COMMENTS BY THE CENTER FOR REGULATORY EFFECTIVENESS (CRE) ON EPA'S APRIL 26-29, 2010 SCIENCE ADVSIORY PANEL ON ATRAZINE, FILED ON APRIL 26, 2010 BY HAND AND ELECTRONICALLY IN WWW.REGULATIONS.GOV

AT DOCKET NUMBER EPA-HQ-OPP-2010-0125

I. INTRODUCTION AND SUMMARY

EPA has failed to advise this Scientific Advisory Panel ("SAP") of the federal requirements and guidelines governing EPA's use of scientific studies for regulatory purposes. EPA cannot regulate on the basis of studies that do not meet the requirements and guidelines.

These requirements and guidelines include reproducibility, *e.g.*:

• EPA's Information Quality Act ("IQA") Guidelines, p. 47 (EPA will "ensure" reproducibility);[1]

•FIFRA Data Requirements, 40 CFR § 158.70(a) (EPA "will evaluate the conduct of each experiment in terms of whether the study was conducted in conformance with the design, good laboratory practices were observed, **and results were reproducible"**);[2] and

• Interagency Coordinating Committee on the Validation of Alternative Methods ("ICCVAM"), Test Validation and Acceptance Criteria, **("**The extent of within-test variability, and the reproducibility of the test within and among laboratories must have been demonstrated. Data must be provided describing the level of intra- and interlaboratory reproducibility and how it varies over time. The degree to which biological variability affects this test reproducibility should be addressed").[3]

EPA has asked this SAP to review an unprecedented number of studies. Yet EPA has not identified a record which shows that all these studies meet the data quality requirements and guidelines set forth above. For example, EPA has not produced a record which shows that all these studies are reproducible.

CRE's comments on the 2002 EPA risk assessment raised a similar validation issue about some tests which the Agency said showed endocrine effects from exposure to atrazine. EPA's response to CRE's comments said (emphasis added):

> "In determining whether data are acceptable, the Agency considers a number of factors, (e.g., study design including sample size, **replication**, use of appropriate controls, etc.)

[1] http://www.epa.gov/quality/informationguidelines/documents/EPA_InfoQualityGuidelines.pdf
[2] http://ecfr.gpoaccess.gov/cgi/t/text/text-idx?c=ecfr&rgn=div5&view=text&node=40:23.0.1.1.9&idno=40#40:23.0.1.1.9.1.1.4
[3] http://iccvam.niehs.nih.gov/SuppDocs/guidelines/MethodGuidelines/criteria.htm,

including the use of GLPs, to determine whether scientifically sound methods were employed." [4]

The same data quality standards should be applied in this current SAP.

CRE asks this SAP to ask EPA a question: where is the record demonstrating that the new atrazine studies which EPA wants this SAP to consider are reproducible and meet the other data quality requirements and guidelines?

These comments are discussed in more detail below. CRE requests and appreciates the SAP's response to our comments in the SAP's minutes for this meeting.

II. STUDIES MUST MEET EPA'S INFORMATION QUALITY ACT GUIDELINES

EPA has not informed the SAP of the federal standards that determine which scientific studies EPA can use to regulate atrazine. Some of these standards are imposed by EPA's Information Quality Act ("IQA") Guidelines.

For Influential Scientific Information, such as an atrazine Risk Assessment, EPA's IQA Guidelines require that EPA "ensure reproducibility for disseminated original and supporting data according to commonly accepted scientific, financial, or statistical methods." [5]

At least some of the new atrazine studies before the SAP, Higley 2010, do not meet the IQA Guidelines because they use the H295R test for endocrine disruption.[6] The H295R test flunked external peer review.

EPA publicly disclosed its external peer review report for H295R after repeated inquiries by CRE representatives and others. EPA did not make the H295R peer review report publicly available until March 26, 2009, even though it was completed in June 2008.

The Peer Review Report includes the following comment, "Overall, the test guideline has the potential to be a screening tool for steroidogenesis but requires further testing and refinement."[7]

EPA's response to this comment is: "No response needed."

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[4] http://epa.gov/quality/informationguidelines/documents/2807Response_03_27_03.pdf, page 20.

