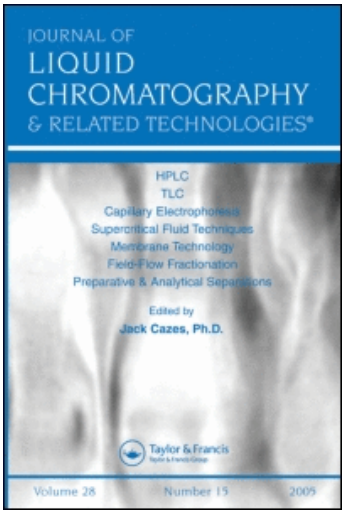

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DETERMINATION OF ESTRIOL-3-SULFATE-16-GLUCURONIDE IN PREGNANCY SERUM USING LC/TANDEM MS

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ABSTRACT

A determination method for estriol 3-sulfate 16-glucuronide (E_3-3S-16G) in pregnancy serum by liquid chromatography/electrospray ionization-tandem mass spectrometry has been developed. E_3-3S-16G gave an intense deprotonated ion in the negative-ion mode, which provided a product ion formed from the elimination of the glucuronic acid moiety by the collision induced dissociation.

The serum specimen was diluted, heated to remove protein followed by a solid phase extraction using an OASIS HLB cartridge, then subjected to liquid chromatography/tandem mass spectrometry. The recovery of E_3-3S-16G from serum was satisfactory and there was no interfering peak derived from the serum components at the elution position of E_3-3S-16G, but the ionization efficiency of the analyte was influenced by some substances from the serum and varied among each specimen.

To overcome this problem, the standard addition method was employed in the present study and the inter-assay coefficient of variation was acceptable. The developed method was applied to serum samples obtained from pregnant women in their third trimester and gave satisfactory results.

INTRODUCTION

In pregnancy, estriol (E_3) is predominantly produced in the placenta from androgen precursors of fetal origin and accounts for the largest part of the total estrogen. It is well-known that most of E_3 exists as conjugates and its major form in blood is a double conjugate, E_3 3-sulfate 16-glucuronide (E_3-3S-16G) (Figure 1).[1] The serum level of this metabolite reflects the function of the fetal/placental unit, therefore, many determination methods have been developed. However, most of the methods required the tedious and often poorly reproducible deconjugation step by enzymic or chemical hydrolysis and/or solvolysis before quantification.[2]

Andreolini et al.[3] and Nambara et al.[4,5] have reported the direct determination methods of E_3-3S-16G without deconjugation by high-performance liquid chromatography with fluorometric detection and radioimmunoassay using a specific antibody, respectively.

On the other hand, liquid chromatography/mass spectrometry (LC/MS) is the most promising analytical method for polar compounds, such as steroid conjugates. Particularly, LC/tandem MS (MS/MS) is an attractive methodology, which is highly specific and helpful for the determination of small amounts of substances in biological fluids.[6] Although, Zhang and Henion reported the determination of estrogen sulfate in human urine by LC/MS/MS,[7] the application of this method to analysis of estrogen conjugates in biological samples has been poorly studied.

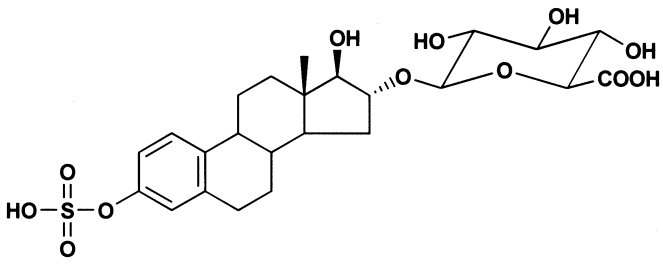

Figure 1. Structure of E_3-3S-16G.

Especially, to our knowledge, there has been no reported instance for LC/MS of estrogen double conjugates in biological fluids. The present paper deals with the development of an LC/MS/MS method using an ion trap mass spectrometer for the determination of E_3-3S-16G in pregnancy serum without deconjugation.

