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PRELIMINARY STUDIES ON THE DETERMINATION OF ESTRIOL-16α-GLUCURONIDE IN PREGNANCY URINE BY DIRECT RADIOIMMUNOASSAY WITHOUT HYDROLYSIS

HERMAN ADLERCREUTZ, TESSA LEHTINEN and MATTI TIKKANEN

Department of Clinical Chemistry, University of Helsinki, Meilahti Hospital, 00290 Helsinki 29, Finland

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SUMMARY

Results of preliminary studies on the determination of estriol-16α-glucuronide in pregnancy urine by radioimmunoassay without hydrolysis are presented. The method was also compared with a colorimetric estriol assay method and a gas chromatographic (g.l.c.) method for specific determination of estriol-16α-glucuronide. Despite a rather high degree of specificity of the antiserum the results obtained suggest that the values obtained are about 24% too high. However, the correlation coefficient when compared to the g.l.c. method was high ($r = 0{\cdot}97$) whereas it was much lower when the method was compared with the colorimetric estriol procedure ($r = 0{\cdot}86$). The results obtained show that the assay of estriol-16α-glucuronide is simple and precise and that the day to day variation (7–11%) in the excretion of this conjugate does not significantly exceed the methodological variation (inter-assay coefficient of variation = 7·7%).

INTRODUCTION

Estriol-16α-glucuronide is quantitatively the main estrogen metabolite excreted in human pregnancy urine. Its urinary level, unlike that of total estriol, is relatively unaffected by the administration of antibiotics like ampicillin [1]. Furthermore, alterations in maternal liver function result in marked changes in the urinary concentrations of other estriol conjugates while the excretion of estriol-16α-glucuronide remains reasonably constant [2]. These observations would suggest that estriol-16α-glucuronide is the most suitable urinary estrogen metabolite for the purpose of monitoring fetal well-being [3]. This communication describes our preliminary results on developing a radioimmunological method for the direct assay of estriol-16α-glucuronide in urine.

EXPERIMENTAL

Material. Twenty-four hour urine samples were collected both from hospitalized pregnant women with various diseases and from healthy pregnant women. The samples were immediately frozen and stored at −18°C unless the analysis was started on the same day.

Reference compounds and reagents. 6,7-^{3}H-estriol-16α-glucuronide was prepared according to the method of Tikkanen and Adlercreutz [4] using human liver preparations; later this compound became available from The Radiochemical Centre (Amersham, Bucks., England). The S.A. of the two preparations were 24 and 32 Ci/mmol, respectively. The following *reference steroids* were used (abbreviations in parentheses): estrone (E_1), estradiol-17β (E_2), estriol (E_3) and estriol-16α-glucuronide (E_3-16Gl) (Ikapharm, Ramat–Gan, Israel), estrone-3-glucuronide (E_3-3Gl), estradiol-3-glucuronide (E_2-3Gl), estriol-3-glucuronide (E_3-3Gl), estradiol-17β-glucuronide (E_2-17Gl), 17-epiestriol-16α-glucuronide (17-epiE_3-16Gl) and 16-epiestriol-16β-glucuronide (16-epiE_3-16Gl) (gift from Dr. A. E. Kellie), 2-hydroxyestradiol-1-S-glutathione (2-OHE_2-1-S-glutathione) (gift from Dr. J. S. Elce), estrone-3-sulfate (E_1-3S) (Orion–Yhtymä, Helsinki, Finland), estriol-3-sulfate ($E_3$3S) (Leo Ab, Hälsingborg, Sweden), pregnanediol-3α-glucuronide (P_2-3Gl) (Sigma Chemical Company, St. Louis, Mo., U.S.A.).

The *assay buffer* used was sodium borate buffer, pH 8·0, containing 0·00001% Tween 20 (w/v) and 0·02% gelatine (w/v). Dextran-coated charcoal (625 mg of Norit A and 625 mg Dextran T70 in 100 ml of assay buffer) was used for the separation of bound and unbound E_3-16Gl.

Antiserum. The E_3-16Gl used for antibody preparation was isolated from 40 l of pregnancy urine. Its purity was assessed by field desorption mass spectrometry and was found to be similar to that of commercially available preparations [5]. The crystalline hapten was linked to BSA as described by Kellie [6] The antigen so prepared was injected, in Freund's adjuvant, into three rabbits, two of which produced antibodies. These rabbits developed antisera which could be used at a dilution of 1:200 (v/v). At this dilution 40–50% binding was obtained in the absence of unlabeled hormone.

