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SHORT COMMUNICATION

DETERMINATION OF TOTAL OESTRONE IN PERIPHERAL SERUM FROM NON PREGNANT HUMANS

KJELL CARLSTRÖM and HOLGER SKÖLDEFORS

Departments of Obstetrics and Gynaecology and of Surgery II, Sabbatsberg Hospital, Karolinska Institutet, Stockholm, Sweden

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INTRODUCTION

Oestrone sulphate is by far the most abundant oestrogen in non pregnant humans. It forms the major part of the circulating oestrone and more than 85% of the biologically active oestrogen (oestradiol-17β + oestrone + oestrone sulphate) in human males [1–3]. Changes in the peripheral levels of total oestrone will therefore mainly reflect changes in the oestrone sulphate concentration. The methods hitherto developed for the determination of oestrone sulphate in blood from non pregnant subjects are rather complicated and include several chromatographic steps, a fact which has precluded the processing of large clinical materials [1–3]. In view of our ongoing studies on age dependent hormonal changes in the human male [4, 5] we have attempted to develop a rapid non-chromatographic radioimmunoassay of oestrone sulphate or alternatively total oestrone in serum. The present communication describes a rapid procedure for the determination of total oestrone in serum from non pregnant individuals, based upon enzymatic hydrolysis with purified *Helix pomatia* extract, ether extraction and radioimmunoassay.

MATERIALS

Amberlite XAD-2, bead size 0.30–0.45 mm (Rohm & Haas Co, Philadelphia, PA, U.S.A.) was washed five times each with methanol, acetone and glass distilled water [10] and was stored in glass distilled water. *Helix pomatia* extract (1000.000 U of sulphatase and 100.000 U of β-glucuronidase per ampoule) was obtained from Industrie Biologique Francaise, Gennevilliers, France and radioactive oestrogens ([2,4,6,7-^{3}H]-oestrone, [2,4,6,7-^{3}H]-oestradiol-17β, [6,7,-^{3}H]-oestrone sulphate (ammonium salt) and [6,7-^{3}H]-oestradiol-17β-glucosiduronate) from New England Nuclear Corp, Boston, MA, U.S.A. Other reagents were of ordinary a.r. grade. Diethyl ether was freshly distilled over acid $FeSO_4$ immediately before use. Anti-oestrone-6-carboxymethyloxime-TBG (Miles-Yeda Ltd, Kiryat Weizmann, Rehovot, Israel) was diluted as recommended by the manufacturer, but with phosphate buffered saline pH 7.0 with 0.1% (w/v) gelatin instead of Tris buffer. For some methodological experiments anti-oestradiol-17β-hemisuccinate–BSA [6] was used at a final dilution of 1:37500 in the same kind of buffer.

METHOD

One ampoule *Helix pomatia* extract is diluted with 0.15 M sodium acetate buffer pH 4.2 to a final vol. of 10 ml. 3 ml of Amberlite XAD-2, from which the water is completely drained off, is added to the enzyme and the mixture is incubated under slow rotation for 15 min. After that the Amberlite XAD-2 is removed by filtration.

To 200 μl of serum 25 μl of 1.5 M sodium acetate buffer pH 4.2 is added, followed by vortex mixing. 400 μl of purified *Helix pomatia* extract is added and after vortex mixing the sample is incubated at 55° for 30 min. After that additional 400 μl of purified enzyme is added, followed by vortex mixing and incubation at 55° for another 30 min. The hydrolyzed sample is cooled to room temperature and the liberated steroids are extracted with two 3 ml portions of diethyl ether. The combined ether portions are evaporated to dryness and subjected to radioimmunoassay.

Quantitation by radioimmunoassay is carried out using appropriate antiserum, ^{3}H tracer and standard curve using the technique described by Hotchkiss *et al.*[7] and Edqvist & Johansson[8].

