How to Ward Off Retinal Toxicity of Perfluorooctane and Other Perfluorocarbon Liquids?

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Purpose. Reactive and underfluorinated impurities are acknowledged as a source of cytotoxicity of perfluorocarbon liquids (PFCLs) used as blood substitutes. To determine whether this is also a relevant factor in retinal toxicity, we analyzed eight PFO batches associated with adverse ocular events.

Methods. (A) The amount of reactive and underflurinated impurities was analyzed by fluoride-selective potentiometry and expressed as H-value. (B) Cytotoxicity of these batches was determined by an ISO 10993-5-compliant extractive test and compared to published data generated with a direct-contact method. (C) A toxic PFO batch (061014) was purified to remove reactive and underfluorinated impurities. (A) and (B) -measurements were repeated after that. (D) The dose dependence of the H-value and cytotoxicity was determined in a dilution experiment.

Results. (A) The batches revealed H-values ranging from 1.400 ppm to 4.500 ppm. (B) All batches induced cell growth inhibition; seven must be classified as cytotoxic. Findings from ISO-conform extractive and direct-contact methods showed no difference. (C) After all reactive and underfluorinated impurities in batch 061014 were removed, the H-value dropped to <10 ppm and cytotoxicity disappeared. (D) Cytotoxicity increases gradually as the H-value rises.

Conclusions. The clinical relevance of the H-value as a safety parameter for PFO endotamponades could be proven. The H-value is a measure for reactive and underfluorinated impurities that cause toxicity of PFCLs and should be incorporated in each endotamponade specification with a limit of 10 ppm to prove the effectiveness of the ultra-purification required and ensure a safe product. Despite the fact that an (ISO)-standard literally is a "standard" only, which cannot cover all imaginable possibilities, the incorporation of the H-value determination into the relevant ISO standard has been initiated. If a thorough risk assessment results in risks that cannot be detected and/or managed by the effective standard, additional investigations have to be performed.

Keywords: perfluorocarbon liquids, perfluorooctane, retinal toxicity, cytotoxicity, H-value, underfluorinated compounds

Perfluorocarbon liquids (PFCLs) have contributed strongly to improving the surgical management of vitreoretinal diseases like complex retinal detachment. Today, many surgeons value PFCLs as an indispensable tool in modern vitreoretinal surgery.[1–8] However, severe complications have been reported after PFCL use in Spain, France, Italy, the Middle East, and South America over the last 5 years, resulting in product recalls caused by the correlation between the reported cases and use of specific product batches.[9–12] In detail, the authors described a distinct visual loss, retinal necrosis and fibrosis, retinal vascular occlusion, and retinal or optic nerve atrophy. The situation has caused concern among vitreoretinal surgeons regarding the safety of this group of medical devices.[9–11,13]

Pastor et al.[11] tested samples of perfluorooctane (PFO) batches associated with the patients in Spain involving diagnoses of severe retinal and optic nerve damage characterized by retinal fibrosis and necrosis, vascular occlusion, macular hole formation, nerve head atrophy, and phthisis bulbi. It is the merit of Pastor et al.[11] to have proven that the batches used have cytotoxic properties. They demonstrated the distinct cytotoxic effects of these batches after developing their own in vitro testing method, which was developed for nonwater-soluble and vaporizable chemical substances and is described in detail in a patent filed (Patent ES2644987). However, the causal link between the cytotoxic effect of the batches and substance-specific properties remained vague.[11,13] It is understandable that the phenomenological evaluation of the batches concerning their cytotoxicity and correlation to the documented clinical findings has not overcome the surgeons' doubts. In particular, the question arose whether new or additional, hitherto unknown risks are related to PFCL products, and whether the toxic effects described were caused by the PFCL itself or by its impurities. However, the fact that PFCLs have

been well investigated for their potential use in medical application has been overlooked in this discussion.