Methods. Estriol in urine was assayed using the extraction procedure and method of Brown and Coyle [7]. This was done in another hospital, in the routine laboratory. Gas chromatographic determination of E_3-16Gl was carried out as described previously [4].

Radioimmunoassay procedure for E_3-16Gl. The urine is suitably diluted (1000 to 100,000 times) with the assay buffer, and 0·5 ml aliquots transferred in duplicate into disposable Eppendorf microtubes. The standard curve is prepared in duplicate using standard E_3-16Gl in amounts ranging from 50 to 2000 pg. After adding 100 μl of the diluted antiserum and labeled E_3-16Gl (5000 c.p.m. corresponding to 10·0 nCi) to each tube the solutions are mixed and equilibrated in an Eppendorf rotamixer 3300. The tubes are then incubated at +4°C overnight. The tubes are now placed in an ice-bath and 0·2 ml of Dextran-coated charcoal added to each. After mixing and incubating for 10 min, at +4°C, the tubes are centrifuged (2·5 min, 8000 *g*, +4°C). Aliquots (0·5 ml) of supernatant are then transferred to counting vials containing scintillation fluid and radioactivity is measured in a Wallac 81000 automatic liquid scintillation counter (Wallac Oy, Turku, Finland).

RESULTS

The standard curve was linear between 50 and 1000 pg. Because of the high concentration of E_3-16Gl in pregnancy urine the absolute sensitivity of the method was not assessed. However, when non-pregnancy urine samples were analysed other urinary constituents interfered with the determination.

Precision. The intra-assay coefficient of variation was calculated from 30 duplicate determinations of urine samples with E_3-16Gl concentrations varying from 20·2 to 55·0 mg/24 h [8] and was found to be 6·3%. The same urine samples were used to calculate the inter-assay coefficient of variation, which was found to be 7·7%.

The accuracy of the method was tested by adding 250 to 5000 pg amounts of reference E_3-16Gl to urine samples containing endogenous E_3-16Gl. The mean recovery was 95·9 ± 4·1% (n = 5).

The specificity of the antiserum was investigated by determining the cross-reaction of a number of unconjugated and conjugated steroids according to the procedure of Abraham [9]. All the following steroids cross-reacted less than 1% with E_3-16Gl: E_1, E_2, E_3, E_1-3S, E_1-3Gl, E_2-3Gl, E_3-3Gl, E_2-17Gl, 16-epiE_3-16-Gl, 17-epiE_3-16Gl, 2-OHE_2-1-S-glutathione and P_2-3Gl. There was a 3% cross-reaction with E_3-3S.

The day to day variation in urinary excretion of E_3-16Gl was investigated in four hospitalized pregnant women by collecting 24-h urine specimens over four consecutive days. The coefficients of variation were 6·6, 7·1, 8·5 and 11·3% (mean 8·4%). The coefficient of variation of E_3-16Gl levels was also calculated for 23 pairs of 23-h urine samples collected on consecutive days, it was 10·2%. Thus the day to day variation in E_3-16Gl excretion does not significantly exceed the methodological (inter-assay) variation.

Comparisons between RIA and gas chromatographic assays of E_3-16Gl. Parallel determinations were made with a specific gas chromatographic method [4] and the RIA method. The correlation coefficient obtained was 0·97 (n = 13) but the RIA method gave about 24% higher values. Assays were made in the range from 0·5 to 62·7 mg E_3-16Gl/24 h and the regression equation was: $y = 1{\cdot}279x + 0{\cdot}832$, where y represents the RIA values. Comparison with the procedure of Brown and Coyle [7] which is used routinely in the hospital from which the urine samples were obtained showed a correlation coefficient of 0·86 (n = 35), but the RIA values were about 50% higher. Assays were made in the range from 7·0 to 62·2 mg E_3-16Gl/24 h and the regression equation was: $y = 1{\cdot}368x + 3{\cdot}011$, where y represent the RIA method.