[5] EPA IQA Guidelines, p. 47, http://www.epa.gov/quality/informationguidelines/documents/EPA_InfoQualityGuidelines.pdf

[6] See Agenda/Charges at http://www.epa.gov/scipoly/sap/meetings/2010/april/042610agenda.pdf[6]

[7] *Disposition to Peer Review Panel Comments on the H295R Steroidogenesis Assay*, available online at http://www.epa.gov/endo/pubs/assayvalidation/h295r_pr.htm .

EPA responds to 10 other separate and significant peer review criticisms of H295R by stating, "This is being investigated and the protocol modified, if appropriate." EPA's responses to the many peer review criticisms of H295R acknowledge that the test needs work and is not final. To the best of CRE's knowledge, EPA is still investigating these peer review criticisms of H295R.

The peer review panel's conclusion is consistent with the following EPA response to criticism of the H295R protocol:

> "There will be a complete and separate H295R protocol after this assay undergoes peer review both by the US-EPA and OECD which will combine all of these aspects."

To the best of CRE's knowledge, H295R has not been peer reviewed by the OECD. Consequently, by EPA's own admission, there is no validated and complete H295R test protocol.

Peer review criticisms (from advisors to EPA) of H295R are included in Appendix A to CRE's comments on the April 26th atrazine SAP.

Under these circumstances, EPA cannot studies that include H295R to regulate atrazine, because EPA cannot demonstrate that H295R will generate accurate, reliable, unbiased and complete information.

III. STUDIES MUST MEET EPA'S FEDERAL INSECTICIDE, FUNGICIDE AND RODENTICIDE ACT (FIFRA) DATA RULES IN 40 CFR PART 158

Before EPA can use studies to regulate atrazine, the studies must also comply with EPA's FIFRA data rules, which state the following "*General policy"* for "*Satisfying data requirements*" (emphasis added):

> " The Agency will determine whether the data submitted or cited to fulfill the data requirements specified in this part are acceptable. This determination will be based on the design and conduct of the experiment from which the data were derived, and an evaluation of whether the data fulfill the purpose(s) of the data requirement. In evaluating experimental design, the Agency will consider whether generally accepted methods were used, sufficient numbers of measurements were made to achieve statistical reliability, and sufficient controls were built into all phases of the experiment**. The Agency will evaluate the conduct of each experiment in terms of whether the study was conducted in conformance with the design, good laboratory practices were observed, and results were reproducible."**

40 CFR § 158.70(a).[8]

IV. STUDIES MUST MEET THE TEST VALIDATION AND ACCEPTANCE REQUIREMENTS OF THE INTERAGENCY COORDINATING COMMITTEE ON THE VALIDATION OF ALTERNATIVE METHODS

EPA is a member of ICCVAM, and EPA is one of the authors of ICCVAM's *Validation Guidelines* and *Validation Criteria*. This ICCVAM document recommends government-wide validation criteria requirements for test reliability and reproducibility.[9]

According to EPA and ICCVAM,

> **"**Before a new or revised test method is used to generate information to support regulatory decisions, it must be (1) validated (its reliability and relevance for its proposed use must be determined) and (2) accepted, (one or more regulatory or research agencies must determine that it fills a specific need). This report describes recommended criteria and processes for the validation and regulatory acceptance of new and revised toxicological testing methods. In addition, it recommends ways to facilitate the development and adoption of new testing methodologies, both nationally and internationally. The ad hoc Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) determined that this Report should be applicable to all proposed toxicological testing methods, including those termed 'alternatives.' This decision was based on the premise that the validation and regulatory acceptance of alternative test methods should be no different than for other test methods."
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> "For a new or revised test method to be considered validated for regulatory risk assessment purposes, it should generally meet the following criteria (the extent to which these criteria are met will vary with the method and its proposed use)."
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> **The extent of within-test variability, and the reproducibility of the test within and among laboratories must have been demonstrated. Data must be provided**

[8] **http://ecfr.gpoaccess.gov/cgi/t/text/text-idx?c=ecfr&rgn=div5&view=text&node=40:23.0.1.1.9&idno=40#40:23.0.1.1.9.1.1.4**

[9] Interagency Coordinating Committee on the Validation of Alternative Methods ("ICCVAM"), Test Validation and Acceptance criteria, http://iccvam.niehs.nih.gov/SuppDocs/guidelines/MethodGuidelines/criteria.htm

describing the level of intra- and interlaboratory reproducibility and how it varies over time. The degree to which biological variability affects this test reproducibility should be addressed."[10]

The complete EPA/ICCVAM standards for test validation and regulatory acceptance are set forth in Appendix B to CRE's comments.

