FINAL REPORT

Development of a Standardized Approach for Evaluating Environmental Chemicals with Low Solubility in the Estrogen Receptor (ER) Binding Assay

Volume I (Report and Index A-C)

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Prepared for

Teresa Wall
Program Analysis (MD-71)
NHEERL, ORD, USEPA
Research Triangle Park, NC 27711
Phone: 919-541-3591
Fax: 919-541-1499
Email: wall.theresa@epa.gov

Prepared by

J. Charles Eldridge, Ph.D.
Professor of Physiology & Pharmacology
Wake Forest University
School of Medicine
Winston-Salem, NC 27157-1083
Phone: 336-716-8570
Fax: 336-716-8501
Email: eldridge@wfubmc.edu

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EXECUTIVE SUMMARY

This report describes results from follow-up studies on binding competitiveness of selected chemicals to estrogen receptor (ER) prepared from rat uterine cytosol (RUC). Earlier studies with these chemicals, prepared in ethanol stock, had revealed that some chemicals competed to a slight degree at very high concentrations, but issues of assay methodology prevented analysis at optimum conditions. The present study consisted of validation of the ER binding assay using dimethyl sulfoxide (DMSO) as the stock solvent, followed by analysis of competition of a subset of 19 chemicals prepared as DMSO stock instead of ethanol.

Overall, the ER assay performed very well when DMSO stock was used. The solvent itself did not affect performance at 20% or less of total assay volume. Binding kinetics of estradiol, estrone, equilin and equilenin were relatively similar to each other ethanol versus DMSO stock, and also to results from published literature. The ability to dissolve much higher concentrations of putative competitors in DMSO, than in ethanol, permitted assessment of binding characteristics beginning at 5 mMolar, versus a maximum of 0.1 mMolar when stocks were prepared in ethanol. On the other hand, concentrations of some solutes in DMSO appeared to interfere significantly with the assay's charcoal separation methodology, at concentrations of 1-10 mMolar.

Nonetheless, competitive properties were measurable for some chemicals in DMSO stock, that had not been possible when using ethanol stock. Some could be identified as extremely weak, true competitors at constants in excess of 100 µMolar (the maximum possible for ethanol stock). Others chemicals displaced ER binding in excess of 1 mMolar but were subsequently identified as not true competitors. A few chemicals displaced more binding from DMSO stock than from ethanol stock; this was believed due to better solubility of the tested substance in DMSO and/or better transfer when serial dilutions were prepared in the assay system's aqueous buffer.

In summary, tests of the ER binding assay system using rat uterine cytosol, showed that the assay performed as well with competitor stock prepared in DMSO as with ethanol. The ability to prepare solutions at much higher concentrations in DMSO (50-fold in our methodology) leads to a conclusion that DMSO stock can be effectively used for routine RUC protocols, while also permitting analysis of competitive properties of extremely weak ER binders, in excess of 1 mMolar.

Introduction:

This report summarizes our findings from studies conducted in accordance with EPA Contract PR-NC-05-10467. The essential purpose of the studies was to determine if the solvent dimethyl sulfoxide (DMSO) could serve as well as, or even better than, a more commonly used solvent (ethanol) in preparing competitor solutions for testing in the estrogen receptor (ER) binding assay. It was noted in earlier studies that the ethanol solvent had a potential to interfere with the ER binding system, which made it difficult to assess competitiveness of chemicals at concentrations of 1 mMolar or above. Because putative competitors are typically more lipophillic than hydrophilic, a more universal solvent than ethanol needed to be considered, and it was decided to examine the efficacy of DMSO.

As described in the Statement of Work (US EPA PR-NC-05-10467), the project had 4 specific tasks to be completed, all involving the use of DMSO instead of, or in comparison with, ethanol. The actual assay reagents, protocols and conditions used for this study were not different from those used in a previous study (SC Laws *et al., Toxicol Sci 94:*46-56, 2006), except that DMSO was substituted for ethanol where described. A basic assay protocol and description of reagents is attached (Appendix A). Chemicals used for this project are shown in Table 1.

Task I: Identify the limit concentration for two chemical solvents (ethanol and DMSO) for the ER competitive binding assay.

Our routine ER binding assay was conducted with rat uterine cytosol (RUC) and [^{3}H]-estradiol (tracer) prepared in the same manner as used in previous studies (SC Laws *et al., Toxicol Sci 94:*46-56, 2006), with either ethanol or DMSO added to the incubations at various concentrations. Incubation and separation steps were conducted accordance with the Protocol (see Assay Protocol for details).

Briefly, fixed amounts of RUC and [^{3}H]-estradiol were incubated in a series of tubes containing a fixed volume of TEG buffer with increasing amounts of either ethanol or DMSO (0% - 32%, at 2% increments). After incubation overnight at 4° C, a dextran-charcoal suspension was added to all tubes, which were then centrifuged. A fixed volume of supernatant (receptor bound hormone) was removed from each tube and counted. Net count in the 0% tubes was set as 100% binding (the maximum possible), and all other sample counts were computed as a percentage of the maximum count.

Table 1. Chemicals Analyzed for Competitive Binding Properties in the Present Study.

Inventory No.[a]	CAS No.	Chemical Name
4	112038	Octadecyl-trimethylammonium chloride
7	112458	Undecylenic aldehyde
8	112630	Methyl lineolate
9	112754	N,N-dimethyltetradecylamine
10	1143722	2,3,4-trihydroxybenzophenone
14	121437	Trimethylborate
15	122372	4-Hydroxydiphenylamine
16	124130	Octyl aldehyde
18	135193	2-Naphthol
24	1987504	4-Heptyl-phenol
25	24650428	2.2-dimethoxy-2-phenylacetophenone
26	27176870	Ddodecylbenzene sulfonic acid
27	27668526	n-Octadecyldimethyl[3-(trimethoxysilyl)propyl}-ammonium chloride
31	3236713	4,4'-(9-fluorenylidene)-diphenol
32	3380345	5-Chloro-2-(2,4-dichlorophenoxyl)-phenol
33	3367257	Sulcofuron-natrium monohydrate
36	59507	4-chloro-3-methyl-phenol
46	80433	Dicumyl peroxide
49	96764	2,4-d-tert-butylphenol

[a] Inventory numbers were assigned to the 50 chemicals originally received, in order to simplify identification in the lab while conducting the competition studies.

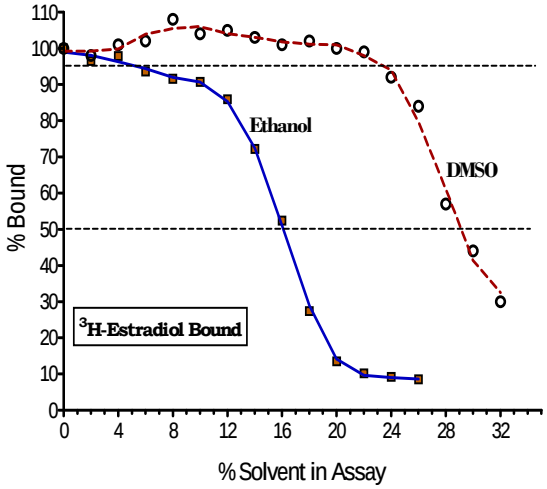

Figure 1. Comparative Effect of Added Ethanol versus Dimethyl Sulfoxide (DMSO) on Estradiol Binding to Estrogen Receptor (ER) in the RUC Assay Incubation.

Results (Figure 1) demonstrated that the effect of DMSO presence was much less than the effect of ethanol presence. The approximate IC_{50} was 16% for ethanol and 29% for DMSO. A perhaps more important measure of the ethanol vs. DMSO effect was the IC_{95} (Figure 1), as an approximation of the lowest concentration capable of producing a significant reduction of tracer binding. The IC_{95} was approximately 5% ethanol and 20% DMSO.

Because our routine ER binding assay protocol results in a 1:3 dilution of competitor solution in the final assay volume, we have interpreted the results represented by Figure 1 to

indicate that a competitor stock prepared in DMSO solvent should not be added directly to the assay system (it would be 33% of the total assay volume). We conclude that it should be diluted at least 1:2 in assay buffer to prepare the highest concentration that would be safe from interference with binding due to solvent effects.

