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Direct determination of estriol 3- and 16-glucuronides in pregnancy urine by column-switching high-performance liquid chromatography with fluorescence detection

Tetsuharu Iwata, Tsuyoshi Hirose, Masatoshi Yamaguchi*

Faculty of Pharmaceutical Sciences, Fukuoka University, Nanakuma, Jonan-ku, Fukuoka 814-80, Japan

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Abstract

An HPLC method for the direct and simultaneous determination of estriol 3- and 16-glucuronides in pregnancy urine is described. The method is based on direct derivatization of the glucuronic acid moiety in estriol glucuronides in urine with 6,7-dimethoxy-1-methyl-2(1H)-quinoxalinone-3-propionylcarboxylic acid hydrazide. The derivatization reaction proceeds in aqueous solution (or urine sample) in the presence of pyridine and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide at 37°C. The resulting fluorescent derivatives were separated by column-switching chromatography using a first column (YMC-Pack C_4) for clean-up of the derivatives and a second column (YMC Pack Ph) for the complete separation of the derivatives. The derivatives were detected spectrofluorimetrically at 445 nm with excitation at 367 nm. The detection limits (signal-to-noise ratio=3) for estriol 3- and 16-glucuronides were 150 and 180 fmol in a 5 μl of urine (14 and 17 ng ml^{-1} urine), respectively. The present method is highly sensitive and simple without any clean-up such as conventional solid-phase extraction. © 1997 Elsevier Science B.V.

Keywords: Estriol glucuronides

1. Introduction

In pregnancy, estriol is formed from maternal and fetal precursors (particularly cholesterol) by various enzymes in placenta or fetus. Estriol is then mainly metabolized to the glucuronide conjugates, estriol-3-glucuronide (E_3-3G) and estriol-16-glucuronide (E_3-16G) and excreted in urine. The relationships between the concentrations of these main estriol glucuronides in urine and pathological disturbances of pregnancy have been widely investigated. However, the detailed relationships have remained unknown. Thus, the measurement of these glucuronides in pregnancy urine might be useful for the investigation and biochemical monitoring of the function of the maternal/fetal unit.

Many high-performance liquid chromatographic (HPLC) methods with ultraviolet absorbance [1], electrochemical [2] and fluorescence [3–5] detection have been developed for the determination of estriol glucuronides in biological fluids. These HPLC methods provide insufficient sensitivity for bioanalytical purposes and, moreover, require the tedious and often poorly reproducible solid-phase extraction step for the extraction, purification and classification of main estriol metabolites in biological fluids. A fully

*Corresponding author.

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automated HPLC method has recently been developed for the determination of urinary E_3-16G [6]. This method is moderately sensitive, but only allows detection of E_3-16G.

The aim of the present investigation was to develop a simple and highly sensitive HPLC method for the simultaneous determination of E_3-3G and E_3-16G without conventional solid-phase extraction. We previously developed 6,7-dimethoxy-1-methyl-2(1H)-quinoxalinone-3-propionylcarboxylic acid hydrazide (DMEQ-hydrazide) as a highly sensitive fluorescence derivatization reagent for carboxylic acids [7]. The reagent reacts selectively with carboxylic acids of glucuronide conjugates even in aqueous solution using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) as the coupling agent under mild temperature. The reagent was successfully applied to the direct determination of urinary glucuronides of etiocholanorone and androsterone [8]. Thus, we examined the use of this reagent for the development of an HPLC method with fluorescence detection for the direct and simultaneous determination of E_3-3G and E_3-16G in pregnancy urine.

