

Linköping University Postprint

Substantial discrepancies in 17β -estradiol concentrations obtained with three different commercial direct radioimmunoassay kits in rat sera

Jakob O. Ström, Annette Theodorsson and Elvar Theodorsson

N.B.: When citing this work, cite the original article.

Original publication:

Jakob O. Ström, Annette Theodorsson and Elvar Theodorsson, Substantial discrepancies in 17β -estradiol concentrations obtained with three different commercial direct radioimmunoassay kits in rat sera, 2008, Scandinavian Journal of Clinical and Laboratory Investigation.

<http://dx.doi.org/10.1080/00365510802254638>.

Copyright © [Taylor & Francis Group](#), an informa business

Postprint available free at:

Linköping University E-Press: <http://urn.kb.se/resolve?urn=urn:nbn:se:liu:diva-15392>

Substantial discrepancies in 17 β -estradiol concentrations obtained with three different commercial direct radioimmunoassay kits in rat sera

Jakob O. Ström¹, Annette Theodorsson^{1,2} and Elvar Theodorsson¹

Department of Clinical Chemistry¹ and Department of Neurosurgery²,

Institute of Clinical and Experimental Medicine, University Hospital, Linköping, Sweden

Corresponding author and reprint request: Elvar Theodorsson, IKE/Clinical Chemistry, University

Hospital, 581 85 Linköping, Sweden; Telephone: +4613223295

E-mail: Elvar.Theodorsson@ibk.liu.se

Running head: RIA for 17 β -estradiol in rat sera

This study was supported by Grants by The County Council of Ostergotland

Disclosure Statement: The authors have nothing to disclose.

ABSTRACT

The extensive use of estrogen for contraception and amelioration of post-menopausal symptoms has made it the subject of substantial recent research efforts. Ovariectomized (ovx) rats treated with exogenous ovarian hormones constitute important tools in the investigation of the effects and mechanisms of estrogen actions. The crucial need to control and to monitor plasma levels of 17 β -estradiol calls for accurate, precise and robust assay methods. The performance of direct radioimmunoassays (RIAs) for measurement of 17 β -estradiol has previously been reported for human samples, but – to our knowledge – not for rat samples. In the current study, 552 serum samples from ovx, native and hormone treated rats were used to compare the performance of three commercially manufactured direct RIAs from the companies DPC (Siemens Healthcare Diagnostics Inc., formerly Diagnostic Products Corporation), DSL (Diagnostic Systems Labs) and MPB (MP Biomedicals, formerly ICN Biomedicals). Substantial differences in results between the three assay methods were found when measuring serum 17 β -estradiol concentrations. The following formulas, describing the relation between the different methods' results, were obtained using weighted Deming's orthogonal regression (all regression formulae in the abstract are based on pg/mL): $DSL = 0.43 * DPC + 12.3$, $MPB = 2.1 * DPC + 84.7$ and $DSL = 4.8 * MPB + 22.2$. Furthermore, a preceding diethyl ether extraction step of the serum appears to impair the RIAs' performances in the present samples: $DPC_{ex} = 0.39 * DPC_{unex} + 0.76$, $DSL_{ex} = 0.32 * DSL_{unex} - 1.7$ and $MPB_{ex} = 0.22 * MPB_{unex} + 1.4$.

Keywords: Estrogens, Validation Studies, Pharmacokinetics, Radioimmunoassay, Rats

INTRODUCTION

17β -estradiol is the subject of extensive recent research efforts due to its widespread use for amelioration of postmenopausal symptoms and as a contraceptive. A substantial part of the experimental animal studies have been conducted in ovariectomized (ovx) rats receiving exogenous 17β -estradiol to maintain stable plasma concentrations. Characterization and control of the experimental conditions requires analysis of 17β -estradiol concentrations in rat sera. Due to the very low serum levels, the measurement is frequently performed by competitive radioimmunoassays (RIA).

17β -estradiol circulates in the blood weakly bound to albumin (60%) and strongly bound to sex hormone binding globulin (SHBG) (38%) which leaves only 2-3% in the free, physiologically active fraction [1]. To measure the free fraction only and for minimizing matrix effects, an extraction step is conventionally employed to eliminate the water-soluble carrier molecules from the sample. Most “in-house” RIAs still employ this technique, commonly using diethyl ether as extraction medium. However, during the last decade the extraction step has been increasingly excluded from most of the available commercially manufactured RIAs (referred to as “direct” contrary to the “indirect” RIAs including an extraction step). These kits offer a fast and convenient way of measuring 17β -estradiol and are also frequently employed in rat models [2-10].

Because of their widespread use, substantial efforts have been made to validate the commercial direct RIAs for use in human samples by comparing with other methods, e.g. enzyme immunoassay (EIA) [11-13], chemiluminescent immunoassays (CLIA) [11,12,14,15] and gas chromatography mass spectrometry (GCMS) [16,17], and also by investigating the correlation between different RIAs [11,12,16-24]. But because of the obvious risk of inter-species differences in the sample matrix, these studies only offer partial guidance to researchers about to measure 17β -estradiol samples from laboratory animals. Methods for analyzing 17β -estradiol have been assessed for use in sera from the

macaque [25], but - to our knowledge - no previous studies has tested the validity of commercial 17β -estradiol RIA kits in rat sera.

