External Letter Peer Review of the "Methodology for Estimating the Area Under Curve (AUC) in the IRIS Toxicological Review for Acrylamide"

Contract EP-C-07-024
Task Order 58

Submitted to:

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Office of Research and Development
U.S. Environmental Protection Agency
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PEER REVIEW COMMENTS FROM

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Response to Charge Questions for Technical Review of
"Methodology for Estimating the Area Under Curve in the IRIS Toxicological Review of Acrylamide"

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Contract No. EP-C-07-024
Task Order No. 58

January 8, 2010

RESPONSE TO CHARGE QUESTIONS

1. *Please comment on the discussion and presentation of the method used to estimate the AUC in the ToxRev. Were the discussion and presentation clear and sufficiently detailed to support an understanding of what was done?*

Response:

The general approach used by EPA is scientifically sound and represents a sophisticated and appropriate use of the detailed dosimetry and toxicokinetic data available from the Doerge and Tareke publications. I have a number of specific comments related to both clarity of presentation and technical details of the implementation of the approach.

Overall, it would be helpful if a schematic or schematics were included that illustrated the necessary steps in the estimation of the internal doses in rats, humans, and conversion to corresponding human intakes. This would be most appropriate at the beginning of Chapter 5 or within the specific RfD and cancer slope factor derivation sections. This would help the reader understand the application of the derived 2nd order rate constants for hemoglobin (Hb) adduct formation, the derivation of the estimates relating AUCs for acrylamide (AA) and glycidamide (GA) to administered doses of AA, and the procedure for estimating human equivalent doses.

Other comments related to clarity of presentation are included below related to several of the detailed analyses.

2. *Is the method that EPA used to estimate AUC (based upon hemoglobin adducts levels as a biomarker of exposure) consistent with previously published methods in the peer reviewed literature that also used hemoglobin adduct levels, adduct formation rates, and what is known about the kinetics of acrylamide?*

Response:

Yes, the procedure is consistent and appropriate. More detailed comments are presented below.

3. *There are a number of calculations and numerical results in the derivation of the AUC and the HEC. Have the equations and the resulting numbers been clearly and transparently presented so that the numbers can be checked for numerical accuracy? Please provide and comment on any errors in the calculations or the equations.*

Response:

The general approach used by EPA is scientifically sound and represents a sophisticated and appropriate use of the detailed dosimetry and toxicokinetic data available from the Doerge and Tareke publications. I have some specific comments that I think will assist in the clarity of presentation of the information. I present the comments on Appendix E first, because this Appendix contains the essential calculations that underlie information presented in Chapters 3 and 5.

Comments on Appendix E

The calculations and analyses presented in Appendix E are central to the calculation of internal dosimetry in rats and humans and therefore to the derivation of the RfD and cancer slope factor; thus, these analyses should be clearly presented. I have several questions and suggestions for improving the clarity of Appendix E.

- In Table E-1, the reference in the title of the table to "Tareke et al. (2008)" should be changed to Tareke et al. *(2006)*. It would also be helpful to reference the specific figures or tables in the original sources that the data are taken from (Figure 3 in Tareke et al. 2006 and Table 1 in Doerge et al. 2005c).
- The presentation of the results of the linear regression that follows Table E-1 should probably be formatted and labeled as a separate table, Table E-2, so that it can be referred to easily in the rest of Appendix E and in the main document.

- In the discussion on p. 39, Hb reaction rate regression results from Tareke et al. (2006) are discussed. The reaction rate constant quoted for GA Val formation in rats, 35, appears to be incorrect; it should be 34 (see Tareke al. 2006, p. 67, second column).
- The presentation of the Adduct Formation Model (pp. 43-46 of Appendix E) needs to be expanded somewhat to clarify a few issues. In particular, Table E-2 is unclear in several respects:

    1. The formation rates appear to be the estimated rates relating adduct formation to AUC. However, the value for AA AUC for males does not seem to correspond to the regression-derived slopes from earlier in Appendix E, while the remaining values do. Can this be clarified? Also, see below in response to question 4 my suggestions for modifications to the derived formation rates.
    2. The elimination rates cited do not match Tareke et al. (2006). Tareke et al. (2006) reports the half-lives for elimination of Hb valine adducts of AA and GA in Figure 12 as 12 days (females and males, AA Val) and 11 and 13 days (females and males, respectively, GA Val). However, in their discussion, they note that these rates are essentially the rate for red blood cell turnover and would be expected to be independent of whether the adduct was AA or GA and independent of sex. A half-life value of 12 days is consistent with the literature on red blood cell lifetime in rats (60 days, corresponding to essentially complete turnover in 5 half-lives). A half-life of 12 days can be converted to an elimination rate, k_e, in hr^{-1}:

        $$k_e = \frac{\ln(2)}{12d * 24h/d} = 2.4x10^{-3}$$

        Similarly, half-lives of 11 and 13 days correspond to elimination rates of $2.6x10^{-3}$ and $2.2x10^{-3}$. So in Table E2, the elimination rates for GA Val are consistent with the values reported by Tareke et al. (2006), but those for AA Val are not (1.3×10^{-3} in the current version of the table; 2.4×10^{-3} is the value from Tareke et al. 2006). Again, I would suggest that a single value corresponding to a 12 day half-life for red blood cell turnover is appropriate for all of the calculations.
    3. The "Predicted AUC" column is particularly unclear. Predicted for what

dose? And are the units correct (um-d)? Previous AUC estimates have been reported in um-h: what does the predicted AUC column correspond to?

I suggest that two or four plots (either combining AA Val and GA Val on each sex-specific plot, or separating them) corresponding to Figure 8 in Tareke et al. (2006) with the data points reported by Tareke et al. (2006) and the fitted adduct formation model results be presented along with the final male and female-specific coefficients relating administered dose of AA in drinking water to AUC of AA and GA. The intake dose rate should be specified. This would clarify the basis of the values presented in the "Summary" at the beginning of Appendix E.