EXPERIMENTAL

Materials and Reagents

E_3-3S-16G was synthesized from E_3 3-benzyl ether 16-glucuronide acetate methyl ester[8] in our laboratories, by the usual methods. Stock solution of E_3-3S-16G was prepared as a 10 μg/mL solution in MeOH and subsequent dilutions were carried out to 1 μg/mL and 0.1 μg/mL with MeOH.

OASIS HLB cartridges (60 mg) were purchased from Waters Co. (Milford, MA, U.S.A.) and prepared before use by successively washing each cartridge with MeOH (2 mL), H_2O (2 mL), and 100 mM $HCOONH_4$ (1 mL).

All other reagents and solvents were commercially available and of analytical grade.

Apparatus

LC/MS was performed using a Finnigan MAT LCQ (San Jose, CA, U.S.A.) liquid chromatograph/ion trap mass spectrometer connected to a JASCO PU-980 (Tokyo, Japan) chromatograph, and electrospray ionization (ESI) was used in the negative-ion mode. The spray needle voltage was 5 kV, and the heated capillary temperature, the sheath gas flow rate, and the auxiliary gas flow rate were set at 290°C, 80 units and 30 units, respectively. The capillary voltage and the tube lens offset were both –10 V.

A Chemcobond ODS-W (150 × 2.1 mm i.d., Chemco Scientific, Osaka, Japan) column was used at a flow rate of 0.15 mL/min at 30°C. MS/MS was performed in the trap with helium as the collision gas, and the relative collision energy was set at 30%.

Serum Samples

Blood was collected from healthy pregnant and non-pregnant women, allowed to stand for 3 h at room temperature, and then centrifuged at 1500*g* for 10 min at 4°C. The serum was stored at –20°C until use. The serum obtained

from a non-pregnant woman was used as the control serum in which E_3-3S-16G was not detected by the present LC/MS/MS system.

Pretreatment of Serum Samples

A standard solution of E_3-3S-16G (2.5–20 ng in MeOH) was transferred to a glass tube and the solvent was evaporated. The serum (50 μL) was put into each tube and mixed, which corresponded to the concentration of 50–400 ng/mL. After standing for 10 min at room temperature, the resulting serum was diluted with 100 mM $HCOONH_4$ (1 mL) and heated for 3 min at 80°C to denature the protein. After centrifugation (1500*g*, 15 min), the supernatant was applied on an OASIS HLB cartridge.

After washing with 1 mM NaOH (1 mL) and H_2O (1 mL), the desired compound was eluted with MeOH (1 mL). The solvent was evaporated and then the residue was redissolved with the mobile phase (50 μL). After centrifugation (1500*g*, 15 min), part of the supernatant (10 μL) was subjected to LC/MS/MS.

Recovery of E_3-3S-16G from Serum and Influence of Endogenous Substances for Ionization of E_3-3S-16G

The control serum (50 μL) spiked with 10 ng of E_3-3S-16G was pretreated as described above and the peak area obtained from this sample was defined as A. The control serum (50 μL) was pretreated in the same way and then spiked with 10 ng of E_3-3S-16G, and the peak area obtained from this sample was defined as B. The peak area C was obtained from the standard 10 ng of E_3-3S-16G.

The recovery was calculated using the formula, A/B X 100 (%). The influence of the endogenous substances for the ionization of E_3-3S-16G was evaluated using the B/C ratio.

RESULTS AND DISCUSSION

LC/MS/MS of E_3-3S-16G

Our initial effort was directed at selecting a mobile phase suitable for the LC/MS of E_3-3S-16G. In the ESI mode, a buffer consisting of a non-volatile salt is not advantageous, therefore, a volatile one, such as $AcONH_4$ and $HCOONH_4$, is commonly used. Furthermore, it is generally known that the mobile phase using MeOH as an organic modifier provides a higher sensitivity for polar compounds than that using MeCN.