2. Experimental

2.1. Materials and solutions

All chemicals were of analytical-reagent grade, unless noted otherwise. De-ionized and distilled water, purified with a Milli-Q II (Millipore, Milford, MA, USA) system, was used for all aqueous solutions. E_3-3G and E_3-16G were obtained from Sigma (St. Louis, MO, USA). DMEQ-hydrazide was prepared as described previously [7]; it is now commercially available from Wako Pure Chemicals (Tokyo, Japan). DMEQ-hydrazide solutions were prepared in N,N-dimethylformamide. Reversed-phase columns, YMC-Pack C_4, C_8 and Ph (Yamamura Chemicals Labs., Kyoto, Japan), L-column ODS (Chemicals Inspection and Testing Institute; Tokyo, Japan) and TSK gel ODS 120-T (Tosoh; Tokyo, Japan) (250× 4.6 mm I.D., particle size 5 μm for all columns) were used.

A stock solution of E_3-3G and E_3-16G [0.10 m*M* (46.5 μg ml^{-1})] was prepared in water and stored at −20°C until use. The solution was diluted further with water to the desired concentrations before use.

2.2. Urine samples

Urine samples were obtained from normal pregnant women. Aliquots of about 20 ml from a single morning urine sample of the volunteers were stored and frozen until analysis.

Urinary creatinine concentrations were determined according to the Jaffé reaction, using a creatinine-test Wako (Wako, Tokyo, Japan).

2.3. Derivatization procedure

To 5 μl of an aqueous test solution (or urine sample) were added 5 μl of water and 50 μl of 4% pyridine in ethanol. The mixture was Vortex-mixed for ca. 5 min and then 50 μl of 50 m*M* DMEQ-hydrazide in N,N-dimethylformamide and 25 μl of 4 *M* EDC in water were added. The resulting mixture was warmed at 37°C for 10 min and centrifuged at 1000 *g* for ca. 5 min. The supernatant (10 μl) was injected into the chromatograph. For the reagent blank, 5 μl of water instead of 5 μl of a test solution was subjected to the same procedure.

The amounts of E_3-3G and E_3-16G in urine were calibrated by means of the standard addition method; the 5 μl of water added to non-pregnancy urine sample in the derivatization procedure was replaced by 5 μl of the E_3-3G and E_3-16G (2.0–500.0 ng each/5 μl) standard solutions. The mixture was allowed to stand at room temperature for 10 min and then treated as described above. The net peak heights of the individual glucuronides were plotted against the concentrations of the spiked glucuronides.

2.4. Column-switching chromatography

A flow-diagram of the column-switching HPLC system and a time program of the system controller are shown in Fig. 1 and Table 1, respectively. The system consisted of a first column (YMC-Pack C_4) separation of the DMEQ derivatives of E_3-3G and E_3-16G from urinary interferences and a second column (YMC-Pack Ph) for a complete separation of the derivatives.

The column-switching HPLC was performed with

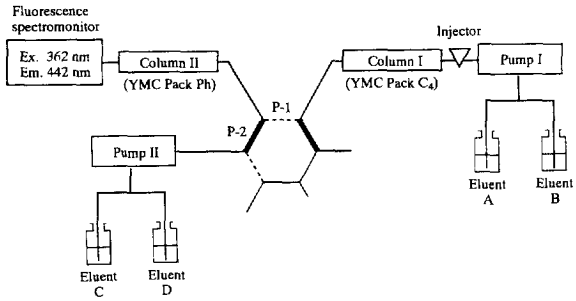

Fig. 1. Flow diagram of the column-switching HPLC system. P-1, valve position 1 (bold line); P-2, valve position 2; (dotted line). The path shown with the thin solid line in the valve was not used.

a Hitachi L-6200 intelligent pump (pump I) (Tokyo, Japan) equipped with a high-pressure sample injector (10-μl loop), a Hitachi L-6200 pump (pump II), a Jasco FP-210 spectrofluoromonitor (Tokyo, Japan) fitted with a 15-μl flow-cell and a Hitachi six-way automatic valve. The column temperature was maintained at 40±0.2°C with a Shimadzu CTO-6A column oven (Kyoto, Japan). Mixtures of methanol and aqueous 0.5% triethylamine [35:65 (eluent A), 45:55 (eluent B), 40:60 (eluent C) and 50:50 (eluent D), v/v] were used as mobile phases. The flow-rates of the four eluents were all 1.0 ml min^{-1}. The fluorescence intensity was monitored at 445 nm (emission) and 367 nm (excitation). Uncorrected fluorescence excitation and emission spectra of the eluate were measured with a Hitachi 650–60 fluorescence spectrophotometer fitted with a 20-μl flow-cell; the spectral bandwidths were 5 nm for both excitation and emission monochromators.

