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SHORT COMMUNICATION

EVIDENCE FOR THE PRESENCE OF 6-KETO-OESTRADIOL-17β IN HUMAN PLASMA—IMPLICATIONS FOR OESTRADIOL-17β RADIOIMMUNOASSAYS

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To enhance assay specificity and thus dispense with the need for chromatographic purification steps, antisera for oestradiol-17β radioimmunoassays are often raised against oestradiol-17β-protein conjugates linked *via* the 6-position of the steroid. On testing a series of such antisera we found that the metabolite 6-keto-oestradiol-17β, which is structurally most similar to the immunogen, showed cross-reactions of 150–500% with respect to oestradiol-17β. Thus, even low levels of this metabolite could constitute a hazard to the specificity of assays based on these antisera. We have measured levels of 6-keto-oestradiol-17β in human plasma by radioimmunoassay and determined the extent of its interference in a radioimmunoassay for oestradiol-17β.

For the 6-keto-oestradiol-17β assay, plasma was extracted once with 10 vol. diethyl ether in a Multivortex mixer (Baird & Tatlock Ltd., London) run for 2 min at 1800 rev./min. After extraction the organic phase was separated, the ether evaporated and the residue dissolved in 0·5 ml carbon tetrachloride. 0·5 ml of 0·05M NaOH was added, mixed as above for 2 min, and 0·2 ml aliquots of the aqueous layer taken for the assay. To these aliquots was added 0·625 pg oestradiol-17β-6(O-carboxymethyl)oxime-^{125}I monoiodohistamine tracer (S.A. 3·7 mCi/μg) (1) in 0·05 ml of 0·05M sodium phosphate buffer, pH 6·0, containing 0·2% gelatin and 0·1% stilboestrol to minimize non-specific adsorption of steroids. Antiserum raised against oestradiol-17β-6-(O-carboxymethyl)oxime-BSA and selected for minimum cross-reaction with other oestrogens, was then added in this diluent at a final dilution (1/100,000) to bind about 70% of the tracer. The total incubate vol. was 0·5 ml. The assay tubes were incubated for 3 h at room temperature before the separation of antibody-bound from free steroid by a double antibody method.

For the oestradiol-17β-assay, samples were extracted as above. A dilution of a solid-coupled antiserum sufficient to bind about 70% of the tracer (2·5 pg tritiated oestradiol-17β) was used and the assay tubes were incubated at room temperature overnight without agitation in a diluent containing 15% sucrose to reduce settling of the antiserum [2]. Antibody-bound was separated from free hormone by centrifugation (1700 *g*, 10 min) immediately after the addition of 1·5 ml of diluent as a wash. The supernatant fraction was decanted into 10 ml scintillation fluid in counting vials and the tritiated steroid tracer extracted into the organic phase for counting.

The 6-keto-oestradiol-17β radioimmunoassay had a working range of 1–200 pg/assay tube (75·4 ± 1·8% binding of tracer with zero antigen, 67·0 ± 1·3% with 1 pg/tube, 39·8 ± 1·0% with 50 pg/tube, 28·6 ± 1·9% with 200 pg/tube, non-specific binding 3·1 ± 0·4%; means ± inter-assay S.D., results from 12 assays), and the recovery of 20–100 pg amounts of 6-keto-oestradiol-17β added per ml plasma was 89·4 ± 7·3% (mean ± S.D., from 8 experiments). The specificity of the assay was tested by the incorporation of cross-reacting steroids in assay diluent directly into the assay. Of the steroids tested only oestradiol-17β gave an appreciable cross-reaction (2·5% when defined as the percentage concentration of steroid displacing 50% of labelled tracer [3]); oestrone, oestriol and 6-α hydroxy-oestradiol-17β, gave cross-reactions of 0·1%, 0·04% and 0·8% respectively.

The oestradiol-17β assay had a working range of 2–100 pg steroid per assay tube (72·3 ± 2·1% binding of tracer with zero antigen, 63·3 ± 2·0% with 2 pg/tube, 36·6 ± 4·6% with 20 pg/tube, 14·9 ± 3·8% with 100 pg/tube, non-specific binding 2·3 ± 1·3%; means ± inter-assay S.D., results from 11 assays). After the partition from carbon tetrachloride into NaOH water blanks were undetectable and thus no corrections for methodological blanks were required. The recovery of 20–100 pg amounts of unlabelled steroid added per ml plasma was 91·1 ± 7·0% (mean ± S.D., n = 11). The cross-reaction of 6-keto-oestradiol-17β was 200%, oestrone was 2·9% and oestriol was 0·3% in this assay.

To validate these assays oestradiol-17β was separated from the 6-keto metabolite in plasma extracts by selective elution from Celite 545 with increasing concentrations of ethyl acetate in 2,2,4-trimethyl pentane. Sephadex LH-20

Table 1. Levels of 6-keto-oestradiol-17β and oestradiol-17β in plasma measured by radioimmunoassay after Celite chromatography of plasma extracts

Plasma sample		Weeks gestation	6-keto-oestradiol-17β pg/ml.	Oestradiol-17β pg/ml.
Pregnant female	1	19	90	7800
	2	20	88	7800
	3	33	125	14300
	4	33	400	15200
	5	40	600	29800
Pregnant female Pool	1	8-12	< 3	1430
	2	36-40	400	25900
Luteal phase pool		-	< 1.2	251
Male pool		-	< 0.6	27

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chromatography did not effect this separation satisfactorily. Concentrations of oestradiol-17β and 6-keto-oestradiol-17β were measured after Celite chromatography in pools of plasma taken from normal men, from women in the luteal phase of the menstrual cycle and in plasmas collected during pregnancy. From the results (Table 1) it can be seen that measurable amounts of 6-keto-oestradiol-17β were found only in late pregnancy. In our assay for oestradiol-17β, using an antiserum selected to minimize cross-sections with the 6-keto compound, these levels would contribute only up to 5% of the apparent oestradiol-17β concentration. It was found that the oestradiol-17β results were not significantly different when samples were assayed without chromatography.

The use of antisera with greater cross-reactions for 6-keto-oestradiol-17β could result in an interference by this compound which might become unacceptable. We have found that one widely used chromatographic purification procedure, Sephadex LH-20, does not satisfactorily separate oestradiol-17β from its 6-keto metabolite and so this potential interference is not limited to assays which avoid chromatographic purification procedures. In those radioimmunoassays for oestradiol-17β which are based on antisera raised against 6-coupled immunogens, it is clearly important to select an antiserum which has a relatively low cross-reaction with 6-keto-oestradiol-17β.

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