RESULTS AND COMMENTS

Technical details

It was found that the hydrolysis of oestrone sulphate was the crucial point in this assay. Conjugate extraction combined with solvolysis or acid hydrolysis were less successful in our hands due to a very poor reproducibility. Enzymatic hydrolysis with *Helix pomatia* extract was far better in this respect, but the use of untreated enzyme yielded unacceptably high blank values. However, it was found that the blank values could be effectively reduced by treating the enzyme preparation with Amberlite XAD-2 prior to use.

The efficient adsorption by the Amberlite XAD-2 resin of steroids and similar compounds from aqueous solutions is well known [9–11]. The nature of the compounds producing blank effects of crude *Helix pomatia* extracts is not known; there was no parallellism between a dilution curve of the crude extract blank and the standard curve in any of the two radioimmunoassay systems tested. However the blank values in both systems are efficiently reduced by the Amberlite XAD-2 treatment. Thus in the oestrone radioimmunoassay, 2 × 400 μl of untreated enzyme + 200 μl of saline gave rise to a blank value corresponding to a total oestrone concentration of 1.0 nM, while the Amberlite XAD-2 treatment reduced this blank effect to 0.15 ± 0.09 nM (n.d.—0.30 nM, $n = 13$). Corresponding figures for the oestradiol-17β system were 1.23 and 0.27 nM respectively.

The amount of resin used seems somewhat critical: Less than 3 ml will sometimes give too high blank values while the use of more than 5 ml sometimes causes a decreased enzyme activity. This latter observation has also been made by other workers (Eneroth, P., personal communica-

Table 1. Total oestrone in peripheral serum from non pregnant individuals

Subjects		Total oestrone, nM Mean	Range
Males 40–54 yr (n = 42)		3.94	(1.00–9.35)
Males 58–79 yr (n = 47)		2.89	(0.88–7.43)
Follicular phase	(5 cycles)	5.00	(3.80–9.50)
Ovulatory phase	(5 cycles)	12.40	(9.50–19.75)
Luteal phase	(5 cycles)	7.50	(5.30–10.20)

tion). In the present assay method we use the enzyme preparation within one hour after purification. We have not tested its stability for longer time.

The two-step hydrolytic procedure is directly taken from the clinical routine method for urinary oestriol described by de la Torre *et al.*[11].

Severe emulsions appear frequently during the ether extraction. Therefore hydrolysis and ether extraction (by vortex mixing) is best done in threaded glass tubes and the phases are separated after screw capping by centrifugation. After freezing at -20 to $-40°$ the ether layer is transferred to the radioimmunoassay tube. This freezing procedure is strongly recommended, since traces of the aqueous phase in the radioimmunoassay tube will produce falsely elevated values.

Recovery, precision and sensitivity

The recovery of [6,7,-^{3}H]-oestrone sulphate and [6,7-^{3}H]-oestradiol-17β-glucosiduronate added to serum prior to hydrolysis and extraction was 77.7 $\pm$ 1.1 and 80.8 $\pm$ 0.7% respectively (corrected for non-enzymatic hydrolysis).

The variation between duplicates expressed as S.D. for 78 duplicate pairs in the range 0.85–9.35 mM total oestrone (mean 3.90 nM) was 0.275 nM = 7.02%. Analysis of one single serum sample in 10 different assays gave a concentration of 5.81 $\pm$ 0.52 nM, i.e. a variation of 8.9%. The least detectable total oestrone concentration significantly different from the reagent blank (difference > 2 S.D.) was 0.33 nM.

Total oestrone concentration in serum

The total oestrone concentrations in peripheral serum from non pregnant individuals are given in Table 1. The values are roughly comparable with those previously given for oestrone + oestrone sulphate by other authors [1–3]. The age-dependent decrease found for males is noteworthy and is in accordance with the corresponding decrease in urinary oestrogen excretion previously reported from this laboratory [4]. The described technique is suitable for total oestrone assay in larger clinical materials. Combined with a selective sulphoconjugate extraction with pyridinium sulphate and chloroform [13] the technique might be used in a specific oestrone sulphate assay. Direct radioimmunoassay on untreated serum samples using antisera against oestrone sulphate will of course be more convenient. An antiserum against oestrone sulphate has recently been described, but is reported as yet being less suitable for a direct assay procedure [14].

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