Based on these results, MeOH-10 mM $AcONH_4$ or $HCOONH_4$ (7:13, v/v) was used as the mobile phase and the effect of these organic salts on the formation of a characteristic ion was elucidated. Ikegawa et al.[9] have reported that in the LC/ESI-MS of double conjugates of bile acids, the ratio of singly charged ion $[M–H]^-$ and doubly charged ion $[M–2H]^{2-}$ was influenced by an acidic component of the salt added to the mobile phase according to the p*K*a value of the acidic functional group, and in the case when the p*K*a value of the acidic functional group was smaller than that of acidic component of salt, the doubly charged ion was mainly formed.

Although, E_3-3S-16G contains glucuronic acid (p*K*a ca. 3.5) and sulfuric acid (p*K*a *ca*. 1.5) groups, the singly charged ion at m/z 543 $[M–H]^-$ was obtained as a base ion, together with a fragment ion at m/z 367 [M–H–glucuronic acid moiety (G)]$^-$ in both mobile phases (AcOH; p*K*a 4.7, HCOOH; p*K*a 4.0); and contrary to our forecast, the intensity of the doubly charged ion (m/z 271) was very weak (Figure 2a). It was convenient for the quantitative analysis that the intense ion was only the deprotonated ion. The ion intensity of the deprotonated ion formed during the use of $HCOONH_4$ was 1.5 times stronger than that during the use of $AcONH_4$; therefore, MeOH-10 mM $HCOONH_4$ (7:13, v/v) was chosen as a mobile phase. Using this mobile phase with a flow rate of 0.15 mL/min, E_3-3S-16G eluted at ca. 5.3 min, therefore, the LC eluent entered the mass spectrometer from 4 to 7 min after injection using a diversion valve.

Among the several parameters of the ion source, the capillary temperature most significantly affected the ionization of the analyte, when a mobile phase containing a high percentage of water as used in this study, and was set at the relatively high temperature of 290°C. Other conditions of the ion source were also examined in order to obtain the higher sensitivity, and set as described in the experimental section.

The collision-induced dissociation using the deprotonated ion as the precursor ion with 30% of the relative activation energy provided the product ion formed from the elimination of the glucuronic acid moiety (m/z 367) as the base ion, besides the ion derived from the loss of sulfuric acid (m/z 463), with a weak intensity (Figure 2b). The intensity of the ion at m/z 463 did not increase, even though the larger activation energy was used. These results suggested that E_3-3S-16G was selectively determined by the MS/MS mode using the ion at m/z 543 and 367 as the precursor and monitoring ion, respectively.

Incidentally, in the ion-trap mass spectrometer, the selected reaction monitoring mode is not always superior to the multiple reaction monitoring (MRM) mode in sensitivity and sometimes less reproducible than the MRM mode. Therefore, the MRM mode monitoring the ion at m/z 367 after scanning at m/z 360–370, was used for the determination of E_3-3S-16G in serum.

