Missing evidence for toxicity of high PFAT5 levels in mixtures of lipids

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Abstract: The compliance of lipid admixtures to physical emulsion stability parameters is extremely important to ensure the safety of patients. For example, admixtures containing a percentage of fat globules larger than 5 μm in diameter (concept known as PFAT5) of more than 0.05% might produce toxic effects in lung and liver. This concern is mainly based on a limited number of animal studies investigating admixtures with high PFAT5 levels resulting from 48 h of admixture storage. However, all effects observed in these studies might as well be attributed to chemical instability like lipid oxidation, which was not analysed and therefore could not be excluded.

Aims: This study aims at investigating the correlation of high levels of PFAT5 in lipid emulsion admixtures with lipid oxidation parameters under different storage conditions.

Methods: We studied the physical (PFAT5 value) as well as the chemical (pH, primary and secondary oxidation parameters) stability of an admixture of a lipid emulsion and an amino acid solution after up to 48 h following different storage conditions (exposure to oxygen, exposure to artificial light).

Abbreviations: ALT, alanine amino transferase; Anv, p-anisidine value; AST, aspartate amino transferase; GST, glutathione-S-transferase; 4-HNE, 4-hydroxynonenal; MDA, malondialdehyde; PN, parenteral nutrition; USP, US Pharmacopeia.

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Results: High levels of PFAT5 were only observed after exposure of the admixture to oxygen. Additional exposure to artificial light led to a parallel increase in the primary and secondary oxidation parameters, while the pH was unchanged.

Conclusions: The admixtures investigated in the former animal studies were obviously both physically and chemically unstable and all effects observed in the studies could just as well be caused by chemical instability, namely the administration of lipid peroxides with the admixture.

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1. Introduction

The stability of lipid emulsions and their admixtures during the infusion period is paramount to the safety of the patients. Thus, the intravenous infusion of lipid emulsions over a certain period of time should comply with pharmacopeia specifications to achieve the potential benefits of the lipid emulsions without the adverse effects consequent to physical or chemical instability.

Physical instability occurs when the net negative charge upon the lipid droplets is reduced by the presence of oppositely charged cations, which are introduced from admixture components such as electrolytes, minerals or amino acids. The domination of the positive attractive forces over the negative repulsive forces favours the coalescence of oil droplets. Infusion of fat globules exceeding the internal diameter of the microvasculature (5 μm) are assumed to induce fat embolism in the lungs, therefore in lipid emulsions the volume-weighted percentage of fat globules greater than 5 μm, the “PFAT5 level”, has to be below 0.05% [1]. The US pharmacopeia (USP), however, does not regulate the PFAT5 limit of admixtures.

The potential toxicity of lipid admixtures with high PFAT5 level has been investigated in several animal studies [2–5]. One of these studies addressed the toxic effects of a high PFAT5 level on the lung. In this study, two admixtures of a lipid emulsion with an amino acid solution were used: One prepared shortly before infusion and one prepared 48 h in advance, leading to the infusion of an “aged”, unstable solution with a high PFAT5 level. Surprisingly, no microvascular thrombosis was reported in the lungs after a 24 h infusion of the unstable admixture to guinea pigs [5]. In contrast, these animals showed an increased cellularity in the lung [5]. This raised the question whether in the respective study a factor other than PFAT5 was responsible for the unexpected type of lung pathology. Interestingly, malondialdehyde (MDA) was also increased in the lungs of the guinea pigs treated with the unstable admixture [5]. In general, MDA results from lipid peroxidation of polyunsaturated fatty acids [6]. It can either arise endogenously during oxidative stress or exogenously from lipid admixtures containing high levels of polyunsaturated fatty acids in the presence of oxygen and/or ambient light during long-term storage [7]. Of note, lipid peroxidation end products are not limited to MDA and also include other aldehydes like 4-hydroxynonenal (4-HNE). Therefore, if MDA was administered to the guinea pigs, the even more toxic 4-HNE was administered as well [8]. Administration of 4-HNE induces mitochondrial dysfunction in human airway epithelial cells in vitro [9] and leads to the accumulation of inflammatory cells in the lung in vivo [10], similar to the observed changes in the guinea pigs [5]. Chemical instability of the admixture infused to the guinea pig therefore is another potential explanation for the observed changes, especially as neither protection from light nor protection from oxygen has been reported in the guinea pig study [5].

