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ORIGINAL RESEARCH

Direct determination of estriol 3- and 16-glucuronides in pregnancy urine by column-switching liquid chromatography with electrospray tandem mass spectrometry

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ABSTRACT: Using column-switching liquid chromatography/tandem mass spectrometry (LC-MS/MS), we developed an improved analytical method of urinary estriol glucuronides. This new method is derived predominantly from maternal and fetal precursors in pregnancy. We used in the following procedure: first, we filtered urine samples with a membrane filter. Next, we directly injected the 50 μL aliquot of urine samples onto a pre-column. Then, after activating the column-switching valve, we backflushed the loaded samples onto the C_{18} analytical column. Urine samples can be assayed within 20 min without any sample preparation steps. We monitored separated estriol glucuronides by negative electrospray ionization (ESI) and selected-reaction monitoring (SRM). The calibration range of estriol-3-glucuronide (E3-3G) and estriol-16-glucuronide (E3-16G) was 0.1–20 μg/mL and the linearity of the method was 0.9984 for E3-3G and 0.9987 for E3-16G. The limits of detection at a signal-to-noise (S/N) ratio of 3 were 10 ng/mL (E3-3G) and 5 ng/mL (E3-16G). The analytical recovery was over 85% and, in general, inter-day and intra-day variability for precision and accuracy were less than 10%. When applied to a pregnancy urine sample to biomedical monitoring of the function of the maternal/fetal unit, the proposed method allowed rapid and sensitive screening for the detection of E3-3G and E3-16G.

KEYWORDS: estriol glucuronides; pre-column concentration; negative ion ESI; selected-reaction monitoring

INTRODUCTION

Estriol (E3), (16α, 17β)-estra-1,3,5(10)-triene-3,16,17-triol, is the biogenesis of estrogens by the fetoplacental unit in late pregnancy. E3 is produced predominantly in the placenta from androgen precursors of fetal 16-hydroxy dehydroepiandrosterone sulfate, and then it is secreted into maternal circulation. For maternal circulation, E3 converts to four conjugates: estriol-3-glucuronide (E3-3G), estriol-16-glucuronide (E3-16G), estriol-3-sulfate (E3-3S), and estriol-3-sulfate-16-glucuronide (E3-SG; Levitz *et al.*, 1984). Thus, researchers commonly biochemically monitor the excretion of total urinary estriol in pregnancy urine to evaluate the function of the maternal/fetal unit. Most of the estriol glucuronide excreted by maternal urine after the missed period reflects some degree of embryonic steroidogenic activity. This activity increases as the embryo develops (Mendizabal *et al.*, 1984).

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Abbreviations used: ESI, electrospray ionization; TEA, triethylamine.

Various direct radioimmunoassay methods (Dipietro, 1976; Lehtinen and Adlercreutz, 1977; Sugar *et al.*, 1977), chemiluminescence assays (Kohen *et al.*, 1980; Barnard *et al.*, 1981) and many high performance liquid chromatographic (HPLC) methods with ultraviolet absorbance (Schöneshöfer *et al.*, 1986), electrochemical (Schimada *et al.*, 1982) and fluorescence (Andreolini *et al.*, 1985; Caccamo *et al.*, 1988; Tiel *et al.*, 1989; Iwata *et al.*, 1997) detection have been developed for monitoring of E3 glucuronides in biological fluid. Recently, a liquid chromatography/mass spectrometric method (Zhang and Henion, 1999; de Alda and Barceló, 2000) for the estimation of E3 metabolites in native urine was described. However, significant analytical interferences exist in certain urine samples. Therefore, time-consuming sample clean-up steps for the specific liquid chromatographic estimation are still mandatory. In this new method we report on a column switching HPLC-MS/MS method that we have developed for the direct and simultaneous determination of estriol 3- and 16-glucuronides in human urine samples. This method is both highly sensitive and simple, and it does not utilize enzymatic hydrolysis or any sample preparation steps, such as conventional extraction or a derivatization reaction.

