Comprehensive Profiling of Free and Conjugated Estrogens by Capillary Electrophoresis-Time of Flight-Mass Spectrometry

Application Note

Clinical Research

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Introduction

The bioavailability and biological activity of estrogens is tightly regulated by phase I/II metabolic transformation processes associated with hydroxylation, glucuronidation or sulfation that are critical to human health, development and fertility. However, there is lack of a specific, sensitive yet robust methodology for comprehensive estrogen profiling given the wide dynamic range and chemical diversity of endogenous/exogenous estrogen metabolites present in complex biological fluids, such as urine [1].

Agilent Technologies

Experimental

Chemicals and Reagents

Twelve standard estrogens were purchased from Sigma-Aldrich (St. Louis, MO, USA):

- Five glucuronide conjugates:
  - estrone 3-glucuronide ($E_1$3G)
  - 17ß-estradiol 3-glucuronide ($E_2$3G)
  - ß-estradiol 17-glucuronide ($E_2$17G)
  - estriol 3-glucuronide ($E_3$3G)
  - estriol 16-glucuronide ($E_3$16G)
- Three sulfate conjugates:
  - estrone 3-sulfate ($E_1$3S)
  - ß-estradiol 3-sulfate ($E_2$3S)
  - estriol 3-sulfate ($E_3$3S)
- Four free estrogens:
  - estrone (E_1)
  - estradiol (E_2)
  - 16-ethynyl estradiol (EE_2)
  - estriol (E_3)

Stock solutions were prepared at concentrations of 3–15 mM in 1:1 MeOH:H_2O and stored at -20 °C. Creatinine, uric acid, sodium chloride, melatonin, and HEPES were also purchased from Sigma-Aldrich. Stock solutions of 10 mM were prepared in water and stored at 4 °C. A simulated urine matrix was used for preparation of estrogen standard solutions containing 15 mM sodium chloride, 1 mM creatinine, 500 µM uric acid, 150 µM melatonin (EOF marker) and 150 µM HEPES (internal standard). Ammonium bicarbonate (Sigma Aldrich Inc.) was prepared as a 500 mM stock in water and used as the background electrolyte for both sheath liquid and CE separations. HPLC-grade methanol (Caledon Labs, Georgetown, ON, Canada) was used for preparation of all stock solutions and sheath liquid. All aqueous buffers and stock solutions were prepared with water purified using a Thermo Scientific Barnstead EasyPure II LF ultrapure water system (Cole Parmer, Vernon Hills, IL, USA).

Instrumentation

All CE-ESI-TOF-MS experiments were performed using an Agilent G7100A CE system (Mississauga, ON, Canada) interfaced with coaxial sheath liquid electrospray ion source to an Agilent 6224 TOF LC/MS orthogonal axis time-of-flight mass spectrometer (Mississauga, ON, Canada). Nitrogen gas was used as the nebulizer gas in ESI and the drying gas for the MS. The system software was 3D-CE ChemStation (CE) and Agilent MassHunter Workstation Data Acquisition (TOF-MS). Data processing was performed using MassHunter Qualitative and MassHunter Quantitative software. All data processing, electrophoregrams, and surface response models were performed using Igor Pro 5.0 (Wavemetrics Inc., Lake Oswego, OR, USA).

CE-ESI-TOF-MS Conditions

An uncoated fused silica capillary (Polymicro Technologies, AZ, USA) with a 50 µm id and 85 cm length was used for all experiments and maintained at 20 °C. Unless otherwise noted, as in the experimental design, all conditions were as follows:

- A 50 mM ammonium bicarbonate buffer adjusted to pH 9.5 with ammonium hydroxide was used as the background electrolyte.
- The sample was injected at 50 mbar for 5 seconds (approximately 5 nL) and the applied voltage was 20 kV.
- The sheath liquid was 5 mM ammonium bicarbonate in 80:20 MeOH:H_2O at a flow rate of 14 µL/min.
- The mass spectrometer was operated in negative-ion mode under the following conditions: ion spray voltage at -4.0 kV, drying gas temperature at 300 °C, drying gas flow rate at 4 L/min, and nebulizer gas flow rate at 10 L/min.
- The MS settings for ion extraction were fragmentor = -145 V, skimmer = -65 V, and Oct 1 RF Vpp = 750 V.
- The mass range scanned was *m/z* 50–1100 with 6800 transients/scan.
- Purine and hexakis(2,2,3,3-tetrafluoropropoxy)phosphazine (HP-0921) were spiked into the sheath liquid at a concentration of 0.02% v and produced corresponding reference ions at *m/z* 119.03632 and 981.9956 that were used for real time internal mass correction allowing for mass accuracy < 2 ppm in most cases.

Experimental Design

Experimental design was used to determine the optimum ESI conditions to maximize the ionization efficiency of free estrogens and their anionic conjugates. The three factors that were selected for optimization were capillary voltage (2.5–5.0 kV), fraction of methanol in the sheath liquid (40–80%), and sheath liquid flow rate (4–20 µL/min) since they have significant impacts on spray stability when using a coaxial sheath liquid interface that impact separation efficiency and concentration sensitivity in CE-MS due to potential suction and

post-capillary dilution effects, respectively[1]. A two level (±1)-three factor (that is, 2^3) central composite design with six axial (± 1.7) and five central (0) conditions for a total of 20 experiments were performed to systematically maximize estrogen detectability[2]. The responses of E_3, $E_3$3S and $E_3$3G were measured by calculating the absolute peak areas or signal-to-noise (S/N) ratios of these ions in each experiment. Multiple linear regression of the data matrix was performed by Excel (Microsoft Inc., Redmond, WA, USA) and used to develop an empirical model based on three main factors and their interactions, which was refined iteratively by eliminating insignificant variables ($P < 0.05$) that had minimal effect on the overall predictive accuracy as reflected by changes in R^2 value. The optimized models were then used to graph 3D surface response plots in the experimental space for the three estriol metabolites. The optimum conditions for maximizing estrogen detection was determined to be a capillary voltage of -4.0 kV with 80% v/v methanol in the sheath liquid at a flow rate of 14 µL/min.