We hypothesized that the admixtures infused in the respective animals studies were chemically unstable despite limited time (48 h) of potential exposure to oxygen and artificial light. Therefore we have studied the physical (PFAT5 level) and chemical (pH, primary and secondary oxidation...
parameters) stability of a comparable admixture composed of an injectable lipid emulsion, Intralipid® 20%, and an amino acid solution in different storage conditions and after different ageing times.

2. Materials & methods

2.1. Materials

The admixture analysed in this study is based on the injectable lipid emulsion Intralipid® 20% in 100 mL sterile bottles (batch no. 16HI0261, Fresenius Kabi Austria GmbH, Graz, Austria). The emulsion was stored at 25 °C prior to the experiments. The additional product used for the admixture preparation was the amino acid solution ProcalAmine® (batch no. J4H018, B. Braun Medical Inc., Irvine, CA, USA). This amino acid solution was also used in the experiments performed by Driscoll and coworkers [2–5]. The admixture was prepared by aseptically adding 3 parts of ProcalAmine® and 1 part of Intralipid® 20% into a sterile glass bottle and gently mixing it before exposing to different ageing conditions. The composition of the admixture in comparison to the admixture used by Driscoll and coworkers is given in Table 1.

2.2. Experimental procedure

The admixture was exposed to different ageing conditions and the stability study was performed at different elapsed times. The different ageing conditions resulted from a combination of protection from light and exposure to oxygen. Samples protected from light were completely covered with aluminium foil and stored in a dark place during the study, while samples not protected from light were stored in transparent bottles in the presence of standard laboratory artificial light (approximately 500 lx). Samples exposed to oxygen were left fully open during the study, while samples protected from oxygen were sufficiently flushed with nitrogen immediately after preparation and tightly sealed. These procedures resulted in the four different ageing conditions, which are listed in Table 2.

To simulate very aggressive ageing conditions, like rocking transport, a set of samples underwent stirring at 800 rpm using laboratory magnetic stirrers during the study. This resulted, however, in obviously visible phase separation of the admixtures after 6 h. Such admixtures could easily be excluded from patient administration and thus this condition was not further investigated.

<table>
<thead>
<tr>
<th>Amount in this study (g/L)</th>
<th>Amount in Driscoll et al., 2005 (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amino acids</td>
<td>22.0</td>
</tr>
<tr>
<td></td>
<td>22.5</td>
</tr>
<tr>
<td>Glycerin (from ProcalAmine®)</td>
<td>22.5</td>
</tr>
<tr>
<td>Sodium acetate</td>
<td>1.5</td>
</tr>
<tr>
<td>Magnesium acetate</td>
<td>0.4</td>
</tr>
<tr>
<td>Calcium acetate</td>
<td>0.2</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>0.9</td>
</tr>
<tr>
<td>Potassium chloride</td>
<td>1.1</td>
</tr>
<tr>
<td>Phosphoric acid NF</td>
<td>0.3</td>
</tr>
<tr>
<td>Potassium metabisulfite NF</td>
<td>0.4</td>
</tr>
<tr>
<td>Soybean oil emulsion</td>
<td>50.0</td>
</tr>
<tr>
<td>Water for injection</td>
<td>qs</td>
</tr>
</tbody>
</table>

Table 2
Different ageing conditions of the 3:1 ProcalAmine®/Intralipid® mixture.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Light exposure</th>
<th>Oxygen exposure</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>C2</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>C3</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>C4</td>
<td>Yes</td>
<td>No</td>
</tr>
</tbody>
</table>
The elapsed times (ageing time) studied were 0, 3, 6, 24, and 48 h. The counting was started immediately after submitting the samples to the different ageing conditions.

2.3. Analytical procedure

2.3.1. Physical stability

Evolution of PFAT5 of the admixture was studied, at the different storage conditions and different elapsed times, with an AccuSizer 780, Automatic Particle Sizer APS (Particle Sizing Systems Inc., Santa Barbara, CA). This equipment claims the use of a Single-Particle Optical Sensor (SPOS) Subsystem technique implemented by Light Extinction (LE) and/or by Light Scattering (LS) methods, that assumes to count and to size individual particles as each passes through a thin “optical-sensing zone”. Experimental data from this apparatus and the PFAT5 concept have been questioned as being representative of emulsion physical stability [11]. It is, however, not the focus of this paper to elaborate on this issue and as the method is in accordance with current USP requirements, the apparatus was considered as an appropriate method for the purpose of our study. The size range selected for the calculation of PFAT5 was from 5 μm to 50 μm. All measurements were made in triplicate.