PFCLs were subjected to fundamental toxicological assessment during the development of PFCL-based blood substitutes between the 1970s and 1990s. Groß et al.[14] and Gervits[15] summarized the latest knowledge on the requirements for the medical use of PFCLs in the early 1990s, demonstrating the relationship between reactivity and the toxicity of impurities. Essential to all medical applications of PFCLs is therefore the control of impurities, especially of incompletely fluorinated by-products, which are unavoidable by-products of the synthesis of PFCLs that must be eliminated by thorough purification.[3] Meinert[16] referred to those findings from blood substitute research to the ophthalmological application of PFCL, relying on them as the starting point to develop PFCLs of sufficient quality for ophthalmic application.

In contrast to completely fluorinated PFCLs, the underfluorinated by-products are reactive. That quality makes these impurities distinguishable from the PFCL, because only these impurities react, for example, with strong bases under the formation of hydrogen fluoride (HF). The latter can then be selectively quantified via fluoride-selective ionometry. Groß et al.[14] demonstrated that this is the most sensitive physicochemical method for assessing the safety of PFCLs, demonstrating convincingly that a fluoride concentration in the range of 10 ppm can be reproducibly detected. Gervits[15] described a reliable correlation between the actual content of underfluorinated compounds determined by fluoride selective ionometry and the results of cell culture tests using specifically prepared solutions of defined impurities in highly purified perfluorodecalin. In addition, there is evidence that a PFCL is nontoxic provided the concentration of underfluorinated compounds is in the range of only 10 ppm.[14–16] It is noteworthy that different hydrogen-containing and unsaturated impurities reveal various toxic effects.[15]

Against this background, Meinert[16] specified the acceptance criterion of underfluorinated compounds as the fluoride-ion equivalent in PFCL to be $5{\cdot}10^{-5}$ mol/L fluoride-ions for use in vitreoretinal surgery. He acknowledged that some cell lines are less sensitive and would enable laxer acceptance criteria. But if this level shall be used as a safety criterion, the level of $5{\cdot}10^{-5}$ mol/L fluoride ions, defined as being "practically free of cleavable fluoride ions," should not be exceeded.[16] PFCLs that were purified according to Meinert's[17] method meet this criterion and were proven suitable for vitreoretinal application.[18]

In this investigation, we used the above-referenced method to determine the amount of underfluorinated compounds in the recalled PFO batches that had been used in the alarming clinical cases in Spain. Furthermore, we aimed to confirm whether the established purity criterion for PFCLs as blood substitutes would remain valid for ophthalmic cases, and how the affected PFO-batches differ from the purity criterion Meinert[16] defined over 20 years ago.

Materials and Methods

Materials

The studies were conducted on original packaged samples of ala octa manufactured by alamedics GmbH (Dornstadt, Germany; Table 1).

A commercial perfluorooctane, batch (PFO 33/15), with an H value <10 ppm, manufactured and highly purified by Pharmpur (Koenigsbrunn, Germany), served as reference material.

Table 1. Original Packaged Units of the Following Lots of Ala Octa Manufactured by Alamedics GmbH (Dornstadt, Germany) Used as Test Samples (Presented Tests in This Paper Were Completed Before the Expiry Date was Reached)

Ala Octa Lot #	Expiry Date
171214	2018-12
061014	2018-10
050514	2018-05
080714	2018-07
150414	2018-04
200114	2018-01
070714	2018-07
041213	2017-12

Methods

Eight different batches of PFO were analyzed for toxic impurities by two validated and GMP-certified analytic methods, fluoride selective ionometry and cytotoxicity testing according to ISO-10993-5.

(A) Fluoride Selective Potentiometry. The perfluorooctane products were analyzed for reactive underfluorinated impurities as an origin of the cytotoxic reaction by fluoride-selective ionometry described by Groß et al.[14] and Gervits.[15] Principle of the method is the quantification of fluoride ions by ion-selective potentiometry after chemical transformation of the reactive underfluorinated impurities according to the formula[5,7]:

R^F = fully fluorinated substituent; Nu = nucleophiles that promote fluoride-cleavage under strong and harsh basic conditions; F^- = fluoride ions

