Determination of Estriol and Creatinine in Urine by High Performance Liquid Chromatography

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A quantitative method for the determination of estriol (E3) and creatinine (C) in random urine by high performance liquid chromatography is described. The mobile phase was a mixed solution of methanol and phosphate buffer (0.025 M, pH 6.5) and the detection wavelength was at 205 nm. The method was simple, rapid and accurate. The OCV for E3 and C using this method were 1.7 – 3.4% and 2.2 – 2.5%, respectively. The RCV for E3 and C were 6.2 – 7.0% and 4.5 – 6.9%, respectively. The recoveries were 87 – 104% for E3 and 98 – 103% for C, respectively. The method has been used for clinical determinations.

INTRODUCTION

In order to lower perinatal mortality and morbidity, many methods for monitoring foetal and pregnancy health have been developed. One of the most reliable indices has been 24-h urinary estriol excretion (Goebelsmann, 1979; Ostergard, 1971). However, a number of problems have complicated its clinical use.

The patient and physician inconvenience, as well as innate collection errors of the 24-h urine specimen, are practical clinical problems. However, a series of studies has culminated in the conclusion that random urine estriol/creatinine ratio is a satisfactory, practical, and reliable index of foetal welfare for the majority of patients (Aubry, 1975; Lockwood, 1974). Hitherto, the determination of estriol and creatinine were mainly using the methods of Brown (1955) and Folin (1914), respectively, then calculating the estriol/creatinine ratio base on the results obtained. Apart from the methods being time consuming and costly, the accuracy was also affected by requiring two separate analytical procedures to obtain the result.

A rapid, simple and accurate HPLC method is described in this paper by which estriol and creatinine in pregnancy urine could be determined simultaneously.

EXPERIMENTAL

Apparatus. The liquid chromatograph consisted of a WGP-5 reciprocating piston pump (Zhijian scientific instrument factory, Hangzhou, China) and a UV/FL-1 UV detector (Nanjing analytical instrument factory, China). Chromatograms were obtained on a recorder, (Dahua, Shanghai, China).

Reagents. Estriol standard solution (1 mg/mL): The solution was prepared by dissolving 100 mg of estriol (Sigma, St Louis, MO, USA) in methanol, diluting to 100 mL and storing in a refrigerator. Creatinine standard solution (1 mg/mL): The solution was prepared by dissolving 100 mg of creatinine (Merck, dried at 100 °C for 2 h) in 0.1 M HCl and diluting to 100 mL, followed by the addition of several drops of chloroform and storing in a refrigerator. Phosphate buffer solution (0.025 M, pH 6.5): 139 mL 0.05 M NaOH was added to 500 mL of 0.05 M KH_2PO_4 and this was diluted to 1000 mL with water. All chemicals, except where otherwise stated, were of analytical grade, and all solutions were prepared in doubly distilled water.

Chromatographic conditions. Stainless-steel column: Nucleosil C_{18} column (5 mm × 200 mm) (Dalian, China). Mobile phase: methanol + phosphate buffer (pH 6.5, 75 : 25 v/v). This solution was passed through a 0.45 μm filter under vacuum before use. Flow-rate: 0.80 mL/min (5.4 MPa). Injected sample volume: 20 μL.

Determination of estriol. 0.5 mL 12 M hydrochloric acid was added to 2 mL urine. The mixture was heated at 100 °C for 60 min in a heating bath and then cooled rapidly under running tap water. 5 mL of diethyl ether was added to the cooled hydrolysed urine and vortex mixed for 2 min. Then 4 mL of the organic layer was collected and washed once with 2 mL of 8% $NaHCO_3$ solution (adjusted pH10 with 20% NaOH solution). 3 mL of the washed organic layer was collected and evaporated to dryness with air at 50 °C, and then 1 mL of the HPLC mobile phase was added to dissolve the residue. After 1 min of votex mixing, 20 μL of the sample solution were injected into the HPLC system.