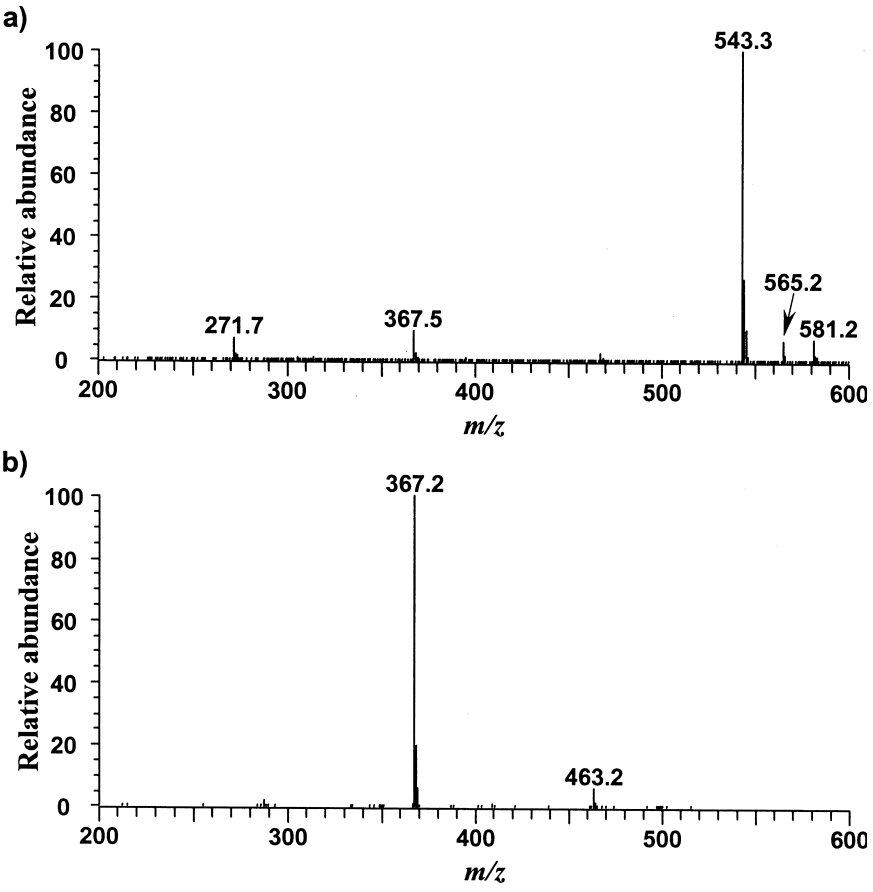

Figure 2. a) ESI mass spectrum of E_3-3S-16G. b) Product ion mass spectrum of E_3-3S-16G. Two nanograms of E_3-3S-16G was injected to LC/MS(/MS). Mobile phase: MeOH-10 mM $HCOONH_4$ (7:13, v/v).

Pretreatment of Serum Sample

We initially used a plasma specimen for the determination of E_3-3S-16G, but the compound could not be detected under any conditions. Although, the reason for this is not clear, we used the serum sample for this purpose.

The diluted serum specimen was heated to denature the proteins, and the resulting solution was purified by solid phase extraction using an OASIS HLB cartridge. This cartridge is packed with the polymer consisting of the hydrophilic

and lipophilic monomers, which is advantageous for highly polar compounds, and has recently been reported to give good results for the analyses of bile acid glucuronides.[10] At first, we used an usual octadecylsilyl (ODS) solid phase extraction cartridge, but the recovery of E_3-3S-16G was very poor due to its weak retention on the ODS.

Incidentally, the deproteination by an organic solvent (MeOH or MeCN) was unsatisfactory in the recovery of the analyte, so the denaturation with heat was employed. After washing the OASIS HLB cartridge with 1 mM NaOH and H_2O, the steroid was eluted with MeOH. The washing with 1 mM NaOH effectively improved the recovery.

The recovery rate of the spiked E_3-3S-16G (200 ng/mL), from the control sera obtained from five non-pregnant women, was 77.6 ± 4.2% [mean ± standard deviation (SD)] and a significant difference among individuals in the recovery was not observed.

The effect of the serum components for the ionization of E_3-3S-16G was examined. Five different control sera were separately pretreated and then 10 ng of E_3-3S-16G was added to the samples. Every one of the peak area values obtained from these samples (the values were defined as B in the experimental section) were smaller than that obtained from the standard sample (C), and the degree of ion suppression widely varied among the five specimens; when the peak area obtained from the standard sample was taken as 1.0, those from the control sera ranged from 0.62 to 0.97 (B/C).

It was obvious that some endogenous substances affected the ionization efficiency of E_3-3S-16G, but the substances could not be removed. In the quantitative analysis using LC/MS, the variation in the ionization efficiency among individual samples is one of the most troublesome problems. It was considered that the use of a compound labeled with a stable isotope as the internal standard (IS) is effective to overcome this problem, but the synthesis of the labeled E_3-3S-16G could not be accomplished.