The aim of the current study was to compare the results of three direct 17β -estradiol RIAs from DPC (Siemens Healthcare Diagnostics Inc., formerly Diagnostic Products Corporation), DSL (Diagnostic Systems Labs) and MPB (MP Biomedicals, formerly ICN Biomedicals) and to investigate how the concentrations measured using these kits were affected by the adding of a diethyl ether extraction step.

MATERIAL AND METHODS

Animals

The one hundred and twenty female rats of the Sprague-Dawley strain used in the current study were purchased from B&K Universal (Sollentuna, Sweden). All procedures were conducted in accordance to the National Committee for Animal Research in Sweden and Principles of Laboratory Animal Care (NIH publication no. 86-23, revised 1985). The study was approved by the Local Ethics Committee for Animal Care and Use at Linköping University. The animals used in this experiment were also included in a study of 17β -estradiol administration methods (separate manuscript simultaneously submitted for publication).

Hormone treatment

Seventy-five animals were ovx by the dorsal route, and subsequently administered 17β -estradiol by three different methods (daily injections, silastic capsules and slow-release hormone pellets). The aims of the different administration regimens were to yield physiological levels of 17β -estradiol in the blood. Remaining 45 animals were divided into an ovx control group (n=15) and three native control groups according to cycle phase at time of blood sampling (proestrus: n=10, estrus: n=10 and diestrus: n=9). One of the thirty animals in the native control groups were excluded due to an

anatomic anomaly making vaginal smear, and thus monitoring of cycle phase, impossible. This part is thoroughly described in another manuscript simultaneously submitted for publication.

Blood sampling

Blood samples of 1 mL were collected on seven occasions during a 6-week period from the animals in the 17 β -estradiol treated groups, and on a single occasion in the control groups. All samples except the last were collected by venipuncture into Serum Tube (Vacuette[®] Serum Tubes, Hettich Labinstrument AB, Sollentuna, Sweden) one for each animal. For the last sampling, the animals were sacrificed and trunk blood collected. After clotting, the blood samples were centrifuged at 2500 rpm for 10 minutes. Then the serum was aspirated, transferred to another vial and frozen until analysis. This resulted in a total of 552 serum samples to be analyzed.

Anesthetics and analgetics

During ovx, blood sampling and administration of pellets and silastic capsules the animals were anesthetized with 1.4% (initially 4.2%) isoflurane (Forene[®] inhal-v 250 mL, Abbott Laboratories, Abbott Park, IL, USA) in an oxygen/nitrous oxide mixture (30%/70%). The animals were also injected with 0.1 mL/kg body weight of Rimadyl[®] (50 mg/ml Pfizer, Dundee, Scotland) dissolved in 1 mL of saline solution subcutaneously in the neck during the ovx. Eye gel (LubriThal[®] 15 g Leo laboratories Ltd., Dublin, Ireland) was utilized for protection of the rats' eyes during anesthesia.

Extraction procedures

To investigate the effects of extraction on the results, serum from more than a hundred (DPC: n=108, DSL: n=105 and MPB: n=110) samples were selected for each kit and subjected the following procedure: First 200 μ L serum was thoroughly vortexed with 2 mL diethyl ether (Merck) in glass test tubes for 30 seconds. The test tubes were subsequently cooled in a bath of 95% ethanol and carbonate ice. When the aqueous fraction was frozen, the diethyl ether was poured into another tube

and then evaporated in a heating block maintained at 40°C. The dried samples were reconstituted using the zero standards from each kit. The extracted samples were analyzed in duplicate and in the same séance as their unextracted counterparts.

Hormone assays

For the purpose of comparing the results of different commercial RIAs, the serum samples were analyzed with the following three kits:

1) DPC ¹²⁵I radioimmunoassay kit (17β-estradiol double antibody, KE2D, Siemens Healthcare Diagnostics Inc., Tarrytown, NY, USA). This method has, according to the manufacturer, a lowest detection limit of 1.4 pg/mL (5.1 pmol/L) and intra- and inter-assay coefficients of variation in the measurement range 50-80 pg/mL (183.6-293.7 pmol/L) of 4 and 4.9% respectively.

2) DSL ¹²⁵I radioimmunoassay kit (17β-estradiol double antibody, DSL-4800, Webster, TX, USA). The reported intra- and inter-assay coefficients of variation are, respectively, 6.5–8.9% and 7.5–12.2%, and the lowest limit of detection 2.2 pg/mL (8.1 pmol/L).

3) MPB ¹²⁵I radioimmunoassay kit (17β-estradiol (E2) Double Antibody - ¹²⁵I RIA Kit, MP Biomedicals, Solon, OH, USA). The reported intra- and inter-assay coefficients of variation are, respectively, 4.7-10.6% and 5.9-11.9%. Lowest detection limit is not reported by the manufacturer.