Equipment and Chemicals. Ion sensitive electrode: Mettler Toledo (Mettler-Toledo GmbH, Gießen, Germany), Perfect Ion Fluoride; Ionometer: Mettler Toledo, pH/Ion Analyzer MA235; fused silica boiling flasks and suitable reflux condensers; nonane for synthesis (Merck KGaA, Darmstadt, Germany); 1,6-diaminohexane, 99.5% (Fisher Scientific GmbH, Nidderau, Germany); hydrochloric acid 32% p.a. (Carl Roth GmbH + Co. KG, Karlsruhe, Germany); TISAB-III-Solution (Sigma-Aldrich Chemie GmbH, Munich, Germany); sodium fluoride p.a. (Merck); phenolphthalein solution 1% in ethanol (Merck); purified water. Blank value = reagent and boiling flask blank value (value after heating without PFCL).

Step 1: Chemical Transformation of the Reactive Underfluorinated Impurities. Ten milliliters of the PFCL are mixed with 3.4 g 1,6-diaminohexane and 15 mL nonane. This mixture is heated in a 100-mL glass flask equipped with a reflux condenser for 8 hours under stirring to a temperature of 120°C. After cooling to room temperature, the solution is vigorously mixed with 30 mL hydrochloric acid (1.3 molar) and the aqueous phase is separated.

Subsequently, 15 mL of the aqueous phase are neutralized with 1.3 molar aqueous hydrochloric acid using phenolphthalein as an indicator and diluted to 25 mL with deionized water. Ten milliliters of this neutralized diluted solution are transferred into a 25-mL glass beaker and 1 mL TISAB-III is added under stirring.

Table 2. Results of Validation of Fluoride-Selective Potentiometry

Parameter	Result of Validation
Specificity	0.7 ppm
Linearity	0.998 (Slope: −0.018544 y-intercept: 0.622344)
Precision	RSD 5.9%
Accuracy	100 % (1st determination) 103 % (2nd determination)
Range	20%–200%

Step 2: Fluoride Selective Potentiometry. Ion-selective potentiometry is used to quantify fluoride ions in the sample solution. Prior to sample measurement, calibration must occur using sodium fluoride solutions with fluoride ion concentrations between 0.005 mmol/L and 0.05 mmol/L as reference standard. In addition, a blank value is recorded. The sample solution is then analyzed.

The amount of reactive underfluorinated impurities is expressed as equivalent to the number of C-H bonds in these impurities—the so-called H-value.

$$c_{F-C-H}[ppm] = \frac{1}{3} \times \left(\left(c_{F^-} \left[\frac{mmol}{L} \right] \times 5 \times \frac{\bar{M}_{PFCL} \left[\frac{g}{mol} \right]}{\rho_{PFCL} \left[\frac{g}{mL} \right]} \right) - bv[ppm] \right)$$

where:
c_{F-C-H} is the concentration of incompletely fluorinated contaminants in the sample;
c_{F^-} is the measured concentration of fluoride ions in the sample;
$\bar{M}_{PFCL}$ is the molecular weight of the measured PFCL;
ρ_{PFCL} is the density of the measured PFCL;
bv is the recorded blank value (reagent and boiling flask blank);
5 is the factor to compensate for the dilution steps; and
$\frac{1}{3}$ is the stoichiometric factor (calculation of number of CH-bonds).

Validation of the Method. This method was validated as a limit test according to the ICH Q2 guidelines[19] proving the specificity of the method and the detection limit by considering both steps in the test. Specificity is ensured by the reaction path in step 1 and the use of fluoride-selective electrodes in step 2 as well (Table 2). The detection limit was determined as the double of the noise-level measured after performing both method steps in the presence of PFCL resulting in 10 ppm. This limit can only be met if the samples are practically free of reactive underfluorinated impurities. Fulfillment of this limit test thus ensures compliance with the quality criteria specified in the literature.[14–16]

Carrying out the method in two separate steps allows not only its use as a pure limit test but also comparative evaluations are applicable, since the method's second step can function as an assay when viewed individually. However, in these cases it is important to consider that the fluoride concentrations detected may originate from the transformation step from different compounds with different reactive and toxic properties, including HF already formed.