By contrast, Figure 1 results indicate that an ethanol stock would need to be diluted at least 7-fold prior to addition in an assay. Indeed, our earlier studies of ER binding competition by selected chemicals (SC Laws *et al., Toxicol Sci 94:*46-56, 2006) routinely used a 1:10 dilution of ethanol stock as the maximum concentration of competitor.

Furthermore, because DMSO can presumably dissolve greater quantities of test competitor, these results bode well for using DMSO as the solvent of choice when preparing the primary stock of the various competitors. In our earlier studies (SC Laws *et al., Toxicol Sci 94:*46-56, 2006), it became necessary to limit the highest competitor concentrations to 0.1 mMolar for several reasons, including the presence of significant interference by ethanol on ER binding.

As will be shown in subsequent sections of this report, it was possible to assess competitiveness of chemicals at much higher starting concentrations (typically 5 mMolar, versus 0.1 mMolar for ethanol), when the initial stocks were prepared in DMSO. Note that because of other issues, principally interference of extremely high solute concentrations with the charcoal separation step, we were unable to examine competitiveness of Molar concentrations, even if they might have dissolved in DMSO at that level. See results in latter sections of Task II.

Task II: Develop a tiered approach for determining the best solvent and limit of solubility for any test chemical.

The objective of this task was to develop a tiered approach for (1) determination of the best solvent for any test chemical, and (2) to identify the maximum concentration of test chemical that can be used without disrupting ER binding kinetics of the assay itself.

ICCVAM's Expert Panel Report (Section 2.1.3) had recommended a sequence of testing with water, then ethanol, then DMSO (http://iccvam.niehs.nih.gov/methods/endodocs/edfinrpt/edfinrpt.pdf)

The original Statement of Work for this contract also stated the following:

However, it is anticipated that the approach for the ER Competitive Binding Assay will also include preliminary information of the physical properties of the test chemical (e.g., partition coefficients, hydrophobicity, solubility, etc.), as well as indicators that a chemical is precipitating out after being added to the assay tube/buffer (e.g., U-shaped curves with increasing chemical concentration; solubility test using light scattering technique and Nepheloskan instrument). The report for this task shall include a flow chart demonstrating the approach for (1) determining the best solvent as evidenced by allowing the highest limit concentration of the test chemical (but not to exceed 1 mM);

and (2) determining the maximum concentration of test chemical that can be used in the assay without chemical precipitation following addition to the assay tube and an overnight incubation at 4 C.

Several introductory points should be made before moving to analysis of the results:

1. The only chemicals available to us for analysis were in a set originally delivered for an earlier completed study (SC Laws *et al., Toxicol Sci 94:*46-56, 2006). These chemicals are low molecular weight organic molecules without appreciable solubility in water, but they were soluble in ethanol and DMSO. Upon initial dilution of the primary ethanol or DMSO stocks in our aqueous assay buffer (TEG, see Protocol), these chemicals typically fell out of solution. Many sorts of chemicals (e.g. proteins) can dissolve in water but perhaps not well in ethanol. Therefore, we chose not to conduct a round of primary stocks prepared in water, for this particular study, because we were certain that none would dissolve nearly as well in water as in a more non-polar system like ethanol or DMSO. Nonetheless, many substances selected for evaluation as ER binders may have chemical structures that permit greater solubility in water, and should be evaluated from an aqueous stock

2. Primary stocks were usually prepared at 30 mMolar concentration in DMSO. Because competitor solutions were diluted 1:3 in the final assay volume, a 30 mMolar stock diluted 1:2 (see discussion of Figure 1) produced an initial concentration in the assay itself of 5 mMolar. Serial dilutions were made at 1:10 in TEG buffer.

3. Most of the chemicals in the available set did precipitate when the primary stock was diluted in assay buffer, whether the stock solvent was ethanol or DMSO. Indeed, estradiol itself was insoluble when prepared in the TEG buffer at 10 or 100 µMolar. Therefore, the standard operating procedure dictated that all dilutions of all competitors be vortexed and thoroughly mixed prior to dispensing into assay tubes.

4. Our laboratory had no equipment to determine solubility. Lack of solubility of chemicals at high concentration in TEG was easily observed to the naked eye, and photos were made of examples (see Figure 3).

Results from Task I (Figure 1) indicated that DMSO at concentrations at or below 16.7% (1:2 x 1:3) would have no effect on the ER binding assay, using our protocol. The initial goal for Task II was to determine whether dilutions of estradiol stock initially prepared in DMSO displayed different binding characteristics in the RUC assay, compared to primary stock prepared in ethanol.

For the experiment shown in Figure 2, primary stocks of estradiol were prepared in ethanol or DMSO, from which dilutions were made in TEG buffer. Estradiol concentrations ranged from 200 nMolar to 20 pMolar in the final assay volume. Results showed virtually identical displacement curves. This test was conducted several times, showing that there is no difference in the performance of estradiol in DMSO or ethanol.

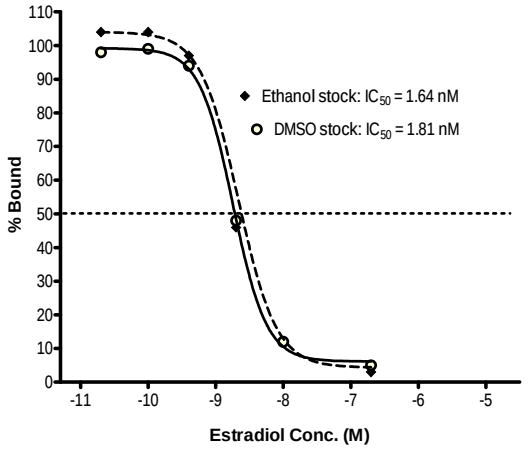

Figure 2. Estradiol Inhibition Curves Using Primary Stocks Prepared in Ethanol versus DMSO.

Another goal for Task II was to establish the limit of solubility for test chemicals. As stated above, it was not possible to complete this task by direct measurement of solutions to determine if any precipitation remained. Nonetheless, some useful observations were made as we began preparing stocks in DMSO and dilutions of the test chemicals in TEG buffer for analysis in the receptor binding assay.

Each of the chemicals that was evaluated with a DMSO primary solvent dissolved completely, by visual inspection. The initial stock concentration of 30 mMolar was routinely possible. When using ethanol as the primary stock solvent, it was seldom possible to prepare stock at concentrations in excess of 1 mMolar.

However, a related solubility issue did routinely occur, when it was attempted to make dilutions (in TEG buffer) from the primary stock, whether from ethanol or DMSO. The RUC assay buffer system is of necessity aqueous; both tracer and cytosol must be prepared in an aqueous buffer (tris-EDTA with glycerol). Yet the tested chemicals were typically very non-polar organic materials, having essentially no solubility in water.

In the examples shown in Figure 3 (below), chemicals were initially prepared at 1 mg/ml in ethanol (far left vial) then diluted in TEG buffer to (left to right) 100, 10, 1, and 0.1 µMolar. Precipitating crystals occurred with TEG dilution of chemicals initially prepared in either ethanol or DMSO. Therefore, when preparing dilutions for competition assays it became necessary to vortex and shake the dilutions well just before transfer to the succeeding dilution vial, or addition to assay tubes.

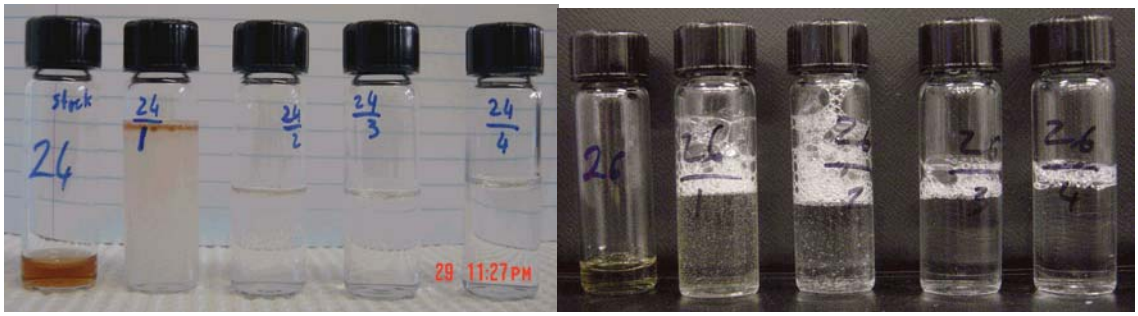

Figure 3. Precipitation of Test Chemicals when the Primary Stock is Diluted in TEGD Buffer. Compound 24 is 4-heptyl-phenol (CAS #1987504), and Compound 26 is dodecylbenzene sulfonic acid (CAS #27176870)

While this finding did not directly address the question of maximum solubility in the primary stock solvent, it did provide a reminder that dilutions for assay in the µMolar range must be carefully prepared, because the compounds being tested are typically poorly soluble in water, and there is low assurance that the dilutions will be accurate.