Molecular Modeling and Prediction of Relative Migration Times

Molecular volume (MV) was calculated as the Connolly solvent-excluded volume after minimization of energy using molecular mechanics (MM2) in Chem3D Ultra 8.0. pK_a values for estrogens were derived from literature[3], which were used to determine z_{eff} in the background electrolyte (pH 9.5) using the Henderson-Hasselbalch equation. From these two physicochemical parameters (MV and z_{eff}), the relative migration time (RMT) for 12 estrogen metabolites (training set) were modeled, using multiple linear regression (MLR)[4]. A 12-fold cross-validation that randomly held out an estrogen metabolite (test set) was then performed to assess overall model robustness. The predictive accuracy of the model was assessed in terms of correlation of determination (R^2) for the training set, the average correlation of determination for the test set (Q^2), and the average absolute bias of predicted RMT relative to average RMT (n=30) values measured experimentally by CE-TOF/MS.

Urine Collection and Sample Pretreatment

Urine samples were collected from three healthy female volunteers who were not taking any regular medication and had provided written informed consent prior to beginning the study. Urine samples were collected in sterile containers and stored at -80 °C until analysis. A recent study has demonstrated that estrogen conjugates have excellent long-term stability when urine samples are stored frozen without addition of chemical preservatives[5]. Urine samples were centrifuged for 10 minutes at 150 g and supernatant urine was then analyzed directly by CE-TOF/MS after 10-fold dilution in de-ionized water. Solid-phase extraction (SPE) was also performed on the urine supernatant using Oasis HLB cartridges (Waters Inc., Milford, USA) with a vacuum manifold. The protocol for SPE of human urine was performed based on recent work reported by Qin *et al*[6]. with a few modifications to make the concentrated eluent compatible with CE-TOF/MS analysis. Before extraction, each HLB cartridge was preconditioned with 1.0 mL methanol followed by 1.0 mL water and 1.0 mL aqueous phosphoric acid (0.3%, v/v). A 5.0 mL aliquot of supernatant urine was mixed with 5.0 mL aqueous phosphoric acid (0.3%, v/v) and then loaded onto the HLB cartridge. After sample loading, the cartridge was washed with 1.0 mL water followed by 1.0 mL methanol:water:acetic acid (60:40:2 v). Elution of the analytes was performed using 1.0 mL methanol without addition of 2% ammonium hydroxide[6], which was found to generate excessive band broadening of estrogen conjugates due to electrokinetic dispersion in CE-TOF/MS. The eluent was evaporated to dryness under a stream of nitrogen gas at 50 °C and the resulting residue was reconstituted in 40 µL acetonitrile:water (75:25 v) containing 50 µM HEPES as an internal standard. By loading 5.0 mL of urine and reconstituting to 40 µL, the effective concentration of urinary estrogens was increased by about 125-fold, which has been found to offer good recoveries when using HLB columns ranging from 90–110%.

Method Calibration and Verification

Calibration standards were prepared as standards in a simulated urine matrix (15 mM sodium chloride, 1 mM creatinine, 500 µM uric acid) at six levels over a 100-fold concentration ranging from 0.5 to 50 µM using 50 µM HEPES as the internal standard to improve precision for quantification in terms of relative peak areas (RPA) and metabolite identification in terms of relative migration times (RMT) for urinary metabolites. This is needed to correct for long-term drift in ion source and random changes in EOF when using CE-TOF/MS, respectively[4]. Ion suppression effects was investigated by comparison of the sensitivity (that is, slope of calibration curve) of three estrogen standards (that is, E_1, E_1-3G, E_1-3S) prepared in the mock urine solutions relative to spiked standards in authentic urine samples that were pooled from three premenopausal female volunteers and subsequently diluted 10-fold in de-ionized water. Intra-day reproducibility was determined by repeated measurements of estrogen standards in mock urine at three different concentration levels (1, 5, and 15 µM) in order to determine reproducibility of the method (n = 30). Limits of detection ($S/N \approx 3$) and limits of quantification ($S/N \approx 10$) for estrogen conjugates determined from the slope of the calibration curve and background noise measured at the specific *m/z* for each ion.

Ethics Approval

This study involving the collection of urine samples from female volunteers was approved by the McMaster University Research Ethics Board.

Table 1. Identification of Estrogens by CE-TOF/MS Based on Accurate Mass and Relative Migration Time (RMT) that is Accurately Predicted Based on Two Intrinsic Physicochemical Properties of an Ion

Estrogen metabolites	Measured RMT[a]	*m/z* $[M-H]^-$	$-z_{eff}$[b] (ph 9.5)	MV[c] (Å3)	Predicted RMT[d]	Bias (%)
Estradiol (E_2)	0.687 ± 0.002	271.1704	0.11	262	0.692	+0.68
Ethynyl estradiol (EE_2)	0.687 ± 0.002	295.1704	0.21	283	0.668	-2.74
Estriol (E_3)	0.688 ± 0.001	287.1653	0.13	268	0.692	+0.52
Estrone (E_1)	0.691 ± 0.002	269.1547	0.14	257	0.710	+2.74
Estriol 3-glucuronide ($E_3$3G)	0.861 ± 0.005	463.1974	1.00	401	0.848	-1.49
Estradiol 3-glucuronide ($E_2$3G)	0.865 ± 0.001	447.2024	1.00	396	0.855	-1.16
Estriol 16-glucuronide ($E_3$16G)	0.880 ± 0.001	463.1974	1.13	395	0.906	+2.98
Estrone 3-glucuronide ($E_1$3G)	0.884 ± 0.001	445.1868	1.00	391	0.862	-2.54
Estradiol 17-glucuronide ($E_2$17G)	0.887 ± 0.002	447.2024	1.14	393	0.913	+2.90
Estriol 3-sulfate ($E_3$3S)	0.960 ± 0.001	367.1221	1.00	307	0.974	+1.49
Estradiol 3-sulfate ($E_2$3S)	0.978 ± 0.001	351.1272	1.00	302	0.980	+0.27
Estrone 3-sulfate ($E_1$3S)	1.019 ± 0.001	349.1115	1.00	297	0.987	-3.14