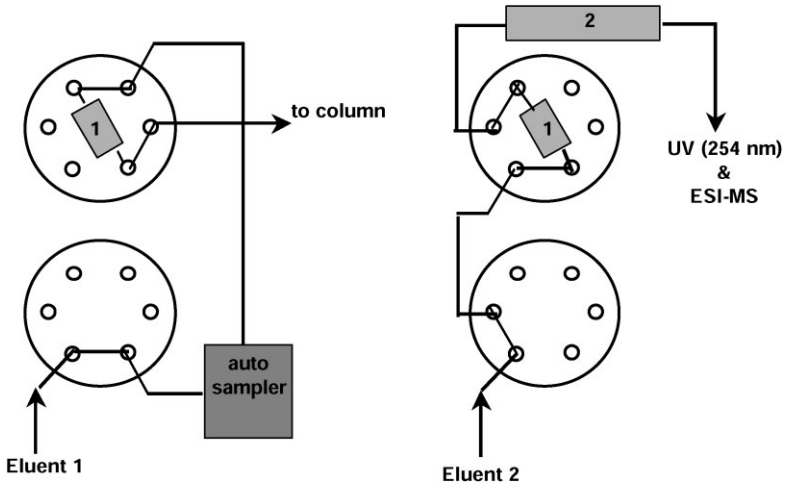

Figure 1. Flow diagram of the column-switching conditions in HPLC. 1, Shodex MS Pak PK-2A (*N*-vinylacetamide copolymer, 2.0 mm i.d. × 10 mm length, Showa Denko K. K., Tokyo, Japan); 2, Capcell Pak C_{18}, UG 120 (1.5 mm i.d. × 250 mm length, 5 μm particle size, Shiseido Fine Chemical Co., Tokyo, Japan). The mobile phase of Eluent 1 was 0.1% triethylamine (pH 6.4 adjusted with 10% acetic acid) at a flow rate of 500 μL/min for 10 min. The mobile phase of eluent 2 consisted of 0.1% triethylamine (A) and 90% acetonitrile (B) at a flow rate of 100 μL/min. The mobile phase B was initially 30% for 2 min, and increased to 100% at 9 min with holding for 4 min.

EXPERIMENTAL

Materials and reagents. All of the chemicals used in this method were of analytical-reagent grade, and all solvents were of HPLC grade. We used de-ionized and distilled water, purified with the Milli-Q (Millipore, Milford, MA, USA) system, for all aqueous solutions. We obtained E3-3G and E3-16G from Sigma-Aldrich (St Louis, MO, USA). The deuterated internal standard d_3-testosterone-glucuronide (T-G-d_3), 16,16,17-$^2H_3$-testosterone-17β-glucuronide, was purchased from NARL Reference Materials (Pumble, Australia).

Stock solutions of E3-3G and E3-16G were prepared at concentrations of 100 μg/mL in 70% methanol and stored at − 20°C until use. We further diluted to the desired concentrations (0.1–20 μg/mL) with 70% methanol before use. The T-G-d_3 was prepared at a concentration of 10 μg/mL in 70% methanol.

