Author Response: Chemical Considerations Regarding the H-Value Methodology and Its Relation With Toxicity Determination

We thank Srivastava and colleagues\(^1\) for their careful and extensive comments on our article, which emphasize the importance of this subject and the urgent need for more clarity.\(^2\) Before going into the questions in detail, we will summarize our main findings.

This article is the first contribution in a planned series of publications aimed at providing tools suitable for ensuring patient safety in the ophthalmological application of perfluorocarbon liquids (PFCL) by reliably identifying, detecting, and controlling the critical contaminants in these products. It goes without saying that product safety cannot be guaranteed by carrying out just one batch release test, due to the need for strict compliance with Good Manufacturing Practice (GMP) regulations that range from preparing the approval documentation for medical products to the routine production of each individual batch. To identify critical quality parameters, the products themselves that are being administered must be considered, as well as potential contamination associated with the manufacturing processes.

In the research cited above, the first question we addressed was whether the quality criteria originating from blood-substitute research in the 1990s also should be applied to ophthalmological products. Our investigations were carried out on batches of perfluorooctane (PFO) that had demonstrably led to severe adverse reactions. There is evidence from blood-substitution research that substance-specific, incompletely fluorinated by-products that inevitably arise during production and are dissolved in the PFCL can, even in minute quantities, lead occasionally to severe toxic reactions. It is mandatory that a specification of this class of contaminants must be created, as they can always arise completely independently of the materials used initially in production and of fluorination processes.

Unlike the extreme chemical stability of fully fluorinated perfluorocarbon (PFC) molecules, incompletely fluorinated impurities can react with chemical and biological material, or they can be transformed into other toxic substances.

The reactivity of incompletely fluorinated impurities and their toxicity are closely related; however, the intended use of PFCL assumes that only completely fluorinated products are used. Only then can they acquire the essential, particular stability required (thus ensuring their compatibility) and their surface properties.

This is why the degree of fluorination must always be measured right down to the trace level—and the aim of the H-value test cited in the article.

The H-value is a quality parameter for perfluorinated compounds in which, ideally, all hydrogen atoms have been replaced by fluorine atoms (PFCL). It indicates the concentration of the remaining hydrogen atoms. Its determination is carried out in two steps. HF is split off by a chemical transformation targeting precisely the (under these conditions) reactive, residual protons. This is quantified potentiometrically in the next analysis step. It should be done as a limit test. The acceptance criterion should be “practically free from,” meaning below the detection limit (10 ppm).

The key to understanding this limit test is its first part: the treatment of the PFCs to be investigated under highly specific conditions. The limit test not only triggers a reaction specifically targeting the incompletely fluorinated impurities, it also distinguishes between reactive and nonreactive compounds under these (extreme, not achievable in vivo) conditions.

What also is important to understand about this test is that the reactivity of a component (here the underfluorinated compounds) defined on the basis of a standardized reaction is a necessary but not sufficient precondition for a toxic effect. That is, a reactive impurity must exist to trigger an acute toxic reaction, but not every reactive impurity will trigger a toxic effect in the same manner.

The great advantage of the H-value test is that it does not target individual contaminants but rather selectively detects the particular structural motif among a wide variety of contaminants that is responsible for their reactivity (toxicity). Thus, the H-value test can be designed as a limit test, and the limit value can be set to the method’s detection limit (reactive residual protons in ppm, related to the examined PFCL’s molecular weight). If this limit has not been exceeded, the perfluorocarbons under investigation can be regarded as being practically free of reactive underfluorinated impurities; in other words, this compound type no longer has the potential to cause damage.

As described in the methods section in the article cited above, the detection limit is determined from the blank value by exposing PFCL’s ultra-high purity to this test. The determined values represent the background noise. The detection limit was set at twice the noise value, which is an even stricter criterion than usual (3-fold noise). The value thus determined for the detection limit is 10 ppm.

The H-value test is still much more highly sensitive than other possible detection methods, as it determines a sum parameter for reactive underfluorinated impurities by combining a specific chemical reaction with a specific potentiometric determination method. For example, the frequently discussed quantitative nuclear magnetic resonance (NMR) investigations of residual protons are limited by various chemical shifts in individual impurity species and the multiple splitting of the signals by the coupling of F and H atoms.

As described above, only PFCL that have been subjected to a multistage, ultra-high purification procedure fulfill the H-value test’s criterion. This has another positive side effect: The process steps of ultra-purification remove both the incompletely fluorinated impurities from the PFCL and other contaminants, and thereby further potential sources of toxic effects.

To the best of our knowledge, we are the first to have demonstrated in the aforementioned article the causal chain, reactive impurities in PFCL → high H-value → cytotoxicity → adverse event, caused by a toxic reaction during ophthalmological application.

To complete our investigations of the toxic batches, we determined the cytotoxicity’s dose-dependence and its correlation to the corresponding H-values. Our most important finding was that it was not the perfluorooctane itself, but rather the impurities dissolved in it that were responsible for the toxic effects. But the fact that our dilution study suggests that a steady increase in cytotoxicity accompanies a rising H-value must not be overinterpreted. In that test, we examined a toxic material with an unchanged impurity profile in combination with an ultra-purified, nontoxic material. On the contrary, the results of batches investigated in the article cited above prove that each batch maintains its own unique relationship between its H-value and cytotoxicity despite originating from the very same raw material.
To elucidate the underlying reasons, we report on the chemical composition of the individual species in the impurity profile and their quantity, and discuss these in a follow-up publication that has already been submitted and accepted.

We now answer the authors' comments in detail:

1. The authors mentioned “When a digestion process to cleavage C-F bonds from a complex molecule is performed, it is important to calculate the yield of the reaction to ensure that all of the fluoride ions are extracted during the digestion.”