Finally, those relationships between drinking water dose and AUC should be converted to dose on a mg/kg-d basis so that the values presented in Tables 3-6 and 3-7 can be directly identified in Appendix E. The current text and equations making this conversion in Chapter 5 (pp. 31 and 35 of the IRIS AUC Methodology sections document) should be moved to Appendix E or Chapter 3, accompanying Tables 3-6 and 3-7.

Comments on Chapter 3

In the discussion presented on p. 5 of the IRIS AUC Methodology sections, the reader should be directly referred to Appendix E for details of the calculations underlying Tables 3-5 through 3-7.

In the titles for Tables 3-6 and 3-7, as well as in text discussing these tables, it should be explicitly noted that AUC per administered dose will depend on the route of exposure as well as, perhaps, the vehicle/mode of administration due to potential differences in bioavailability. This is why the estimates of AUC per administered dose based on the adduct data following drinking water exposure for 7 weeks from Doerge et al. (2005) is most appropriate for use in estimating internal doses in the Johnson et al. (1986) and Friedman et al. (1995) studies, both of which administered AA in drinking water. Then, in the first column in these two tables, the route and mode of administration for each of the calculations should be explicitly and consistently noted.

The information is mostly there, except in relationship to the data from Fennel et al. (2005) for humans.

Comments on Chapter 5

Repetition of the full set of tables from Chapter 3 (Tables 3-5 to 3-7) is somewhat confusing here. It would be more useful to have a summary table that reports the coefficients relating administered dose of AA in drinking water in rats to AUC (sex- and adduct-specific values) as well as the values used to relate the AUC values in humans to the corresponding administered dose, and then refer the reader to Appendix E where the details are presented. It is also unnecessary and somewhat confusing to reproduce from Appendix E Table 5-8 and the corresponding regression results (see above for detailed comments on these).

4. EPA derived hemoglobin adduct formation rate constants based on recently published data from in vivo studies to replace the formation rates based on more dated in vitro data. The rationale is that rate constants based upon levels of acrylamide (or glycidamide) and adducts in the blood from test animals receiving an oral exposure of acrylamide support a more accurate and relevant estimate of the internal exposure to the test animals in the two chronic (2-year) drinking water studies (Friedman et al., 1995; Johnson et al., 1986) compared to the use of formation rates based upon in vivo studies where acrylamide is directly added to blood.

Please comment on whether or not you agree with EPA's rationale that rate constants based on the in vivo data are preferable to the use of rate constants based upon in vitro data. Please also comment on the appropriateness of the method that EPA used to derive the in vivo rate constants.

Response:

I think that the overall approach and decision to use the in vivo-derived second order rate constants is a scientifically sound approach, and I congratulate EPA on synthesizing the detailed available data in a useful way.

However, I think that the details of the derivation of rate constants presented in Appendix E need to be reviewed and revisited. In Appendix E, a table of regression results for development of rate constants for Hb adducts in rats is presented. In that table of regression results, it would also be helpful to include standard errors on the regression coefficients as well as a formal assessment of whether the fitted slopes for the two genders are significantly different from one another; figures might be helpful in seeing the differences. This is particularly important in light of the striking

coherence among the overall data relating Hb adducts to AUC from male and female rats and mice demonstrated in the Tareke et al. (2006) Figure 4.

If EPA chooses to use gender- and species-specific reaction rates in deriving a relationship between male rat AUC and administered dose, these differences in adduct formation rate should be formally demonstrated and justified. The observed gender differences in AUC following administration of AA do not necessarily imply a difference in second order reaction rates for Hb adduct formation, contrary to the discussion on pp. 29 and 39 of the IRIS AUC Methodology sections document. Gender differences in AUC following a given dose are possible due to differences in absorption, volume of distribution, etc. However, there does not appear to be a good biological justification to expect a gender-related difference in second-order reaction rates with Hb, particularly if one is extrapolating and applying these reaction rates among rats, mice, and humans.

I pulled the reported standard deviations on the mean AUC from Doerge et al. (2005c) and adduct levels from Tareke et al. (2006) (estimated from error bars on the graph); these are tabulated in a revised version of Table E1 below. I plotted the AA Val vs. AA AUC and GA Val vs. GA AUC by gender in the two figures below (no standard deviations were reported for the IV AUC measurements). The tabulated and plotted data for GA Val also include the results relating GA Val to GA AUC following GA IV and gavage administration, which are entirely coherent with the data from AA administration.

Suggested Revised Table E1. Additional data on standard deviations and adduct formation following GA administration are highlighted:

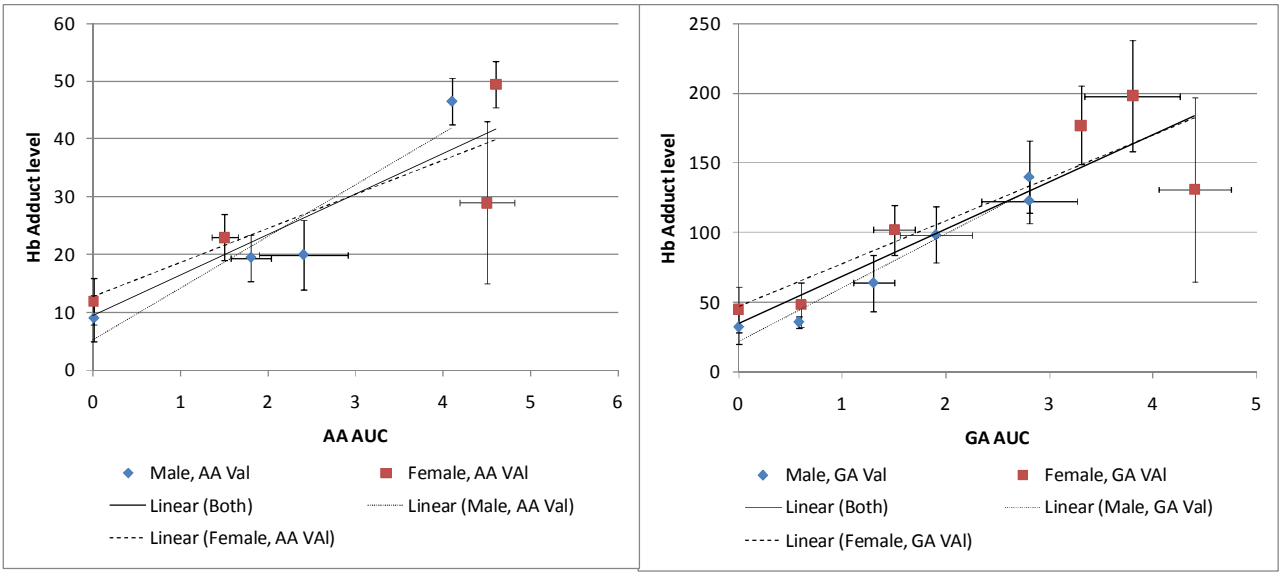
			Tareke et al. (2006) Figure 3		Doerge et al. (2005c) Tables 1 and 3	
Type of adduct	Dosed compound	sex-route	Hb adduct level (fmole/mg globin)	(SD)	AUC (uM-hr)	(SD)
AA-Val	AA	M-control	9	4	0	0
AA-Val	AA	M-Diet	19.5	4	1.8	0.23
AA-Val	AA	M-gavage	20	6	2.4	0.51
AA-Val	AA	M-IV	46.5	4	4.1	0
AA-Val	AA	F-control	12	4	0	0
AA-Val	AA	F-Diet	23	4	1.5	0.15
AA-Val	AA	F-gavage	29	14	4.5	0.31

AA-Val	AA	F-IV	49.5	4	4.6	0
GA-Val	AA	M-control	32.5	12	0	0
GA-Val	AA	M-IV	36	4	0.58	0
GA-Val	AA	M-gavage	64	20	1.3	0.2
GA-Val	AA	M-Diet	98.5	20	1.9	0.35
GA-Val	GA	M-IV	140	26	2.8	0
GA-Val	GA	M-gavage	123	16	2.8	0.46
GA-Val	AA	F-control	45	16	0	0
GA-Val	AA	F-IV	48.5	16	0.6	0
GA-Val	AA	F-Diet	102	18	1.5	0.2
GA-Val	AA	F-gavage	131	66	4.4	0.46
GA-Val	GA	F-IV	177	28	3.3	0
GA-Val	GA	F-gavage	198	40	3.8	0.5

Below are the fitted slopes and standard errors for the trend lines in these plots.

Gender	AA Val (fmoles per mg globin) per µM-h AA AUC Slope (SE)	GA Val (fmoles per mg globin) per µM-h GA AUC Slope (SE)
Male	8.9 (2.2)	38.4 (3.9)
Female	5.9 (2.5)	30.6 (8.7)
Both	7.0 (1.5)	33.8 (4.9)

I would suggest that the lower slopes for females are due primarily to a single data point in each of these sets, the adduct levels in females observed following gavage administration of AA.



These are also the most uncertain points, given the large standard deviations on the mean values reported. Without these two points, clear gender differences in the slopes of either the AA Val or

GA Val lines are not necessarily apparent. I would suggest that there is no clear biological or data-driven reason to use gender-specific Hb adduct formation rates when dealing with the rat data.

If that conclusion is accepted, then I would suggest that the overall second-order rates based on data from mice and rats, fitted to Figure 4 of Tareke et al. (2006), as discussed on p. 41 of the IRIS AUC Methodology sections, are appropriate for use in both the rat and human dose calculations. These values, 7.5 and 32.5 for AA Val and GA Val formation as a function of AA and GA AUCs, respectively, are entirely consistent with the rat data presented here, and, in my opinion, provide the most robust basis for estimation of the second order rate constants.

If this comment is accepted, the Adduct Formation modeling for rats using the adduct level data for the Doerge et al. (2005a) drinking water 7 week exposure presented in Appendix E should be redone with a) a single, non-sex-specific formation rate each for AA Val and GA Val, and b) a single coherent rate for "elimination" of adducts corresponding to the turnover rate for red blood cells in rats (see my comments in response to Question 3 above). This will result in sex-specific coefficients relating exposure in drinking water to serum AUC for AA and GA.

5. Please provide any additional comments you may have on the method used to estimate the AUC and the HEC.

Minor text comments:

- In Chapter 3, "Aylward" is consistently misspelled as Alyward- please correct.
- The use of the terms "Human Equivalent Concentration" and HEC in Chapter 5 is distracting. A more intuitive set of terms would be "Human Equivalent Dose" and HED, which can be defined up front. The estimates are not concentrations; they are dose rates.

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Review on the „Methodology for Estimating the Area Under Curve in the IRIS Toxicological Review of Acrylamide“

The following chapters of the *Draft IRIS Toxicological Review of Acrylamide* were provided for a review of the methodology used for estimating the area under the curve (AUC):

3.5. Hemoglobin adducts and urinary metabolites as biomarkers of exposure

3.6. Physiologically based toxicokinetic models

5.1.2. Methods for analysis – including models (BMD, equivalent AUCs, in vitro rate constant, etc.)

5.1.3. RfD derivation – including application of uncertainty factors

5.1.4. Previous RfD assessment

5.4.4 Human Equivalent Concentration (HEC) – based on equivalent areas under the time-concentration curve (AUC) for serum AA or GA

5.4.5. Oral slope factor and inhalation unit risk (containing the chapter 5.4.5.1 „Oral slope factor“)

Appendix E. Derivation of in vivo second order rate constants and the adduct formation simulation model

References cited (and additional relevant references)

General remarks

Owing to the technical relevance of acrylamide, there is a considerable body of information on its toxicity, metabolism and toxicokinetics. Several toxicokinetic modellings have been published to bridge experimental animal and human toxicity data. Furthermore, a multiplicity of toxicity endpoints is to be observed (in first instance carcinogenicity and neurotoxicity), to be linked with metabolic pathways.