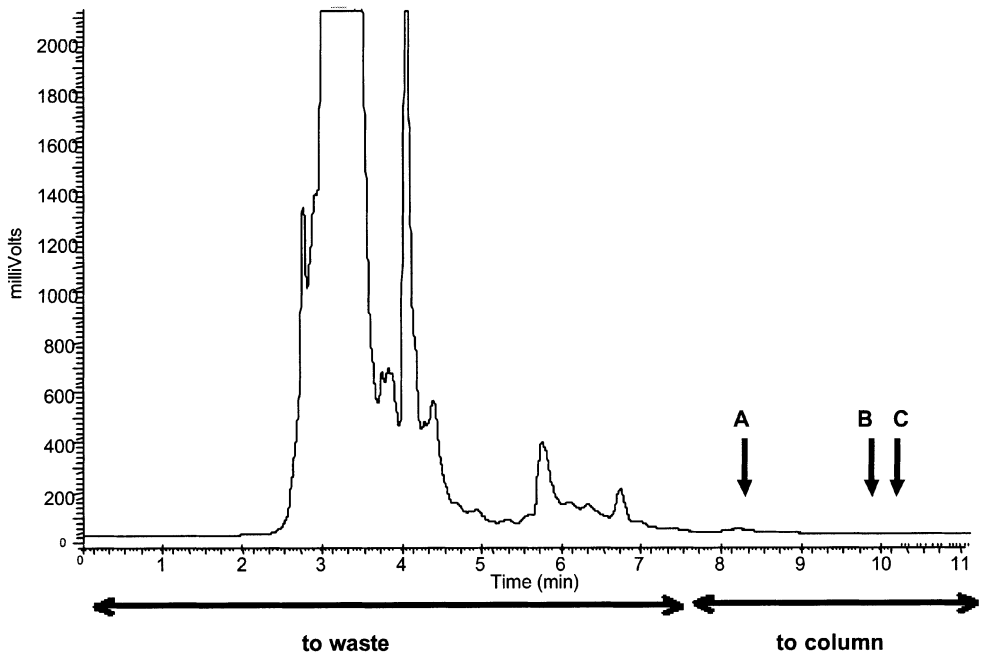

Figure 2. UV chromatogram of normal urine. A, Estriol-3-glucuronide at 8.15 min; B, Estriol-16-glucuronide at 9.85 min; C, Testosterone-glucuronide-d_3 (ISTD) at 10.05 min.

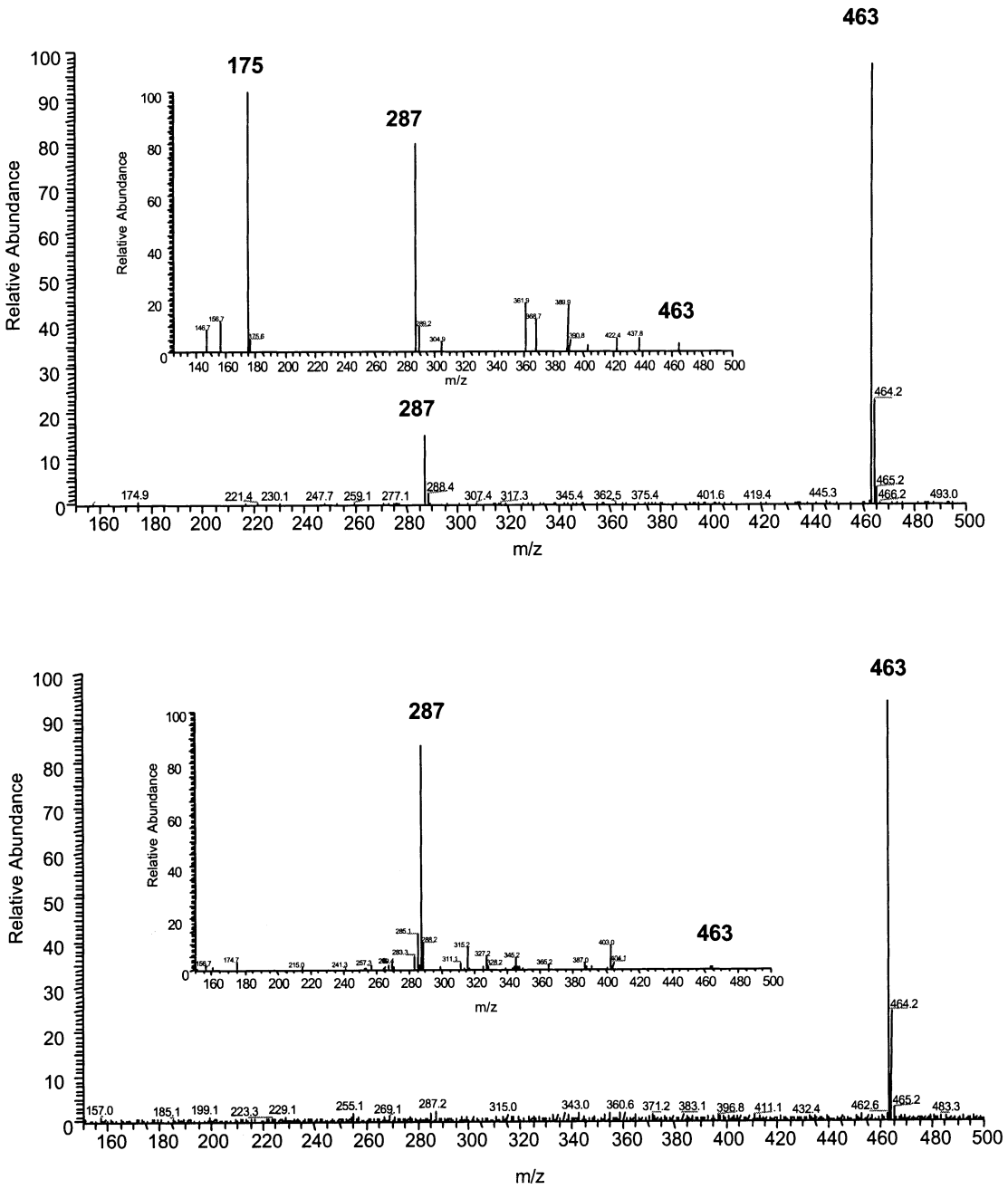

Figure 3. Negative-ion electrospray tandem mass spectra of estriol-3-glucuronide (up) and estriol-16-glucuronide (down).