2.3.2. Chemical stability

To assess the chemical stability of the admixture, primary and secondary oxidation parameters, namely Peroxide number (PO) and p-anisidine value (Anv), were determined at the different storage conditions and elapsed times. These parameters were measured using a built-in spectrophotometer (FOODLAB fat, CDR s.r.l, Florence, Italy). This device uses CDRs’ dedicated test kits for each parameter. All test kits provided by the CDR are well calibrated in accordance with valid international standards AOCS (American Oil Chemist Society) controlled by the company Neutron Laboratory (Italy).

Since this device employs pure oil and/or fat in order to determine the oxidation parameters, the oil part of the admixture had to be extracted from the remaining water phase part. For this purpose, we used pure ethanol (99.5%) as an extraction agent. Among other oil/fat extraction methods, such as lyophilization, extraction using ethanol was chosen because of its simplicity, fast extraction time, and ease of handling.

2.3.3. Statistical analysis

All measurements for the physical parameters were made in triplicate, the final value of the peroxide value was taken from a single measurement, and the final value of the p-anisidine value is an average of duplicate measurements. Due to the limited number of values in this exploratory study, no statistical analysis was performed.

3. Results

This section will be divided into two parts. The first part shows the results regarding the physical stability for the admixture in the different storage conditions and ageing times. The second part gathers the results concerning the chemical stabilities.

3.1. Physical stability parameters

Table 3 presents the PFAT5 values of the admixtures for the different storage conditions and elapsed time studied. The evolution of PFAT5 values is also presented in Fig. 1.

According to the results obtained, the highest PFAT5 values were observed following storage conditions C2 and C3, where samples were exposed to oxygen. For these samples, a small oil layer was observed at the top of the admixture after 48 h, visually indicating instability. However, it is important to remark that even for one of these two last conditions (C2), PFAT5 values of the admixture were only slightly outside the USP specification after 24 h elapsed time and that an exponential increase occurred only after 48 h ageing.

The measurement of PFAT5 for admixtures is complex, as there is high probability of bubble inclusions during the admixing, which can be “assigned” as oil droplets and thus contribute to the PFAT5
value. Considering this, the physical stabilities for up to 24 h under clinical storage conditions (C4: exposure to light, but no exposure to oxygen) could be considered as excellent.

3.2. Chemical stability parameters

Studies on the oxidative stability of vegetable oils and their admixtures require the measurement of the evolution of the primary and secondary oxidation products over time. Primary oxidation products of lipid oxidation are determined by the peroxide value during oil production and storage, and also for marketing purposes. Secondary oxidation products, such as aldehydes, carbonyls, trienes, and ketones are determined by the p-anisidine value. Together with the peroxide value, the p-anisidine value can give relevant information about the oxidation process of the oil [12].

Tables 4 and 5 show the peroxide values and the p-anisidine values, respectively, as a function of ageing conditions and elapsed time for the admixtures studied. As can be seen in these tables, both oxidation parameters seem to be affected by the condition “exposure to oxygen” in combination with “exposure to artificial light” for ageing times longer than 24 h. This effect is also clearly depicted in Figs. 2 and 3.

A relevant influence of the condition “exposure to oxygen” on the p-anisidine values is also observed when it is combined with the condition “exposure to artificial light” (see Table 5 and Fig. 3). Regarding the pH values, there are not significant changes during ageing time at any of the storage conditions studied.