All three abovementioned kits have been used for assaying 17β-estradiol levels in rats in several previous studies [3-8].

Different kits from the same manufacturer were of the same LOT-number, and before the analysis, the reagents were pooled to avoid inter-assay variation. The three analyses (DPC, DSL and MPB) were performed in single séances. For each assay three standard curves were analyzed, and all samples were read against to their closest standard curve. The serum volumes used in each tube, both for standard curve and samples, were 100 μL for the DPC and DSL RIAs, and 25 μL for the MPB

RIA. Standards and samples were analyzed in duplicate and measured for 300 seconds in a gamma counter (Gamma Master 1277; Wallac-Pharmacia, Turku, Finland).

All 552 unextracted samples were analyzed with the DPC kit, 350 with the DSL kit and 395 with the MPB kit. Due to scarce sample volumes, the samples analyzed with DSL and MPB were not completely the same, but overlapping and with similar concentration ranges.

Sources of error

Due to limited availability of sample material the analyses in this study were performed in half the recommended serum and standard volumes. A concern was that this could affect the result and limit the possibility of drawing conclusions, especially for the MPB RIA where the recommended volume already was small (50 μ L compared to the 200 μ L recommended for the DPC and DSL RIAs). To investigate this 46 samples were analyzed with full recommended volume with the MPB RIA, and the obtained values were compared with those from the half-volume MPB RIA. Analysis with weighted orthogonal regression gave the following relation: $MPB_{50\mu l} = 1.1 * MPB_{25\mu l} + 2.8$ (based on pg/mL), $MPB_{50\mu l} = 1.1 * MPB_{25\mu l} + 10.3$ (based on pmol/L) and the correlation coefficient was found to be $r=0.98$. In other words the halving of volumes in the MPB RIA did not seem to have any substantial effect on the results, and it is reasonable that this could be extrapolated to the DPC and DSL RIAs.

Statistical analysis

The comparison between methods and extraction procedures were performed using Pearson's correlation coefficient and weighted orthogonal Deming's regression (Method Validator, version 1.1.9.0, Philippe Marquis, 1999, Metz, France). The results obtained with different RIA methods were analyzed for significant differences using ANOVA (Systat version 11, Systat Software, Inc. San Jose, California).

Excluded test results

The test results of ten samples were excluded due to an unacceptable discrepancy between the duplicates. This was the case for one unextracted sample analyzed with the DPC kit, three extracted and three unextracted samples analyzed with the DSL kit and for three unextracted samples analyzed with the MPB kit.

Another four test results obtained with the unextracted DSL method were excluded from the analysis because of technical problems when performing the assay.

Three samples turned out to have concentrations much higher than the highest concentration of the standard curve in all of the three unextracted analyses. These results ran the risk of unmotivated impact of the statistical analyses and were therefore excluded (large concentration range).

RESULTS

Comparisons of results from different unextracted RIAs

DPC vs. DSL

The mean of the test results for the 340 samples visualized in Fig. 1 A and B were significantly higher with the DPC RIA than with the DSL RIA ($p < 0.001$). The bias was mainly proportional but also had a minor non-proportional component, and the relation between results obtained with the two methods is described by the following formula: $DSL = 0.43 * DPC + 12.3$ (based on pg/mL), $DSL = 0.43 * DPC + 45.2$ (based on pmol/L). Correlation: $r = 0.91$.

DPC vs. MPB

As seen in Fig. 1 B and C the DPC RIA gave significantly lower results for these 391 samples than MPB did ($p < 0.001$). There was a clear non-proportional error, indicated by the high intercept of the regression line on the Y-axis, and also a proportional error. The relation is described by the following formula: $MPB = 2.1 * DPC + 84.7$ (based on pg/mL), $MPB = 2.1 * DPC + 311.0$ (based on pmol/L). Correlation: $r = 0.95$.