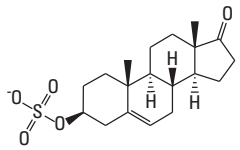
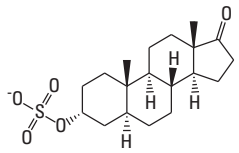
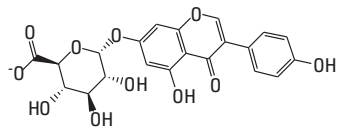
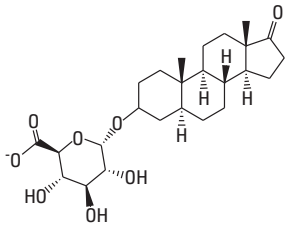
a. Measured RMTs for metabolites were measured in mocked urine samples (n = 10), where error represents ±1.
b. Calculated from measured estrogen pKa at pH 9.5, where $z_{eff} = 1/(10^{[pH-pK_a]}+1)$
c. Chem3D Ultra 8.0 after MM2 energy minimization as Connelly Solvent-Excluded Volume
d. Predicted RMTs determined by MLR based on two physicochemical properties (z_{eff}, MV), where model equation was $y = (0.8407 \pm 0.0062) + (0.173 \pm 0.010)z_{eff} - (0.080 \pm 0.010)MV$, where $R^2 = 0.9750$ and $Q^2 = 0.9543$ when performing 12-fold cross-validation.

Table 2. Figures of Merit for Estrogen Conjugate Analysis by CE-TOF/MS Under Negative Ion Mode Detection

Estrogen metabolites	Measured RMT[a]	*m/z* $[M-H]^-$	LOD (S/N=3, µM)	Linearity[b] (R^2)	Precision RPA[c] (CV, n=30)	Precision RMT[c] (CV, n=30)
Estriol 3-glucuronide ($E_3$3G)	0.861 ± 0.005	463.1974	0.17	0.991	10.9	0.09
Estradiol 3-glucuronide ($E_2$3G)	0.865 ± 0.001	447.2024	0.07	0.993	13.2	0.11
Estriol 16-glucuronide ($E_3$3G)	0.880 ± 0.001	463.1974	0.15	0.988	11.8	0.11
Estrone 3-glucuronide ($E_1$3G)	0.884 ± 0.001	445.1868	0.17	0.989	11.2	0.12
Estradiol 17-glucuronide ($E_2$17G)	0.887 ± 0.002	447.2024	0.13	0.997	10.8	0.17
Estriol 3-sulfate ($E_3$3S)	0.960 ± 0.001	367.1221	0.23	0.996	11.2	0.14
Estradiol 3-sulfate ($E_2$3S)	0.978 ± 0.001	351.1272	0.12	0.997	8.6	0.06
Estrone 3-sulfate ($E_1$3S)	1.019 ± 0.001	349.1115	0.07	0.995	10.0	0.04

a. Measured RMTs for metabolites were measured in mocked urine samples measured as 10 replicates at three different concentrations (n=30), where error represents ±1σ.
b. Calibration curves derived from linear regression of average normalized ion responses (n=3) of estrogens relative to internal standard at six different concentrations over a 100-fold range from 0.5–50 µM
c. Precision was determined by performing 10 replicate analysis of estrogen standards at three different concentration levels (1, 5, and 15 µM).

Table 3. Identification of Putative Urinary Steroid Conjugates Based on Accurate Mass (< 3 ppm) and Relative Migration Time (RMT) by CE-TOF/MS After 125-fold Off-Line Preconcentration Using Solid-Phase Extraction

Putative urinary metabolite	Empirical formula	2D Chemical structure	*m/z* $[M-H]^-$	δ_{mass} [a] (ppm)	Measured RMT
Dehydroepiandrosterone 3-sulfate (DHEAS)[b]	$C_{19}H_{28}O_5S$		367.1576	-1.29	1.021 ± 0.002
Androsterone 3-sulfate[a] (A-3S)	$C_{19}H_{30}O_5S$		369.1740	0.27	1.015 ± 0.003
Genistein 7-glucuronide (Gen-7G)	$C_{21}H_{18}O_{11}$		445.0774	0.45	1.175 ± 0.017
Androsterone 3-glucuronide (A-3G)[b]	$C_{25}H_{38}O_8$		465.2506	-2.58	0.950 ± 0.002

a. Refers to mass error (δ_{mass}) defined as the relative difference between measured (TOF/MS) and theoretical *m/z* as derived from its putative chemical structure

b. Putative steroid conjugates identified in urine also possess low abundance/isobaric ions, namely (epi)testosterone 3-sulfate (*m/z* 367.1585), etiocholanolone 3-sulfate (*m/z* 369.1741) or etiocholanolone 3-glucuronide (*m/z* 465.2494)