**Answer:** The question raised in the letter about the yield of the elimination of fluoride ions would be relevant only if the corresponding test were designed as an assay, that is, the concentration of individual toxic compounds (impurities) should be determined. However, the test presented here is not an assay, but rather a limit test, and it only responds to the structural entity (structural motif) of reactive underfluorinated compounds. This approach is based on recognizing the correlation between reactivity and toxicity. In the first step, when determining the H-value (chemical transformation), the test-samples are subjected to a predefined, standardized “test reaction.” Depending on this test reaction, the samples are divided into reactive samples and nonreactive samples. There is no point to discussing a yield with this approach, as none is possible.

2. The authors stated that the reaction was not evaluated “to ensure that the three fluoride ions were always released.”

**Answer:** If the formula stated in our “Methods” is formally broken down further into individual steps, it becomes obvious that a substitution reaction occurs at the resulting double bond on initial HF elimination (F⁻ no. 1) with the formation of two further ions (HF). In its given form, the formula is a widespread, comprehensive representation of the reactive principle of chemical transformation.

3. The authors also mentioned “Further, it is doubtful that the amine used in the protocol could easily break the C-F bonds.”

**Answer:** It is common knowledge that this reaction occurs under specific (alkaline) conditions, as specified in the first step of the test. In addition, one of the driving forces is the energy gain from various binding energies: from C-F (489 kJ/mol) to H-F (567 kJ/mol).

4. The authors comment “Moreover, as the methodology is described, it seems to be specific to those compounds with only one or two C-H bonds in the perfluorocarbon molecule.”

**Answer:** As shown under item 1, the presented H-value test is used to characterize the reactive and thus potentially toxic impurities. A strictly standardized, extremely harsh test reaction (here referred to as transformation reaction) is used. Compounds that do not react under these conditions do not meet the criterion “reactive” and are unable to trigger acute toxic reactions due to their inertia. It goes without saying (and is a basic rule of GMP for all other medical devices and drugs) that potential impurities possibly occurring during manufacture be specified and appropriately controlled additionally via the appropriate tests (residues of starting material, leachables, extractables...). Contamination by perfluorooctanoic acid (PFOA) is plausibly traceable to an unreacted starting material (a key point addressed in the discussion in our follow-up paper already submitted).

5. The authors also mentioned that “The method also does not detail the molecular weight and density that the authors used in the equation.”

**Answer:** Allow us to point out that this is a sum parameter executed as a limit test; therefore the reference value is obviously the fully fluorinated target molecule (PFOA), as indicated in the methods section.

6. Another comment is “Moreover, according to the article, 10 ppm was the toxicity detection limit, which is the most important parameter described. Therefore, to validate this parameter, all details of how the detection limit was calculated should be disclosed.”

**Answer:** Our paper clearly states: if the H-value is below the detection limit, then the investigated PFCLs are practically free of reactive underfluorinated impurities. Both a potentially acute and latent toxic reaction caused by this contamination class is thus excluded. We do not use the term toxicity detection limit in this way in our article in connection with the H-value. This detection limit concurs with the values published by various authors.

7. The authors also stated “Finally, the authors tested the dose dependency of the H-value and cell growth inhibition (Table 5) by adding different amounts of an ultra-purified sample into the toxic batch 061014 sample. However, in doing so, it is clear that the so-named H-value (related to the incompletely fluorinated compounds) will be reduced.”

**Answer:** The problem mentioned above exists with each and every dilution test. Note that the relationships between concentration and cytotoxicity derived from the dilution curves of batch 061014 are not transferable to other batches: there, the correlation between H-value and cytotoxicity is different. This suggests differences in the impurity profiles of the individual batches, as mentioned in the article. We clarify these effects in one of our aforementioned follow-up studies in which each batch’s impurity profile is characterized individually. The most important finding from our description of dose dependency is the strong evidence that it is not the main component, but rather the impurities dissolved in the main component that are responsible for the cytotoxic effect.

8. Another comment is that “The analytical method to determine the concentration of incompletely fluorinated compounds was developed earlier than 1990, as referenced by Groß et al.”

**Answer:** It is true that the triggering element for the reactivity of impurities and thus their toxic potential are the remaining (defective) hydrogen atoms in compounds that should, ideally, be fully fluorinated. As we show, determining the H-value must be a two-step process in which the remaining (defective) hydrogen atoms are first cleaved off as HF by the transformation reaction, and then the amount of fluoride is determined in the reaction solution is analyzed in the second step. Specificity is ensured by the reaction path in step 1 and the use of fluoride-selective electrodes in step 2 as well. We kindly refer again to the work of Gervits as an example.

9. The authors also stated “Currently, there are other well-known analytical techniques, such as nuclear magnetic resonance, to determine and quantify hydrogen in the samples.”

**Answer:** It is correct in principle that there are other suitable methods for detecting remaining hydrogen atoms. However, they do not allow the generation of a sum parameter corresponding to the H-value according to uniform evaluation criteria. There is no way the NMR tests mentioned above (which we have used for years as a supplemental investigative method) even begin to reach the detection limit. This is, on the one hand, because different impurity species engage in different chemical shifts, and on the other hand, the H atoms couple with the F atoms (over several chemical bonds) with corresponding coupling constants (e.g., 0-130 Hz [geminal] 0-100 [vincinal]). As a result, their signals disappear in the noise.
To the best of our knowledge, nothing has changed: H-value determination remains by far the most sensitive method for detecting remaining (defective) hydrogen atoms as the common structural motif of various reactive, underfluorinated, impurity species, as stated already in the article referred to above (Groß et al.).

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