V. CONCLUSION AND RECOMMENDED ACTION

Scientific studies must be reproducible before EPA can use them to regulate atrazine. This SAP should ask EPA to identify the record which demonstrates that the new atrazine studies that the Agency wants this SAP to review are reproducible.

Respectfully Submitted,

Scott Slaughter
The Center for Regulatory Effectiveness

[10] **VALIDATION AND REGULATORY ACCEPTANCE OF TOXICOLOGICAL TEST METHODS, A Report of the ad hoc Interagency Coordinating Committee on the Validation of Alternative Methods, Executive Summary (emphasis added), http://iccvam.niehs.nih.gov/docs/about_docs/validate.pdf**

APPENDIX A

Peer Review Comments on the H295r Test

(From *Disposition to Peer Review Panel Comments on the H295R Steroidogenesis Assay*, finally available online at http://www.epa.gov/endo/pubs/assayvalidation/h295r_pr.htm)

Comment

"It is important to stress that chemicals that generate a negative result in the H295R steroidogenic test guideline could be false negatives and they should not be considered safe without a complete evaluation of them with the other Tier 1 battery test guidelines. This in vitrosystem lacks that ability to study complex interactions that could occur in vivo such as metabolism of tested compounds, biodistribution, interaction with other endocrine systems that may modulate sex hormones steroidogenesis, etc."

Comment

"One major question is whether any small change in hormone production in an isolated in vitro system has any relevance for the health outcome of an exposed organism. This remains unaddressed in the documents available for review."

Comment

"As the steroidogenesis assay only looks at one final outcome, namely the amount of estradiol and testosterone secreted, it is not possible to make biologically meaningful statements on the relevance of any observed disruption for the organisms as a whole. There are so many factors not directly related to steroidogenesis that could influence the assay system as it is currently described and intended to be used, that the issue of 'false positives' is likely to be an important concern, particularly once dealing with unknown complex environmental samples."

Comment

“The implications of the presence of these other pathways (aldosterone, cortisol synthesis) may be far reaching for the reliable application of the proposed H295R steroidogenesis assay, as all these pathways are interconnected (at least in adrenocortical cells, not necessarily in gonadal cells). There is no critical discussion of the potential drawbacks of choosing an adrenocortical cell line to study effects of chemicals on gonadal testosterone and estradiol production. There is no scientifically supported discussion of the possible differences in regulation of steroidogenesis in adrenocortical cells and gonadal cells, yet it is known these are qualitatively and quantitatively very different. Several of the above points have been discussed in detail in several publications from my own lab in recent years (Sanderson and van den Berg, 2003; Sanderson, 2006).”

Comment

“The reproducibility of the test system appears to be relatively poor. This may be partly due to the variability inherent in the use of cell lines in culture, but is also likely to be due to the various immunoassay-based hormone analysis methods used. The latter influence may be reduced by selecting a single method of detection, preferably not immunoassay guideline based.”

Comment

“For the most part the assay is sufficiently repeatable and reproducible. However, I am concerned with the high CV among laboratories and also within laboratories. The within lab CV is particularly high for prochloraz and this could be because it is inhibiting the basal steroid production. As the constitutive levels are being inhibited this may lead to error as the levels may

differ due to autoregulation that is inherent in this system. I would recommend using a test group where the inhibition is tested using acute-stimulated (forskolin or 8bromocamp) steroid production as a model. This might reduce the variability and make the data set more comparable between the laboratories. For instance there is a large variability in EC50 for foskolin between the different labs (Table 10.3)."

Comment

"The way the H295R cell system is being proposed to be used is like a black box. It will be difficult to interpret the meaning of any outcomes that may be observed on testosterone and estradiol levels, and this is further compounded by the drawbacks of using immunoassay-based detection methods. A more focused definition of the purpose of a tier 1 assay for steroidogenesis would be recommendable; allowing for the development of a H295R cell-based steroidogenesis test guideline that would provide less ambiguous information about the steroidogenesis disruption potential of chemicals or unknown environmental extracts."

Comment

"The huge CV reported for between laboratory comparisons may have to do with the difference in basal hormone production and associated differences in the magnitude of response to know inducers and inhibitors as well as test substances."

