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Note

The use of oestradiol-17β antiserum covalently coupled to Sepharose to extract oestradiol-17β from biological fluids

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Using radioimmunoassay, Glencross et al. [1] reported oestradiol-17β concentrations in bovine blood plasma, collected during the oestrous cycle, to be in the 1—10 pg/ml range. As the limit of reliable estimation of this steroid in the assay was 10—15 pg, it was necessary to use large samples of plasma (10—20 ml). This entailed extraction of the oestradiol-17β with solvent and extensive chromatographic purification of the extract to concentrate the steroid sufficiently for assay. Such preliminary purification procedures increase the error of the assay and also make it very laborious and time consuming.

A much simpler, yet efficient, method of extracting endogenous oestradiol-17β from large samples of bovine blood plasma and milk has now been devised, based on its binding to oestradiol-17β antiserum covalently coupled to Sepharose. The oestradiol-17β can be conveniently recovered for radioimmunoassay and the antiserum for re-use.

MATERIALS AND METHODS

Steroids and reagents

Steroids, assay buffer and dextran-coated charcoal suspension were as described previously [1].

Radioactivity counting

Radioactivity was measured by liquid scintillation counting (5 min) in glass vials in a 7-ml solution of 2,5-diphenyloxazole (POP; 5 g/l) and 1,4-di[2-(5-phenyloxazolyl)]-benzene (POPOP; 0.1 g/l) in toluene (MI 92 scintillation fluid; Packard Instruments, Reading, Great Britain), using a Packard Tricarb Spectrometer (Model B2450) and a ^{226}radium external standard.

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Plasma and defatted milk samples

Blood plasma samples (50 ml) taken by the method of Glencross et al. [1] from heifers in early pregnancy (7—25 days of pregnancy) when plasma oestradiol-17β levels are known to be low [2] were pooled.

Milk samples (30 ml) collected from non-pregnant cows and cows in the first 60 days of pregnancy were centrifuged (1900 *g*) for 20 min at 4°C and the cream layer removed. The defatted samples, which contained < 0.05% fat as measured by the Gerber method (British Standard 696), were pooled.

Antiserum

The antiserum (G510/6) was prepared against a 6-oxo-oestradiol-17β, carboxymethyloxime, bovine serum albumin conjugate provided by Dr. D. Exley [3] and raised in a castrated male goat (G510 of NIRD herd). It was highly specific for oestradiol-17β with a 6% cross-reaction to oestrone and less than 1% to oestradiol-17α.

Preparation of Sepharose-coupled antiserum

The antiserum was covalently coupled to Sepharose 6B (Pharmacia Fine Chemicals, Uppsala, Sweden) after activation with cyanogen bromide [4]. After washing with the buffers, the Sepharose-coupled antiserum was stood in 1 *M* ethanolamine, pH 9.0 for 2 h at room temperature to block any active sites remaining on the gel. The Sepharose-coupled antiserum was then washed with water and stored in gelatin-free assay buffer at 4°C in the dark.

Operation of columns of Sepharose-coupled antiserum

Sepharose-coupled antiserum in gelatin-free assay buffer was placed into columns having 13-mm sintered discs (porosity 1), obtained from Corning (Stone, Great Britain), to give 1-ml settled gel volumes. The columns of Sepharose-coupled antiserum were stored in the gelatin-free assay buffer at room temperature.

Before first use, the Sepharose-coupled antiserum column was washed with water (10 ml). An aqueous sample (water, diluted plasma or defatted milk) to which tritium-labelled oestradiol-17β had been added as tracer, was passed through the column, followed by water washings (3 ml, 10 ml) and the eluates discarded. Excess water was removed by briefly applying pressure from a nitrogen supply to the top of the column and also discarded. The antibody bound oestradiol-17β was then eluted with 3 ml water—acetone (5:95, v/v). The aqueous acetone, collected in a glass assay tube, was evaporated to dryness under nitrogen ready for radioimmunoassay.

The Sepharose-coupled antiserum column, after washing with water (10 ml) was ready for re-use. If not so required, it was stored at room temperature under gelatin-free assay buffer.

Radioimmunoassay

Oestradiol-17β, extracted and recovered by the above method, was then submitted to radioimmunoassay [1] using the oestradiol-17β antiserum (G510/6) at a dilution of 1:500,000.

RESULTS AND DISCUSSION

Specificity of the Sepharose-coupled antiserum

The specificity of the Sepharose-coupled antiserum was tested by submitting [^{3}H] oestradiol-17β and [^{3}H] progesterone dissolved in water (10 ml) to the extraction and recovery procedure and estimating the radioactivity in the water (10 ml) and aqueous acetone (3 ml) eluates. The results (Table I) show high recovery of [^{3}H] oestradiol-17β in the aqueous acetone fraction, whereas [^{3}H]-progesterone was not bound to the oestradiol-17β specific antibody.

TABLE I

RECOVERY OF [^{3}H]STEROIDS IN WATER AND AQUEOUS ACETONE FRACTIONS AFTER PASSING THROUGH SEPHAROSE-COUPLED ANTISERUM COLUMNS

Steroid added	% recovery (mean ± S.E.M., n = 3) of [^{3}H]steroid in water fraction	% recovery (mean ± S.E.M., n = 3) of [^{3}H]steroid in aqueous acetone fraction
[^{3}H] Oestradiol-17β ($5.64 \cdot 10^5$ dpm; 665 pg)	4.2 ± 1.8	94.1 ± 3.2
[^{3}H] Progesterone ($1.07 \cdot 10^5$ dpm; 165 pg)	90.6 ± 3.8	3.7 ± 0.3
[^{3}H] Progesterone and progesterone ($1.02 \cdot 10^4$ dpm; 80 ng)	97.8 ± 0.4	3.9 ± 1.7

Efficiency of recovery of oestradiol-17β added to water, plasma or milk and extracted by the Sepharose-coupled antiserum; re-use of the antiserum columns

The efficiency of the procedure for extraction of oestradiol-17β from water, plasma or milk and the possible re-use of the Sepharose-coupled antiserum preparation were then investigated.