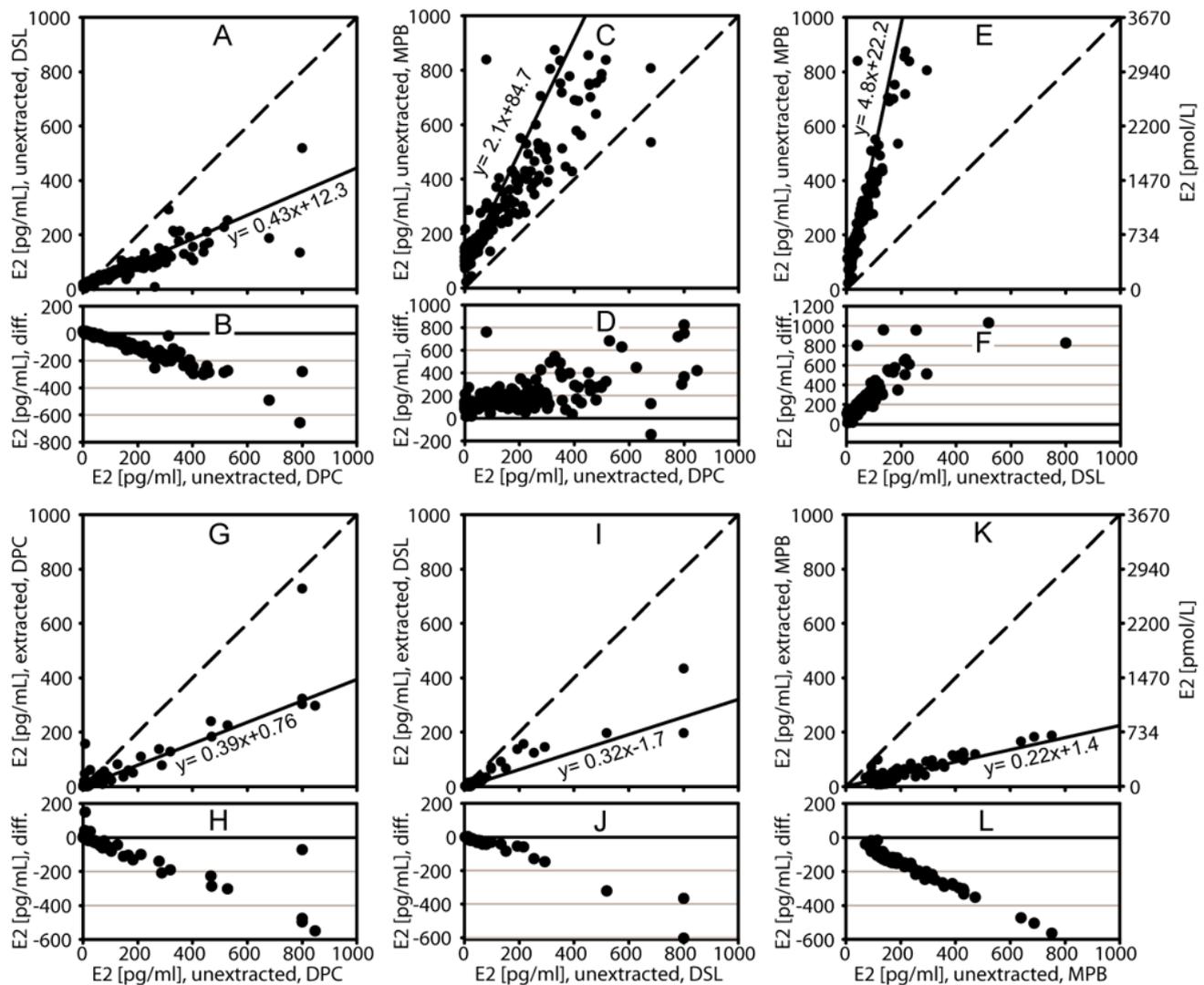


Fig.1. Each comparison is visualized by a correlation plot with a Deming's regression line (A, C, E, G, I and K) and with a bias plot (B, D, F, H, J and L). Conversion from pg/mL to the SI-unit pmol/L is displayed in the far right part of the figures. 17 β -estradiol is abbreviated "E2" in the figure. The discrepancies in the Y-axis values in the bias plots should be noted. The samples plotted in the different comparisons are not the same, but overlapping. A, B: Unextracted DPC against unextracted DSL. C, D: Unextracted DPC against unextracted MPB. E, F: Unextracted DSL against unextracted MPB. G, H: Unextracted against extracted DPC. I, J: Unextracted against extracted DSL. K, L: Unextracted against extracted MPB.

DSL vs. MPB

The most substantial differences between results in the RIA comparisons were found when comparing the values for the 227 samples in Fig.1 E and F analyzed with both DSL and MPB. MPB gave significantly higher values ($p < 0.001$), and the relation is described by the following formula:

DSL= 4.8*MPB+22.2 (based on pg/mL), DSL= 4.8*MPB+81.5 (based on pmol/L). The error is proportional but also has a minor non-proportional component. Correlation: $r=0.91$.

Effect of extraction

As is depicted in Fig.1 G-L, the adding of an extraction procedure rendered significantly lower concentrations for all three methods (DPC: $p<0.05$; DSL: $p<0.05$; MPB: $p<0.001$). Following formulas and correlation values describe the relation between values obtained with (index: ex) and without (index: unex) extraction for each method, and as can be seen, the errors are almost entirely proportional:

$DPC_{ex} = 0.39 * DPC_{unex} + 0.76$ (based on pg/mL), $DPC_{ex} = 0.39 * DPC_{unex} + 2.8$ (based on pmol/L),
correlation: $r=0.89$

$DSL_{ex} = 0.32 * DSL_{unex} - 1.7$ (based on pg/mL), $DSL_{ex} = 0.32 * DSL_{unex} - 6.2$ (based on pmol/L),
correlation: $r=0.93$

$MPB_{ex} = 0.22 * MPB_{unex} + 1.4$ (based on pg/mL), $MPB_{ex} = 0.22 * MPB_{unex} + 5.1$ (based on pmol/L),
correlation: $r=0.93$

(Fig.1)

Differences in concentrations of 17 β -estradiol in native rats

The 17 β -estradiol concentrations of the 29 native control rats in this study were measured with all three kits, extracted and unextracted, and the values shown in table 1 were obtained. (Table 1)

DISCUSSION

Substantial differences were found when measuring rat serum 17 β -estradiol concentrations by means of three commercial direct RIAs. However, all three methods properly reflected the basic 17 β -estradiol – related biological processes. Furthermore, preceding the measurements with diethyl ether extraction decreased the absolute concentrations measured, but did not improve the concordance between the methods.