3. Results and discussion

The derivatization and HPLC conditions were examined using a standard solution of E_3-3G and E_3-16G [5.0 nmol (2.3 μg) ml^{-1} each].

3.1. Single-column HPLC and derivatization conditions

For first HPLC column, the separation of E_3-3G and E_3-16G was studied on reversed-phase columns, L-column ODS, TSK gel ODS 120-T and YMC-Pack C_4, C_8 and Ph, with aqueous methanol and aqueous acetonitrile as mobile phases using stepwise gradient elution. Moreover, the effect of triethylamine in mobile phase on the tailing of the peak due to the reagent DMEQ-hydrazide was examined. The tailing of the peak due to the reagent was minimized by addition of triethylamine to the mobile

Table 1
Program for controlling the column switching system

Time (min)	Valve position	Eluents (A–D)	Effect
0	P-1	A	The reagent blank components were eluted from column I.
21.0	P-2	C	Column II was equilibrated with eluent C.
		A	The fluorescent derivative of E3-3G retained on column I was transferred to column II.
		C	Wasted.
24.0	P-1	C	The fluorescent derivative of E3-3G (peak 1) was separated on column II.
		A	Interfering substances (peaks at retention times of ca. 35.0–0.48 min in Fig. 2B were removed from column I.
48.8	P-2	B	The fluorescent derivative of E3-16G (peak 2) was transferred from column I to column II.
		D	Wasted.
51.8	P-1	D	E3-16G (peak 2) was separated on column II.
		A	Interfering substances (Peaks at retention times of ca. 51.8–80.0 min in Fig. 2B were removed from column I.
80.0			End of analysis

phase as a counter-ion against the reagent. The best separation was obtained on YMC-Pack C_4 by step-wise gradient elution with mixtures of methanol and aqueous 0.5% triethylamine [0–35 min: eluent A (35:65, v/v); 35–60 min: eluent B (45:55, v/v)]. Fig. 2A shows a typical chromatogram obtained with the E_3-3G and E_3-16G standard solution. The peaks for E_3-3G and E_3-16G were completely separated from those due to the blank. The fluorescence excitation (maximum, 367 nm) and emission (maximum, 445 nm) spectra of the DMEQ derivatives of E_3-3G and E_3-16G in the eluates from the column were virtually identical to those of DMEQ-hydrazide in the mobile phase. E_3-3G and E_3-16G gave single peaks in the chromatogram.

The most intense peaks were obtained at concentrations greater than 10 m*M* of DMEQ-hydrazide solution for both the glucuronides; 50 m*M* was used in the procedure. EDC and pyridine were used to facilitate the derivatization of the glucuronides with DMEQ-hydrazide. Maximum and constant peak heights could be obtained at pyridine concentrations in the range of 1–5%; 4% was selected as optimum. The peak heights for the glucuronides were maximal and constant at concentrations of EDC higher than 2.0 *M*; 4.0 *M* was employed.

The derivatization reaction of the glucuronides with DMEQ-hydrazide apparently occurred even at 0°C; higher temperature allowed the fluorescence to develop more rapidly. However, at 60°C, peak heights decreased with the increase of heating time. At 37°C, the peak heights for both glucuronides were almost maximal after heating for 7 min. Thus, heating for 10 min at 37°C was employed in the procedure. The DMEQ derivatives in the final mixture were stable for at least 24 h in daylight at room temperature.