Table 3

<table>
<thead>
<tr>
<th>Storage condition</th>
<th>Ageing time (h)</th>
<th>0</th>
<th>3</th>
<th>6</th>
<th>24</th>
<th>48</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>0.030</td>
<td>0.008</td>
<td>0.007</td>
<td>0.131</td>
<td>0.914</td>
</tr>
<tr>
<td></td>
<td>SD</td>
<td>0.009</td>
<td>0.001</td>
<td>0.001</td>
<td>0.007</td>
<td>0.069</td>
</tr>
<tr>
<td>C1 without light/</td>
<td>Mean</td>
<td>0.032</td>
<td>0.039</td>
<td>0.049</td>
<td>0.062</td>
<td>3.180</td>
</tr>
<tr>
<td>without oxygen</td>
<td>SD</td>
<td>0.002</td>
<td>0.003</td>
<td>0.002</td>
<td>0.006</td>
<td>0.050</td>
</tr>
<tr>
<td>C2 without light/</td>
<td>Mean</td>
<td>0.015</td>
<td>0.023</td>
<td>0.017</td>
<td>0.660</td>
<td>3.710</td>
</tr>
<tr>
<td>with oxygen</td>
<td>SD</td>
<td>0.002</td>
<td>0.001</td>
<td>0.005</td>
<td>0.039</td>
<td>0.042</td>
</tr>
<tr>
<td>C3 with light/</td>
<td>Mean</td>
<td>0.022</td>
<td>0.038</td>
<td>0.039</td>
<td>0.067</td>
<td>0.250</td>
</tr>
<tr>
<td>with oxygen</td>
<td>SD</td>
<td>0.002</td>
<td>0.003</td>
<td>0.004</td>
<td>0.005</td>
<td>0.012</td>
</tr>
</tbody>
</table>

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Fig. 1. Evolution of the PFAT5 values of the admixture with ageing time for different storage conditions.
4. Discussion

The stability and quality of parenteral nutrition (PN) products like lipid emulsions and their admixtures during the infusion period is very important for the safety of the patients. Infusion of unstable lipid emulsions and admixtures may lead to severe side effects, especially in critically ill patients. Therefore, it is important to make sure that the PN products comply with the pharmacopeia specifications. According to the USP, lipid injectable emulsions should have a pH between 6 and 9, and the maximum values of oil mean droplet diameters must be lower than 0.5 μm. In regard to the permitted percentage of fat globules larger than 5 μm (PFAT5), the USP has limited it to a maximum value inferior
to 0.05% of the total fat globules. It has been suggested that admixtures should also meet these requirements [13], as they are intended to be administrated intravenously; however, the USP does not mention this specific case [1]. According to this, the current study was performed to determine how external ageing conditions and elapsed time could affect the final quality/stability of the admixture of a lipid emulsion with an amino acid solution.

Our results clearly show that a remarkable increase in PFAT5 levels within 48 h after preparation only occurs in the respective admixture after exposure to oxygen. If exposure to oxygen is combined with exposure to artificial light, increases in PFAT5 levels coincide with increases in primary and secondary oxidation parameters and thus increased levels of aldehydes like MDA and 4-HNE. The most relevant investigations regarding the toxicity of high levels of PFAT5 include three rat studies and one study in guinea pigs [2–5]. None of these studies reported protection of the samples from light or from oxygen. It is therefore reasonable to assume that the infused admixtures of those studies both showed a high PFAT5 as well as a high lipid oxidation level. Here we discuss the results of the former animal studies in light of this new finding.

First of all, as mentioned in the introduction, 24 h after infusion of an admixture with a high PFAT5 level, diffuse cellular infiltrates as well as increased levels of MDA were observed in the lung, but not in the liver of guinea pigs [5]. According to our results, those diffuse cellular infiltrates in the lung are more likely caused by 4-HNE, an end product of lipid oxidation. Our study has the limitation that an absolute quantification of the levels of single lipid oxidation products in the aged admixture is not feasible due to the indirect measurement in form of the peroxide value, a common stability parameter of lipid emulsions. Nevertheless, the fact that MDA is present in both lung and liver of the guinea pigs in the micromolar level (up to 73 micromol per gram of wet tissue), rather than in the nanomolar level as observed in other studies of oxidative stress [14], further corroborates the hypothesis that MDA and other lipid peroxidation products were rather infused than endogenously produced.

In addition to the study in the guinea pig, lipid admixtures with high levels of PFAT5 were also investigated in several studies in rats [2–4]. The rats showed increased plasma levels of the enzymes asparagine amino transferase (AST) and glutathione-S-transferase (GST), as well as increased liver values of MDA after 24 h infusion of an “unstable” admixture [2]. After 72 h infusion, the MDA levels of the liver were still increased, and same was true for AST in plasma [4]. The changes observed in these studies were interpreted as hepatotoxic effect, mainly based on increased plasma levels of AST and GST, and higher levels of MDA in the liver [4,5].

