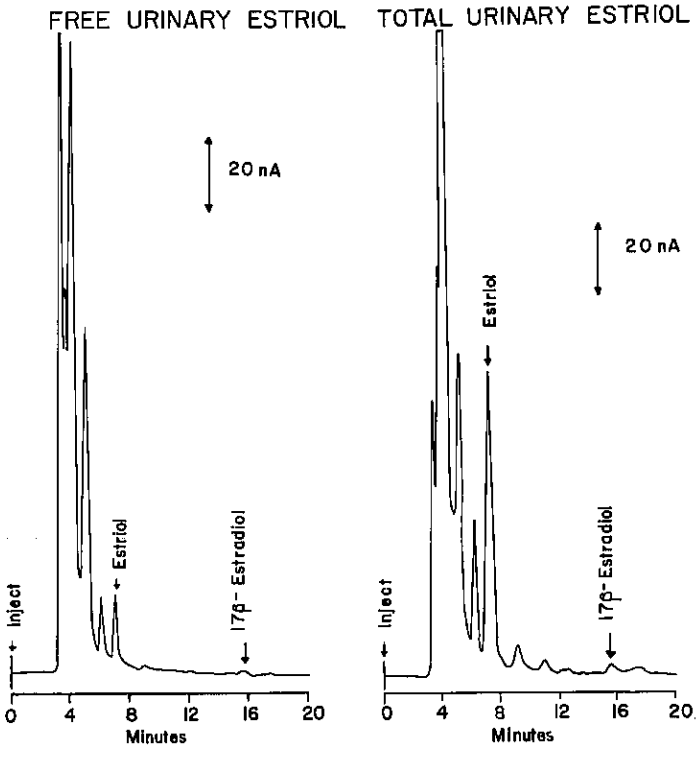

Figure 4. (A) Urinary chromatogram, prior to hydrolysis, 12.1 mg/day. (B) Urinary chromatogram, post-hydrolysis, 53.1 mg/day.

Other solvents were investigated for their extraction efficiency and selectivity in urinary specimens. Ethyl acetate, methylene chloride, chloroform, and a mixture containing 90% methlene chloride/10% isopropanol were investigated, but none were superior to diethyl ether (recoveries range from 20.4 to 64.1%).

To streamline the assay further, a procedure using Clin-Elut® tubes (Analytichem International, Inc., Lawndale, CA) was investigated, again using ether as the extracting solvent. These extraction tubes are packed with cellulosic material. The urine is poured into the tube and the aqueous sample immobilized on the cellulose. Organic solvents may then be poured through, extracting the solutes of interest. However, using this scheme, the recovery of estriol was not as high as the proposed procedure; 73% was extracted.

A mixture of 90% methylene chloride/10% isopropanol was also used as the extracting solvent with these cellulosic tubes. This mixture has been frequently recommended when working with urine and serum specimens containing various drugs. The recovery improved, but the liquid chromatography demonstrated a peak interfering with estriol. To alter the chromatography, a 25 cm cyano column was investigated. The column resolved the interfering peak from estriol, and the recovery increased to 97.0%. To compensate for the more polar bonded phase, less solvent in the mobile phase was necessary: 50 mM $NaClO_4$, 0.5% $HClO_4$, 10% methanol. However, the electrode quickly became passivated because there was not a sufficient concentraton of organic solvent in the mobile phase to adequately rinse the electrode surface. The peak heights were not reproducible during the day, decreasing from previous injections. When the concentration of organic solvent in the mobile phase was increased to overcome this problem, resolution of estriol was lost.

The procedure of choice, therefore, is that outlined in the Experimental section, using the OCTYL column. It provides the required precision and accuracy for assaying large numbers (30) of samples per day.

Liquid chromatography/electrochemistry is a highly useful tool for biomedical research. As with any instrumental technique, the precision and accuracy of the measurement depends on the instrumentation, the skill and knowledge of the operator and the integrity of the sample preparation procedure. Use of these techniques for medical diagnosis and accountability for the same rests entirely with the user of this equipment.

References

1. *The Bio-Science Handbook, 12th edition, Bio-Science Laboratories, Van Nuys, CA.*

2. *O. Hiroshima, S. Ikenoya, M. Ohmae, K. Kawabe, Chem. Pharm. Bull., 28(1980) 2512-2514.*

3. *J.T. Taylor, J.G. Knotts, G.J. Schmit, Clin. Chem., 26(1980) 130-132.*

4. *R.J. Dolphin, P.G. Pergande, J. Chromatogr., 143(1977) 267-274.*

5. *T.H. Mourey, S. Siggia, Anal. Chem., 52(1980) 881-885.*

Table 1. Calculated Urinary Estriol Levels

Patient	Week Pregnancy	Mg/day
JB	19	2.4
JB	24	3.8
CK	38	54.3

Table 2. Sample Calculations

Excretion rate = conc x vol x (time between successive excretions)[1]

$$\text{Excretion rate (mg/day)} = \frac{18.5\ \mu g}{mL} \times \frac{1\ mg}{1000\ \mu g} \times 210\ mL \times \frac{1}{105\ min} \times \frac{1440\ min}{day} = 54.3\ mg/day$$

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