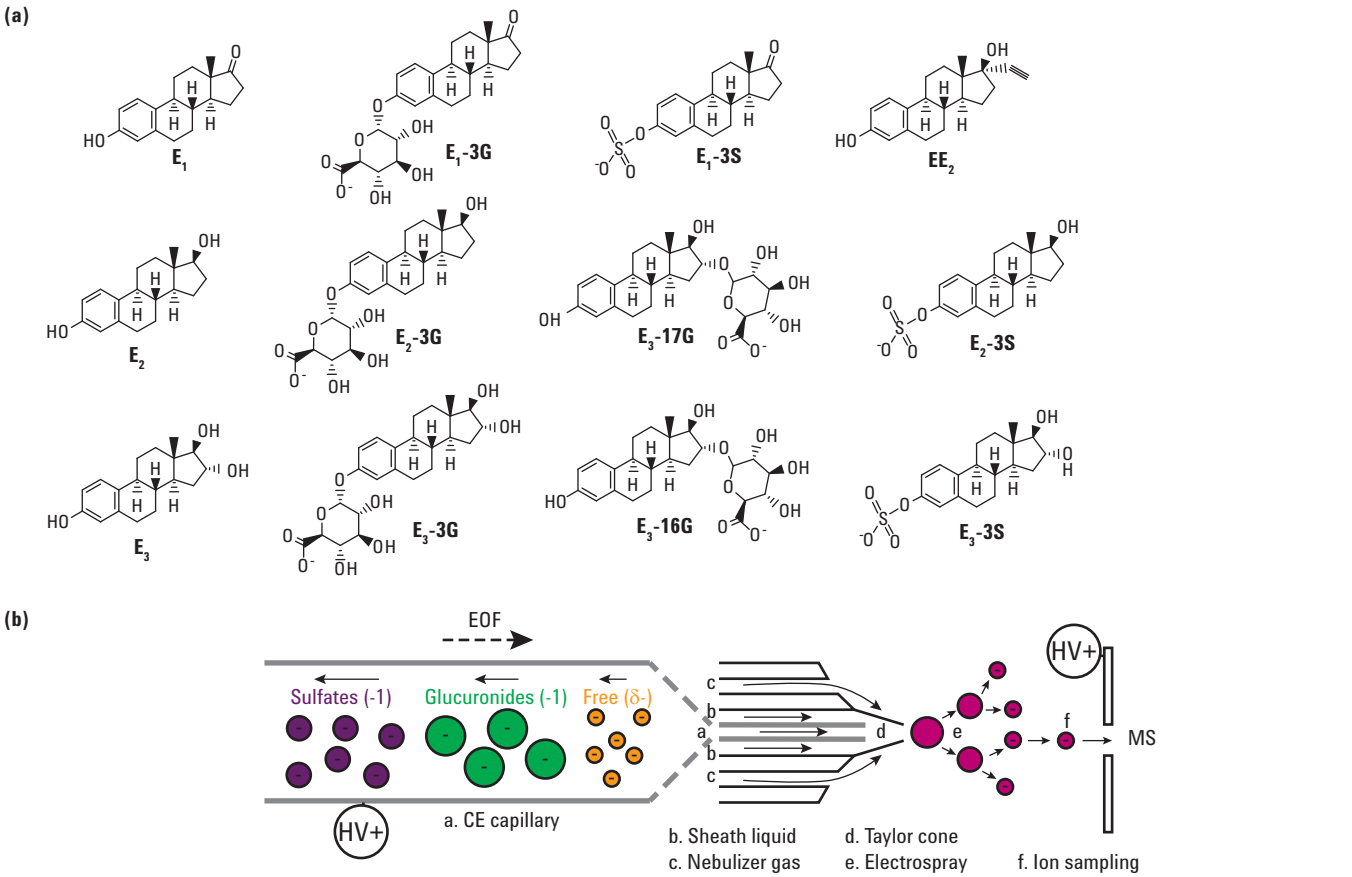


Figure 1. *(a) 2D chemical structures of model weakly acidic estrogens and their anionic conjugates examined in this study, including estrone (E_1), estrone 3-glucuronide ($E_1$3G), estrone 3-sulfate ($E_1$3S), 17α-ethynylestradiol (EE_2), 17ß-estradiol (E_2), 17ß-estradiol 3-glucuronide ($E_2$3G), ß-estradiol 17-glucuronide ($E_2$17G), ß-estradiol 3-sulfate ($E_2$3S), estriol (E_3), estriol 3-glucuronide ($E_3$3G), estriol 16-glucuronide ($E_3$16G) and estriol 3-sulfate ($E_3$3S). (b) schematic depicting the resolution of anionic urinary metabolites by CE-TOF/MS based on their effective charge density, where weakly acidic estrogens and their ionic conjugates are desorbed into the gas-phase through a coaxial sheath liquid interface.*

