DIRECT RADIOIMMUNE ASSAY OF 16-GLUCOSIDURONATE METABOLITES OF ESTRIOL IN HUMAN PLASMA AND URINE

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1. Introduction

It has been established that the 16-glucosiduronate metabolites of estriol comprise the major estriol component in pregnancy plasma [1], urine [1,2] and amniotic fluid [3]. However, little is known about their physiological or pathological correlations. One reason is the fact that assays for individual metabolites of estriol either do not exist or are unsuitable for practical processing of large numbers of clinical samples. The possibility that immune assays might provide a useful means of measuring individual estriol metabolites prompted us to develop and investigate various antigens. A non-specific 16α-glucosiduronate antiserum has been reported [4]. This report describes radioimmune experiments in which picogram quantities of the 16-glucosiduronate metabolites of estriol were measured in the presence of vast excesses of estriol, other estrogens and/or their metabolites. Antibody specificity together with the natural preponderance of the 16-glucosiduronate metabolites made possible their direct measurement in plasma and urine.

2. Materials and methods

The majority of these studies were performed using [^{3}H] estriol-16-glucosiduronate (15 Ci/mM) obtained as a gift from Dr U. Goebelsmann.* The crossreactivity with estriol-3-sulfate-16-glucosiduronate was determined with [^{3}H] estriol-16-glucosiduronate (50 Ci/mM) obtained from Amersham Searle. Non-radioactive steroids were obtained from Sigma Chemical Co. and Steraloids Inc. except for estriol-3-sulfate-16-glucosiduronate which was obtained from Dr U. Goebelsmann.

2.1. *Preparation of antigen*

A bovine serum albumin (BSA)–estriol-16-glucosiduronate conjugate was prepared using methods similar to those previously described for estriol [5,6]. The conjugate was purified by initial dialyses against 0.001 N HCl followed by dialysis against phosphate buffer (pH 7.6). Adult male albino rabbits were immunized with the steroid conjugate according to previously published schedules [6]. Antiserum obtained after a 2-month course of immunization was used in the following experiments.

2.2. *Radioimmune experiments*

Antibody binding curves were derived as follows: various dilutions of antiserum (0.3 ml) were incubated at 4°C with 110 pg (5000 cpm) of [^{3}H] estriol-16-glucosiduronate. After 2 h, saturated ammonium sulfate (0.5 ml) was added. The solution was mixed, incubated for an additional 15 min and pelleted by centrifugation. A portion (0.6 ml) of the supernate was transferred to a scintillation counting vial to which was added 10 ml aquasol and 0.5 ml of deionized distilled water. Samples were counted for 10 minutes in a Nuclear Chicago Mark II Liquid Scintillation Counter (50% counting efficiency for ^{3}H).

The standard inhibition curve was derived from an

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experiment in which 0.3 ml of an initial 1 : 120 antiserum dilution bound 50% of added [^{3}H] estriol-16-glucosiduronate. Known quantities of unlabeled estriol-16-glucosiduronate or other steroids were dissolved in buffer and a 0.05 ml aliquot of each sample was allowed to pre-incubate for 30 min at 4°C with 0.3 ml of the selected dilution (1 : 120) of antiserum. The [^{3}H] estriol-16-glucosiduronate was then added, mixed and incubated for an additional 2 h. Separation of the bound fraction was accomplished by saturated ammonium sulfate precipitation and a sample of the supernate was counted as described above.

The procedure was modified slightly for handling plasma samples. To generate the standard curve 5 μl of 'stripped' male plasma (7) containing known amounts of estriol-16-glucosiduronate and 0.3 ml of the 1 : 120 dilution of antiserum were incubated at 4°C for 30 min. The subsequent steps were identical to those of assays in buffer described above. Unextracted plasma samples were obtained from several subjects and assayed directly for estriol-16-glucosiduronate. To compare estriol-16-glucosiduronate levels with unconjugated estriol concentrations in the same subjects aliquots (200 μl) of the plasma samples were also extracted with ether and assayed [8] for neutral estriol.

Procedures for urine assay were identical to assays in buffer described above.

3. Results

3.1. *Sensitivity*

This was defined as the point on the standard curve distinguishable from 0.0 pg by two standard deviations and was 25 pg/tube. Intra-assay variance evaluated using ten duplicate determinations with values ranging from 50 to 1000 pg/tube gave coefficients of variation equal to or less than 15%. Interassay variance determined in eight duplicate determinations in two different assays resulted in a coefficient of variation of 20.5% [9].

3.2. *Specificity*

The association constant (K_a) was 3.48×10^9 M^{-1} as determined by a Scatchard plot [10]. Hapten inhibition results (fig.1, table 1) demonstrate that the antiserum is highly specific for estriol-16-glucosiduronates. Cross-reactivity with estriol, various estriol metabolites and other steroids ranged from 2.2% to less than 0.001% except for estriol-3-sulfate-16-glucosiduronate which was 100%.