(B) Cytotoxicity Testing According to ISO-10993-5. Cytotoxicity of these batches was investigated according to an ISO 10993-5-compliant extraction test method and those results were compared to published results generated via a recently developed method for testing PFCL cytotoxicity.[13] Cytotoxicity was tested according to ISO-10993-5 on extracts of PFO samples using mouse fibroblasts L929 for the cell growth-inhibition test. The test material was extracted for 24 ± 2 hours at 37 ± 1°C using Dulbecco's modified Eagle's medium (DMEM). Extraction was performed with 1 g test material in 3.3 mL DMEM. The incubation took place under CO_2 atmosphere to prevent the medium's acidification. After incubation, the aqueous phase was separated. A cell suspension of L929 mouse fibroblast cells was prepared in DMEM. The L929 cell suspension was brought in contact with the extract (aqueous phase) for 68 to 72 hours at an extract concentration of 0.2 g/mL.

As negative control, a polypropylene material was extracted using 1 g/5 mL DMEM 10% fetal calf serum (FCS) for 24 ± 2 hours at 37 ± 1°C. An extract of latex gloves was used as positive control (6 cm^2/mL DMEM 10% FCS, 24 ± 2 hours, 37 ± 1°C). A triple determination was made in each case.

After incubation, the protein content was measured. Each cell culture's protein content was compared to the protein content of the solvent. Cell growth was thus determined in the presence of the test material. To differentiate dead cells from living cells, bicinchoninic acid (BCA) staining was used (reduction of Cu(II) to Cu(I) by proteins) followed by complex formation with bicinchoninic acid).

(C) Multistep Ultra-Purification of PFO. Cytotoxic batches were purified following Meinert's[17] procedure to remove underfluorinated and reactive compounds. (A) and (B) -measurements were repeated for this purified material.

(D) Dose Dependency of H-Value and Cytotoxicity. H-value's and cytotoxicity's dose dependence were determined using a concentration series produced by mixing different portions of a toxic, unpurified batch of PFO with a purified, nontoxic batch free of reactive and underfluorinated compounds. H-value and cytotoxicity was determined according to the method described under (A) and (B).

Table 3. H-Value and Cell Growth Inhibition of Ala Octa Lots

Ala Octa Lot #	Cytotoxicity [% Growth Inhibition] ISO10993-5 (Extractive Method (B))	H-Value [ppm]
171214	96	4500
061014	94	2800
050514	50	2100
080714	48	2100
150414	40	1400
200114	39	2600
070714	33	1800
041213	23	3200
Pharmpur PFO 33/15	0	<10
Ultra purified 061014	0	<10

Results

(A) Determination of the H-Value

We had access to eight PFO batches associated with the adverse events in Spain. The concentration of underfluorinated impurities was determined in all these batches and expressed as H-value. Our results are summarized in Table 3. Only the high-purity material batch PFO 33/15 met the <10 ppm limit in the test. The results for the batches in question are two orders of magnitude above the limit value (1400 ppm to 4500 ppm).

Table 4. Comparison of the Results of Cytotoxicity Testing Using Direct Contact Method According to [1] and Extraction Method [This Paper Method (B)] According to ISO 10993-5

	Cytotoxicity [% Growth Inhibition] ISO10993-5	
Ala Octa Lot #	Results According to Reference 11 (Direct Contact)	Results According to Method (B) (Extractive)
171214	99	96
061014	99	94
050514	50	50
080714	47	48
150414	66	40
200114	44	39
070714	47	33
041213	18	23
Pharmpur PFO 33/15	–	0

(B) Determining Cytotoxicity Using an Extraction Method

The same batches were tested for cytotoxicity using an extraction method in accordance with ISO 10993-5. Aqueous extracts of the batches were prepared and tested on mouse fibroblasts L929. Cell growth inhibition and its corresponding H-value are compared in Table 3. Again, the mean values of at least two parallel measurements per batch were listed. According to ISO 10993-5, any product that exceeds the threshold of 30% growth inhibition is formally classified as cytotoxic. Except for batch 041213, all the other batches we investigated exceeded the limit of 30% growth inhibition. In case of batches 171214 and 061014, cell growth was practically zero. However, batch 041213 also revealed remarkable growth inhibition (23%).