The precision was established from reproducibility measurements using a standard E_3-3G and E_3-16G solution [25 pmol (11.6 ng)/5 μl each); the relative standard deviations; ($n=10$) were 1.1 and 1.4%, respectively. The limits of detection for E_3-3G and E_3-16G were 10 and 12 fmol (4.7 and 5.6 pg) per injection volume (10 μl) at a signal-to-noise ratio of 3, respectively.

Glucuronide conjugates of several steroids

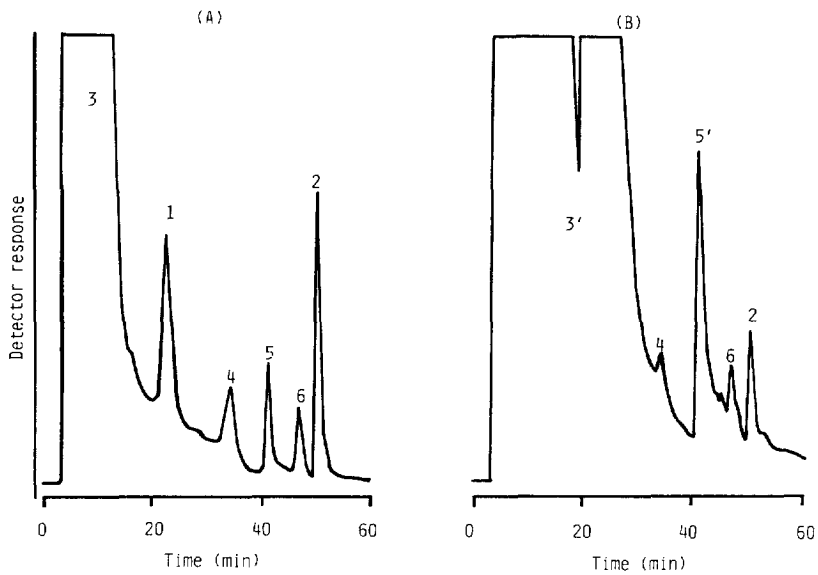

Fig. 2. Chromatograms of the DMEQ derivatives of (A) a standard mixture of E_3-3G and E_3-16G and (B) a pregnancy urine sample, obtained by using only column I and eluents A and B. A portion (5 μl) of a E_3-3G and E_3-16G standard solution [100 nmol (45.6 μg) ml^{-1} each] (or pregnancy urine) was treated according to the derivatization and single-column HPLC procedures. Peaks: 1, E_3-3G; 2, E_3-16G; 3–5, DMEQ-hydrazide components; 3′ and 4′, DMEQ-hydrazide component(s)+endogenous urinary substance(s). Step-wise gradient elution (0–35 min, eluent A; 35–60 min, eluent B). E_3-3G and E_3-16G concentrations in the pregnancy urine were 2.10 and 18.2 μg ml^{-1}, respectively.

(estrone-3-,17β-estradiol-3-, β-estradiol-17-, testosterone-17-, dehydroisoandrosterone-3-, etiocholanolone-3- and androsterone-3-glucuronides) and uronic acids (glucuronic acid and galacturonic acid) reacted with DMEQ-hydrazide under the derivatization conditions recommended to give the corresponding fluorescent derivatives. However, under the preliminary HPLC conditions, the DMEQ derivatives of these compounds were eluted at retention times different from those of E_3-3G and E_3-16G. Accordingly, the compounds did not interfere with the determination of E_3-3G and E_3-16G. The DMEQ derivatives of biogenic acids, such as dicarboxylic acids (oxalic, malonic succinic and adipic acids), hydroxycarboxylic acids (lactic and malic acids) and 17 α-amino acids were co-eluted with DMEQ-hydrazide under the HPLC conditions.