Table 1

17 β -estradiol concentrations measured in sera from native rats			
Method _{unextracted/extracted}	Proestrus, n=10, mean \pm SEM, in pg/ml (and pmol/L)	Estrus, n=10, mean \pm SEM, in pg/ml (and pmol/L)	Diestrus, n=9, mean \pm SEM, in pg/ml (and pmol/L)
DPC _{unex}	63.1 \pm 7.7 (231.6 \pm 28.3)	5.9 \pm 1.4 (21.7 \pm 5.1)	13.3 \pm 2.8 (48.8 \pm 10.3)
DPC _{ex}	26.8 \pm 3.1 (98.4 \pm 11.4)	5.2 \pm 1.3 (19.1 \pm 4.8)	12.8 \pm 6.2 (47.0 \pm 22.8)
DSL _{unex}	33.5 \pm 2.5 (123.0 \pm 9.2)	14.5 \pm 0.56 (53.2 \pm 2.1)	18.1 \pm 1.5 (66.4 \pm 5.5)
DSL _{ex}	13.4 \pm 1.9 (49.2 \pm 7.0)	2.6 \pm 0.47 (9.5 \pm 1.7)	2.1 \pm 0.94 (7.7 \pm 3.5)
MPB _{unex}	168.5 \pm 9.4 (618.6 \pm 34.5)	114.7 \pm 7.0 (421.1 \pm 25.7)	113.9 \pm 7.1 (418.1 \pm 26.1)
MPB _{ex}	47.7 \pm 3.9 (175.1 \pm 14.3)	38.0 \pm 9.0 (139.5 \pm 33.0)	27.5 \pm 3.5 (100.1 \pm 12.8)

Different RIA kits

Of the RIA kits from DPC, DSL and MPB tested, MPB rendered the highest concentrations of 17 β -estradiol and DSL the lowest. Using the DPC kit to measure unextracted samples resulted in 17 β -estradiol concentrations in the native rats that best resembled concentrations observed in earlier studies where conventional RIAs, including an extraction step, have been used [26-29]. The differences in concentrations measured by the kits were indeed large enough to complicate comparisons between studies where kits from different companies are used. However, the relatively high degree of correlation ($r=0.91-0.95$) indicate that the regression line formulae strongly predict concentrations from one method to another.

The three kits were evidently properly calibrated by 17 β -estradiol calibrators, and 17 β -estradiol obviously has an identical chemical structure in humans and rats. The crucial question therefore concerns the causes of the observed differences between the kits. The most likely explanation is the

difference in reaction of the immunochemical reagents in the kits to the rat serum matrix of the samples. The RIA kits were designed for use in human samples and may therefore not be optimal for rat serum samples. Even if matrix effects in human sera are negligible, it could cause a problem in rat sera because of inter-species matrix differences.

Several previous studies employing human samples have compared direct RIAs with one another and with other methods, frequently showing substantial differences [11-14,16-24]. A representative example is a study by Stanczyk in which three commercial direct 17β -estradiol RIAs, from DPC, DSL and Pantex, two indirect RIAs (one commercial and one “in-house”), two CLIAs and three EIAs were compared in human sera. There was a good concordance between the two indirect RIAs, but substantial differences among the other tested methods when a broad range of concentrations was included. In agreement with the current study, the concentrations obtained with the DPC RIA were higher than those obtained with the DSL RIA [11]. Of course, all studies do not reveal substantial differences between analytical methods for 17β -estradiol. As an example, Tummon reported good correlation, in particular for high concentrations, between a CLIA from Chiron diagnostics and a coat-a-count RIA from DPC in 505 human samples [15].

Gas chromatography mass spectrometry (GCMS) is the current golden standard for analysis of 17β -estradiol, and would certainly be the best way of testing any other analytical method's performance in rat sera. To our knowledge such study remains to be done. In 2006 a comparison was performed using human samples measured by four direct commercial RIAs, among them exactly the two RIAs from DPC and DSL tested in the current study, three indirect “in house” RIAs and GCMS. The samples were exclusively from postmenopausal women, and thus the levels of 17β -estradiol measured were very low (<25 pg/mL, <91.8 pmol/L). It was shown that the direct RIAs differed more from GCMS than the indirect RIAs did. Of the direct RIAs DPC correlated best (GCMS= $0.91 \cdot \text{DPC} + 2.7$ (based on pg/mL), GCMS= $0.91 \cdot \text{DPC} + 9.9$ (based on pmol/L), $r=0.83$) while DSL correlated a bit less (GCMS= $0.60 \cdot \text{DSL} - 2.0$ (based on pg/mL), GCMS= $0.60 \cdot \text{DSL} - 7.3$ (based on

pmol/L), $r=0.70$) against the GCMS. In this study DSL rendered higher values than DPC, which is consistent with the current study for the low concentrations in question [17].