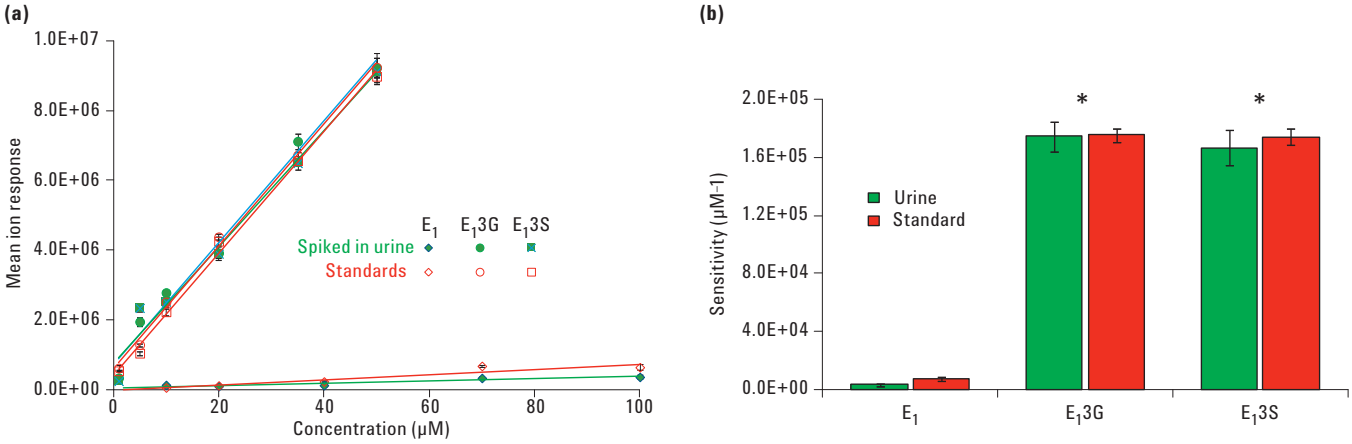


Figure 2. *(a) Overlay of representative calibration curves for native and conjugates estrone (E_1) prepared as standard solutions in a simulated urine matrix (15 mM sodium chloride, 1 mM creatinine, 500 μM uric acid) and spiked standards in pooled female urine (n=5) (b) Bar graph comparing the apparent sensitivity for model estrogens derived from the slope of calibration curves after a 10-fold dilution in de-ionized water. Overall, no significant matrix-induced ion suppression (* $P < 0.01$) was observed for estrogen conjugates in diluted urine samples. Note the 50-fold lower ion response for free E_1 under negative ion mode detection relative to bulkier and strongly ionic estrogen conjugates.*

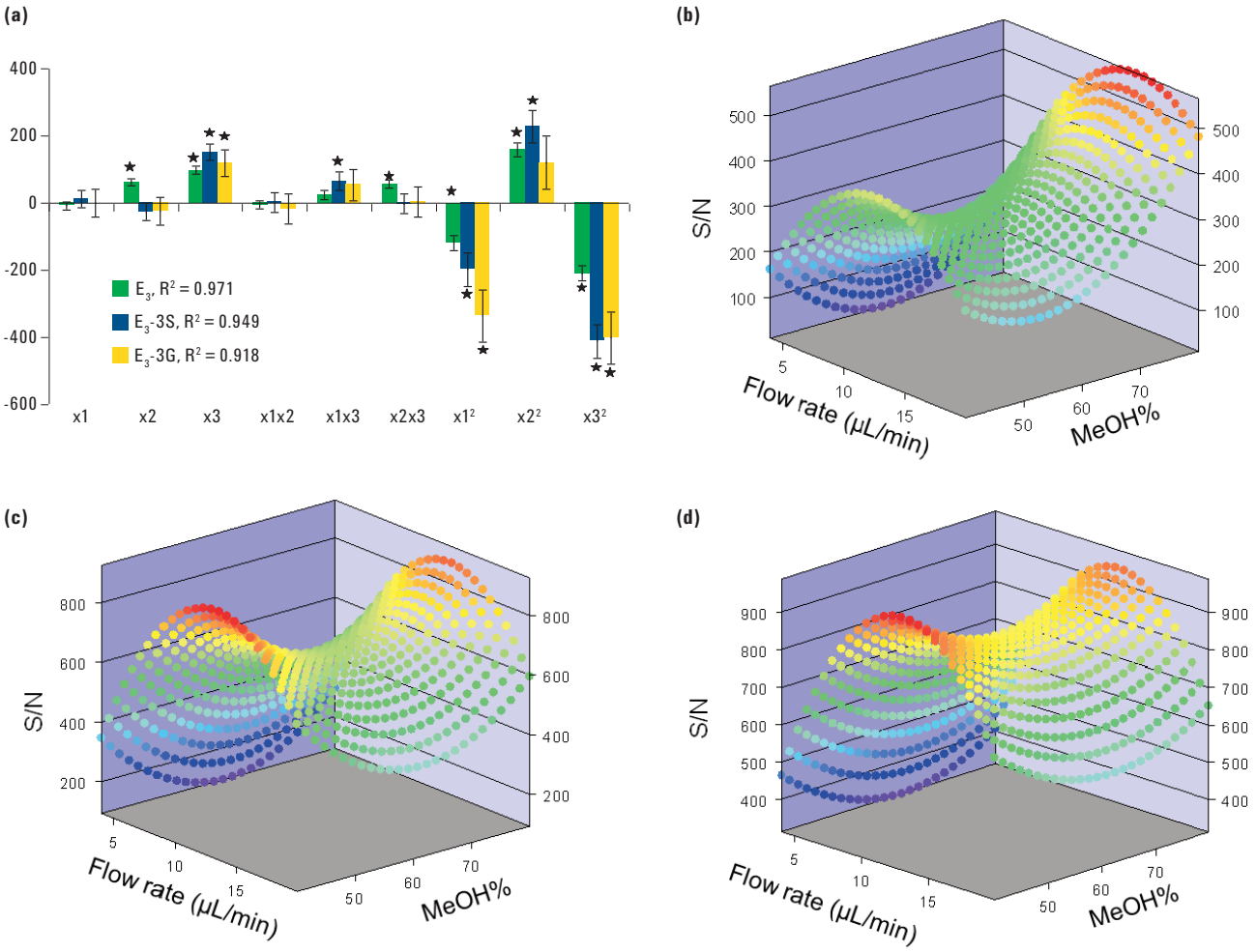


Figure 3. *(a) Experimental design for optimization of ionization conditions for estrogens when using a coaxial sheath liquid interface in CE-ESI-TOF/MS under negative ion mode, where coefficients from multiple linear regression represent three factors (x1 = ESI voltage, x2 = MeOH% in the sheath liquid, x3 = sheath liquid flow rate; *= significant at 95% CL) and their second-order terms. Representative bimodal surface response curves as a function of signal/noise (S/N) for b) E_3 (m/z 287.1653) c) $E_3$3S (m/z 367.1221) and d) $E_3$3G (m/z 463.1974) are depicted, where optimal conditions to enhance ionization efficiency of estrogens were a cone voltage of 4.0 kV using 80% MeOH as the sheath liquid at a flow rate of 14 µL/min.*