3.2. Column-switching chromatography and determination of urinary E_3-3G and E_3-16G

Pregnancy urine was treated according to the established derivatization procedure and the reaction mixture was subjected to the pre-column HPLC (Fig. 2B). The first column HPLC permitted the quantification of E_3-16G in the urine. However, the large peaks due to endogenous substances (t_R=0–40 min in Fig. 2B) interfered with the determination of E_3-3G in human urine. Thus, a column-switching HPLC was used to improve the analytical separation by combining two different chromatographic modes.

Various parameters of the second HPLC separation combined with the first column HPLC were studied using reversed-phase columns, such as L-column ODS, TSK gel ODS 120-T, YMC Pack C_4, C_8 and Ph with 0.5% triethylamine, methanol, acetonitrile and their mixtures as mobile phases. The most effective separation was obtained on YMC Pack Ph with mixtures of methanol and aqueous 0.5% triethylamine as the second HPLC. A typical chromatogram obtained with normal pregnancy urine is shown in Fig. 3A. The peaks for E_3-3G and E_3-16G in the urine were attributed on the basis of their retention times and the fluorescence excitation and emission spectra of the eluates in comparison with the standard compounds, and also by co-chromatography of the standards and urine sample with aqueous 20–100% acetonitrile or aqueous 25–100% methanol as mobile phases. When a male urine sample was treated according to the procedure, no peaks were observed at the retention times peaks 1 and 2 in the chromatogram (Fig. 3B), though peaks 1 and 2 were observed in the chromatogram of pregnancy urine (Fig. 3A). In addition, peaks 1 and 2 did not appear in the chromatogram of non-pregnancy urine. These results indicates that peaks 1 and 2 might be due to urinary E_3-3G and E_3-16G, respectively.

The recoveries (n=8) of E_3-3G and E_3-16G [35 and 100 pmol (16.3 and 46.5 ng) per 5 μl] added to a pooled normal urine was 96.2 and 95.1%, respectively. We could not find a suitable internal standard. However, losses of E_3-3G and E_3-16G throughout

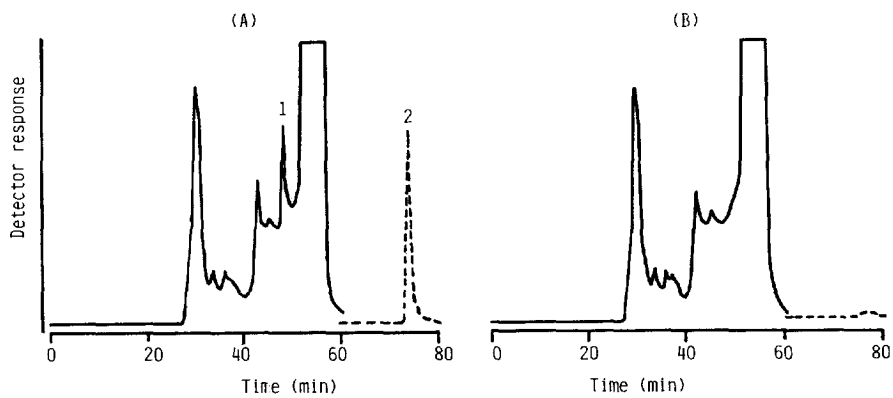

Fig. 3. Chromatograms obtained with human urine from (A) pregnant woman and (B) normal male. A portion (5 μl) of pregnancy urine (or male urine) was treated according to the procedure. Peaks: 1, E_3-3G; 2, E_3-16G; others, endogenous urinary substances. Detector sensitivity: solid line, ×4; dotted line, ×1. The same pregnancy urine as that in Fig. 2 was used.