Indeed, estradiol and other natural steroid hormones, are themselves poorly soluble in water. Transport of significant levels in blood is possible only because the steroids are able to accomplish a low-affinity association with carrier plasma proteins. Once inside cells, steroids bind quickly to target receptors that contain strong hydrophobic clefts in the hormone binding domain region. Bioactive steroids typically bind to target receptors in the sub-nMolar range, and suspensions of these tiny concentrations are possible in cytoplasm. On the other hand, the *in vitro* binding protocols used in the present work tested much higher (mMolar to µMolar) concentrations of chemicals that associate very weakly with receptors. These highly non-polar substances do not dissolve or suspend effectively in the aqueous buffers that are necessary to maintain the 3-dimensional receptor configuration. There is no universal solvent system that can accommodate to every type of molecule, so preparations of assay reagents will always be a challenge.

Yet another problem, uncovered during our studies of maximum solubility for Task II, was that the process of separating bound and free tracer had its own limits. When the concentration of some competitors began to exceed 100 µMolar, the sheer mass of chemical appeared to saturate the separation process. An example of this problem occurred when extremely high concentrations of sulcofuron-natrium monohydrate (abbreviated as SFM, CAS No. 3367257) were tested for competition against estradiol binding in the RUC assay.

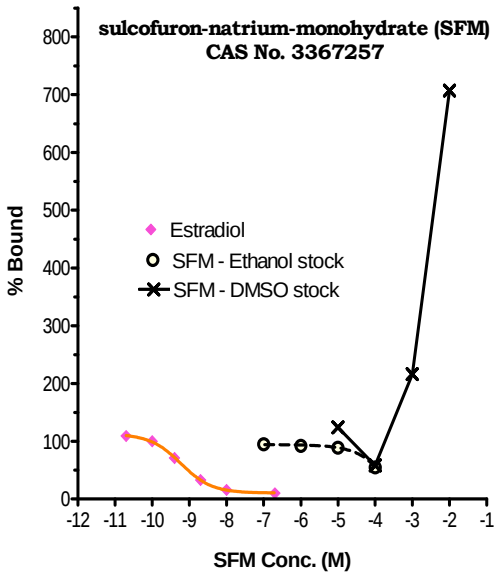

Figure 4. Interference of Extremely High Concentrations of Test Chemical (sulcofuron-natrium monohydrate) in RUC Assay Separation Process.

In earlier studies, this chemical had shown a modest degree of displacement at 0.1 mMolar, (SC Laws *et al.*, *Toxicol Sci 94:*46-56, 2006), which made it a high priority for testing at a higher concentration fom a DMSO stock. Assay incubations were set up at concentrations as high as 10 mMolar, using DMSO stock.

However, as shown in Figure 4, the counts of tracer remaining in the supernatant rose sharply at 1 and 10 mMolar SFM. This was believed due to saturation of the dextran-charcoal separating agent by the chemical being tested, leaving some unbound (to ER) tracer in the supernatant instead of precipitated in the pellet. It was then decided to conduct additional studies on this property to ascertain if the effect might occur with other chemicals.

Primary stocks of 7 chemicals (Table 2) were prepared in DMSO, and dilutions made in TEG buffer at 30, 3, 0.3 and 0.03 mMolar, each diluted 1/3 in the assay system. Aliquots were dispensed into tubes with ^{3}H-estradiol and TEG buffer (at the volume of receptor that was not being used in these experiments).

TABLE 2. Compounds Tested in Charcoal Separation Analysis (Figs. 5, 6. 7)

Inventory No.[a]	CAS No.	Chemical Name
16	124130	octyl aldehyde
18	135193	2-naphthol
25	24650428	2.2-dimethoxy-2-phenylacetophenone
27	27668526	n-octadecyldimethyl[3-(trimethoxysilyl)propyl}-ammonium chloride
31	3236713	4,4'-(9fluorenylidene)-diphenol
32	3380345	5-chloro-2-(2,4-dichlorophenoxyl)phenol
33	3367257	sulcofuron-natrium monohydrate

[a] Inventory numbers were assigned to the 50 chemicals originally received, in order to simplify identification in the lab while conducting the competition studies.

After a 30 min incubation at 4° C, ice-cold dextran charcoal suspension was added, the tubes were centrifuged, and the supernatants were counted. Results are presented in Figure 5 (5 mg charcoal/tube, our standard protocol), Figure 6 (10 mg/tube) and Figure 7 (15 mg/tube).

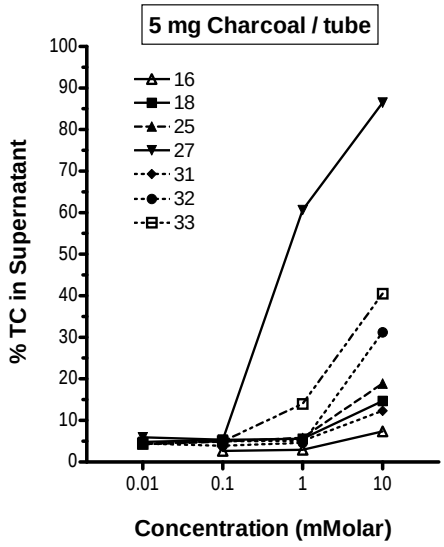

Figure 5. Interference of 5 mg Charcoal Separation by High Concentrations of Test Chemical.

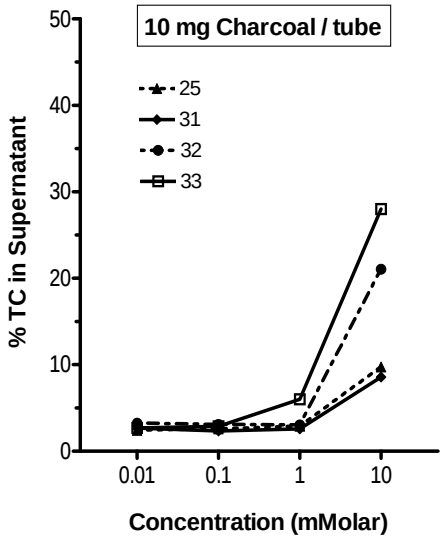

Figure 6. Interference of 10 mg Charcoal Separation by High Concentrations of Test Chemical.

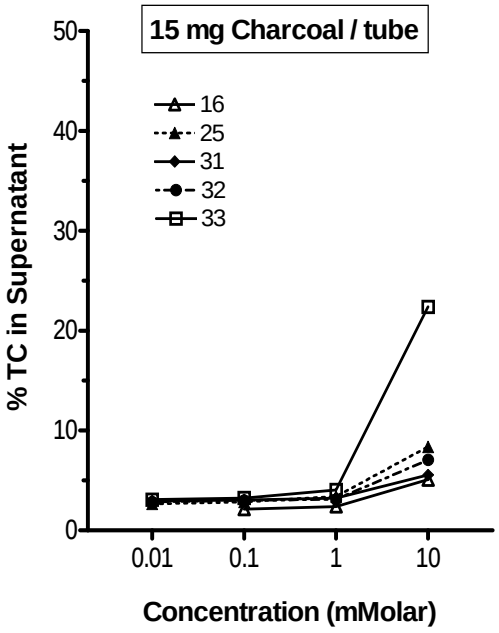

Figure 7. Interference of 15 mg Charcoal Separation by High Concentrations of Test Chemical.

Results clearly demonstrated that extremely high concentrations of test chemical impede the assay separation step. Our routinely used addition of 5 mg charcoal (Figure 5) was able to precipitate essentially all radiolabeled tracer when the concentration of test chemical was 10 or 100 µMolar. When concentrations of reached 1 mMolar, the charcoal became saturated with some test chemicals, and all of the tested chemicals impeded separation at 10 mMolar.