In the available toxicokinetic modellings, different aspects and methods of modelling were employed, which makes a comprison difficult. Calculations were based sometimes on only limited experimental observations/data.

The current Draft IRIS Toxicological Review addresses these problems and offers a solution in that it extracts AUC data out of the available information sources, compares and discusses these data and deviations, and finally uses the data to derive HECs.

I very much support this general procedure to estimate an AUC in animals and humans that can be used to derive a human HEC.

In the present case, of haemoglobin adduct data play a pivotal role, related to the adducts of both acrylamide (AA) and glycidamide (GA). The way to base toxicokinetic calculations on adduct values, as presented here, is very much straightforward.

Note: Many of the references cited in the chapters provided for review do not appear in the attached list of „References Cited (and additional relevant references)". It is therefore recommended to again re-check all citations.

Specific remarks on the draft chapters provided

- Chapter 3.5: The use of „Biomonitoring Equivalents" (BEs) is actually not new. The way this is presented here reads as if this is new since the workshop reported by Hayes and Alyward in 2008. [Remark on spelling of the 2nd author: see below.]
- On the 2nd page of chapter 3.5., the basic AUC formula is introduced. In this context, also the „second order rate constant" is introduced, but without any additional information. In order to avoid possible confusion, I recommend to speak here of the „second oder rate constant of adduct formation".
- 4th page of chapter 3.5: A reference „Hartmann et al. 2008" is mentioned here twice. In the list of references cited, the date is 2009!
- Table 3-8: The way of presenting the citations to the table is confusing and probably incorrect (not in a final state?)
- 10th page of chapter 3.5: There is confusion on the spelling of the auhor Aylward (in the references list: „Hays and Aylward, 2008b"). In the body of the text, it reads both Alward and Alyward! Editing is needed!
- Chapter 3.6, 3rd page (page #13): There is confusion about the key reference(s) Kopp and Dekant 2008 and 2009. In effect, both citations appear to be related to the same publication (i.e., Toxicol Appl Pharmacol 235(2), 135-142, 2009), which had been e-published before, in 2008. This one publication describes data from both rats and humans!
- Chapter on the Walker et al. (2007) model: 6th line from the end of the chapter: in the Kopp and Dekant (2009) study...
- Appendix E, last para of „In vivo adduct formation rate constants for humans" (p. #42), citation of „Hartmann et al. 2009": There are two different papers by this group, namely (1) Hartmann EC, Boettcher MI, Bolt HM, Drexler H, Angerer J (2009) N-Acetyl-S-(1-carbamoyl-2-hydroxy-ethyl)-L-cysteine (iso-GAMA) a further product of human metabolism of acrylamide: comparison

with the simultaneously excreted other mercapturic acids. Arch Toxicol. 83(7):731-4, and (2) Hartmann EC, Boettcher MI, Schettgen T, Fromme H, Drexler H, Angerer J (2008) Hemoglobin adducts and mercapturic acid excretion of acrylamide and glycidamide in one study population. J Agric Food Chem. 56(15):6061-8. As the 2009 paper provides data from just a single person (i.e., the author), it appears that it is the 2008 paper, which is meant here.

A major point, related to chapters 5.1.2 and 5.1.3

For acrylamide, the most important toxicity endpoints are carcinogenicity/genotoxicity and neurotoxicity. For both endpoints the aspect of metabolism and toxicokinetics is important. For genotoxicity, it is plausible that this is realated to the oxidative metabolic pathway, via GA. For neurotoxicity, however, this may well be different. This aspect is not discussed in the chapters provided for review. It would mean that species comparisons of effect estimates for carcinogenicity/genotoxicity should be orientated towards AUC estimates of GA. For neurotoxicity, there could be alternative estimates, based on AUCs of AA (for me the more likely case), or of GA as an alternative.

The aspect of a differential relevance of AA and/or GA AUC for the different toxicological endpoints should be made more clear in chapters 5.1.2 and 5.1.3, in order to provide transparency of the background of the calculations.

<u>Response to the Charge Questions</u> (e-mail dated Dec 30, 2009)

1. I agree with the discussion and presentation of the method used to estimate the AUC in the Toxicological Review. Detailed remarks are entered above.

2. The method that EPA used to estimate AUC based on Hb adducts is consistent with previously published methods and appears to me as the best possible way at present to bridge the animal and human data.

3. The equations and numbers presented appear clear and transparent.

4. I very strongly agree with the view of EPA that in vivo data are preferable to in vitro data in deriving the rate constants.

5. Additional comments: see above.

Dortmund, Jan 10, 2010

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QUESTION 1. Comments on the presentation

General comments

The presentation is unfortunately confusing and difficult to follow and needs extensive editing before being acceptable. It would simplify the evaluation of the used methodology for estimating the Area under curve (AUC) and the further extension of this approach, if the terms and expressions are as consistent as possible with earlier literature related to these issues. The impression is that the authors are new in the field of dosimetry by means of adduct measurements. References to the basic literature on the use of haemoglobin adducts are lacking (see suggestions in Appendix to Charge Question 1).