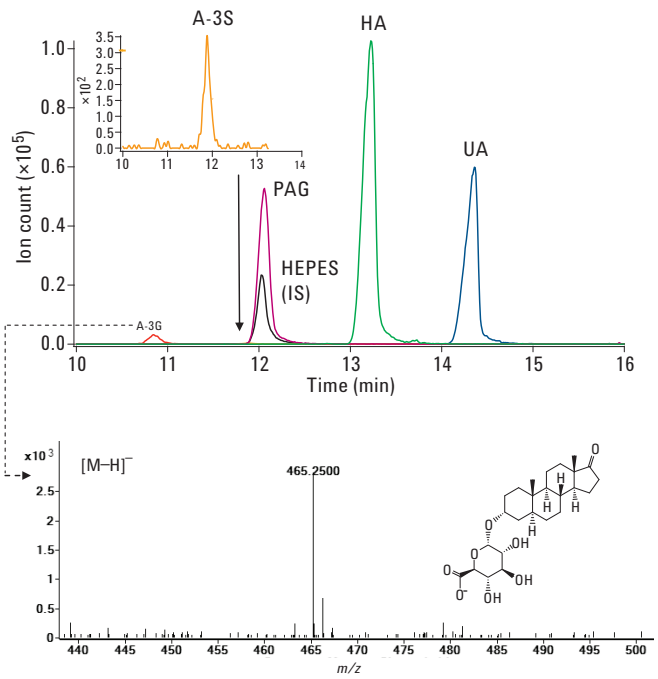


Figure 4. Representative extracted ion electropherogram overlay of major urinary metabolites and low abundance steroid conjugates over a 10^3 dynamic range in a pre-menopausal female urine specimen directly analyzed by CE-TOF/MS after a 10-fold dilution in deionized water. Analyte acronyms refer to androsterone 3-glucuronide (A-3G, m/z 465.2494, RMT 0.910 ± 0.015), androsterone 3-sulfate (A-3S, m/z 369.1741, RMT 0.994 ± 0.010), phenylacetylglutamine (PAG, m/z 263.1038; RMT 1.001 ± 0.012), hippuric acid (HA, m/z 178.0510, RMT 1.105 ± 0.010), uric acid (UA, m/z 167.0211, RMT 1.200 ± 0.008) and HEPES as internal standard (IS). Accurate mass and isotope ratio measurements by TOF/MS together with RMT predictions by CE can be used to confirm the identity or exclude isobaric/isomeric candidate ions selected after a database search (for example, KEGG and Human Metabolome Database). Low abundance estrogen conjugates were not reliably detected in diluted urine specimens from non pregnant females due to inadequate sensitivity when using CE-TOF/MS in full-scan negative ion mode detection (LOD ≈ 0.4 µM).

Results and Discussion

Optimization Interference-Free Region

Urine contains high concentrations of salt and abundant metabolites, including Na^+,Cl, urea, and hippuric acid. Optimization of background electrolyte(BGE) conditions is crucial in method development in CE to ensure reproducible migration times without ion suppression of low abundance estrogens by major urinary co-ions.

CE offers a natural desalting feature, where small inorganic salts migrate with higher positive mobilities than most urinary metabolites thereby improving sensitivity without complicated off-line sample pretreatment.

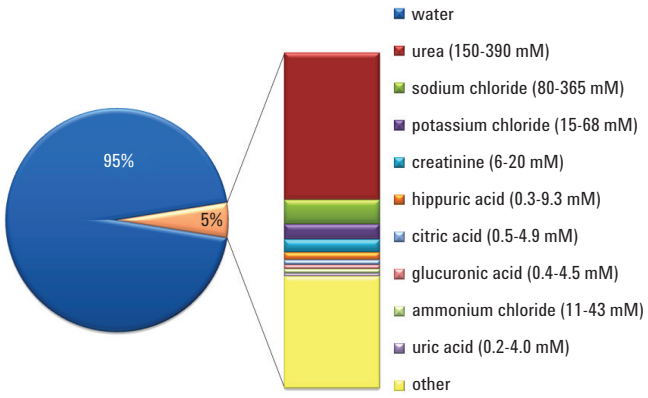


Figure 5. Composition of urine, showing high concentration of salts, neutral compounds, and acidic analytes.

Resolution of Estrogen Conjugates Isomers

Conjugation at different hydroxyl sites generates various structural isomers for estrogen conjugates. Separation can be readily achieved by optimization of BGE (pH>9.5) using CE to partially ionize phenolic moiety on $E_2$17G and $E_3$16G.

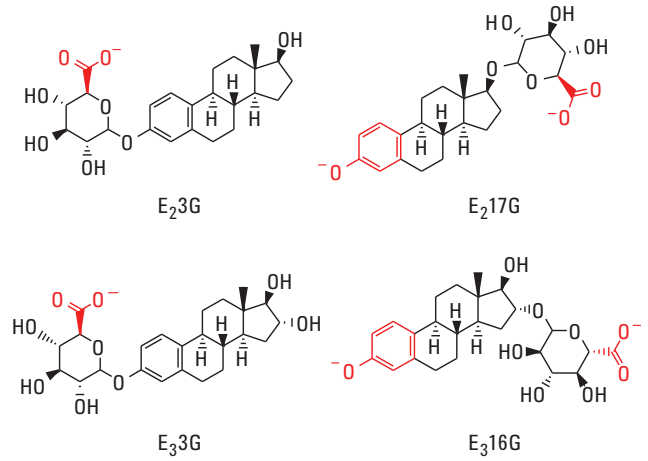


Figure 6. Representative structural isomers of estrogens with glucuronidation at different sites of estradiol (E_2) and estriol (E_3).