Furthermore, doubling (Figure 6) or tripling (Figure 7) the amount of charcoal was unable to resolve the problem completely. Although more tracer was precipitated by additional charcoal, some interference continued to occur. Results also indicate that some chemicals clearly interfered with the separation process more than others. Thus, it would not be possible to "estimate" a non-specific background count for all chemicals tested for ER binding in the range of 10 mMolar.

For additional studies (Tasks III and IV), we did not increase the amount of charcoal in the separation steps, because results suggested that using higher amounts could not appreciably resolve the saturation issue. Furthermore, our experience with this protocol has shown us that using more charcoal per assay tube creates one additional problem when receptor is present, namely a propensity to "strip" bound tracer from receptor. Adding more charcoal requires a more rapid completion of the separation process, to avoid stripping and precipitating radiolabeled tracer that should remain bound to ER. A more rapid separation process in harmful to assay precision, when larger sets of tubes are being analyzed.

These important results clearly identify a practical limit of the assay methodology for assessing competitive binding of various chemicals in the RUC assay. With these reagents and protocols, one might expect a significance interference with assay kinetics when the concentration of some test chemicals exceeds 1 mMolar. A concentration of 0.1 mMolar seems safe and free of impediment; evaluationg concentrations of 10 mMolar may always be impossible in this assay system.

In summary, while there would be interest in evaluating the competitive properties of putative binders at the highest possible concentrations, the problem of using extremely

high concentrations (e.g., in excess of 0.1 mMolar) is not likely to be found in the degree of solubility of the test substances in ethanol or DMSO. A problem of managing high concentrations is more likely to occur when the stock solution (in ethanol or DMSO) is diluted for assay in aqueous buffer. Many chemicals of interest will not stay in solution (or suspension) at millimolar and micromolar levels. In addition, the mechanics of the ER binding assay itself, particularly the separation steps, can become compromised when competitor concentration exceeds 0.1 mMolar. While some environmental contaminants with a very weak competitive property for ER binding may be found in millimolar concentrations, the necessities of the present RUC assay design may preclude an accurate evaluation of their competitive degree.

TASK III. Compare ER Binding Affinity of Several Chemicals Prepared as Stock in Ethanol and DMSO.

The objective for this task was to determine if binding characteristics of selected chemicals were different if analyzed from original stock prepared in ethanol and DMSO. We selected 19 chemicals (Table 1) from a set of 50 previously provided to us for earlier studies. The majority of these had displayed very low competition against ER binding in earlier studies, and was therefore of interest to evaluate at higher concentrations made possible by using DMSO stock. A report of those "low-affinity" competitors is presented in TASK IV, below. This portion of the report will focus on a comparison of higher affinity natural compounds, closer to the range of estradiol itself.

The ability of estradiol to display similar competitive characteristics in DMSO and ethanol was described earlier (Figure 2), in the report of Task II. In addition to estradiol we evaluated the inhibitory property of other known estrogens in the RUC receptor binding assay.

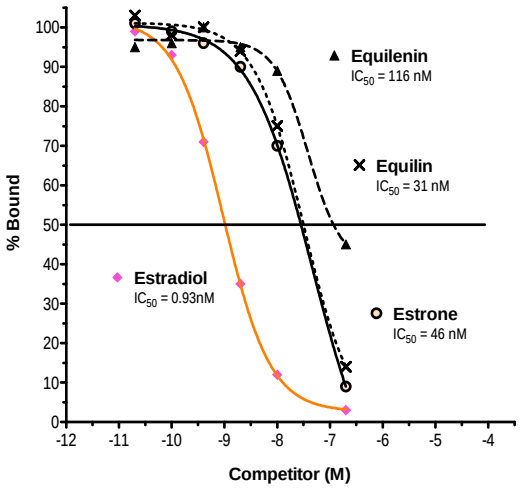

Figure 8. Displacement of Radiolabeled Estradiol Binding from Rat Uterine Cytosol Estrogen Receptor by 4 Natural Estrogens Prepared in DMSO Stock.

Concentrations of 4 estrogens (20 pMolar to 200 nMolar, made from DMSO stock) were incubated with radiolabeled estradiol and RUC in the routine competitive binding assay. Results from the standard displacement assay (Figure 8) showed that estrone had a fairly strong ability to displace estradiol tracer from ER. Equilin and equilenin, two weak estrogens that are a frequent component of post-menopausal hormone replacement, also inhibited binding with IC-50's in the range of .01 – 0.1 µMolar. These results are all very similar to published literature on ER binding kinetics (BR Bhavnani, *J Steroid Biochem Mol Biol 85:*473, 2003).

Additional analysis of the competitive nature of estradiol and estrone binding were conducted, from stock prepared in DMSO, and then compared to plots made from stocks prepared in ethanol. Incubations of RUC, several concentrations of radiolabeled estradiol and 3 concentrations of competitors (estradiol or estrone, from ethanol stock) were prepared in a manner previously described. See Assay Protocol for details, and also SC Laws *et al., Toxicol Sci 94:*46-56, 2006.

The plots in Figure 9, called Lineweaver-Burk plots or "double reciprocal plots", show the expected series of linear slopes when a true competitor for ER binding is incubated at different concentrations near the EC_{50}.

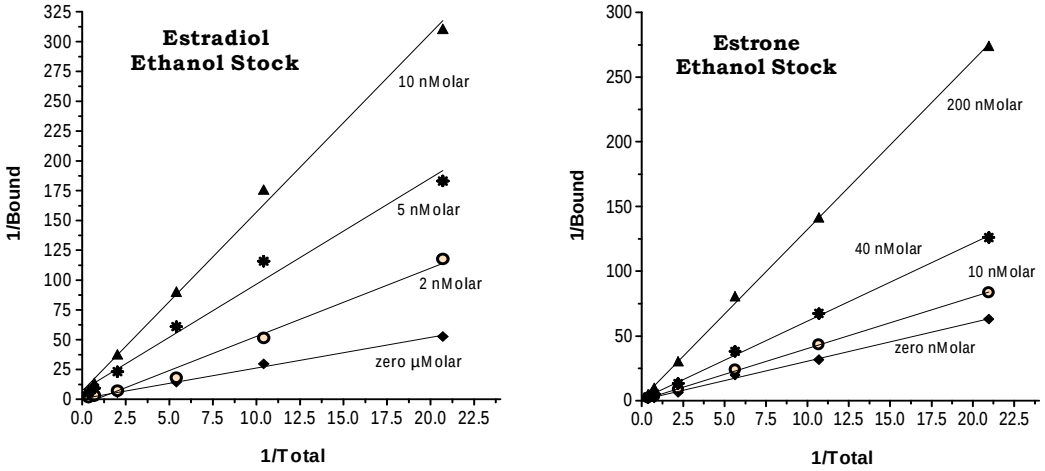

Figure 9. Lineweaver-Burk Plots of Estradiol and Estrone in RUC Assay, from Ethanol Stock.

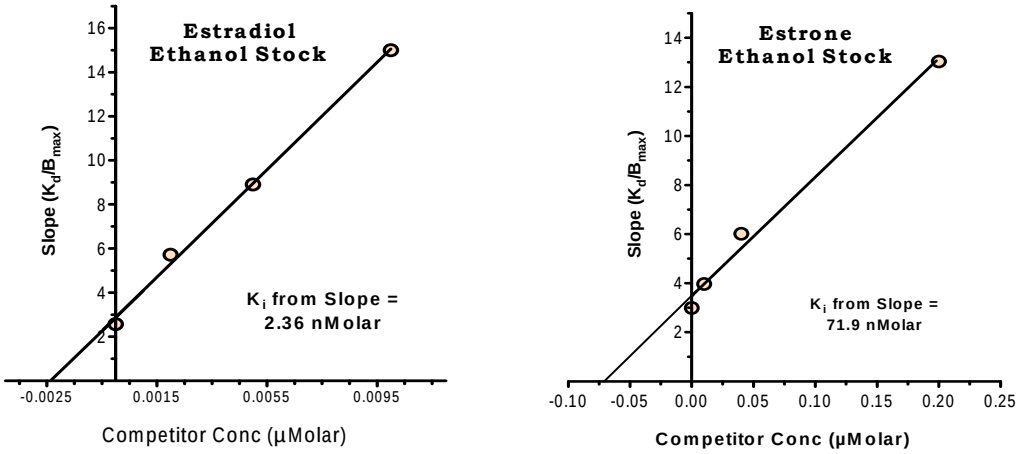

Figure 10. Plots of Lineweaver-Burk Plot Slopes for Estradiol and Estrone, Prepared From Ethanol Stock, in RUC Receptor Binding Assay.