- It might be good to tighten up the text and maybe structure the material in some other way.
- It seems as if the different equations and constants should be described before they are used in the various studies presented (or may be collected in a panel).
- It is also important that the equations are presented in a more straightforward way and that the units are correct and internally consistent (see the Specific comments to Question 1).
- Some of the equations are not written in a mathematically correct way.
- It is not clear if the equations presented on page 3 for steady state adduct levels were used for calculation of daily adduct increments (or daily AUC) from data from the 49-day drinking water study. If so, it would be inappropriate since the adducts apparently are eliminated via a first order process in addition to the normal cell turnover. (see further below)
- Equations from page 34 and 35 in the IRIS document have been rewritten and are presented as examples in the Specific comments to Question 1.
- The equations should be numbered to allow cross-references.
- Furthermore, there are many repetitions in the text, for instance the tables 3-5 to 3-7 need not be repeated as 5-5 to 5-7.
- It is very disturbing that different units and abbreviations are used (not internally consistent and sometimes erroneous). Some examples, with recommended expressions are given in the Specific comments to Question 1.

Specific comments

Some examples of different units used are given below, with recommended expressions in bold and within parenthesis.

- for adduct levels (pmol/g, nmol/g, fmol/mg, pmol/mg, nM/g globin; see comment below) **(pmol/g globin or nmol/g globin)**
- for AUC (AA_{AUC}, AA AUC, AA-AUC) **(AUC_{AA} or AA-AUC)**
- for intake (mg/kg bw/day, mg/kg-day) **(mg/kg bw per day or mg $(kg\ bw)^{-1}day^{-1}$)**
- AAVal. AA-Val, AA –Val **(AAVal)**
- AA, AM **(AA)**
- mMoles-hr, mM – hr, uM – hr, mM AA – hr **(mM×h or μM×h)**
- uM, μM **(μM)**
- L or l for liter
- HG, globin **(globin)**
- HEC POD or HEC_{POD} etc. (**HEC_{POD}**)
- POD-AUC or AUC_{POD} (**AUC_{POD}**)

The authors seem to be unaware of the use of M for concentration. For example 1 mM means 1 mmol/L (not 1 mmol) (as an example; see the equation on page 3).

It is **necessary** to "clean up" the equations and make them understandable.
Example: The first equation, page 34, is used here as an example. Suggestion:

$$GA\text{-}AUC_{BMD,\ male\ rat} = BMD \times \frac{GA\text{-}AUC_{male\ rat}}{mg\ AA/kg\ bw}$$

$$GA\text{-}AUC_{BMD,\ male\ rat} = 0.238\ mg\ AA/kg\ bw \times \frac{15\ \mu M\times h}{mg\ AA/kg\ bw} = 3.57\ \mu M\times h$$

Example: The last two equations, page 35. Suggestion on rewriting:

$$HEC_{BMD} = GA\text{-}AUC_{BMD,\ rat} \div \frac{GA\text{-}AUC_{human}}{mg\ AA/kg\ bw}$$

$$HEC_{BMD} = 3.57\ \mu M\times h \div \frac{12.5\ \mu M\times h}{mg\ AA/kg\ bw} = 0.285\ mg\ AA/kg\ bw$$

Page 3-4

- Before the equation for steady state adduct levels it might be appropriate to explain when and why steady states may be obtained. It should also be explained that the equations (page 3 and 4) are valid if the adducts are chemically stable and the rate of elimination is determined by erythrocyte turnover only. These equations are important since, in practice, dosimetry by means of haemoglobin adducts in humans often are based on steady state adduct levels of chemically stable adducts.

- Equations for the relation between steady state haemoglobin adduct levels (from long-term exposures) and daily AUC (page 3) and daily intake of AA (page 4) are presented. However, for the average reader, it may not be obvious how these equations are related. The equation on page 4 (para 4) might be divided into two parts – the first corresponding to the equation on second last line on page 3, and the second describing the calculation of daily uptake from daily AUC using information of clearance rate (abbreviated as E_k here) of AA in humans (0.15 h^{-1} from Calleman) and volume of distribution.

- Further, it should be made clear that the volume of distribution, 0.38 L/kg, is not experimentally established but merely a crude estimate from Fennell et al. (2005).

- It is not clear why just the study by Hartmann et al. (2009, should be 2008) is mentioned among the many studies on haemoglobin adducts in humans. The Hartmann study is mentioned again on page 11 where also the resulting intake 0.43 μg/kg bw per day is given (should be given also on page 4).

- The last sentence of page 4 is not clear. (This sentence, slightly modified is repeated several times in the text, e.g. page 38). The "in vivo" rate constant is determined from AUC and adduct level. The relation between intake and AUC depends on metabolism and volume of distribution.

- The equation taken from Törnqvist et al. (2008) (page 3) refers to a specific experimental situation and could possibly be used as a footnote in Tables 3-6 and 3-7. The layout of the experiment should be described briefly. This equation and the figure 1.07 are derived from a 7-day drinking water study (which is unclear from this text).

- With regard to earlier literature and descriptions of relation between AUC and Hb adduct levels, here for simplicity the reference to only one paper is given (Törnqvist, M. and Hindsø Landin, 1995). There are many others as well. In this reference some important early literature concerning Hb adducts is referred to. The reference gives a rather pedagogic explanation of the relation between AUC (dose) and Hb adduct

level in chronic and acute exposure, respectively, which partly could be a suggestion for an improved description in this report. Though it is recommended that throughout use AUC for (in vivo) dose.

References to Question 1

Törnqvist, M. and Hindsø Landin, H. Hemoglobin adducts for in vivo dose monitoring and cancer risk estimation. J. Occup. Environ. Med. 37 (1995) 1077-1085.

QUESTION 2. The appropriateness of the "EPA" method to estimate AUC from haemoglobin adducts

General comments

The model used to calculate AUC from Hb adduct level as described in the equations for adduct level after acute exposure in the expression on page 2 in the review is consistent with previous literature. (The expressions could have been better and more clearly described as noted in comments to Question 1.) The approach to use Hb adduct measurement for AUC measurements was developed in 1970-ies (at Stockholm University), and has been used for several simple alkylating agents. The basic method to measure Hb adducts to N-termini is well established for a range of compounds since its publication 1986.