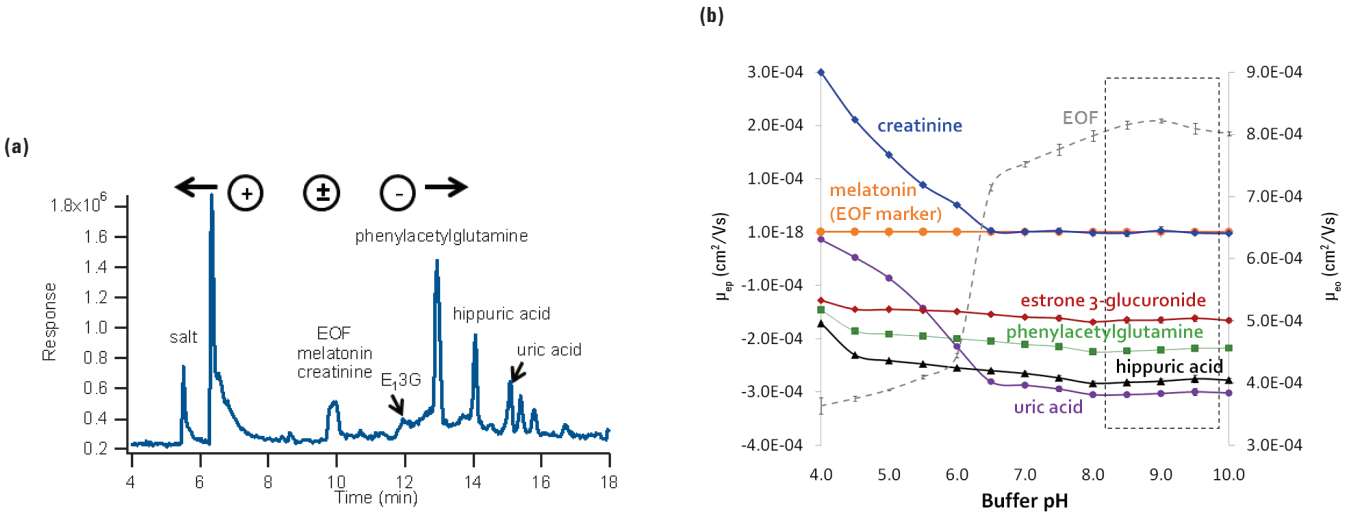


Figure 7. a) Total ion electropherogram showing migration order of major urinary metabolites. b). Mobility plot showing resolution of metabolites in urine based on changes in pH of BGE for interference-free analysis of estrogens ($E_1$3G) by CE-ESI-TOF/MS.

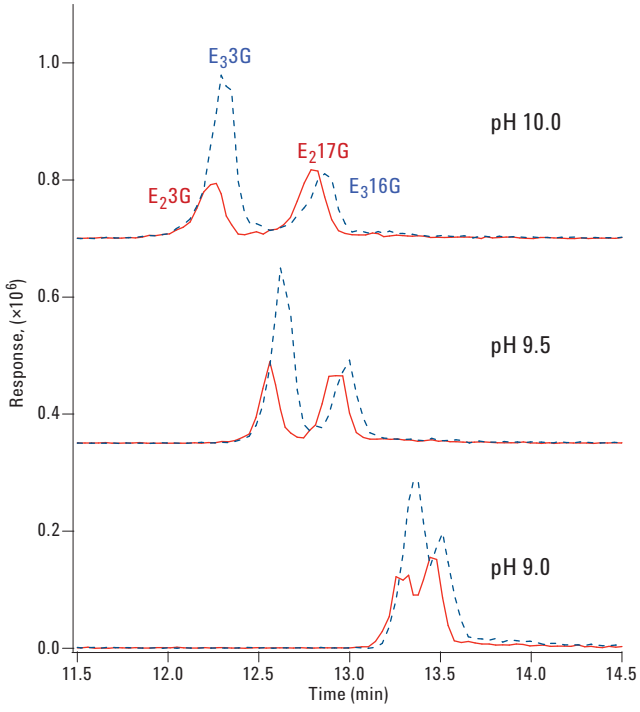


Figure 8. Resolution of two pairs of estrogen glucuronide isomers by CE within creasing pH of BGE.

Unambiguous Identificationby CE-ESI-TOF/MS

Relative migration times (RMTs) in CE can be used as a qualitative tool to support identification of unknown estrogens together with accurate mass, isotope ratios, and databases. Predicted RMTs are determined in silico based on two intrinsic physicochemical properties of an analyte (MV, pKa) when using multiple linear regression.

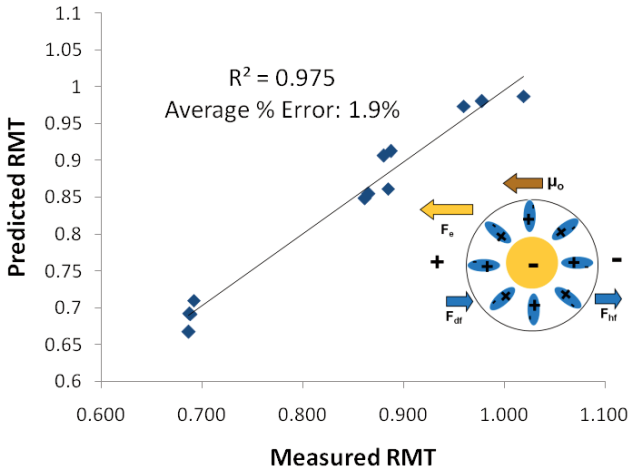


Figure 9. Correlation plot of predicted to measured RMTs for model estrogens. Equation used to determine RMT is based on two intrinsic parameters: RMT=0.99912-0.00133(MV)-0.38408(z_{eff}).