The plots of the slopes are shown in Figure 10 and indicate estrone has a somewhat less affinity for ER than estradiol, by perhaps 1.5 orders of magnitude. This finding was also expected, from earlier literature reports (BR Bhavnani, *J Steroid Biochem Mol Biol 85:*473, 2003). Estrone is a true competitive inhibitor of estradiol. After these expected results on the competitive properties of estradiol and estrone, from ethanol stock as a point of reference, the analyses were repeated using steroids prepared from DMSO stock.

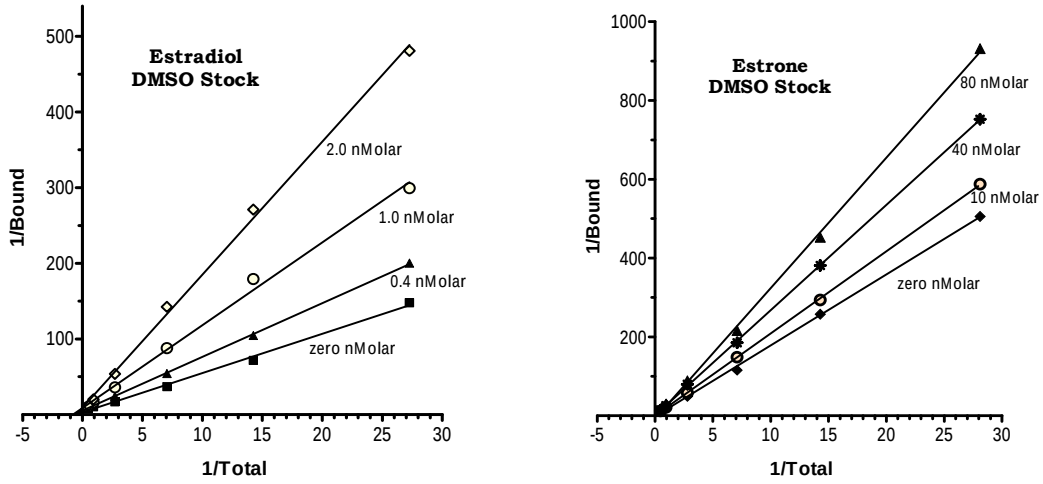

Figure 11. Lineweaver-Burk (L-B) Plots of Estradiol and Estrone Prepared in DMSO Stock and Assessed for Competitive Binding to Estrogen Receptor in RUC Assay.

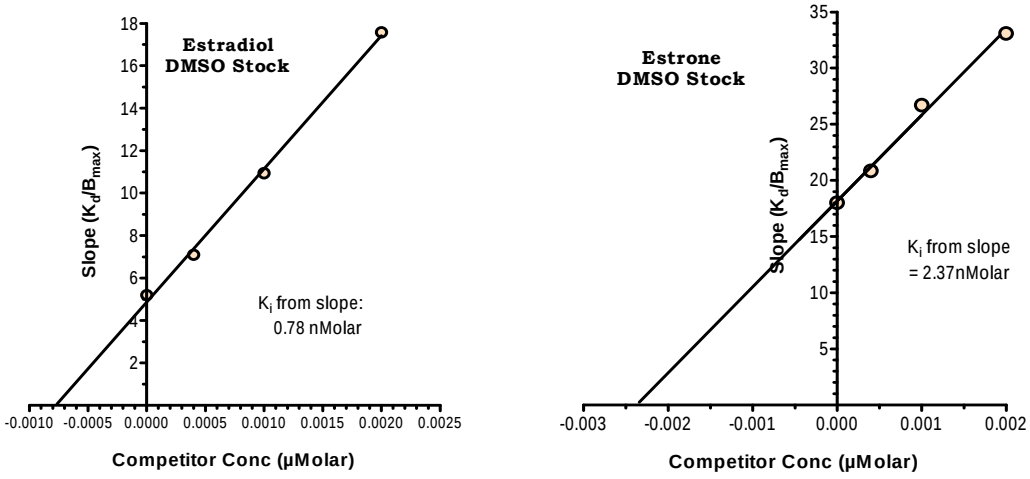

Figure 12. Plots of Lineweaver-Burk Plot Slopes for Estradiol and Estrone, Prepared from DMSO Stock, in RUC Receptor Binding Assay.

Results (Figs. 11 and 12) showed that the competitive kinetics of estrone and estradiol demonstrated a slightly greater affinity of these two hormones for ER, when stock solutions were initially prepared in DMSO, compared to ethanol.
Once again, estrone was about 1 order of magnitude weaker than estradiol for competition with ER binding. Both hormones displayed true competitive inhibition.

The observation that both steroids had higher affinity readings in DMSO, compared to earlier readings made from ethanol stock, is a bit puzzling. It is possible that both estrogens were better dissolved in DMSO stock and therefore diluted to slightly higher concentrations in assay buffer (see earlier discussion of the solubility issues, in Tasks I and II). It is also possible that the different RUC preparations used in these studies, conducted at different times, had slightly variable binding characteristics.

Finally, we were able to analyze equilin, one of the equine estrogens, a bit further and determined that it is a true competitor by our method, with an apparent K_i about 2-3 orders of magnitude lower than estradiol (Figure 13). This was again quite consistent with expectations.

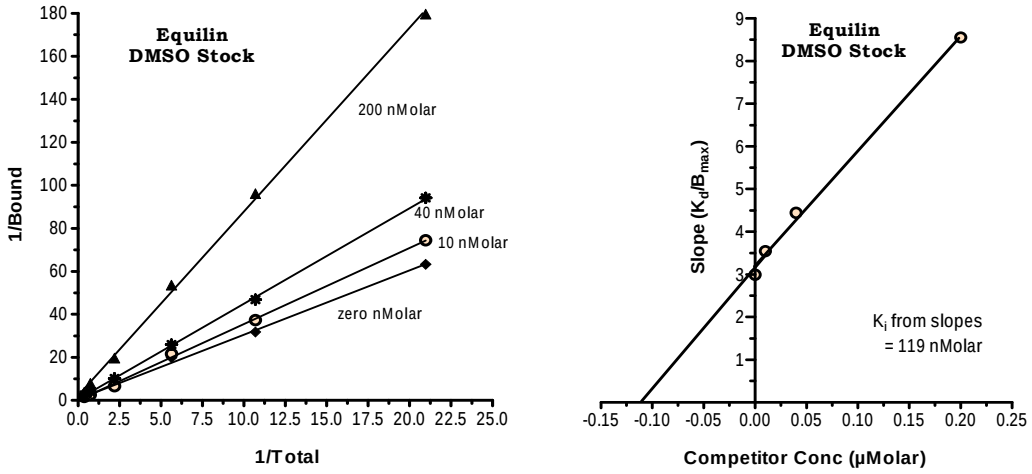

Figure 13. Lineweaver-Burk (L-B) Plots, and Plot of the L-B Plot slopes, of Equilin Prepared in DMSO Stock and Assessed for Competitive Binding to Estrogen Receptor in RUC Assay.

In summary, results from these studies showed that several natural estrogens that have been characterized very well over many years behaved as expected when prepared for assay in stocks of DMSO solvent. Traditionally, ethanol has been used as a "universal solvent" when preparing primary stocks of these organic chemicals for assay in receptor binding protocols. Because DMSO seems able to dissolve greater quantities of the tested substances, and the RUC binding assay behaves in a consistent manner, whether ethanol or DMSO is used as the primary stock solvent, one might conclude that DMSO is preferable for making initial stock solutions.

TASK IV. Evaluate the utility of ethanol or DMSO solvent for a selected group of chemicals with low but significant competitiveness at 0.1 to 1 mMolar.