The results of our cytotoxicity study using the extraction method according to ISO 10993-5 are consistent with the data published by Pastor et al.[11] Table 4 shows our comparison of results, which are obtained using different approaches in two different laboratories.

(C) Multistep Ultra-Purification of a Toxic PFO Sample and Analytical Results

Batch 061014 was treated in a multistep ultra-purification process ensuring the removal of all reactive underfluorinated impurities. H-value and cytotoxicity of this ultra-purified batch were determined again. By separating the reactive underfluorinated contaminants during the ultra-purification, the H-value dropped from 3100 ppm to < 10 ppm. The ultra-purified sample of the batch 061014 exhibited no cell-growth inhibition during the cytotoxicity test (refer to Table 3).

(D) Evidence of the H-Value's and Cytotoxic Effect's Dose-Response

Dilutions of batch 061014 with ultra-purified PFO (H value $<$ 10 ppm) were prepared in several defined steps. Samples of this dilution series were tested for cytotoxicity via our extraction method. The Figure and Table 5 illustrate those results. The threshold of 30% cell growth inhibition was no longer exceeded provided the ultra-purified material contributed more than 75% of the total volume.

Discussion

In the present study, we confirmed the relevance of well-documented knowledge about reactive underfluorinated impurities being a risk factor for PFCL safety for medical application as a blood substitute[14,15,17] for ophthalmic use. The correlation between the H-value and cytotoxic effects of the individually analyzed batches is overwhelming. In addition, we demonstrated that a multistage ultra-purification process of the cytotoxic batch 061014, which eliminated underfluorinated impurities completely, transformed the batch into a well-tolerable material not triggering any cell-growth inhibition. Via the dilution experiment, our evidence reveals that the cytotoxicity increases gradually in conjunction with a rising H-value.

Beginning in 2013, repeated cases of toxic reactions of perfluorooctane used in vitreoretinal surgeries have been reported. A new series of reports on vision loss was published after use of ala octa in Spain in 2015.[11] The focus of the present work was the investigation of affected ala octa batches, because of the best documentation of the cases and the availability of original samples of the affected batches in sufficient quantity. In addition, samples of identical batches were the basis of the publication of Pastor et al.,[11] which clearly identified the PFO batches used as the trigger for the toxic effects. However, the causal link between the cytotoxic effect and their substance specific properties remained vague. Nearly three decades ago, research on the use of various PFCL for medical use as blood substitutes revealed the content of reactive and underfluorinated impurities as being the most important source for PFCL toxicity. To discover whether this is also relevant to the retinal toxicity observed in Spain, we analyzed the PFO batches reportedly associated with those adverse events. Our test results confirm the cytotoxic effects of the PFO batches involved in the severe adverse events in Spain. This proof is even more valuable because a second independent test method for evaluating material cytotoxicity from a second independent organization confirmed findings of the Pastor et al.[11] group, who were the first to confirm by in-vitro testing the clinically diagnosed toxic reactions. Our results on cell growth inhibition are consistent with the findings of Pastor et al.[11] (see Table 4). This is an important fact, as Pastor et al.[11] claimed that there is no alternative to their direct contact method using ARPE-19 and porcine neuroretina explants, for which a patent was filed.[13] Furthermore, for the determination of cell growth inhibition the established and recognized sensitive cell line L929 was used for the characterization of the toxic potential to allow a good comparability to a large number of substances already tested to evaluate their cytotoxicity. Biocompatibility studies require an extended test protocol compared to pure cytotoxicity studies. Possible advantages of using retinal cell lines, such as ARPE 19, only become apparent in this context.[11]