Comprehensive Estrogen Profiling

Under optimal conditions, partially ionized free estrogens are readily separated from their ionic conjugates due to their weak acidity ($pK_a \approx 10.4$), where as glucuronides are resolved from sulfates due to differences in molecular volume (MV). Estrogen conjugate isomers are also resolved by CE due to differences in their effective charge (z_{eff}).

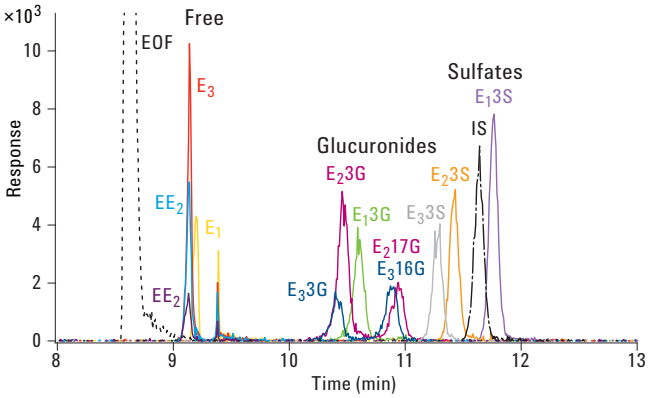


Figure 10. Extracted ion electropherograms showing the resolution of 10 µM spiked estrogen conjugates and 20 µM free estrogens at pH 9.5. The glucuronide conjugates are fully resolved from the sulfate-conjugates, including major structural isomers, namely $E_2$3G and $E_2$17G (m/z 447.2024) and $E_3$3G and $E_3$16G (m/z 463.1974.).

Table 4. Physicochemical Properties of the 11 Model Estrogens

Estrogen	Acronym	*m/z* [M-H]⁻	MV (Å3)	pK_a	z_{eff} (pH = 9.5)
Estrone	E_1	269.1547	257	10.34 ± 0.05[3]	-0.13
Estradiol	E_2	271.1704	262	10.46 ± 0.03[3]	-0.10
Estriol	E_3	287.11653	268	10.38 ± 0.02[3]	-0.12
Estrone 3-sulfate	$E_1$3S	349.1115	297	-3[4]	-1.00
Estradiol 3-sulfate	$E_2$3S	351.1272	307	-3[4]	-1.00
Estriol 3-sulfate	$E_3$3S	367.1221	393	-3[4]	-1.00
Estrone 3-glucuronide	$E_1$3G	445.1868	302	~3-4[5]	-1.00
Estradiol 3-glucuronide	$E_2$3G	447.2024	396	~3-4[5]	-1.00
Estradiol 17-glucuronide	$E_2$17G	447.2024	391	~3-4[5], 10.46[3]	-1.10
Estriol 3-glucuronide	$E_3$3G	463.1974	401	~3-4[5]	-1.00
Estriol 16-glucuronide	$E_3$16G	463.1974	395	~3-4[5], 10.38[3]	-1.12

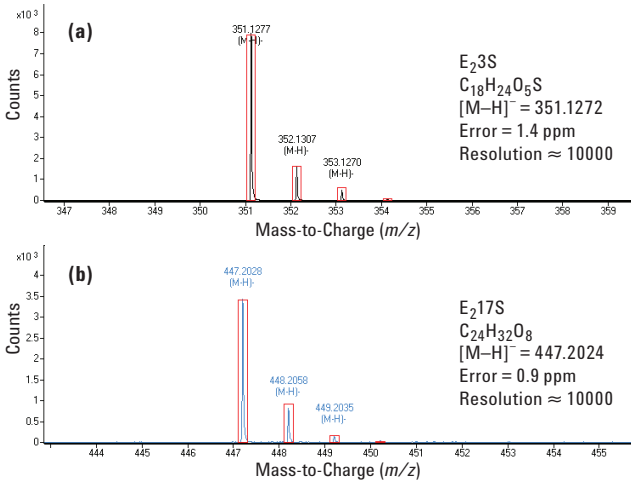


Figure 11. TOF/MS spectra and corresponding predicted isotope ratios (red boxes) based on empirical formulas for a) estradiol 3-sulfate and b) estradiol 17-glucuronide.

Conclusions

This application note is one of the first reports of estrogen analysis by CE that allows for direct speciation of intact and highly polar estrogen conjugates with high selectivity and minimal sample handling. Qualitative identification of unknown steroid conjugates and their positional isomers can be realized by high mass accuracy TOF/MS in conjunction with prediction of ion migration behavior by CE that is relevant to applications in clinical chemistry, environmental analysis and food science.

References

1. N. L. Kuehnbaum, P. Britz-McKibbin, *Anal. Chem*. **2011**, 83, 8063-8068.
2. M. Mokaddem; P.Gareil; J.-E. Belgaied; A. Varenne, Electrophoresis 2009, 30, 1692-1697.
3. C. Kaiser; G. Segui-Lines; J. C. D'Amaral; A. S. Ptolemy; P.Britz-McKibbin, *Chem. Comm*. **2008**, 338-340.
4. A. R. Hurwitz; S. T. Liu, *J. Pharmaceutical Sci*. **1977**, 66, 624-627.
5. L. A. D'Agostino; K. P. Lam; P. Britz-McKibbin *J. Proteome Res*. **2011**, 10, 592-603.
6. B. J. Fuhrman; X. Xu; R. T. Falk; S. E. Hankinson; T. D. Veenstra; L. K. Keefer; R. G. Ziegler, Inter. *J. Biol. Markers* **2010**, 25, 185-194.
7. F. Qin; Y.-Y. Zhao; M. B. Sawyer; X.-F. Li, *Anal. Chem.* **2008**, 80, 3404-3411.

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