The method to measure Hb adducts has been applied in several studies of acrylamide (AA), and its metabolite glycidamide (GA), to monitor exposure and to clarify metabolism in experimental studies. These compounds could relatively easy be studied with this approach, as they are reactive and at the same time sufficiently long-lived in vitro and in vivo. Their chemical properties permit a relatively even distribution in the body and there seems not be any problems like sinks or trapping in membranes etc.

So the principle as such is well-founded, though there are calculations presented in the review that could be improved, see the specific comments and the Appendix below.

One difficulty returned to in Question 4, is the determination of rate constants for adduct formation. There are uncertainties in the determination and it should be noted, that part of the discrepancy in the in vitro determined reaction rate constants obtained between different research groups is certainly related to the standards for quantification of adduct levels (different internal and external standards).

Specific comments

It should also be explained that the equations (page 3 and 4) are valid if the adducts are chemically stable and the rate of elimination is determined by erythrocyte turnover only. However they can not be used for calculation of daily adduct increments from the apparent steady state levels obtained in the 49-day drinking water study as the adducts were shown to be unstable. (The apparent half-time of the adducts in the 21-day drinking water study was considerably shorter than would have been predicted from normal cell turnover only. It was surprising to see that the Hb adducts had that short half-life.)

The model used (described in Appendix E) to simulate Hb adduct accumulation in rats in the 49-day drinking water study is not founded in biological theory and it is unfortunate if this type of model is established for this particular purpose (it is not generally applicable to describe haemoglobin adduct accumulation). The elimination of haemoglobin adducts is determined by a zero order process for elimination of erythrocytes (t_{er} in rats is about 60 days) with possible contribution from other processes such as chemical instability of the adducts. In long-term experiments the growth of the animals results in a dilution of adducts and the adduct level appears to decrease for this reason (Osterman-Golkar et al., 2003). Models that are applicable to repeat-exposure experiments and that incorporate different mechanisms of adduct removal have been developed (Granath et al. 1992; Fennell et al., 1992; Osterman-Golkar et al., 1999; Osterman-Golkar and Vesper, 2006). In principle a model of this kind could be used for calculation of daily adduct increments using data from the 49-day drinking water study. Actually it is not clear from the text if (and how) this has been done.

The adduct data in the 21-day drinking water study in rats by Tareke et al. (2006) is not inconsistent with a first order process for elimination of the adducts with a half-life of about 13 days and the proposed model gives a reasonable fit to the time course adduct data in the 49-day drinking water study. However, application of the model of Osterman-Golkar and Vesper (2006), using t_{er} = 60 days together with a first order rate constant, gives a better fit. See Fig 1 in Question 2.

The "Modeling Approach" described in Appendix E, page 45 is unclear. It is unclear how the data on AUC relates to the data in Tables 3-6 and 3-7 (see for instance for females). For the transparency of the calculations this has to be clarified.

The predicted AUC in Table E-2 are for the total exposure period of 49 days. The table should include values recalculated to daily AUC (in µM×h per day).

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HG is not a consistent abbreviation. Use Hb or globin.

Elimination rate: Add unit.

Some information about exposure should be added.

Figure to QUESTION 2

The simulation model presented in appendix E (Figure 1, p. 46) is based on the assumption that elimination of haemoglobin adducts is a first order process. The erythrocytes have a restricted life time (in the rat about 60 days). The simulation model should reflect this phenomenon. The graph below (Figure 1, Question2) shows a simulation where a first order rate constant of 0.03 d^{-1} has been used in addition to the cell turnover.

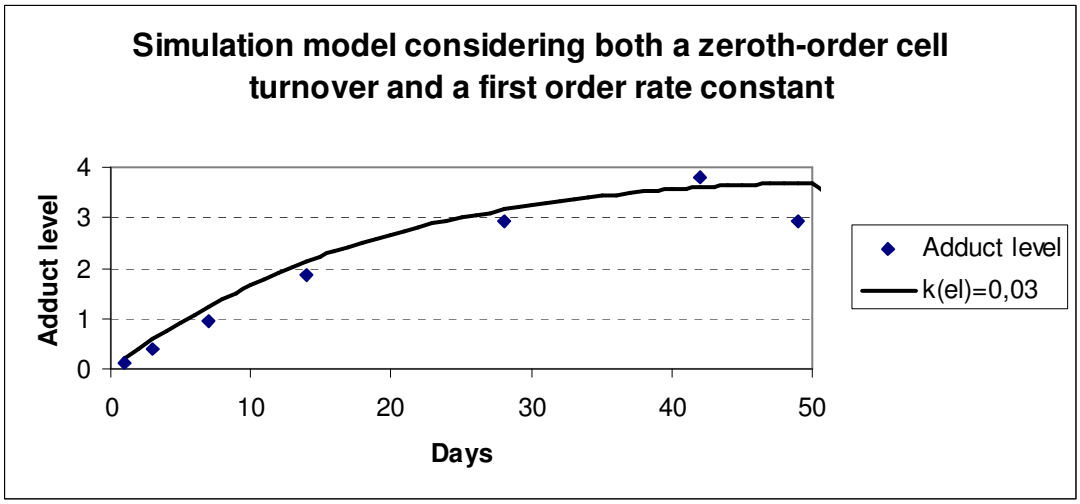

Figure 1.

References to Question 2

Granath, F., Ehrenberg, L. and Törnqvist, M. Degree of alkylation of macromolecules in vivo from variable exposure. Mutat. Res. 284 (1992) 297-306.

Fennell TR, Sumner SC, Walker VE. A model for the formation and removal of hemoglobin adducts. Cancer Epidemiol Biomarkers Prev. 1 (1992) 213-9.