the total procedure are negligible. Thus, external calibration is feasible for the quantification. The within-day precision was determined using a normal pregnancy urine containing E_3-3G and E_3-16G at concentrations of 4.31 and 37.3 nmol (2.0 and 17.3 μg) ml^{-1} urine, respectively. The relative standard deviations were 3.5% and 3.4% for E_3-3G and E_3-16G, respectively ($n=10$ in each case). Linear relationships were observed between the peak heights and the amounts of the concentrations of E_3-3G and E_3-16G (up to at least 100 μg ml^{-1} each) added to 1.0 ml of non-pregnancy urine (Fig. 4); the linear correlation coefficients were 0.995 and 0.998, respectively. The coefficients and the slopes of the calibration curves were independent of the urine samples used. The detection limits (signal-to-noise ratio=3) for estriol 3- and 16-glucuronides were 150 and 180 fmol in 5 μl of urine (14 and 17 ng ml^{-1} urine), respectively.

The concentrations of E_3-3G and E_3-16G in pregnancy urine from healthy volunteers determined by this direct derivatization method are given in Table 2. The concentrations of E_3-3G and E_3-16G in pregnancy urine were in good agreement with the published data [3,4].

The column-switching HPLC method with DMEQ-hydrazide permits the direct and simultaneous determination of E_3-3G and E_3-16G in pregnancy urine without any clean-up procedures such as solid-phase extraction. Although the method requires a long time for HPLC separation, it is highly sensitive and reliable and much simpler than the previous methods [1–6]. Furthermore, it does not require the time-consuming hydrolysis and/or solvolysis of the estriol glucuronides in the determi-

Table 2
Urinary concentrations of estriol-3- and 16-glucuronides in normal late pregnancy

Weeks of gestation	Age (years)	E_3-3G (mg g^{-1} creatinine)	E_3-16G (mg g^{-1} creatinine)
41	30	8.5	33.1
39	32	6.1	19.9
38	30	3.6	18.6
38	29	3.4	15.8
36	31	1.7	21.5
35	29	2.8	13.4
34	35	4.3	8.6
34	30	2.1	15.7
33	29	2.7	15.7
32	27	6.6	20.6
31	28	2.1	6.2
30	30	1.7	12.3
30	26	2.4	9.9
28	28	4.1	16.0
28	27	3.9	11.6
Mean value		3.7	15.9
S.D.		1.9	6.3

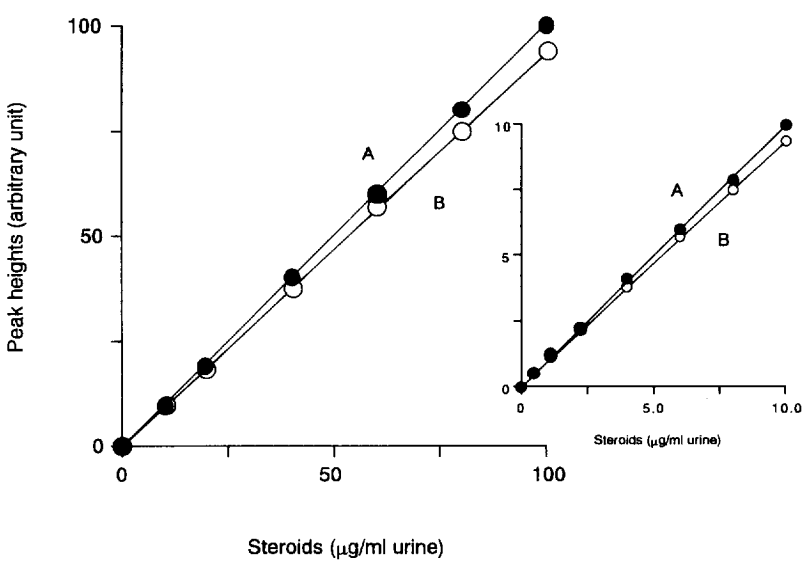

Fig. 4. Calibration graphs of urinary E_3-3G and E_3-16G. Curves: A, E_3-3G; B, E_3-16G.

nation of total urinary estriol. This method could be applied to the diagnosis, monitoring and biomedical investigations of the function of the maternal/fetal unit for routine use.

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