Urine samples. Drug-free human samples were obtained from six normal pregnant women and five non-pregnant women. All-day urine samples of the volunteers were stored and frozen until analysis.

Column-switching chromatography. Figure 1 shows a flow diagram of the column-switching HPLC system and solvent systems. Two columns were connected in a backflush manner through a six-port switching valve (V1). The second valve (V2) was used to divert unnecessary portions of the eluent to minimize contamination of the electrospray ion source. Poly ether ether ketone (PEEK) tubing of 0.13 mm i.d. was used for all transfer lines. The system consisted of a first column, Shodex MS Pak PK-2A (*N*-vinylacetamide copolymer, 2.0 mm i.d. × 10 mm length, Showa Denko K. K., Tokyo, Japan), for separation of E3-3G and E3-6G from urinary interferences, and a second column, Capcell Pak C_{18}, UG 120 (1.5 mm i.d. × 250 mm length, 5 μm particle size, Shiseido Fine Chemical Co., Tokyo, Japan), for a complete separation of E3-3G and E3-6G. We removed the urinary interferences with 0.1% triethylamine (TEA, adjusted to pH 6.4 with 10% acetic acid) at a flow rate of 500 μL/min. Then we flushed the concentrated E3-Gs into the second column with a

Table 1. Intra- and inter-day assay tests with a pre-determined pregnant woman urine fortified with known amounts of estriol-3-glucuronide and estriol-16-glucuronide

		Intra-day ($n = 3$)			Inter-day ($n = 3$)		
Estriol	Amount added (μg/mL)	Amount found (mean ± SD)	CV[a] (%)	Recovery (%)	Amount found (mean ± SD)	CV (%)	Recovery (%)
3-Glucuronide	0.00	1.01 ± 0.11	10.62		1.45 ± 0.09	6.39	
	1.00	2.07 ± 0.13	6.32	106.00	2.25 ± 0.21	9.17	80.00
	3.00	3.92 ± 0.18	4.53	97.00	4.28 ± 0.24	5.61	94.33
	10.00	10.82 ± 0.42	3.92	98.10	10.92 ± 0.65	5.40	94.70
16-Glucuronide	0.00	3.86 ± 0.09	2.30		4.14 ± 0.31	7.58	
	1.00	4.91 ± 0.48	9.45	105.00	5.22 ± 0.35	6.63	108.00
	3.00	7.02 ± 0.35	4.95	105.33	7.24 ± 0.53	7.26	103.33
	10.00	13.00 ± 0.65	4.99	91.40	13.89 ± 0.96	6.92	97.50

[a] CV coefficient of variation.