DISCUSSION

The antiserum to estriol-16α-glucuronide used in the present methodological study showed a rather high degree of specificity and surprisingly did not cross-react with unconjugated estrogens. However, despite this it gave about 24% higher values than a specific g.l.c. procedure, but the correlation coefficient was high (r = 0·97). It also gave 50% higher values than the urinary estriol method of Brown and Coyle [7] but in this case the correlation coefficient was much lower (r = 0·86). In the latter procedure no corrections are made for losses incurred during hydrolysis and purification. We observed the same phenomenon when we compared a mass fragmentographic (MF) and a radioimmunoassay for plasma unconjugated estriol in pregnancy. Despite the use of a specific antiserum to estriol the RIA gave 40–100% higher values than the MF technique and comparison of normal values cited in the literature with those obtained with these two methods indicated the same discrepancies [10]. The reason for these are not obvious and need further investigation. In this connection it may be mentioned that if different dilutions of the same urine sample were assayed the results were the same. It must be emphasized that the specificity of most published RIA methods has only been tested with reference standards with regard to cross-reactivity and direct positive proof of specificity is rarely given.

The theoretical advantages of measuring urinary estriol-16α-glucuronide instead of estriol or total estrogens was briefly outlined in the introduction. In addition, this conjugate has a renal clearance 3–4 times that of creatinine, thus its urinary excretion should not be greatly influenced by alteration in glomerular filtration. Specific g.l.c. assay of estriol in pregnancy urine shows a day-to-day variation of 18·6% [11]. Other investigators using less specific as-

says for estriol have found day-to-day variations from 20 to over 50% [12–15]. In the present investigation we found mean day-to-day variations of 7–11%, the inter-assay coefficient of variation of the method being only slightly less (7·7%). As these figures are lower than reported for total urinary estriol it would suggest that the excretion of estriol-16α-glucuronide in urine is quite constant and would verify our previous theoretical considerations based on observations made on estrogen levels in various disease states [1–3].

A very large sample number would be needed to demonstrate any superiority of estriol-16α-glucuronide assay over that of estriol or total estrogen measurements. Thus we have immunized some sheep in order to raise more antibodies. In addition, we are trying to simplify the technique to make it more suitable for routine clinical use. Preliminary studies suggest that a shorter incubation time (about 3 h) can be used.

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REFERENCES

1. Tikkanen M. J., Pulkkinen M. O. and Adlercreutz H.: *J. steroid Biochem.* **4** (1973) 439–440.
2. Tikkanen M. J. and Adlercreutz H.: *Am. J. Med.* **54** (1973) 600–604.
3. Adlercreutz H. and Tikkanen M. J.: In *Hormone Investigations in Human Pregnancy* (Edited by R. Scholler). Editions Sepe, Paris (1974) pp. 33–51.
4. Tikkanen M. J. and Adlercreutz H.: *J. steroid Biochem.* **3** (1972) 807–818.
5. Adlercreutz H., Soltmann B. and Tikkanen M. J.: *J. steroid Biochem.* **5** (1974) 163–166.
6. Kellie A. E., Samuel V. K., Riley W. J. and Robertson D. M.: *J. steroid Biochem.* **3** (1972) 275–288.
7. Brown J. B. and Coyle M. G.: *J. Obstet. Gynaec. Brit. Cwlth.* **70** (1963) 219–224.
8. Snedecor G. W.: *Biometrics* **8** (1952) 85–88.
9. Abraham G. E.: *J. clin. Endocr. Metab.* **29** (1969) 866–870.
10. Adlercreutz H., Nylander P. and Hunneman D. H.: *Biomedical Mass Spectrometry* **1** (1974) 332–339.
11. Heikkilä J.: *J. steroid Biochem.* **2** (1971) 83–93.
12. Klopper A.: In *Research on Steroids* (Edited by C. Cassano). Il Pensiero Scientifico, Rome, Vol. 1 (1964) pp. 119–132.
13. Shelley T. F., Cummings R. V., Rourke J. E. and Marshall L. D.: *Obstet. Gynec.* **35** (1970) 184–190.
14. Bergsjø P., Bakke T., Salamonsen L. A., Støa K. F. and Thorsen T.: *J. Obstet. Gynaec. Brit. Cwlth.* **80** (1973) 305–310.
15. Scholler R., Castanier M., Fortin M., Veinante A. and Avigdor R.: In *Hormonal Investigations in Human Pregnancy* (Edited by R. Scholler). Editions Sepe, Paris (1974) pp. 87–117.