Osterman-Golkar S, Czene K, Lee MS, Faller TH, Csanády GA, Kessler W, Pérez HL, Filser JG, Segerbäck D. Dosimetry by means of DNA and hemoglobin adducts in propylene oxide-exposed rats.Toxicol Appl Pharmacol. 191 (2003) 245-54.

Osterman-Golkar SM, Vesper HW. Assessment of the relationship between glucose and A1c using kinetic modelling. J Diabetes Complications. 20 (2006) 285-94.

QUESTION 3. Comments on the calculations of AUC and HEC

Comments on Question 3 are also included in comments to Questions 1, 2 and 4.
Section 3.6 "Physiologically based toxicokinetic models" is not really commented on by this reviewer, as partly outside my area of expertise.

Some specific points where the text is unclear are given below.

Page 34, Para 2:
The BMD10 values taken from where? Refs. to Table in main report needed.
Also unclear where the values 1.44×10^{-4} μM×h and 1.3×10^{-4} μM×h arrive from!

Page 35
The first equation on page 35 is not correct: $x / (y/z) = (x \cdot z) / y$

The second equation on the page: It is not mathematically correct to put in the factor 1000 (without the units) into the equation. The example below aims to illustrate this point.

3 kg = 3000 g (correct statement)

3 kg × 1000 = 3000 g (incorrect statement)

Page 36, 5.4.5.1. Para 2:
Should it be 1.8×10^{-1} mg AA/kg per day?
Line 4-3 from bottom: Simplify the expression. Suggestion, write:
0.1 / 0.18 (mg AA/kg bw *per day?*) = 0.56 (mg AA/kg bw)$^{-1}$ per day?

QUESTION 4: Appropriateness of EPA's rationale to calculate in vivo rate constants from relation between AUC and hemoglobin adduct level

General background

In earlier work, on simple alkylating agents, measured Hb adduct levels have been used to calculate the AUC in vivo using a second order reaction rate constant determined in vitro. The determination of an in vitro reaction rate constant could be difficult and, further, it is difficult to mimic the exact in vivo conditions. The in vitro rate constant is therefore associated with uncertainties. Usually the second-order rate constants for the reaction with N-terminal valines determined in vitro within the same lab and with the same standards etc. show good agreement. There might be differences between species, but data for instance for ethylene oxide show minor species-differences in the reaction rate towards N-termini in Hb (see Appendix to Question 4). Though, the measurement of Hb adduct levels in vitro (for determination of reaction rate) and in vivo is dependent on the internal standards and calibration, which might differ between laboratories (as long as no inter-calibration is performed).

Specific background

There is now a large amount of data for AUC and Hb adducts for AA and GA available from experiments in mouse and rats. The in vivo data on AUC offer a unique possibility to evaluate the relation between in vivo data for Hb adduct levels and AUC. Furthermore, considering the differences in the published data on in vitro rate constants for the reaction of AA and GA with N-termini in Hb, the alternative to use this "in vivo rate constant" gives a possibility to improve estimates of AUC from Hb adduct data.

General comments

- The approach applied in the IRIS review to derive the second order rate constants from AUC in single dose experiments in vivo (in rats and mice) seems to be appropriate given the differences of the determined in vitro rate constants.

- The approach to estimate AUC in humans from data on haemoglobin adduct levels in subjects exposed to single doses of 13C-carbon-substituted AA (Fennell et al. 2005) appears to be sound.

- In principle, the method that EPA used to derive the in vivo rate constants seems to be sound.

- There are some minor errors observed in the calculations and in the assumptions according to the specific comments below.

- There are some inherent difficulties to be observed.

Specific comments

- Doerge et al. (2005) observe remaining AA and GA in serum of rats even 12 hours after exposure to a single dose of AA in the diet. Adducts have been measured 8-10 h after the administration. Thus, the adduct level reflect AUC up to 8-10 h. Has this been considered?

- The data from treatment with GA for adducts and AUC could well have been included in this regression (not included according to para 2, page 29 and page 39).

- There is no reason to believe that the second order rate constants for haemoglobin adduct formation in male and female rats would differ. The application of a lower rate constant for females than for males may have exaggerated the difference in AUC between genders. Differences in AUC at a given administered dose are related to differences in metabolism and possible (assumed minor) differences in VD which may be gender-dependent. However, this does not imply that there is a difference between the male and female haemoglobin (same chemical structure) in reactivity towards a chemically reactive compound.

- A plot of data from Table 5-8 page 28 (or the same Table E-1 at page 38) shows that the data points are scattered and do not allow a firm conclusion about a difference between male and female rats concerning the slopes of AUC vs. Hb adduct levels. The r^2-values are lower than given in the Table (not numbered) on page 39. Please, recalculate values in this table. See Figure 2 in Appendix to Question 4.
Recommendation: Use the gender average.

However, differences between species in the reactivity of N-terminal valine in haemoglobin may exist, even if earlier data indicate that these are not large (see Appendix to Question 4). This possible difference may possibly motivate a value of an uncertainty factor $UF_{A\text{-}TK}$ of 1.5 for species-extrapolation.

QUESTION 4. Appendix

Calculations of in vivo rate constants

The data from Table E-1on hemoglobin adduct levels as a function of AUC do not allow a firm conclusion concerning a possible difference between genders with regard to the reactivity of hemoglobin (as reflected in the second order rate constant). The graph below shows data on AAVal.