We synthesized and tried to use the regio-isomer of E_3-3S-16G, estriol 16-sulfate 17-glucuronide, as an IS, which is not an endogenous compound and clearly separated from E_3-3S-16G in the above LC conditions (retention time: 4.6 min), but its ionization efficiency varied more widely than E_3-3S-16G among individual samples and it was not suitable as an IS. Based on these results, we abandoned the IS method, and used the standard addition method for the determination of E_3-3S-16G in serum.

Every one of the regression lines obtained from five control sera spiked with E_3-3S-16G (50–400 ng/mL) showed good linearity with a correlation coefficient (r^2) of more than 0.995, and nearly passed through the origin, though the slope varied among each serum, some of which are shown in Figure 3.

Typical chromatograms are shown in Figure 4. Figure 4a was the chromatogram obtained from a control serum without the addition of E_3-3S-16G, and

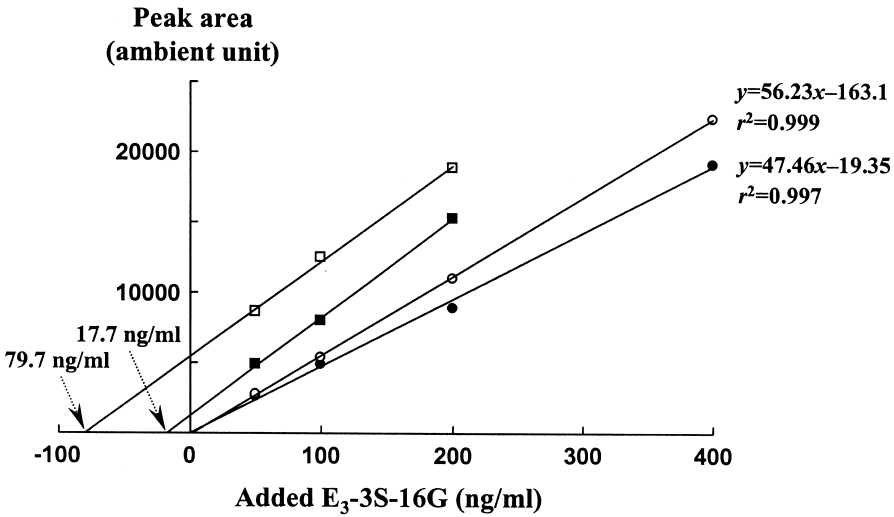

Figure 3. Examples of determination of E_3-3S-16G in human serum by standard addition method. ● and ○: control sera (non-pregnancy sera), ■ and □: pregnancy sera containing 17.7 and 79.7 ng/mL of E_3-3S-16G, respectively.

revealed that there was no interfering peak derived from serum components at the elution position of E_3-3S-16G. The chromatograms obtained from the control sera spiked with 50 and 200 ng/mL of E_3-3S-16G are shown in Figures 4b and 4c, respectively.

Although, the lower limit of quantification was not determined because the ionization efficiency varied among each serum as mentioned above, 50 ng/mL of E_3-3S-16G could be reproducibly quantified in all examined sera.

Determination of E_3-3S-16G in Pregnancy Serum

The present method was applied to the determination of E_3-3S-16G in the pregnancy serum. The standard E_3-3S-16G (2.5, 5.0 or 10.0 ng) was added to 50 μL of the serum specimen and the resulting serum was pretreated then subjected to LC/MS/MS. The peak area (y) was plotted versus the added amount of E_3-3S-16G (x) to obtain the calibration line. The concentration of E_3-3S-16G in the pregnancy serum was determined from the distance between the intersection of the line with the x axis and the origin of the coordinates (Figure 3).