This section describes results from analysis of chemicals that were considered to be "very poor competitors" in earlier studies (SC Laws *et al., Toxicol Sci 94:*46-56, 2006). These compounds typically displaced between 20 and 50 % of radiolabeled estradiol at high micromolar concentrations, when prepared in ethanol as the primary stock. Because an IC_{50} could not be identified, it was not possible to subject the chemicals to further analysis using Lineweaver-Burk plots, and none were identified as true competitors in ethanol stock. It was hoped that higher concentrations, made possible with DMSO as the stock solvent, could yield better information about the competition.

There was no attempt to re-analyze the chemicals that had displayed sufficient solubility and competition from ethanol stock. More than 20 of the original set of 50 chemicals had achieved an acceptable IC_{50} in earlier studies and were tested for true competitiveness in follow-up studies (SC Laws *et al., Toxicol Sci 94:*46-56, 2006).

In addition, a small number of the original set of 50 chemicals displayed no ability at all to displace estradiol binding to ER and these were likewise not re-tested in the present studies. Results from Task II had illustrated that, because of interference in the separation steps, only a marginal increase of competitor concentration would be feasible for binding assays (e.g., 1-2 orders of magnitude) using DMSO stock. Hence, the DMSO studies continued only for compounds that had demonstrated some degree of competition at the 0.1 mMolar level. Of the original 50, nineteen compounds were tested from DMSO stock.

1. Improved Analyses When Using DMSO Stock.

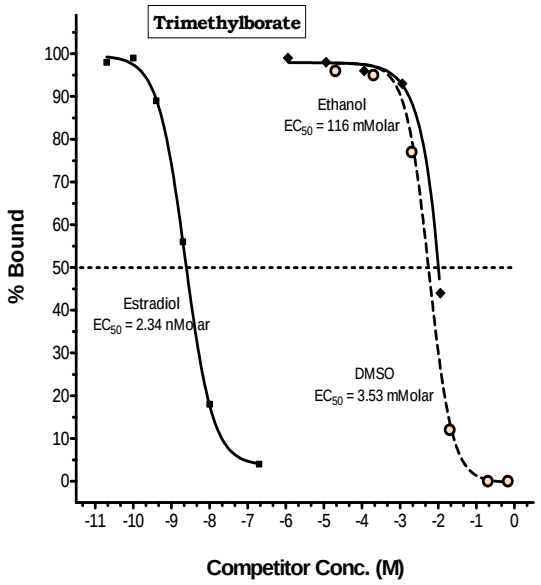

Figure 14. Displacement of 3H-Estradiol Binding to ER by Trimethylborate in the RUC Assay.

One compound, trimethylborate, worked well in DMSO. As shown in Figure 14, the higher concentrations made possible by DMSO (up to nearly 1 Molar) produced a complete displacement curve. The ethanol stock dilutions could only be prepared at concentrations as high as 1 mMolar, which displaced only about 50% of tracer (Fig. 14) at the highest concentration. Dilutions prepared from DMSO stock displaced about the same as from ethanol stock, but higher concentrations were also possible.

The more complete displacement curve from DMSO stock permitted a confident selection of an IC_{50} that yielded

Lineweaver-Burk plots revealing a true competitive inhibition for this compound, in the millimolar range (Figure 15). Lineweaver-Burk plots were not possible for trimethylborate prepared from ethanol so, in this instance, a true competitive binder was identified and quantified from DMSO stock, that was not possible in earlier studies.

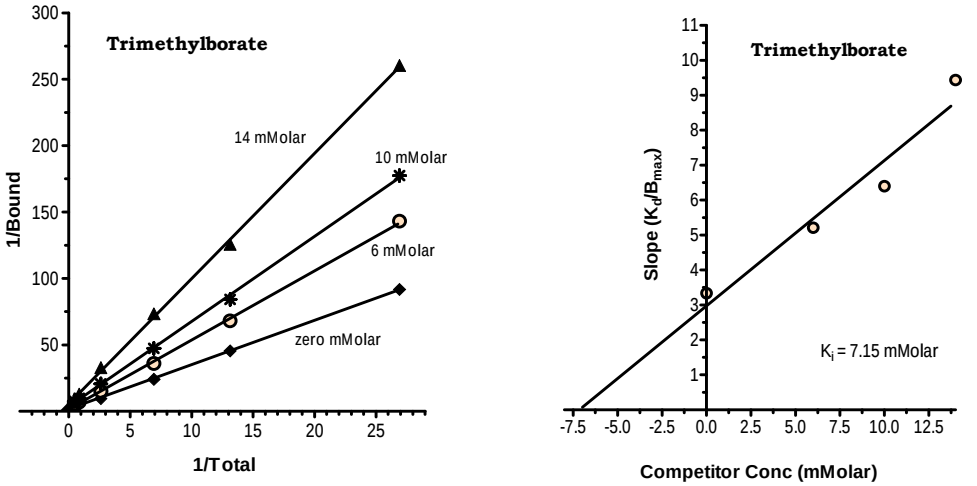

Figure 15. Lineweaver-Burk (L-B) Plots, and Plot of the L-B Plot slopes, of Trimethylborate Prepared in DMSO Stock and Assessed for Competitive Binding to Estrogen Receptor in RUC Assay.

Another compound that performed much better with DMSO stock was 2-naphthol. While displacement of estradiol binding to ER was extremely weak from the ethanol stock, an IC_{50} of significant displacement was observed from DMSO stock (Figure 16). This was most likely due to better solubility of the chemical in aqueous buffer when stock was prepared in DMSO..

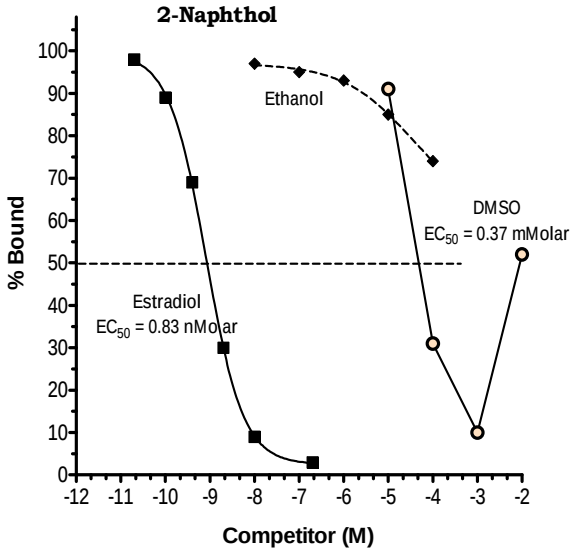

Figure 16. Displacement of ^{3}H-Estradiol Binding to ER by 2-Naphthol in the RUC Assay.

Lineweaver-Burk plots showed that 2-naphthol, like trimethyl borate (Figs. 14, 15) is very likely a true competitor of ER binding (Figure 17). However, demonstration of these properties would not have been possible from stock prepared in ethanol.

Another compound that performed much better in DMSO, compared to ethanol, was 4,4'-(9-fluorenyldiene)-diphenol. As shown in Figure 18, earlier studies using dilutions from ethanol stock displayed little potential

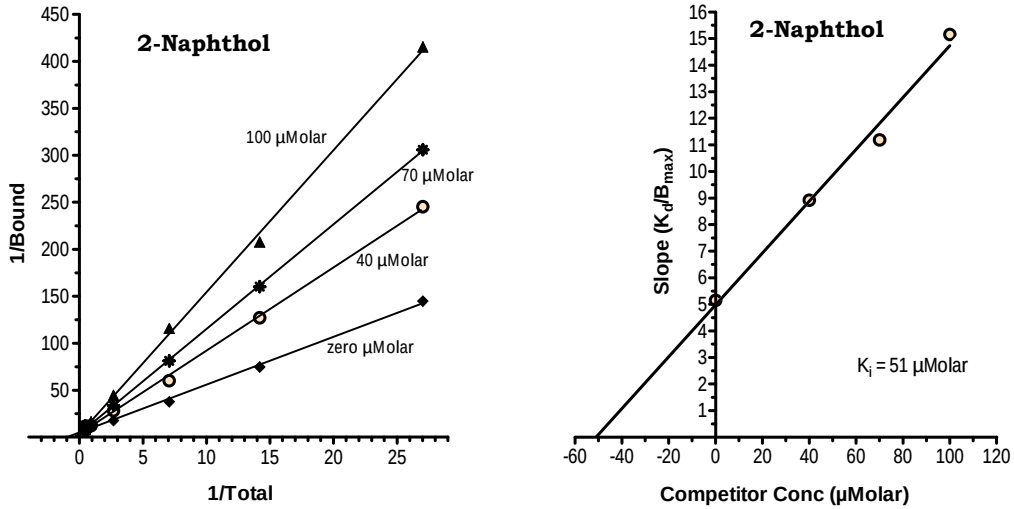

Figure 17. Lineweaver-Burk (L-B) Plots, and Plot of the L-B Plot slopes, of 2-Naphthol Prepared in DMSO Stock and Assessed for Competitive Binding to Estrogen Receptor in RUC Assay.