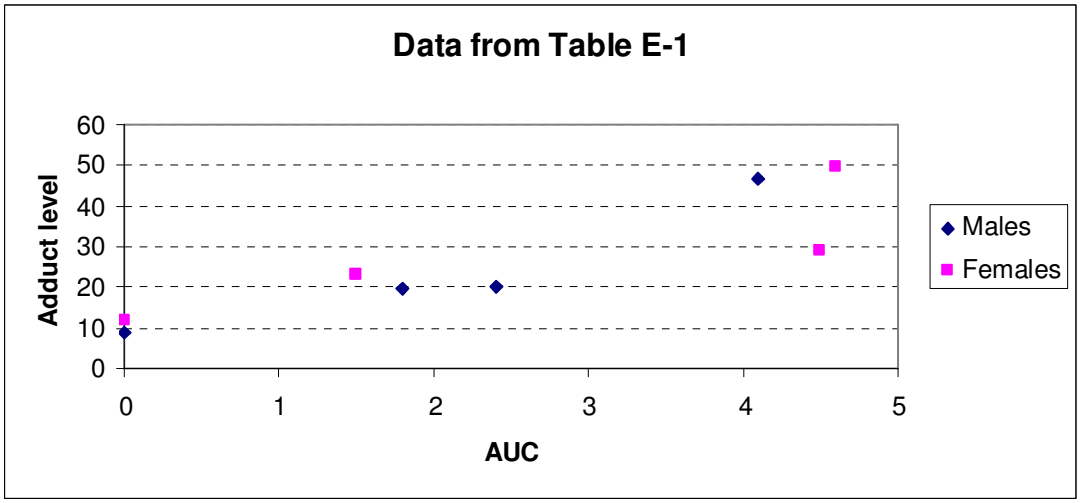

Figure 2.

Differences in vitro rate constants for Hb adduct formation in earlier studies

With regard to differences in reactivity to N-termini in Hb in earlier studies, one example is given here from determination within the same laboratory using the same standards. The well-studied ethylene oxide showed in vitro rate constants in the range $5.1 - 6.4 \times 10^{-5}$ ($l \times g^{-1} \times h^{-1}$) for mice (lowest), rat, human and monkey (highest) (Granath et al., 1999; Couch et al., 1996). In this case no large differences between species in the reaction rate towards N-termini in Hb are indicated. However, in some cases there for certain exist large species-differences in the rate of formation of Hb adducts. This concerns the formation of adducts to cysteine in Hb, where there are a very reactive cysteine in β-chains of the rat Hb. So species-differences cannot be excluded and also is dependent on the reactive compound.

References to Question 4

Couch, R., Ehrenberg, L., Magnusson, A.-L., Nilsson, R., de la Rosa, M.E., and Törnqvist, M. In vivo dosimetry of ethylene oxide and propylene oxide in the cynomolgus monkey. Mutat. Res. 357 (1996) 17-23.

Granath, F., Vaca, C. E., Ehrenberg, L. G. and Törnqvist, M. Å. (1999) Cancer risk estimation of genotoxic chemicals based on target dose and a multiplicative model. Risk Analysis 19, 309-320.

QUESTION 5. Additional comments on the methods and detailed comments on the text

- The Toxicological Review of Acrylamide needs to be rewrited.

- To improve the estimations on AUC from the published data, it is recommended to make some recalculations as suggested in the answers to the Charge Questions above, and to use the "in vivo" rate constants (for the formation of Hb adducts from acrylamide (AA) and glycidamide (GA) which are the average for gender.

- One uncertainty is the extrapolation between species, which in this report also includes "extrapolation" of Hb adduct data obtained at different laboratories. This would be improved with an intercalibration of adduct data. As Hb adduct measurement now has been taken up by so many research groups in the context of AA exposure there is really a need for intercalibrations between laboratories. Though the data on background levels like in Table 3-8 indicate that the there are not so large differences between laboratories in the determined adduct levels.

- Another question concerns between which dose intervals one would like to make the species-extrapolation. In the context of cancer risks from exposure to AA in food the most relevant with regard to estimation would be to calculate AUC in rodents corresponding to the exposure in cancer tests and then extrapolate down to doses at background exposure to AA from food in humans. The human data from Fennell et al. (2005) concern very high exposure doses, 0.5 – 3 mg AA/kg. Though, some preliminary data from analysis of high occupational exposures to AA and from analysis of background Hb adduct levels from dietary exposure of ca 0.5 µg AA/kg indicates no large deviations (on average) in the ratio between adducts from GA and AA, when samples analysed in the same laboratory with the same method, standards etc. (Paulsson, 2003).

- It should also be considered that it might not always be true that an in vivo rate constant would give a better estimate of the relation between AUC (in vivo), and Hb adducts, than the calculation from an in vitro rate constant for Hb adducts formation. It is largely depending on with which exactness the AUC in vivo, respective the in vitro rate constant could be determined.

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This referee, as a representative of the group at Stockholm University originally working with the development of the approach to use Hb adducts for AUC measurement, is pleased to see that the value of the approach has been recognized and that the approach is further developed. Indeed this approach was used to trace that AA in food constitutes an important background exposure (Bergmark, 1997; Törnqvist et al., 1998; Tareke et al., 2000; 2002.)

References to Question 5

Bergmark, E., (1997) Hemoglobin adducts of acrylamide and acrylonitrile in laboratory workers, smokers and nonsmokers. Chem. Res. Toxicol. 10, 78-84.

Paulsson, B. (2003) Dose Monitoring for Health Risk Assessment of Exposure to Acrylamides. Ph.D. thesis, Dept. of Environmental Chemistry, Stockholm University.

Tareke, E., Rydberg, P., Karlsson, P., Eriksson, S. and Törnqvist, M. (2000) Acrylamide - a cooking carcinogen? Chem. Res. Toxicol. 13, 517-522.

Tareke, E., Rydberg, P., Karlsson, P., Eriksson, S., Törnqvist, M. (2002) Analysis of acrylamide, a carcinogen formed in heated foodstuffs. J. Agric. Food Chem. 50, 4998-5006.

Törnqvist, M., Bergmark, E., Ehrenberg, L. and Granath, F. (1998) Riskbedömning av akrylamid. (Risk Assesment of Acrylamide) PM Nr. 7/98, (1998) Kemikalieinspektionen, Solna, 28 pp. (in Swedish, with illustrations in English).