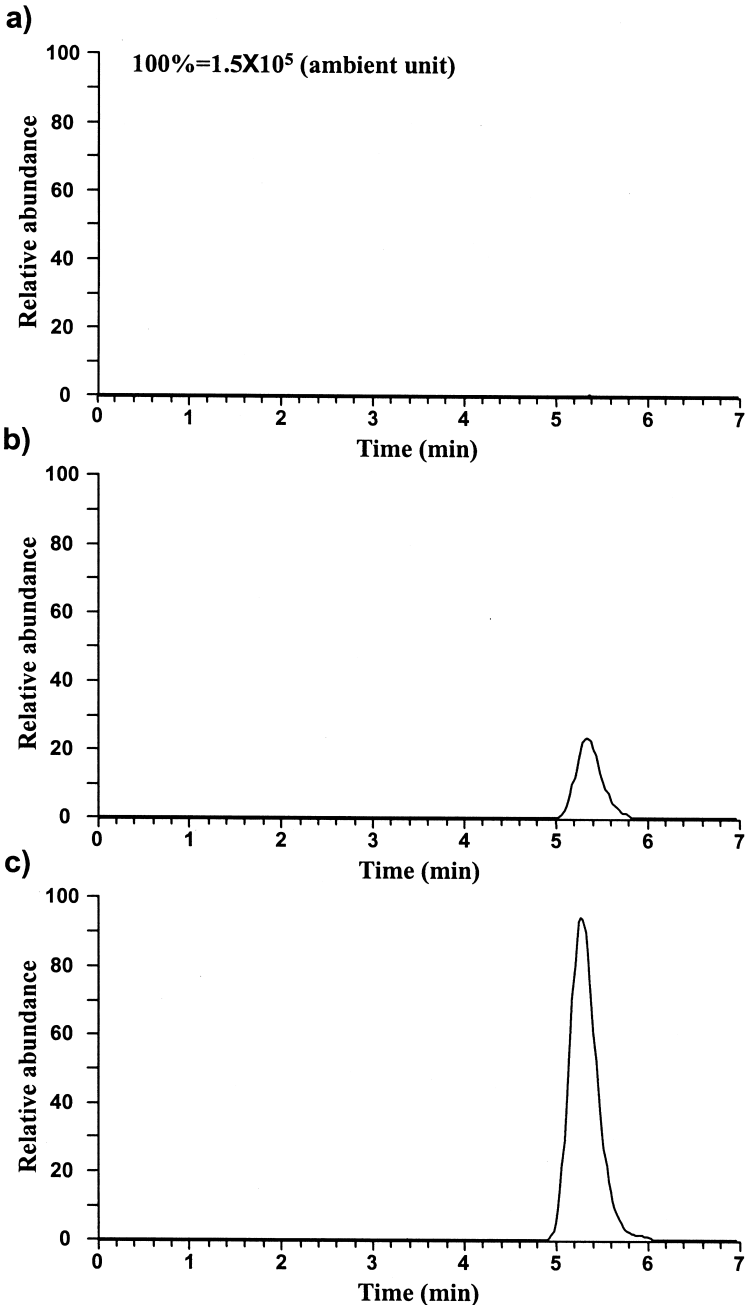

Figure 4. Typical LC/MS/MS chromatograms of E_3-3S-16G in human serum. a) Extract of control serum (non-pregnancy serum). b) and c) Extract of control serum spiked at the concentration of 50 ng/mL and 200 ng/mL E_3-3S-16G, respectively.

The mean serum concentration of E_3-3S-16G in the 36–41 weeks of gestation was 34.3 ng/mL (n=10) with the range of 5.2–79.7 ng/mL, which was compatible with previously reported results.[3,11]

Two different pregnancy sera were assayed five times. The concentrations were 27.9 ± 2.2 ng/mL [coefficient of variation (CV, SD/mean X 100%): 7.7%] and 32.8 ± 2.9 ng/mL (CV: 8.9%). These data demonstrated that the present method had an acceptable reproducibility.

In conclusion, we have developed the LC/MS/MS method for the determination of E_3-3S-16G in the pregnancy serum without prior deconjugation. The MS/MS mode was highly selective, but the problem that the ionization efficiency of E_3-3S-16G was influenced by some serum components developed. To overcome the problem, we employed the standard addition method, which was not always superior in the run capacity, but very useful in the case when a suitable IS was not available.

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