[^{3}H] Oestradiol-17β (3700 dpm; 4.4 pg) and oestradiol-17β standards (0, 5, 10, 13, 17, 20, 23, 28, 35, 45, 60 and 80 pg) were added to water (7 ml), vortex mixed (30 sec) and left to stand for 30 min at room temperature. Samples were passed through twelve Sepharose-coupled antiserum columns (each through a different column) and the oestradiol-17β eluted with aqueous acetone as previously described.

The same quantities of oestradiol-17β standards, including the [^{3}H] oestradiol-17β, after solution in plasma (5 ml) to which water (2 ml) had been added, were then extracted using the same twelve columns of Sepharose-coupled antiserum. The diluted plasma eluates as well as the oestradiol-17β containing eluates were collected. The procedure was repeated but with the same quantities of oestradiol-17β added to the diluted plasma eluates (each 7 ml) from the previous operation, again re-using the same twelve antiserum columns.

Standard quantities of [^{3}H] oestradiol-17β (3480 dpm; 4.1 pg) and oestradiol-17β (0, 5, 7, 10, 15, 25, 40, 65, 100 and 125 pg) added to samples of defatted milk (7 ml) were then submitted to extraction and recovery on ten of the same twelve columns of Sepharose-coupled antiserum. Similar quantities of oestra-

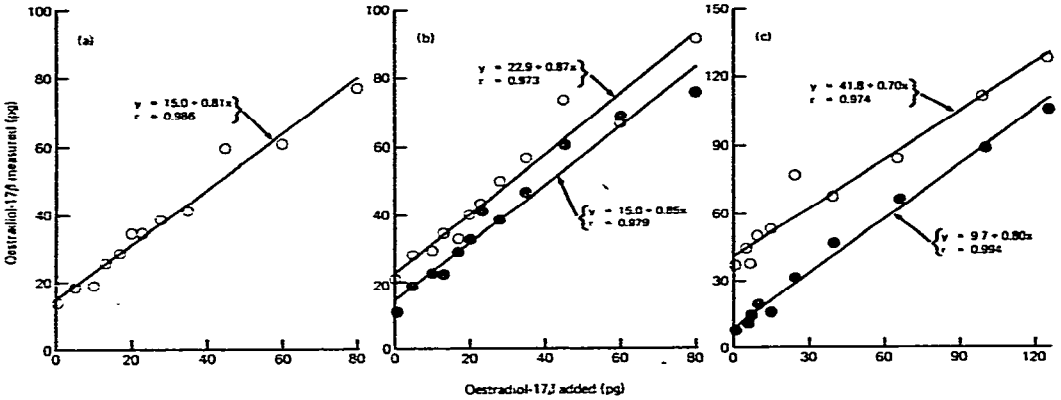

Fig. 1. Oestradiol-17β added to (a) water, (b) diluted bovine plasma or (c) defatted milk, extracted by Sepharose-coupled oestradiol-17β antiserum, recovered in aqueous acetone and estimated by radioimmunoassay. Oestradiol-17β standards added to samples from which endogenous oestradiol-17β had not been removed (○) or to samples from which oestradiol-17β had been removed (●) by prior passage through column.

diol-17β but added to the defatted milk eluates (each 7 ml) were then extracted and recovered on the same ten columns.

The application of oestradiol-17β containing samples in a batch of ten or twelve to the ten or twelve antiserum columns was randomized.

The extracts obtained from evaporation of all 56 aqueous acetone eluates were submitted to oestradiol-17β radioimmunoassay. The correlation between added and estimated oestradiol-17β was excellent (Fig. 1) with evidence of a "reagent blank", i.e. the estimate when no oestradiol-17β is present, of 10—15 pg and of an endogenous oestradiol-17β concentration of 2—3 pg/ml in the pooled plasma and defatted milk.

There was no evidence of deterioration of the Sepharose-coupled antiserum due to storing at room temperature in gelatin-free assay buffer or during successive use. In studies of the variation in the levels of oestradiol-17β in plasma and milk, to be reported elsewhere, it has been confirmed that the columns of Sepharose-coupled antiserum were stable at room temperature for twelve months and after at least 50 times of repeated use.

This extraction procedure using Sepharose-coupled oestradiol-17β antiserum is a simple, efficient and highly specific method of extracting and recovering oestradiol-17β from large volumes of bovine body fluid for accurate measurement by radioimmunoassay. The fact that the antibody preparation can be conveniently recovered for re-use adds to the overall value of the method.

Although this new procedure has been developed for the assay of oestrogens in bovine plasma and defatted milk, it may be generally valuable in analytical and possibly preparative procedures in which a compound (e.g. hormone, vitamin, drug) is present in biological fluid in very low concentrations, provided that the appropriate antiserum is available.

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REFERENCES

1 R.G. Glencross, I.B. Munro, B.E. Senior and G.S. Pope, Acta Endocrinol. (Copenhagen), 73 (1973) 374.
2 R.G. Glencross, Ph.D. Thesis, University of Reading, 1978.
3 P.D.G. Dean, D. Exley and M.W. Johnson, Steroids, 18 (1971) 593.
4 A.E. Bolton, K.K. Dighe and W.M. Hunter, in E.H.D. Cameron, S.G. Hillier and K. Griffiths (Editors), Steroid Immunoassay; Proc. 5th Tenovus Workshop, Cardiff 1974, Alpha Omega Publ., Cardiff, 1975, p. 257.