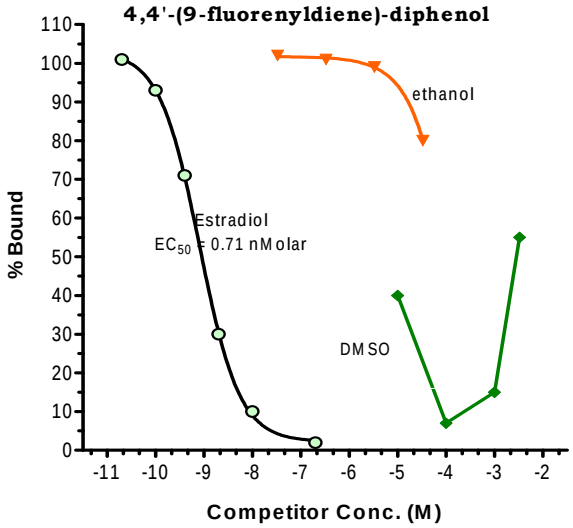

Figure 18. Displacement of ^{3}H-Estradiol Binding to ER by 4,4'-(9-fluorenyldiene)-diphenol in the RUC Assay.

for displacement , but solutions from DMSO stock were more active. Because dilution of DMSO stock to 10 µMolar inhibited binding substantially more than 50%, an IC_{50} was estimated in the range of 1-10 µMolar.

Indeed, successful Lineweaver-Burk plots resulted in this concentration range, which made it possible to elucidate a true competitive inhibition (Fig. 19). This result was impossible with the ethanol stock. As before, it was concluded that increased solubility of the test substance in DMSO, and in the subsequent dilutions in TEG buffer, most likely contributed to this successful outcome.

Several other chemicals in the provided set also performed acceptably well in DMSO stock, compared to ethanol stock, and were identified as true competitive inhibitors at very high concentrations. These included 4-chloro, 3-methyl phenol; 2,3,4-trihydroxybenzophenone; and phenyl,2,4-bis(1,1-dimethylethyl; 2,4-d-tert-butylphenol.

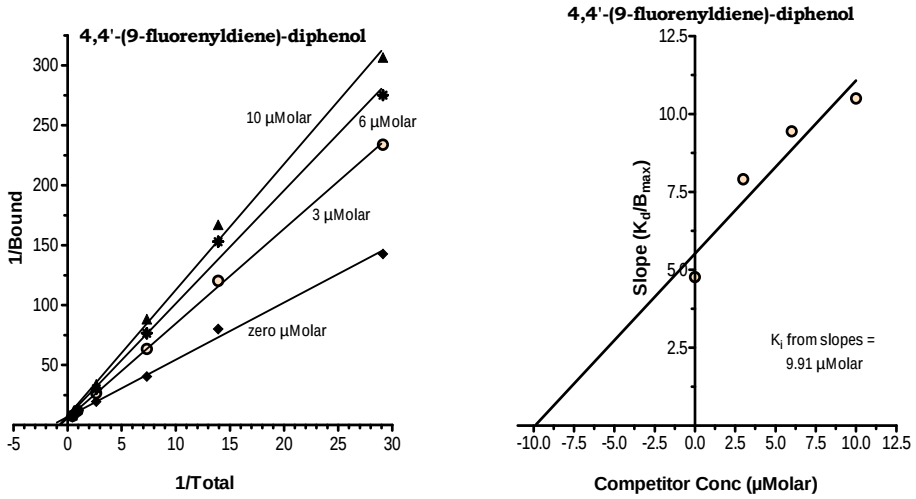

Figure 19. Lineweaver-Burk (L-B) Plots, and Plot of the L-B Plot slopes, of 4,4'-(9-fluorenyldiene)-diphenol Prepared in DMSO Stock and Assessed for Competitive Binding to Estrogen Receptor in RUC Assay.

2. Less Favorable Performance with DMSO Stock

Not every tested chemical performed better in DMSO stock, compared to ethanol. One example was dicumyl peroxide, which yielded an abnormal but functional inhibition plot (Figure 20) at higher concentrations than possible in ethanol stock.

However, attempts at a Lineweaver-Burk plot, using concentrations estimated around an EC_{50}, failed to produce convincing evidence of true competitive inhibition (Fig. 21). ANOVA indicated that the plot of slopes was not significantly linear or non-zero (p = .112).

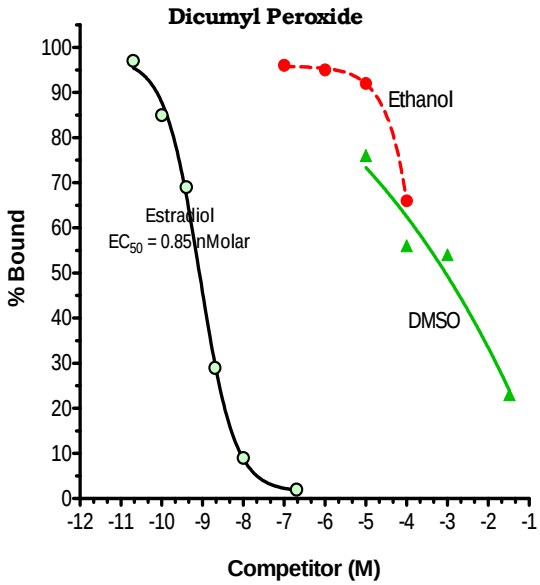

Figure 20. Displacement of 3H-Estradiol Binding to ER by Dicumyl Peroxide in the RUC Assay.

A similar outcome occurred with 5-chloro-2-(2,4-dichlorophenoxyl)-phenol. Dilutions from the DMSO stock had a very potent ability to displace ^3H-E_2 binding in the range of 0.1 to 1 mMolar, but a replot of slopes from Lineweaver-Burk plots was not significantly linear (p = 0.078). Graphs are not shown here. Earlier attempts to determine competition from ethanol stock had also failed to establish a linear plot, even though an IC_{50} had been determined with this compound from ethanol. DMSO was no help.

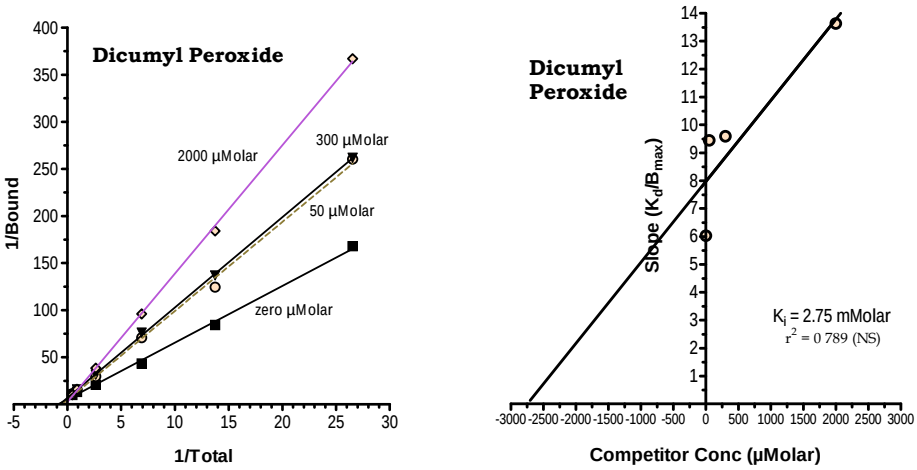

Figure 21. Lineweaver-Burk (L-B) Plots, and Plot of the L-B Plot slopes, of Dicumyl Peroxide Prepared in DMSO Stock and Assessed for Competitive Binding to Estrogen Receptor in RUC Assay.