Comment

"The implications of the presence of these other pathways (aldosterone, cortisol synthesis) may be far reaching for the reliable application of the proposed H295R steroidogenesis assay

guideline, as all these pathways are interconnected (at least in adrenocortical cells, not necessarily in gonadal cells). There is no critical discussion of the potential drawbacks of choosing an adrenocortical cell line to study effects of chemicals on gonadal testosterone and estradiol production. There is no scientifically supported discussion of the possible differences in regulation steroidogenesis in adrenocortical cells and gonadal cells, yet it is known these are qualitatively and quantitatively very different. Several of the above points have been discussed in detail in several publications from my own lab in recent years (Sanderson and van den Berg, 2003; Sanderson, 2006)."

Comment

"Little is known about the impact of most of the test substances on steroid production. The lack of response to a known inducer of sex steroid production in gonadal tissue, for instance human chorionic gonadotropin (hcG), suggests that this system has limitations because of the type of tissue involved (adrenal carcinoma)."

Comment

"One of the most important aspects of the H295R steroidogenesis assay, the analysis of testosterone and estradiol, is poorly defined in the provided documents. The choice of analysis method is left entirely to the implementing laboratory. It is known that ELISAs and RIAs can have very different outcomes dependent on the sample dilution, kit and antibodies used, not to mention the numerous confounding factors (solvent, cross-reactive components). The issues of cross-reactivity, how to deal with conjugated metabolites, and how to reliably compare between hormone levels determined by RIA or LC-MS are left undiscussed. It is highly inconsistent that

there is an elaborate protocol for the ‘consistent’ use of a standard method such as the LIVE/DEAD cytotoxicity kit while no detailed attention is given to the crucial hormone analysis methodology.”

Comment

“For instance this assay will only detect changes that happens post-receptor activation. This is a drawback to this cell system because in vivo the steroidogenic cells secrete steroids in response to trophic hormone stimulation. This assay completely bypasses the receptor signaling which is an essential step in steroid biosynthesis. So substances that can affect steroid production by altering trophic hormone signaling will not be evaluated by this cell system. Also, the high constitutive production of the hormone is abnormal in vivo as this usually happens only in response to trophic hormone stimulation. So it is unknown whether the changes seen with the test substances can be mimicked in vivo to the same extent (or may be even greater) and will require confirmation with animal models or other relevant cell or tissue systems.

Also, the high constitutive levels of steroids, for instance testosterone, may deplete the precursor available for steroid synthesis and may be limiting the steroid biosynthetic capacity in response to test (inducer) substances. The changes in the magnitude of steroid synthesis with forskolin, smaller change for testosterone because basal secretion is high and higher for E2 because of lower basal secretion, clearly support this contention. This requires testing perhaps by supplementing the medium with cholesterol”

Comment

“Analysis of sex hormones. The greatest weakness in the protocols is the lack of detail on sex

hormone analysis methodology. This reviewer is of the opinion that LC-MS would be, by far, the preferred analysis tool for the detection of testosterone and estradiol. LC-MS would avoid the problems that will be (and already have been) encountered with inappropriate cross-reactivity of test samples/chemicals with the antibodies used in sex steroid ELISAs and RIAs. Please see also comments on trenbolone under point 7. The validation of a sensitive LC-MS method should be a logical part of the H295R steroidogenesis test guideline as currently defined. Furthermore, a single LC-MS analysis could detect a number of steroids in addition to estradiol and testosterone at little additional effort/expense, thus improving the 'expandability' of the H295R tool for other hormone endpoints"

Comment

"There is a brief discussion of strengths and weaknesses, but lacks detail and supporting scientific references. The main strength mentioned in the interim report is that the H295R cell line is a pluripotent cell lines that expresses all the enzymes necessary for the production of testosterone and estradiol. However, the fact that numerous other steroid hormone synthesis pathways are also present, although acknowledged, is not discussed."

APPENDIX B

ICCVAM Regulatory Test Validation and Acceptance Criteria

VALIDATION AND REGULATORY ACCEPTANCE OF TOXICOLOGICAL TEST METHODS, A Report of the ad hoc Interagency Coordinating Committee on the Validation of Alternative Methods, Executive Summary,
http://iccvam.niehs.nih.gov/docs/about_docs/validate.pdf

"Before a new or revised test method is used to generate information to support regulatory decisions, it must be (1) validated (its reliability and relevance for its proposed use must be determined) and (2) accepted, (one or more regulatory or research agencies must determine that it fills a specific need). This report describes recommended criteria and processes for the validation and regulatory acceptance of new and revised toxicological testing methods. In addition, it recommends ways to facilitate the development and adoption of new testing methodologies, both nationally and internationally. The ad hoc Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) determined that this Report should be applicable to all proposed toxicological testing methods, including those termed 'alternatives.' This decision was based on the premise that the validation and regulatory acceptance of alternative test methods should be no different than for other test methods.