Our analytical evidence closes the gap where a causal link between the phenomenon "cytotoxicity" and a measurable material property of the notorious PFO batches had been sought. The missing explanation caused—quite understandably—uneasiness among users and regulatory authorities, as there seemed to be some mysterious thing about PFCL, which, since unknown, could not be controlled and use of PFCL in vitreoretinal surgery would have been (if at all) safe only by coincidence. Such fears can now be allayed by the results of the present study, which confirm the requirement established for PFCL use as blood substitute, that they should be practical free of reactive underfluorinated impurities (below $5{\cdot}10^{-5}$ mol/L cleavable fluoride-ions) and allow for safe use in vitreoretinal surgery.[16] To the best of our knowledge, we are the first to have demonstrated the causal chain—reactive impurities in PFCL → high H-value → cytotoxicity → adverse event caused

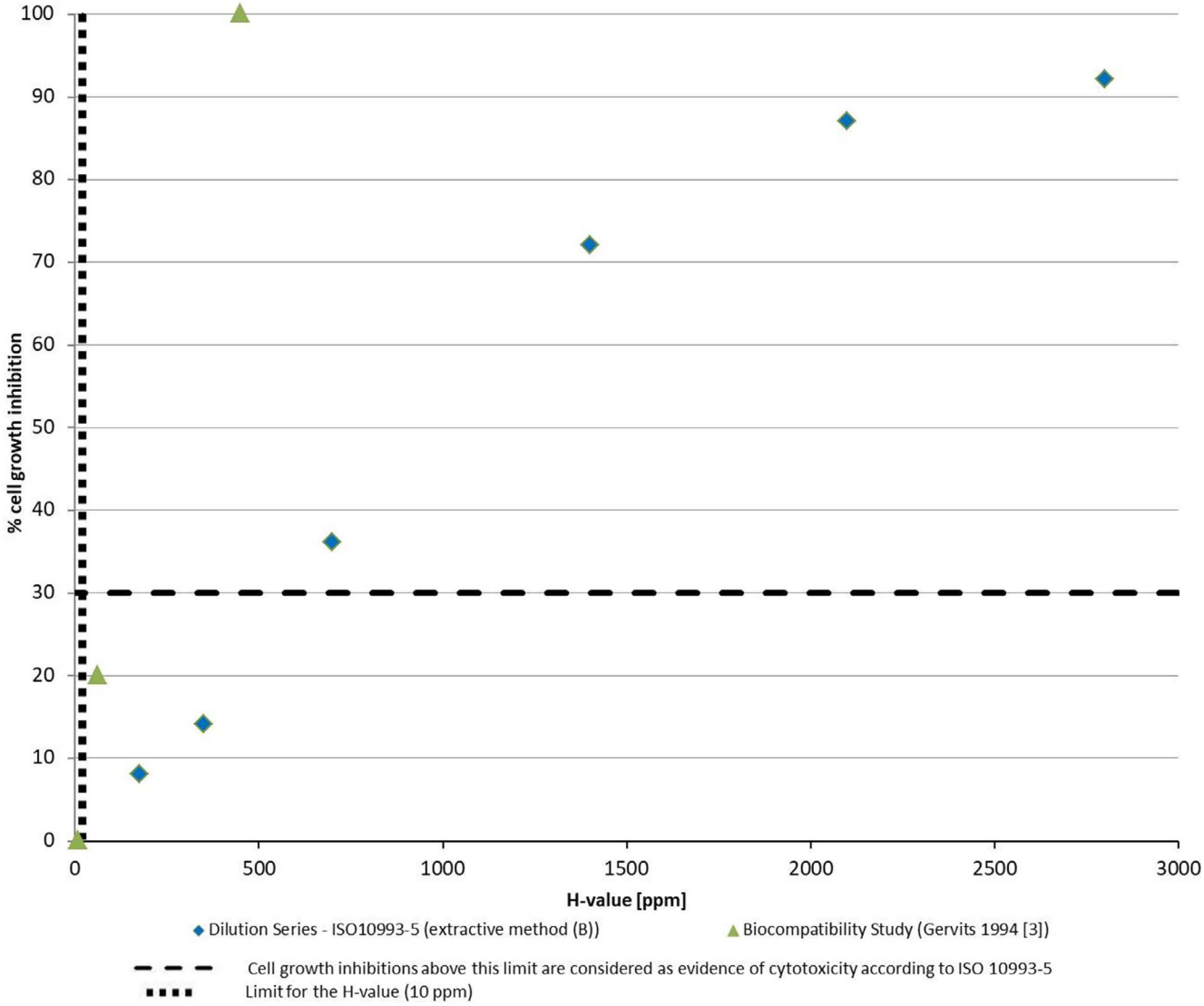

Figure. Correlation of dose dependent H-value and cell growth inhibition in a dilution series using batch 061014 and ultra-purified PFO in the ratio of 100:0; 75:25; 50:50; 25:75; 12,5: 87,5; 6.25:93.75 and comparison to literature data.