Sample extraction

The purpose of extraction of serum samples in this case is to minimize the influence of all other molecules than 17β -estradiol on the final results. This is e.g. achieved by solid phase extraction or by precipitating the proteins using heat, high ionic strength, lowering pH or (as in the current study) by the adding of a highly hydrophobic reagent [30]. When extracting prior to 17β -estradiol analysis the hydrophobic reagent does not only denature the proteins, but also captures the measurand. Rationally, this seems like a good idea especially for analysis in sera from another species where the matrix is a probable confounder, but, as it turned out in our study, it was not. As aforementioned, the adding of a preceding extraction step generally halved the concentrations measured, which for the native rats in this study gave concentrations that do not agree with what has been seen in previous studies where conventional extraction RIAs have been used [26-29]. This suggests that the commercial RIAs tested in the current study are not suited for pre-analysis extraction, at least not using the method used in the present study. A possible explanation for this could be that when analyzing unextracted samples the antibody competes with the plasma proteins for binding to 17β -estradiol, thus enabling it to interact with more 17β -estradiol compared to the extracted samples. Extraction precipitates the plasma proteins and leads to net loss of 17β -estradiol from the samples. An alternative explanation is that the direct RIA kits used have been specifically calibrated for use in the matrix of human samples.

Earlier studies show much variation in results regarding the effect of extraction on RIA's performances. In one study the same DPC RIA as in this study was used for measurement of 17β -estradiol levels in rats, with and without extraction with diethyl ether. A lowering of the obtained values by approximately 20-30% was observed. This comparatively slight decrease is not consistent

with the current study, and the explanations for this could possibly be found in differences in the extraction procedure, which regrettably is not explicitly described in the previous study [2]. In another study three direct 17 β -estradiol RIAs (none of the ones used in the current study) were tested in human serum samples with and without organic solvent extraction. It was found that for all tested RIAs, *higher* concentrations were obtained when extracting, which is not coherent with the current study [20]. One investigation, showing great similarities in results with the current study, was performed by Cook and Read, where, among others, a direct double antibody 17 β -estradiol RIA from DPC (as in the current study) was tested for results in extracted and unextracted human serum samples. Sample extraction resulted in a decrease in the measured concentrations, described by the linear regression formula $DPC_{ex} = 0.357 * DPC_{unex} + 47.9$ (based on pg/mL), $DPC_{ex} = 0.357 * DPC_{unex} + 175.8$ (based on pmol/L), which is very close to the formula describing the corresponding comparison in the current study: $DPC_{ex} = 0.39 * DPC_{unex} + 0.76$ (based on pg/mL), $DPC_{ex} = 0.39 * DPC_{unex} + 2.8$ (based on pmol/L) [12].

Contemplating whether or not to employ an extraction step, it is important to realize that adding extraction to the direct RIA procedures adds an extra source of variability. This calls for strong arguments in favor of the extraction for choosing it to be a part of the procedure. A major advantage may be that extracted plasma samples possibly reflect closer the concentrations of biologically active 17 β -estradiol. It has been shown that the plasma protein SHBG lowers the 17 β -estradiol values measured in human plasma with a direct double antibody RIA from DPC [31]. Another argument in favor of extraction was suggested by Dighe and Sluss for the measurement of low levels of 17 β -estradiol, namely that the reconstitution part of the extraction step offers a possibility of concentrating the sample 17 β -estradiol to a level where the used assay method has higher validity and precision [32]. Dowsett & Folkerd also argued in favor of extraction by simply suggesting better correlation between an extracted than an unextracted procedure of a commercial RIA (DSL-39100, not the DSL-4800 tested in the current study) with a reference in-house RIA in twenty human

samples. As in the current study the observed differences were substantial, but as mentioned, it was stated that it was the method employing *extracted* samples that correlated best with the in-house RIA [21]. Other studies have also reported better concordance with reference in-house RIAs when adding an extraction step: Schioler and Thode tested a number of direct RIAs against an in-house RIA in human samples, and two of the direct RIAs (none of the ones used in the current study) were also assessed using diethyl ether extraction (of both samples and standards). It was found that the direct RIAs tested in this study generally overestimated the 17β -estradiol concentration, but that this overestimation decreased when using extraction [22]. Pazol et al. assessed a direct double antibody RIA from DPC (as in the current study) in macaque serum, and with an extraction step in human serum, comparing the results with the results of an in-house RIA. Both the direct RIA in macaque serum and with extraction in human serum underestimated the concentrations compared with the in-house RIA, but extraction resulted in a more consistent underestimation along the concentration range studied [25].

To further complicate the issue it has been shown that the effect of extraction differs between samples collected from women taking hormone replacement therapy (HRT) through different administration routes. The concentrations found in the samples from women on oral HRT were substantially decreased by extraction, while concentrations in samples from women using transdermal HRT were not. This is probably a consequence of the oral therapy resulting in higher levels of circulating estrogen bound to carrier molecules [33].