"For a new or revised test method to be considered validated for regulatory risk assessment purposes, it should generally meet the following criteria (the extent to which these criteria are met will vary with the method and its proposed use). However, there needs to be flexibility in assessing a method given its purpose and the supporting database (see Sections 2.3 and 2.4):

The scientific and regulatory rationale for the test method, including a clear statement of its proposed use, should be available.

The relationship of the test method's endpoint(s) to the biologic effect of interest must be described. Although the relationship may be mechanistic or correlative, tests with biologic relevance to the toxic process being evaluated are preferred.

A detailed protocol for the test method must be available and should include a description of the materials needed, a description of what is measured and how it is measured, acceptable test performance criteria (e.g., positive and negative control responses), a description of how data will be analyzed, a list of the species for which the test results are applicable, and a description of the known limitations of the test including a description of the classes of materials that the test can and cannot accurately assess.

The extent of within-test variability, and the reproducibility of the test within and among laboratories must have been demonstrated. Data must be provided describing the level of intra- and interlaboratory reproducibility and how it varies over time. The degree to which biological variability affects this test reproducibility should be addressed.

The test method's performance must have been demonstrated using reference chemicals or test agents representative of the types of substances to which the test method will be applied, and should include both known positive and known negative agents. Unless it is hazardous to do so, chemicals or test agents should be tested under code to exclude bias.

Sufficient data should be provided to permit a comparison of the performance of a proposed substitute test with that of the test it is designed to replace. Performance should be evaluated in relation to existing relevant toxicity testing data, and relevant toxicity information from the species of concern. Reference data from the comparable traditional test method should be available and of acceptable quality.

The limitations of the method must be described; for example, in vitro or other non-animal test methods may not replicate all of the metabolic processes relevant to chemical toxicity that occur in vivo.

Ideally, all data supporting the validity of a test method should be obtained and reported in accordance with Good Laboratory Practices (GLPs). Aspects of data collection not performed according to GLPs must be fully described, along with their potential impact.

All data supporting the assessment of the validity of the test method must be available for review.

Detailed protocols should be readily available and in the public domain.

The method(s) and results should be published or submitted for publication in an independent, peer-reviewed publication.

The methodology and results should have been subjected to independent scientific review.

Because tests can be designed and used for different purposes by different organizations and for different categories of substances, the determination of whether a specific test method is considered by an agency to be useful for a specific purpose must be made on a case-by-case basis.

Validation of a test method is a prerequisite for it to be considered for regulatory acceptance."

REGULATORY ACCEPTANCE CRITERIA

Validated methods are not automatically accepted by regulatory agencies; they need to fit into the regulatory structure. Flexibility is essential in determining the acceptability of methods to ensure that appropriate scientific information is considered in regulatory risk assessment. A test method proposed for regulatory acceptance generally should be supported by the following attributes.....

The method should have undergone independent scientific peer review by disinterested persons who are experts in the field, knowledgeable in the method, and financially unencumbered by the outcome of the evaluation.

There should be a detailed protocol with standard operating procedures (SOPs), a list of operating characteristics, and criteria for judging test performance and results.

Data generated by the method should adequately measure or predict the endpoint of interest and demonstrate a linkage between either the new test and an existing test, or the new test and effects in the target species.

There should be adequate test data for chemicals and products representative of those administered by the regulatory program or agency and for which the test is proposed.

The method should generate data useful for risk assessment purposes, i.e., for hazard identification, dose-response assessment, and/or exposure assessment. Such methods may be useful alone or as part of a battery or tiered approach.

The specific strengths and limitations of the test must be clearly identified and described. The test method must be robust (relatively insensitive to minor changes in protocol) and transferable among properly equipped and staffed laboratories.

The method should be time and cost effective.

The method should be one that can be harmonized with similar testing requirements of other agencies and international groups.

The method should be suitable for international acceptance.

The method must provide adequate consideration for the reduction, refinement, and replacement of animal use."