by toxic reaction during ophthalmological application. One has to keep in mind that these acute toxic reactions are completely different from the previously described and known side effects of long-term applications of PFO/PFCL.

Since the transferability of the toxicological investigations of PFCL for use as blood-substitutes to vitreoretinal PFCL use has now been proven, the importance of determining the H-value cannot be overemphasized.

Table 5. Dose Dependency of H-Value and Cell Growth Inhibition

Non Purified Lot # 061014 PFO [%]	H-Value [ppm]	Cell Growth Inhibition [%]
100	2800	94
75	2100	87
50	1400	72
25	700	36
12.5	350	14
6.25	175	8

The root-cause of the retinal toxicity is the reactivity of the impurities. In contrast to the extreme chemical stability of fully fluorinated PFC molecules, the impurities can react with chemical and biological material, or they can be converted to other toxic substances. The determination of the H-value relies on exactly this reactivity of the impurities. To detect all reactive compounds completely, the conditions of the transformation reaction must be very harsh, meaning that any potential toxic impurity will be converted and thereby detected. At the same time, all of the transformations that already occurred in the sample under HF formation are also revealed through fluoride-selective potentiometry.

Any fully fluorinated PFCL, by its inert nature, will pass through the transformation reaction without any change. To exclude any latent risk of toxicity, PFCLs should be practically free of reactive impurities. Applying the method for determining the H-value described here, an H-value not exceeding 10 ppm is equivalent to this stringent criterion, because 10 ppm is the validated, robust limit of this test method. Higher values indicate the presence of reactive underfluorinated compounds in the PFCL material we investigated, and hence the potential risk of material toxicity.

A further conclusion from these studies should be discussed: we acknowledge that the validated limit of 10 ppm for the H-values derived from the detection limit is a sufficient criterion to conclude that a PFCL is not cytotoxic. However, that does not mean that every noncytotoxic PFCL must necessarily have an H-value of $\leq$10 ppm. As referenced in the introduction, different hydrogen-containing and unsaturated impurities reveal different toxic effects.[15] The fact that our dilution study suggests a steady increase in cytotoxicity with an increasing 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 in Table 3 prove that each batch has its individual relationship between the H-value and cytotoxicity despite originating from the same raw material.

To elucidate the underlying reasons, we plan to analyze the chemical composition of individual species in the impurity profile and discuss these in a follow-up publication. It is also important to clarify why the determination of the cytotoxicity of PFCL samples revealed reproducibility problems as reported by Pastor et al.[11] A larger variety of PFCL products for vitreoretinal surgery should also be investigated to evaluate their overall quality and the safety of products currently on the market.

In conclusion, the determination of the H-value is an indispensable tool for assessing the suitability of PFCL for ophthalmic use. The H-value not only represents a random product property, it is the key parameter to evaluate the long-term toxicological potential of a PFCL. This means that the H-value test goes far beyond the phenomenological assessment of a given batch, which would only describe its quality as a snapshot, as this is the case, for example, with an evaluation relying solely on a cytotoxicity measurement.

In conclusion, the recent case series of severe side effects and vision loss after PFO use could have been prevented had the H-value parameter already been the standard parameter for assessing the product quality of PFO or any similar PFCL substance, for example, perfluorodecalin. Therefore, the H-value should be established routine as part of the product specification and final release, and surgeons should request it.

Completely purified and characterized PFCL used as an ocular endotamponade are still safe devices. The toxicity described in connection with individual batches was caused by effects from reactive underfluorinated impurities.

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