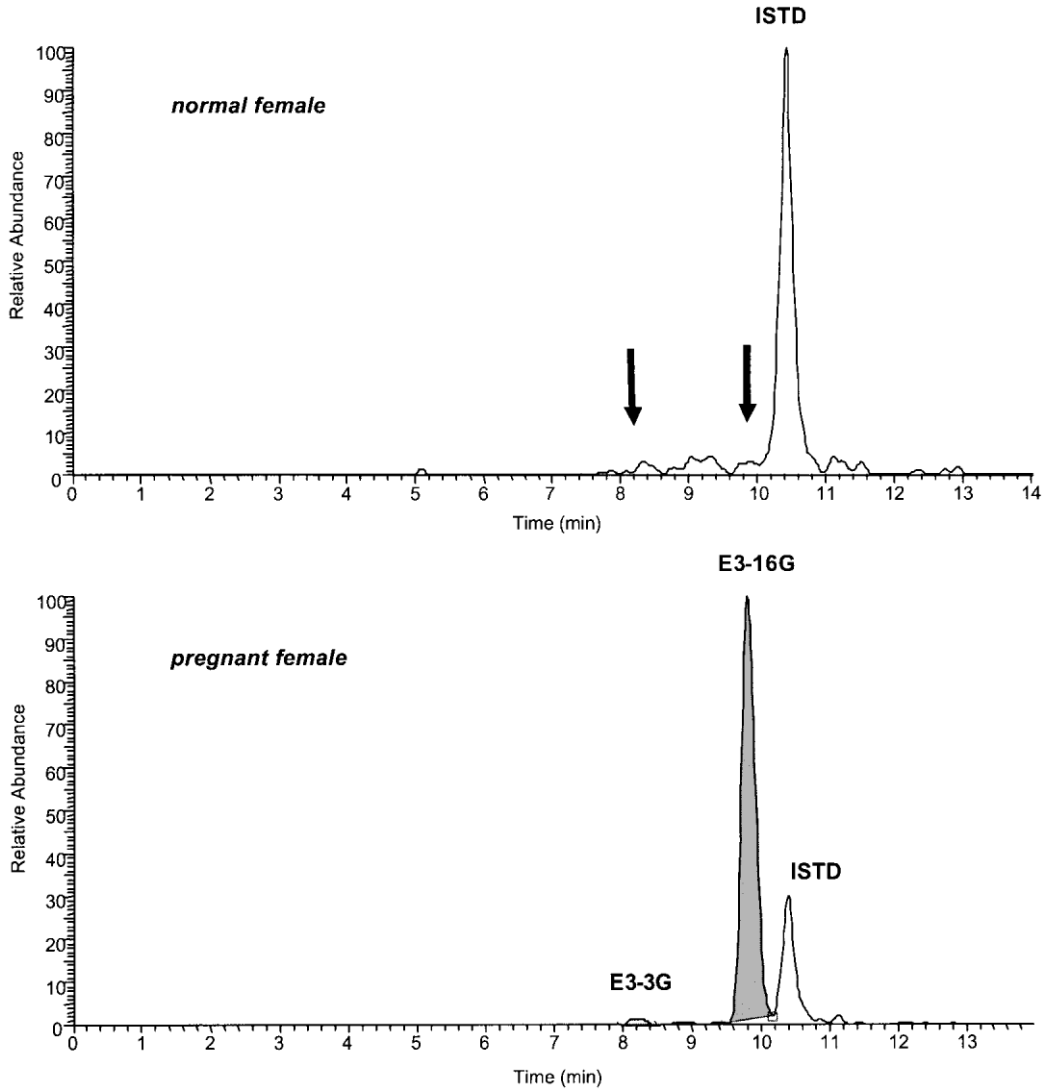

Figure 4. Total ion chromatogram of normal and pregnancy urine. E3-3G, estriol-3-glucuronide at 8.15 min; E3-16G, estriol-16-glucuronide at 9.85 min; and ISTD, testosterone-glucuronide-d_3 at 10.05 min.