Some of the tested chemicals failed to demonstrate sufficient displacement of tracer from either ethanol or DMSO stock. In the case of 2.2-dimethoxy-2-phenylacetophenone (Figure 22), similar displacement occurred at 10 and 100 µMolar from either DMSO or ethanol stock. However, lesser dilutions of the DMSO stock, at 1 and 10 mMolar, failed to show greater displacement in excess of 50%, which was necessary to identify a useful IC_{50} for further analysis of competitive binding. Similar results were observed with other chemicals in the test set, among them 4-hydroxy-diphenylamine and N,N-dimethyl-tetradecylamine.

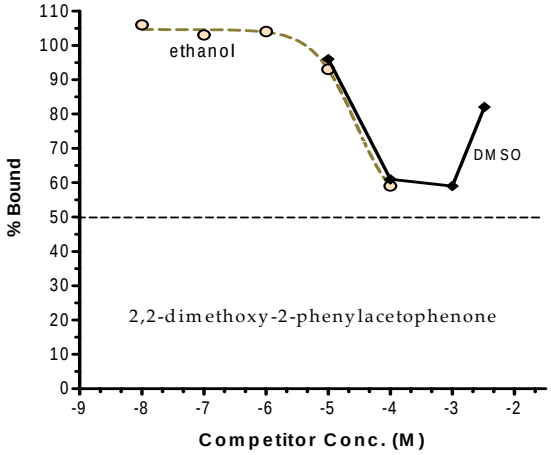

Figure 22. Displacement of 3H-Estradiol Binding to ER by 2,2-dimethoxy-2-phenylacetophenone in the RUC Assay.

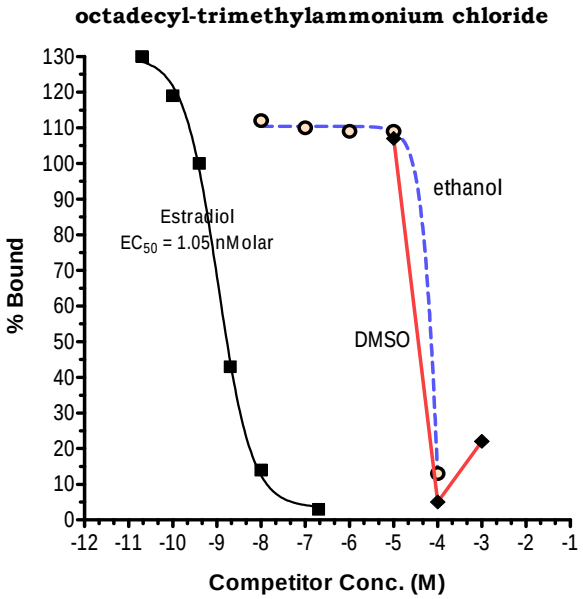

Figure 23. Displacement of 3H-Estradiol Binding to ER by Octadecyltrimethylammonium Chloride in the RUC Assay.

Finally, several compounds failed to demonstrate a sigmoid dose-response curve when increasing concentrations were used, yet they did produce a complete inhibition at some point. An example is shown here for octadecyl-trimethyl ammonium chloride (Figure 23). Binding of tracer to ER dropped from 100% at 10 µMolar to essentially zero at 100 µMolar. The same result occurred when this compound was prepared in ethanol stock, at exactly the same concentrations. Furthermore, Lineweaver-Burk plots to examine for type of competition, also failed (results not shown here). An explanation for this occurrence, which was observed with several other chemicals in both the ethanol and DMSO series, can only be speculated; perhaps the initial dilutions from stock form an aggregate that denatures the receptor structure and prohibits estradiol binding to its binding domain.

Table 3. Comparison of Binding Properties of 19 Chemicals Prepared in Ethanol versus DMSO Stock

CAS No.	Chemical Name	EC_{50} Ethanol	K_i Ethanol	EC_{50} DMSO	K_i DMSO
112038	Octadecyl-trimethylammonium chloride	> 100 µM	Plot failed	~ 200 µM	Plot failed
112458	Undecylenic aldehyde	> 100 µM	---	11.9 mM	---
112630	Methyl lineolate	> 100 µM	---	355 mM	---
112754	N,N-dimethyltetradecylamine	1.9 µM	Plot failed	~ 1 mM	Plot failed
1143722	2,3,4-trihydroxybenzophenone	31.9 µM	102.6 µM	17.0 µM	40.0 µM
121437	Trimethylborate	> 100 µM	110 µM	3.7 mM	5.80 mM
122372	4-Hydroxydiphenylamine	> 100 µM	---	> 1 mM	---
124130	Octyl aldehyde	> 100 µM	---	~ 5 mM	---
135193	2-Naphthol	> 100 µM	---	~ 50 µM	51.1 µM
1987504	4-Heptyl-phenol	19.0 µM	0.8 µM	~ 1 mM	---
24650428	2.2-dimethoxy-2-phenylacetophenone	25.8 µM	136 µM	> 1 mM	---
27176870	Ddodecylbenzene sulfonic acid	Insol.	---	~ 10 µM	----
27668526	n-Octadecyldimethyl[3-(trimethoxysilyl)propyl}-ammonium chloride	1.1 µM	Plot failed	~ 1 mM	---
3236713	4,4'-(9-fluorenylidene)-diphenol	> 100 µM	---	~ 5 µM	9.9 µM
3380345	5-Chloro-2-(2,4-dichlorophenoxyl)-phenol	> 100 µM	Plot failed	~ 100 µM	156.3 µM
3367257	Sulcofuron-natrium monohydrate	> 100 µM	Plot failed	~ 10 µM	Plot failed
59507	4-chloro-3-methyl-phenol	> 100 µM	382 µM	55.2 µM	67.5 µM
80433	Dicumyl peroxide	> 100 µM	---	~ 5 mM	2.7 mM
96764	2,4-d-tert-butylphenol	> 100 µM	---	59.3 µM	40.0 µM

SUMMARY:

To summarize the results of these studies, dimethylsulfoxide (DMSO) can be used to prepare primary stocks of chemicals for analysis of competition for binding to the estrogen receptor (ER) in the rat uterine cytosol (RUC) assay system. From the standpoint of assay performance, DMSO is possibly superior to ethanol for stock preparation. Our report presents evidence in support of the following statements:

1. Use of DMSO solvent made it possible to produce very high concentrations of competitor solutions, in most cases in the range of 1 – 50 mMolar.

2. The presence of DMSO does not interfere with performance of the assay when RUC is used, up to about 20% of total assay volume. For the RUC protocol used, it is reasonable to expect no interference if the material being analyzed contains as much as 50% DMSO.

3. Ethanol solvent demonstrated significant interference at less than 10% of total assay volume, indicating that primary ethanol stocks must be diluted to a greater extent than DMSO stocks for assay with a safe margin.

4. At extremely high concentrations made from DMSO stock, many but not all solutes interfered with the RUC assay separation protocol. It was concluded that the solute chemicals saturated the binding capacity of the separating agents and thus prevented precipitation of excess radioligand that is the protocol requires.

5. Using DMSO solvent for preparation of stock, the RUC assay for ER binding performed as expected for determination of binding parameters of natural estrogens. Competition kinetics and results were consistent with other published literature.

6. Using DMSO solvent for preparation of stock, the RUC assay was able to identify competitive properties of some very weak binders, that had remained unidentified when ethanol stock was used. It was possible to assay DMSO stocks at much less dilution and to observe binding properties at 1 to 10 mMolar.

7. When assayed at similar concentrations, most of the tested chemicals yielded the same competition results with dilutions from ethanol or DMSO stock,

8. Some chemicals inhibited radioligand binding to ER to a greater extent from DMSO stock, than from ethanol stock. This was concluded to be the result of greater solubility of solute in DMSO stock and subsequent dilutions using assay buffer.

9. Some chemicals appeared to not dissolve well in dilutions from either DMSO or ethanol, and also displayed very poor ability to displace radioligand from ER.

CITED REFERENCES:

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Bhavnani BR. Estrogens and menopause: pharmacology of conjugated equine estrogens and their potential role in the prevention of neurodegenerative diseases such as Alzheimer's. *J Steroid Biochem Mol Biol 85:*473-82. (2003)