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**Analysis of Endogenous Estrogen Metabolites in Postmenopausal Urine by HPLC-Electrospray Ionization-Tandem Mass Spectrometry**

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**Introduction:**

There is overwhelming evidence indicating that endogenous estrogen levels and patterns of estrogen metabolism play an important role in the development of breast, endometrial, ovarian, and other human cancers. Despite the importance, few studies have compared the prediagnostic exposure levels of endogenous estrogens in cancer patients and matched controls or in low and high risk populations. A major reason for the lack of relevant research in this important area is the absence of suitable analytic methods, especially for postmenopausal women whose estrogen levels are extremely low. We have developed a simple and rapid derivatization that by forming estrogen-*p*-toluenesulfonhydrazones can greatly enhance sensitivity and separability of endogenous estrogen metabolites by HPLC-ESI-tandem mass spectrometry.

**Methods**

Urine samples were collected from healthy postmenopausal women who were not using exogenous estrogens. Ascorbic acid and sodium azide were added to prevent auto-oxidation and bacterial growth. Deuterated estrogen metabolites were used as internal standards. Ten-ml aliquots of pooled urine sample were hydrolyzed by β-glucuronidase/sulfatase followed by one $C_{18}$ SPE and one anion exchange column. *p*-Toluenesulfonhydrazone derivatives were made to improve ionization and HPLC separation of estrogen metabolites. Liquid chromatography was carried out on a reverse phase Luna C18(2) column (150 × 2.0 mm, 3 µm). The mobile phase consisted of methanol and water (6:4) with 0.1% (v/v) formic acid at the flow rate of 200 µl/min. HPLC-ESI-MS-MS analysis was performed on a Finnigan LCQ$^{DECA}$ ion trap mass spectrometer.

**Preliminary Data**

All estrogen metabolites with D-ring carbonyl group were derivatized and gave intense protonated molecular ion [$MH^+$] when analyzed by electrospray ionization. The parent [$MH^+$] ions were selected and fragmented by colliding with helium gas in the ion trap at the relative collision energy of 30%. The mass spectra resulting from these fragmentations were acquired in the full scan mode ranged from *m/z* 100 to 500. The most abundant and specific product ions from each parent ion were chosen for quantitation through selected reaction monitoring (SRM) mode. The monitored SRM transitions for 2-(OH)$E_1$ and 4-(OH)$E_1$ are *m/z* 455→173 and 269; 2-(OH)$E_1$-$d_4$ and 4-(OH)$E_1$-$d_4$ are *m/z* 459→175 and 273; $E_1$ is *m/z* 439→157 and 253; $E_1$-$d_4$ is *m/z* 443→159 and 257; 16-Keto$E_2$ is *m/z* 455→299; 16α-(OH)$E_1$ is *m/z* 455→213, 257 and 284; 2-Methoxy$E_1$ is *m/z* 469→187 and 283. *p*-Toluenesulfonhydrazide derivatization coupled with tandem mass spectrometry has greatly enhanced method sensitivity and specificity. An injection of 25 pg estrogen metabolite on column resulted in the signal to noise ratios typically greater than 120 for 2-(OH)$E_1$, 4-(OH)$E_1$, 16-Keto$E_2$, and 16α-(OH)$E_1$, and greater than 200 for $E_1$ and 2-Methoxy$E_1$, respectively (Figure 1), which provided an adequate lower limit of quantitation for endogenous estrogen metabolite analyses in postmenopausal urine (Figure 2). Standard curves were linear over a 100-fold calibration range (0.5-64 ng each estrogen metabolite per sample) with correlation coefficients for the linear regression curves typically greater than 0.996. The reverse phase liquid chromatography on $C_{18}$ column with a simple isocratic elution gave excellent separation between all analytes of interest. Further validation of the assay is currently being conducted, which will provide us a highly specific and sensitive method for elucidating the mechanism of estrogen carcinogenesis in humans.

**Figure 1. Estrogen Metabolite Working Standard (25 pg each on column)**

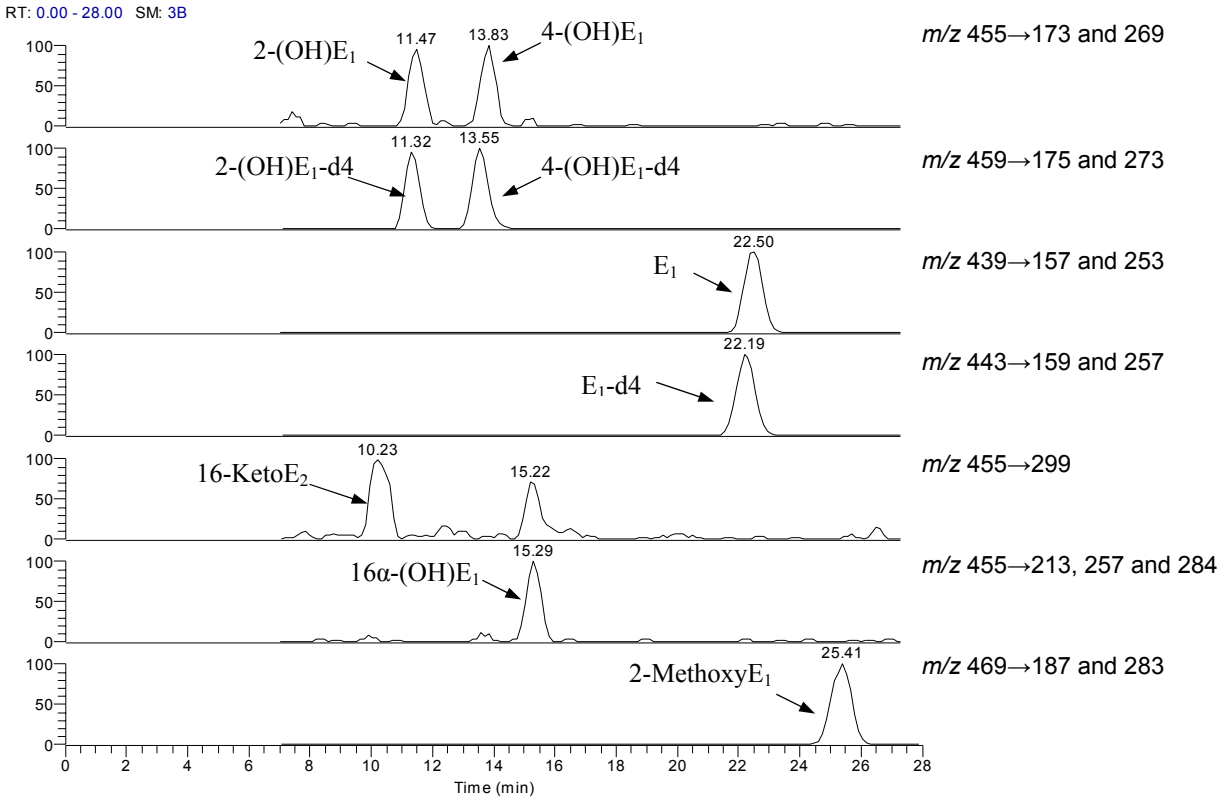


**Figure 2. Postmenopausal Urine**