The question of whether the most true and biologically relevant 17β -estradiol values using the commercial direct RIAs are obtained with or without extraction, either for use in rat or human samples, remains elusive. Comparing a direct RIA's results in extracted and unextracted serum samples with the results obtained from GCMS is probably needed to finally assess the method's suitability for the addition of a preceding extraction step

Dissolving samples after extraction

In earlier experiments conducted in our laboratory the same DPC RIA used in the current study was employed [7,34,35]. Extraction with diethyl ether was performed and then the samples were reconstituted with a phosphate buffer containing 0.2% bovine serum albumin (BSA) and 0.1% triton X100, i.e. not with kit zero standard as in the current study. A problem was encountered in that our measured 17β -estradiol concentrations, both in native and hormone manipulated animals, were an order of magnitude higher than what has been shown in other studies. In a pre-trial to the current study the cause of this problem was found to be in the reconstitution step of our procedure (unpublished data). It is evident that the phosphate buffer containing BSA inhibits the binding between the antibody and the radioligand, and since only the samples and not the standard curve were extracted and reconstituted, the resulting 17β -estradiol values turned out falsely high. It could not be determined that any single constituent (e.g. BSA or triton X) was the culprit, so the source of error seems to have been the buffer's impact as a whole on the antibodies' binding properties.

CONCLUSION

The commercial direct RIA kits tested in the present study differ substantially in their results when measuring 17β -estradiol concentrations in rat sera. Furthermore, extraction by means of diethyl ether substantially decreases the concentrations measured and dampen the biological effects observed. When measuring 17β -estradiol in animals subjected to hormone treatment, it is essential that the levels are compared to native control animals in the same study.

ACKNOWLEDGEMENTS

The expert technical assistance of Lovisa Holm is gratefully acknowledged.

REFERENCES

- [1] Korach KS, Migliaccio S, Davis VL. Estrogens. In: Munson PL, editor. Principles of pharmacology. New York: Chapman & Hall, 1995. pp. 809-25.
- [2] Liu JW, Dawson DD, Peters CE, Baker MA, Walker AM. Estrogen replacement in ovariectomized rats results in physiologically significant levels of circulating progesterone, and co-administration of progesterone markedly reduces the circulating estrogen. *Endocrine* 1997;6 (2):125-31.
- [3] Ziegler DR, Gallagher M. Spatial memory in middle-aged female rats: assessment of estrogen replacement after ovariectomy. *Brain Res* 2005;1052 (2):163-73.
- [4] Kensicki E, Dunphy G, Ely D. Estradiol increases salt intake in female normotensive and hypertensive rats. *J Appl Physiol* 2002;93 (2):479-83.
- [5] Dubal DB, Wise PM. Neuroprotective effects of estradiol in middle-aged female rats. *Endocrinology* 2001;142 (1):43-8.
- [6] Garza-Meilandt A, Cantu RE, Claiborne BJ. Estradiol's effects on learning and neuronal morphology vary with route of administration. *Behav Neurosci* 2006;120 (4):905-16.
- [7] Hilke S, Theodorsson A, Fetissoff S, Aman K, Holm L, Hokfelt T, Theodorsson E. Estrogen induces a rapid increase in galanin levels in female rat hippocampal formation--possibly a nongenomic/indirect effect. *Eur J Neurosci* 2005;21 (8):2089-99.
- [8] Edwards HE, Burnham WM, Mendonca A, Bowlby DA, MacLusky NJ. Steroid hormones affect limbic afterdischarge thresholds and kindling rates in adult female rats. *Brain Res* 1999;838 (1-2):136-50.
- [9] Mannino CA, South SM, Inturrisi CE, Quinones-Jenab V. Pharmacokinetics and effects of 17beta-estradiol and progesterone implants in ovariectomized rats. *J Pain* 2005;6 (12):809-16.
- [10] Bottner M, Wuttke W. Chronic treatment with low doses of estradiol affects pituitary and thyroid function in young and middle-aged ovariectomized rats. *Biogerontology* 2005;6 (4):261-9.
- [11] Stanczyk FZ, Cho MM, Endres DB, Morrison JL, Patel S, Paulson RJ. Limitations of direct estradiol and testosterone immunoassay kits. *Steroids* 2003;68 (14):1173-8.
- [12] Cook NJ, Read GF. Oestradiol measurement in women on oral hormone replacement therapy: the validity of commercial test kits. *Br J Biomed Sci* 1995;52 (2):97-101.
- [13] Munro CJ, Stabenfeldt GH, Cragun JR, Addiego LA, Overstreet JW, Lasley BL. Relationship of serum estradiol and progesterone concentrations to the excretion profiles of their major urinary metabolites as measured by enzyme immunoassay and radioimmunoassay. *Clin Chem* 1991;37 (6):838-44.
- [14] Rojanasakul A, Udomsubpayakul U, Chinsomboon S. Chemiluminescence immunoassay versus radioimmunoassay for the measurement of reproductive hormones. *Int J Gynaecol Obstet* 1994;45 (2):141-6.
- [15] Tummon I, Stemp J, Rose C, Vandenberghe H, Bany B, Tekpetey F, Martin J. Precision and method bias of two assays for oestradiol: consequences for decisions in assisted reproduction. *Hum Reprod* 1999;14 (5):1175-7.
- [16] Nisbet JA, Jomain PA. Discrepancies in plasma estradiol values obtained with commercial kits. *Clin Chem* 1987;33 (9):1672.
- [17] Lee JS, Ettinger B, Stanczyk FZ, Vittinghoff E, Hanes V, Cauley JA, Chandler W, Settlege J, Beattie MS, Folkler E, Dowsett M, Grady D, Cummings SR. Comparison of methods to measure low serum estradiol levels in postmenopausal women. *J Clin Endocrinol Metab* 2006;91 (10):3791-7.
- [18] Mikkelsen AL, Borggaard B, Lebech PE. Results of serial measurement of estradiol in serum with six different methods during ovarian stimulation. *Gynecol Obstet Invest* 1996;41 (1):35-40.