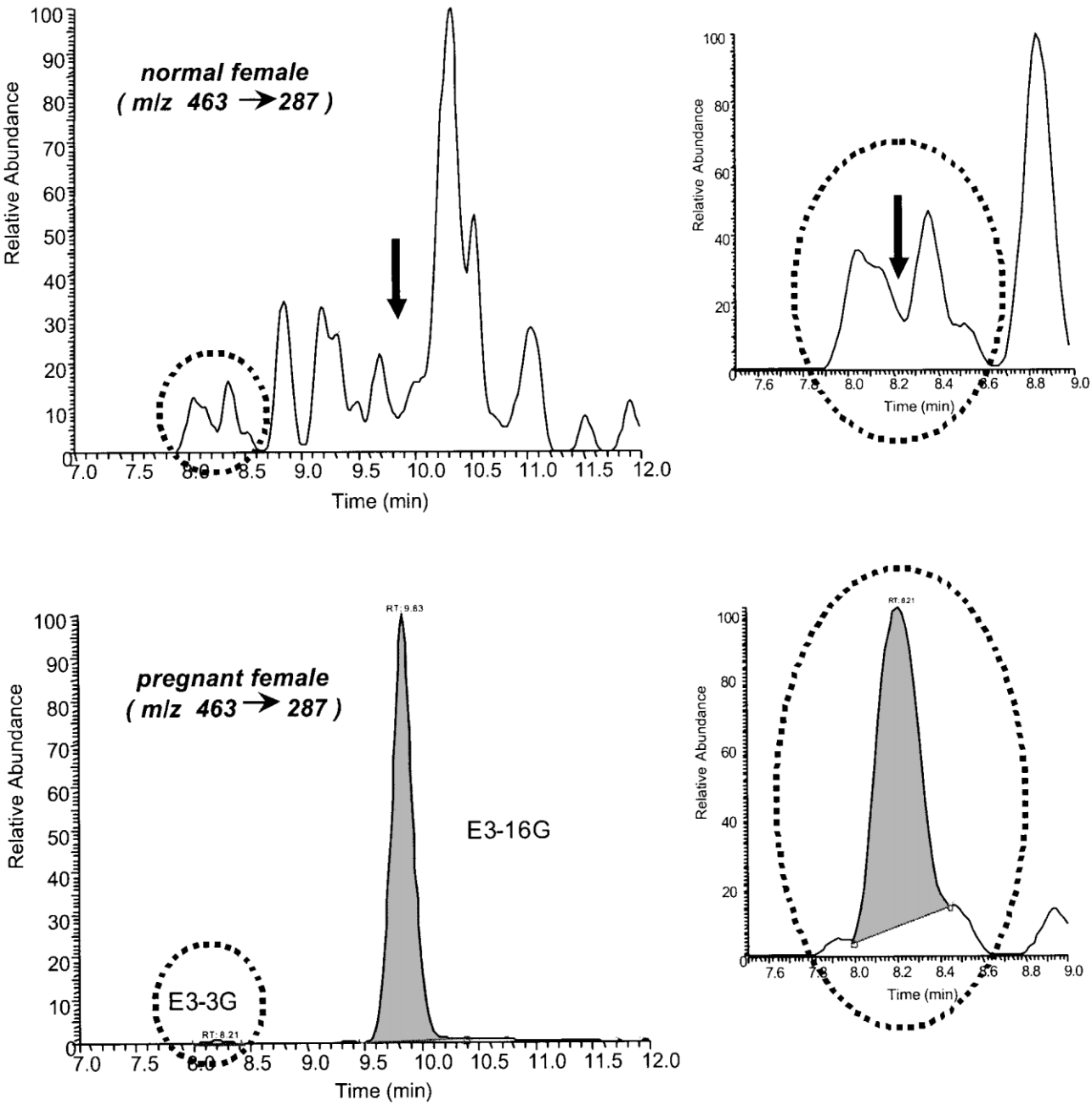

Figure 5. The comparison of normal and pregnancy case using selected-reaction monitoring at *m/z* 287.

gradient solvent system of 90% acetonitrile and 0.1% TEA at a flow rate of 100 μL/min. The column-switching HPLC (Shiseido Nanospace SI-2 HPLC; Tokyo, Japan) was performed with model 3001 pumps equipped with an auto injector (200 μL loop; model 3023), a UV detector (model 3002) and a dual, six-way switching valve unit (model 3012). The column temperature was maintained at 40°C with a model 3004 column oven. The UV wavelength was monitored at 254 nm.

Mass spectrometry. We employed ThermoFinnigan LCQ Advantage mass spectrometry (ThermoFinnigan, San Jose, CA, USA) with an electrospray ion source (ESI) fitted with a pneumatically assisted electro-spray probe. The orthogonal electrospray interface allowed the entire column effluent from the LC to be directed into the source without flow-splitting, which contributed to the greatly enhanced sensitivity. In the negative mode, we used typical ion source parameters as follows: ion spray voltage, 4.5 kV; capillary voltage, −10 V; tube lens offset, −50 V; capillary temperature, 250°C; and multiplier voltage, −880 V. Nitrogen was used as the sheath gas at 50 arbitrary units.

Method validation. For quality control and recovery tests, we applied several steps for method validation. We obtained 50 μL of pooled urine from normal, healthy pregnant women. Before obtaining the urine from those women, we pre-determined their E3-3G and E3-6G levels. We fortified the urine with 1.0, 3.0 and

10 μg/mL of E3-3G and E3-6G. After simple filtration with a PDVH filter (Milles-GV, 0.22 μm pore size, Milipore, Korea Co., Seoul, Korea), we directly injected the urine samples onto a pre-column. After activating column-switching valve, we backflushed the loaded samples onto the C_{18} analytical column (Fig. 1). Over a 3 week period, we analyzed three aliquots at each concentration. The calibration samples were fortified with the range 0.1–20 μg/mL for each E3-Gs.