3.3. *Plasma assays*

As shown in table 2, a significantly higher level ($p < 0.005$) of 16-glucosiduronate metabolites than estriol was measured in plasma samples from several subjects. The correlation coefficient between estriol measured in ether extracts of plasma and estriol-16-

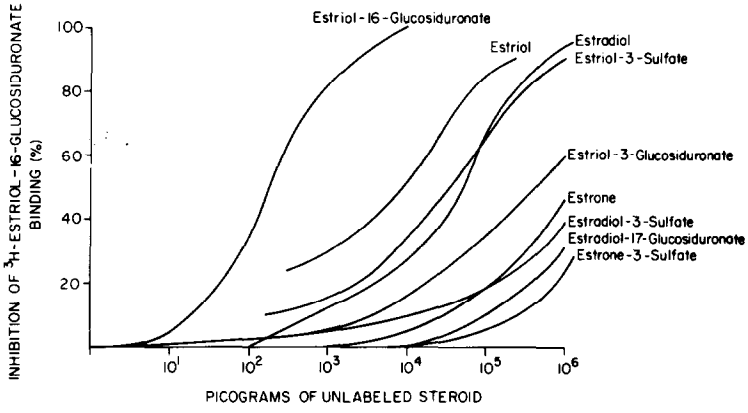

Fig.1. Inhibition of [^{3}H]estriol-16-glucosiduronate binding to estriol-16-glucosiduronate-BSA antisera by related steroid haptens.

Table 1
Cross-reactivity[a] of estriol-16-glucosiduronate antiserum with various steroids

	pg Steroid at 50% inhibition	Cross-reactivity (%)
Estriol-16-glucosiduronate	175	100
Estriol-3-sulfate-16-glucosiduronate	175	100
Estriol	8×10^3	2.2
Estriol-3-sulfate	1.16×10^6	0.015
Estriol-3-glucosiduronate	4.38×10^5	0.04
Estradiol	5×10^4	0.35
Estradiol-3-sulfate	$> 1 \times 10^6$	< 0.0175
Estradiol-17-glucosiduronate	$> 1 \times 10^6$	< 0.0175
Estradiol-3-sulfate-17-glucosiduronate	$> 1 \times 10^6$	< 0.0175
Estrone	1×10^6	0.0175
Estrone-3-sulfate	$> 1 \times 10^7$	< 0.001
Progesterone	$> 1 \times 10^7$	< 0.001
Cortisone	$> 1 \times 10^7$	< 0.001

[a] Defined as $\frac{\text{pg of estriol at 50\% inhibition} \times 100}{\text{pg of other steroid at (50\%) inhibition}}$

Table 2
Comparison of estriol[a] and estriol-16-glucosiduronate levels

Subject No.	Estriol (ng/ml)	Estriol-16-glucosiduronate (ng/ml)
1	27	214
2	20	152
3	28.7	224
4	3.5	19.8
5	4.0	34.5
6	4.8	27.0
7	3.0	17.4
8	6.0	87
9	7.7	67.5
10	15.8	54.0
11	13.8	58.0
12	8.7	65.5
13	10.2	111
14	13.3	60.5
15	5.1	10.4
16	14.0	84
17	19.4	86
Mean	12.058	80.741

[a] Ether extracts

glucosiduronate assayed in unextracted plasma is 0.884 (table 2). An extensive clinical study at different stages of gestation is in progress.

3.4. *Urine assays*

Samples of male urine, which did not inhibit [^{3}H] estriol-16-glucosiduronate antiserum binding (unpublished data) were spiked with varying amounts of estriol-16-glucosiduronate and assayed (table 3). The levels of estriol-16-glucosiduronate measured did not significantly differ from the amount added. Having thus established the feasibility of a direct urine assay, samples of urine from non pregnant females were tested. The levels measured in 7 individuals varied from 0.3–20 ng/ml estriol-16-glucosiduronate.

Table 3
Accuracy of estriol-16-glucosiduronate measurements in spiked urine samples

Estriol-16-glucosiduronate added/tube (pg)	Estriol-16-glucosiduronate measured/tube (pg)
100	120
200	200
500	450
1000	1000

4. Discussion

These studies demonstrate a radioimmunoassay of the 16-glucosiduronate metabolites of estriol directly in plasma and urine. As evidenced by inhibition experiments, none of the closely related metabolites cross-reacted appreciably with the antiserum. The cross-reactivity of the antiserum with estriol-3-sulfate-16-glucosiduronate is probably due to the fact that estriol-16α-glucosiduronate exceeds the antibody combining site which thus could be expected to recognize the estriol -16α-glucosiduronate portion of the diconjugate. This does contribute to the total 16-glucosiduronates in plasma. However, estriol-16α-glucosiduronate is the predominant estrogen metabolite in urine, the sulfoglucosiduronate constituting less than 4% [1].

The plasma levels of 16-glucosiduronate metabolites measured in this study were 6–7 times greater than those of estriol. These higher levels are consistent with the antiserum crossreactivity with estriol-3-sulfate-16-glucosiduronate and are further evidence of the measurement of the latter in the assay as well. The total 16-glucosiduronate levels measured are about one and a half times those previously reported [1] following counter current distribution, enzyme hydrolysis chromatography and fluorometry. The difference may be less real than procedural. There is far less experimental manipulation in the simple immune assay described here.

Simplified metabolite assays made feasible by specific metabolite antibodies have many obvious uses. The higher relative concentrations of estriol-16-glucosiduronates may allow detection of changes in fetoplacental function prior to the appearance of increasing levels of estriol, provided maternal liver and kidney functions are normal.

Since conjugation occurs predominantly in the liver [11,12] and 16-glucosiduronates are excreted in the urine, abnormal plasma or urine metabolite levels may reflect impaired liver or kidney (glomerular) function. In fact derangement of estriol-3-glucosiduronation has been reported in patients with liver cholestasis [2]. Urine steroid metabolites such as estriol-16-glucosiduronate may signify more accurate ovulatory patterns than presently at hand.

Before meaningful diagnostic/prognostic conclusions based on individual levels or ratios can be made native steroids and their metabolites have to be measured in a large diverse clinical population. Such studies are now being initiated in our laboratories.

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