- [19] Lee CS, Smith NM, Kahn SN. Analytic variability and clinical significance of different assays for serum estradiol. *J Reprod Med* 1991;36 (3):156-60.
- [20] Thomas CM, van den Berg RJ, Segers MF. Measurement of serum estradiol: comparison of three "direct" radioimmunoassays and effects of organic solvent extraction. *Clin Chem* 1987;33 (10):1946-7.
- [21] Dowsett M, Folkerd E. Deficits in plasma oestradiol measurement in studies and management of breast cancer. *Breast Cancer Res* 2005;7 (1):1-4.
- [22] Schioler V, Thode J. Six direct radioimmunoassays of estradiol evaluated. *Clin Chem* 1988;34 (5):949-52.
- [23] Rinaldi S, Dechaud H, Biessy C, Morin-Raverot V, Toniolo P, Zeleniuch-Jacquotte A, Akhmedkhanov A, Shore RE, Secreto G, Ciampi A, Riboli E, Kaaks R. Reliability and validity of commercially available, direct radioimmunoassays for measurement of blood androgens and estrogens in postmenopausal women. *Cancer Epidemiol Biomarkers Prev* 2001;10 (7):757-65.
- [24] Haning RV, Jr., Meier SM, Boehnlein LM, Gerrity M, Shapiro SS. Two direct radioimmunoassays for 17 beta-estradiol evaluated for use in monitoring in vitro fertilization. *Clin Chem* 1984;30 (5):787-90.
- [25] Pazol K, Kaplan JR, Abbott D, Appt SE, Wilson ME. Practical measurement of total and bioavailable estradiol in female macaques. *Clin Chim Acta* 2004;340 (1-2):117-26.
- [26] Wise PM, Ratner A. Effect of ovariectomy on plasma LH, FSH, estradiol, and progesterone and medial basal hypothalamic LHRH concentrations old and young rats. *Neuroendocrinology* 1980;30 (1):15-9.
- [27] Goodman RL. A quantitative analysis of the physiological role of estradiol and progesterone in the control of tonic and surge secretion of luteinizing hormone in the rat. *Endocrinology* 1978;102 (1):142-50.
- [28] Butcher RL, Collins WE, Fugo NW. Plasma concentration of LH, FSH, prolactin, progesterone and estradiol-17beta throughout the 4-day estrous cycle of the rat. *Endocrinology* 1974;94 (6):1704-8.
- [29] Kalra PS, Kalra SP. Temporal changes in the hypothalamic and serum luteinizing hormone-releasing hormone (LH-RH) levels and the circulating ovarian steroids during the rat oestrous cycle. *Acta Endocrinol (Copenh)* 1977;85 (3):449-55.
- [30] Henry RJ, Szustkiewicz CP. The Preparation of Protein-Free Filtrates. In: Henry RJ, Cannon DC, Winkelman JW, editors. *Clinical Chemistry Principles and Technics*. Hagerstown, Maryland: Harper & Row, 1974. pp. 390-404.
- [31] Key TJ, Moore JW. Interference of sex-hormone binding globulin in a no-extraction double-antibody radioimmunoassay for estradiol. *Clin Chem* 1988;34 (6):1357-8.
- [32] Dighe AS, Sluss PM. Improved detection of serum estradiol after sample extraction procedure. *Clin Chem* 2004;50 (4):764-6.
- [33] Diver MJ. Monitoring of hormone replacement therapy. *Lancet* 1992;340 (8833):1471.
- [34] Theodorsson A, Hilke S, Rugarn O, Linghammar D, Theodorsson E. Serum concentrations of 17beta-estradiol in ovariectomized rats during two times six weeks crossover treatment by daily injections in comparison with slow-release pellets. *Scand J Clin Lab Invest* 2005;65 (8):699-705.
- [35] Theodorsson A, Theodorsson E. Estradiol increases brain lesions in the cortex and lateral striatum after transient occlusion of the middle cerebral artery in rats: no effect of ischemia on galanin in the stroke area but decreased levels in the hippocampus. *Peptides* 2005;26 (11):2257-64.