RESULTS

Column-switching HPLC with tandem mass spectrometry

In order to find the best column-switching condition, we used the UV detector was used to monitoring of E3-3G and E3-6G at 254 nm. The urine sample was injected, and eluent 1 (0.1% TEA at pH 6.4) was passed through the first column to remove interferences and concentrate the E3-3G and E3-6G. Eluent 2 (90% acetonitrile and 0.1% TEA) backflushed the concentrated analytes, which went into the analytical column after column-switching (Fig. 1). Figure 2 shows a large amount of waste passing through within 7.40 min. It also shows E3-3G, E3-6G and T-G-d_3 as internal standards that are separated at 8.15, 9.85 and 10.05 min, respectively, after the column-switching.

Figure 3 depicts the negative-ion ESI-MS/MS spectra of E3-3G and E3-6G. The mass spectra of E3-3G and E3-6G demonstrated a deprotonated molecular ion ($[M\text{-}H]^-$) at *m/z* 463. In the SRM mass spectra, when the collision energy was 50%, the ion peak at *m/z* 287 ($[M\text{-}OG]^-$), corresponding to the loss of the *O*-glucuronide group from the molecular ion, commonly appeared in both glucuronide forms. The prominent ion peak at *m/z* 175, the fragment of *O*-glucuronide, was observed in the E3-3G.

Validation data

The linear responses to E3-3G and E3-6G were obtained in the range of 0.1–20 μg/mL with a correlation coefficient of 0.998 and 0.999, respectively. The equations for the linear regression lines were $y = 0.840x - 0.0167$ for E3-3G and $y = 1.393x - 0.0452$ for E3-16G, respectively. The limit of detection was 10 ng/mL (E3-3G) and 5 ng/mL (E3-16G).

The intra- and inter-day assay variances from normal, healthy pregnant whose E3-3G and E3-6G levels had been predetermined were less than 10% (Table 1). The recoveries ranged from 80.11 to 108.00%. The overall precision appears to be satisfactory for the quantification of E3-3G and E3-16G in pregnancy.

Patients' studies

We directly measured the E3-3G and E3-16G in urine samples from six healthy, pregnant women and five normal, healthy, non-pregnant females. Figure 4 depicts the total ion chromatograms of the urine sample. Most of interference backgrounds were clearly diminished, and E3-Gs did not appear in the case of the normal, healthy, non-pregnant women. However, E3-3G and a large amount of E3-6G did appear in the case of the normal, healthy pregnant women. We compared the non-pregnancy cases with the pregnancy cases using SRM (Fig. 5), which is the selected parent ion at *m/z* 463 and the produced product ion at *m/z* 287. In comparing these cases, we clearly detected E3-Gs in the case of pregnancy. From the developed method, the mean concentration of E3-3G and E3-16G in the pregnancies was 3.02 μg/mL (range 1.39–5.51) and 11.14 μg/mL (range 4.25–20.94), respectively (Table 2).

DISCUSSION

In 1997, Iwata *et al.* reported a column-switching HPLC with fluorescence detection method for the determination of E3-3G and E3-16G in the pregnancy urine, which was amenable to diagnostic application because of its simplicity and reproducibility. We have developed a simple method to analyze some E3-Gs without any sample preparation step. As shown in Figs 2 and 4, the interferences were clearly removed, and E3-3G and E3-16G were effectively separated. In the patients' study we could detect E3-3G and a large amount of E3-16G in the pregnancy case. This finding means that the increasing E3-Gs levels are caused by increasing steroid production by the fetoplacental unit, and reflect fetal well-being (Peter *et al.*, 1994).

Using column-switching LC-ESI-MS/MS, we developed a direct, simultaneous, simple, rapid and sensitive method to determine E3-3G and E3-16G in pregnancy urine. This direct mass-spectrometric method provides accurate data for diagnosing of congenital disorders

Table 2. Concentrations of estriol-3-glucuronide and estriol-16-glucuronide found in pregnancy women

Sample no.	Estriol-3-glucuronide	Estriol-16-glucuronide
1	4.50	14.51
2	2.47	11.89
3	1.39	4.25
4	5.51	20.94
5	1.80	6.50
6	2.43	8.76
Median	2.39	10.34
Range	1.80–5.51	4.25–20.94
Mean	3.02 ± 1.62	11.14 ± 6.04

instead of the RIA method. This method can be adapted to monitoring fetal well-being and is accepted as a useful indicator of fetoplacental status. In future studies this proposed method also allows for the evaluation of variations in the excretion pattern of estrogen conjugates in normal and high-risk pregnancies and diagnosis of the urogenital symptoms and vaginal cytology in the menopause women who are treated with exogenous estriol.

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