Determination of creatinine. The urine sample was centrifuged at 3000 rev/min for 10 min and diluted 50-fold with the mobile phase. Then 20 μL of the mixture were injected into the HPLC system. Figure 1 shows the results obtained for the analysis of the standard and urine samples.

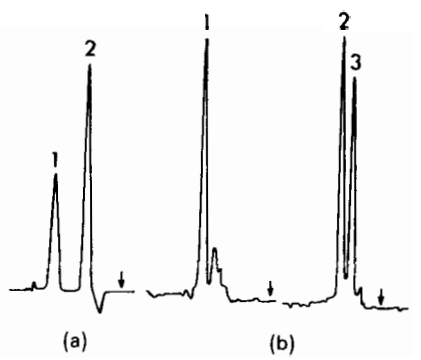

Figure 1. Chromatograms of estriol and creatinine in standard solution (a) and urine (b). 1, Estriol (4 min 09 s); 2, creatinine (2 min 56 s); 3, unknown substance (2 min 20 s).

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RESULTS AND DISCUSSION

Selection of detector wavelength

The absorptions of estriol and creatinine were scanned from 200 to 260 nm. As the scan moved towards shorter wavelengths, both the absorptions increased rapidly (Fig. 2). For optimum sensitivity, the detector wavelength was selected at 205 nm.

Selection of mobile phase

A study was made of the effects upon the chromatographic peaks of varying the composition and acidity of the mobile phase. The results indicated that the retention time of estriol obviously is reduced with increase in methanol content (Fig. 3). However, the retention time of creatinine was unaltered. Thus, estriol and creatinine could be separated satisfactorily by selecting a suitable ratio for the mobile phase. On the other hand, neither retention time changed noticeably with varying acidity of the mobile phase. Both the peaks tended to decrease when the acidity was below pH 5, however. For the above reason, the ratio of methanol and phosphate buffer was selected as 75:25 (v/v), pH 6.5.

Reproducibility

The reproducibility of the method was assessed based on repeated analysis of a solution containing equal concentrations of estriol and creatinine. Intraday reproducibility studies, evaluated by 7 assays each of 8 μg/mL concentrations of estriol and creatinine, yielded coefficients of variation of 3.4% and 2.5% for estriol and creatinine, respectively. Interday reproducibility studies, evaluated by assaying the same concentrations 7 times over a 7-day period, were 7.0% and 6.9% for estriol and creatinine, respectively. At a concentration of 35 μg/mL, the intraday coefficients of variation for estriol and creatinine were 1.7% and 2.2%, respectively. The interday coefficients of variation for estriol and creatinine were 6.2% and 4.5%, respectively.

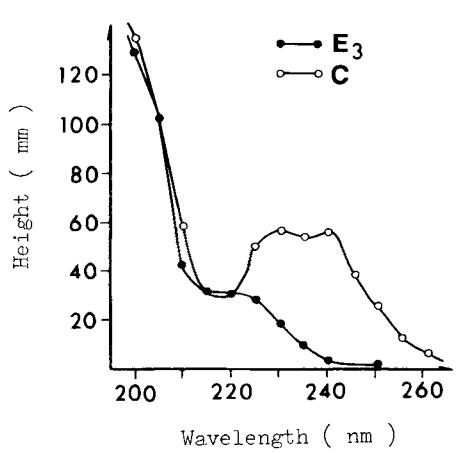

Figure 2. UV spectra of estriol and creatinine.

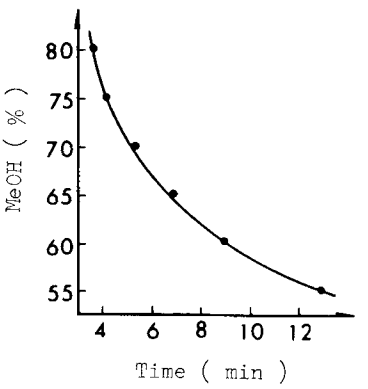

Figure 3. Relation between retention time of estriol and composition of mobile phase.