Of note, AST is a sensitive, but unspecific liver parameter [15]. The specific liver parameter alanine amino transferase (ALT) was not increased in any of the rat studies [2,4]. In humans, an in vivo isolated elevation of AST is usually due to injury to non-liver cells, mostly muscle cells [16]. Appropriately, fixation of animals during an experiment leading to a minor extent of muscle injury can cause increases in AST activity without increases in ALT activity [17]. Of note, the reference values for plasma AST in Sprague-Dawley rats range from 55.0 to 180.6 U/L with a mean value of 117.8 U/L (http://www.envigo.com/resources/data-sheets/sprague-dawley-6-8-wk-males-cbc.pdf). The plasma levels measured in

*Fig. 3. Evolution of the anisidine values of the admixtures as a function of ageing time for the different storage conditions (n = 2).*
the respective studies were out of the reference range in both groups 24 h after start of treatment with partially high interindividual variability [2], which might be due to unintentional differences in handling and fixation. This is further corroborated by the fact that, after 72 h [4] of continuous infusion, the AST plasma levels of the rats are within the normal range in both treatment groups.

Another plasma parameter of liver injury determined in the rat was the enzyme GST [2]. In general, GST is responsible for conjugation of substances like lipid peroxides (MDA, 4-HNE) with glutathione to detoxify them [18]. GST-alpha is a specific isoenzyme in the liver, and its elevation in plasma is commonly interpreted as a biomarker for liver injury. In the respective study, total GST and not GST-alpha was investigated. The increase in plasma GST could therefore be based on increases in GST in almost all organs or even erythrocytes, which have been shown to possess increased levels of GST during hyperlipemia similar to that evoked by administration of a high volume of lipids as in the rat studies [19]. Furthermore, an assumed increase in plasma GST could easily be explained in groups receiving a high level of lipid peroxides like 4-HNE and MDA as in the “unstable” admixture group [18]. High levels of lipid peroxides lead to high intracellular levels of the detoxifying enzyme GST and thus in animals receiving high amounts of lipid peroxides, any single necrotic cell will release more GST than a cell that does not have to deal with high levels of lipid peroxides.

As mentioned for the lung of the guinea pigs, the high MDA level in the liver of animals receiving the “unstable” admixture is most likely due to administration of exogenous MDA with the lipid admixture. In summary, none of the reported changes are indicative of liver injury and specific parameters indicative of liver injury were either unchanged (ALT) or not investigated (for example GST-alpha, bilirubin, etc.).

In addition to the parameters of liver injury, parameters of inflammation were determined in the rat. Apart from the fact that administration of lipid peroxides would result in oxidative stress and thus an inflammatory reaction of the body is expected, the selection and evaluation of these parameters has already been criticized [20] and is not in the focus of this discussion.

In conclusion, none of the three animal studies provides unambiguous evidence for an adverse effect resulting from high levels of PFAT5 in a lipid admixture. In contrast, an increase in PFAT5 levels to up to 1% did not change plasma total GST levels significantly after 96 h of infusion [3], but merely led to slight changes in the unspecific parameter plasma AST (see above). This calls into question the applicability of the pharmacopeia PFAT5 limit of 0.05% for admixtures. An animal study with admixtures exclusively consisting of a high PFAT5 or a high lipid oxidation value could substantiate our results and help to elucidate the actual cause of the observed changes. Nevertheless, our results show already today that if admixtures with Intralipid® 20% are kept under good storage conditions without exposure to oxygen, they can be well preserved and considered safe at least until 24 h after preparation.

Statement of authorship

Melanie K. Bothe: Design of the study, interpretation of data, drafting of article.
Lida-Andrea Quinchia-Bustamante: Design of study, acquisition of data, drafting and critical revision of article.
Getachew Assegehegn: Design of study, acquisition of data.
Crispulo Gallegos-Montes: Design of study, critical revision of article.
Edmundo Brito de la Fuente: Design of study, critical revision of article.
Johannes Harleman: Design of study, critical revision of article.

Conflict of interests statement and funding sources

MKB, LAQ, GA, CGM, EB DLF and JH are all employees of Fresenius Kabi Deutschland GmbH.

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References