Relationship between peak height and concentration

Under selected conditions the relationship between the peak height and the concentrations of estriol and creatinine were studied. The results showed that the peak height was linearly related to the estriol concentration for the range (2×10^{-6}) - (1×10^{-4})g/mL (Fig. 4). The detection limit was 8×10^{-7} g/mL. For creatinine, the linear range was (1×10^{-6}) - (9.5×10^{-5}) g/mL (Fig. 5) and the detection limit was 3×10^{-7} g/mL.

Recovery

After estriol and creatinine in urine samples had been determined ($n=6$), different amounts of estriol and creatinine were added to test the analytical recovery. The formula for calculating this was $[(A-B)/S]\times 100\%$, where A is the amount measured, B is the amount present and S is the amount added. Results showed that the recovery was satisfactory (Table 1).

CONCLUSION

Estriol and creatinine in random urine samples from pregnant patients were determined simultaneously by

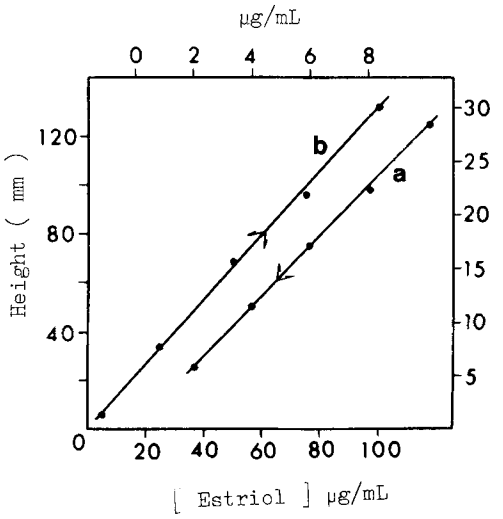

Figure 4. Relation between peak height and concentration of estriol. Line a, sensitivity 2; line b, sensitivity 4.

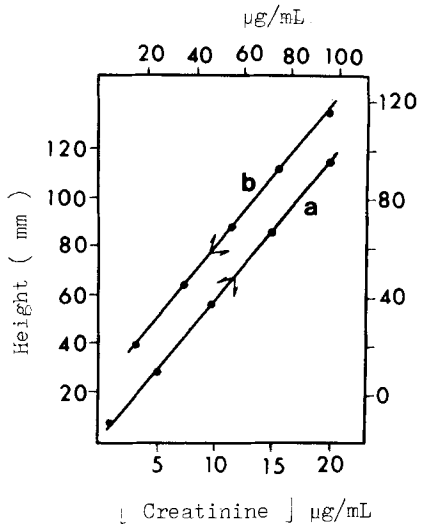

Figure 5. Relation between peak height and concentration of creatinine. Line a, sensitivity 2; line b, sensitivity 4.

high performance liquid chromatography. The mobile phase was a mixture of methanol and phosphate buffer (75:25, v/v) and the detection wavelength was 205 nm. Under selected conditions, estriol and creatinine exhibited sensitive UV absorptions. The peaks of the chromatogram were well defined, and separated from other compounds in urine. The analytical recovery of various amounts of estriol and creatinine added to urine were 87 - 104% for estriol and 98 - 103% for creatinine, respectively. The method was rapid, simple, sensitive and accurate, and has been used in clinical determinations.

Table 1. Recovery test of estriol and creatinine ($n = 6$)

	Amount present	Amount added	Amount measured	Recovery ±SD%
1 Estriol (μg/mL)	15.4	20.0	36.25	104.3±5.4
Creatinine (mg/mL)	2.21	2.00	4.18	98.4±7.6
2 Estriol (μg/mL)	11.2	20.0	30.30	95.5±7.6
Creatinine (mg/mL)	0.69	1.00	1.72	103.0±4.3
3 Estriol (μg/mL)	3.30	5.00	7.65	87.0±5.3
Creatinine (mg/mL)	0.56	1.00	1.58	102.1±2.2

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