Influence of the Calcium Concentration in the Presence of Organic Phosphorus on the Physicochemical Compatibility and Stability of All-In-One Admixtures for Neonatal Use

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ABSTRACT

Background

Preterm infants need high amounts of calcium and phosphorus for bone mineralization, which is difficult to obtain with parenteral feeding due to the low solubility of these salts. The objective of this study was to evaluate the physicochemical compatibility of high concentrations of calcium associated with organic phosphate and its influence on the stability of AIO admixtures for neonatal use.

Methods

Three TPN admixture formulas were prepared in multilayered bags. The calcium content of the admixtures was adjusted to 0, 46.5 or 93 mg/100 ml in the presence of a fixed organic phosphate concentration as well as lipids, amino acids, inorganic salts, glucose, vitamins and oligoelements at pH 5.5. Each admixture was stored at 4 °C, 25 °C or 37 °C and evaluated over a period of 7 days. The physicochemical stability parameters evaluated were visual aspect, pH, sterility, osmolality, peroxide formation, precipitation, and the size of lipid globules.

Results

Color alterations occurred from the first day on, and reversible lipid film formation from the third day of study for the admixtures stored at 25°C and 37°C. According to the parameters evaluated, the admixtures were stable at 4°C; and none of them presented precipitated particles due to calcium/phosphate incompatibility or lipid globules larger than 5 μm, which is the main parameter currently used to evaluate lipid emulsion

stability. The admixtures maintained low peroxide levels and osmolarity was appropriate for parenteral administration.

Conclusions

The total calcium and calcium/phosphorus ratios studied appeared not to influence the physicochemical compatibility and stability of AIO admixtures.

Keywords: stability, total parenteral nutrition, neonates, globule size, calcium, phosphate.

BACKGROUND

Preterm infants, children born before 37 weeks of pregnancy, are the major recipients of parenteral nutrition therapy. This group is frequently intolerant of enteral feeding due to anatomic and functional immaturity of the digestive tract, added to other clinical conditions that affect cardiovascular function in the preterm post-natal life[1-3]. These patients need a calcium/phosphorus ratio higher than 1 (42 mg/Kg/d Ca and 36 mg/Kg/d P) in order to allow bone mineralization. Studies on calcium and phosphorus retention in neonates propose that 1.7/1 (75 mg/Kg/d Ca and 45 mg/Kg/d P) calcium/phosphorus ratio is closer to that observed for intra-uterine life, allowing greater retention of these ions [4]. This ratio is difficult to obtain with parenteral feeding, because the availability of these ions offer is limited by their solubility [5].

Considering the low volumes used in neonatology, the most daunting problem today for pharmaceutical preparation practices of parenteral nutrition is related to ensuring both physicochemical compatibility and adequate calcium and phosphorus supply. The compatibility depends on factors such as solution pH, temperature, material of the parenteral nutrition container, oxygen, light exposure, composition of trace elements, presence of vitamins, peroxidation, relative concentration of each ion, and order of addition of divalent ions such as calcium [6, 7].

Elevated concentrations of cationic electrolytes such as calcium also interfere in the total parenteral nutrition (TPN) admixture stability and may influence directly the size of lipid globules, contributing to the formation of unstable phases in the lipid emulsion (LE) such as aggregation, flotation, coalescence and phase separation [8]. The main parameter currently used to evaluate lipid emulsion physicochemical stability is the percentage of particles larger than 5 μm. According to pharmacopeial specifications, this

81 should not exceed a total of 0.05% of lipid globules in the admixture [9, 10]. Lipid
82 globules of this size may clog the pulmonary capillaries, producing an embolic syndrome
83 and causing cell death mainly in preterm infants. In addition, an incompatibility between
84 calcium and phosphorus parenteral salts, forming an insoluble dibasic calcium
85 phosphate precipitate, may similarly produce a potentially fatal respiratory arrest [11].

86 Organic phosphate was introduced on the pharmaceutical market about two
87 decades ago, and was immediately approved in some European countries. Due to the
88 high cost, its use was restricted to patients such as preterm infants who needed high
89 calcium and phosphorus concentrations [12]. The use of organic phosphate is not
90 permitted in the United States, so compatibility and stability studies on the TPN
91 admixture in this country are exclusively associated with preparations containing
92 inorganic phosphate [13, 14].

93 Since the introduction of organic phosphate on the market, some authors have
94 shown, in clinical comparative studies, the benefits of the use of organic phosphate in
95 relation to inorganic phosphate. However, there are divergences concerning the
96 utilization of this product, mainly in regard to the physicochemical TPN admixture
97 compatibility with high calcium levels, as well as bioavailability. In addition there is still
98 the possible interference of high calcium concentrations, which may also affect the
99 emulsion stability.

100 Due to the clinical relevance of compatibility with high calcium and organic
101 phosphate concentrations and emulsion stability issues posed by the action of calcium
102 and other cationic electrolytes upon negatively charged lipid droplets, this paper was
103 aimed at evaluating these factors in neonatal all-in-one admixtures. Compatibility and
104 stability were measured over time at different temperatures, with a view to detecting the

105 formation of dibasic calcium phosphate precipitate, variations in the size of the lipid
106 droplets, peroxide formation, pH changes, and visual aspect. The goal was to attain a
107 higher safety level for these preparations and safe concentrations in TPN admixtures.
108 We also tested the safety of the proposed admixtures in this work by comparing the
109 measured osmolarity (a factor in venous phlebitis), the level of lipid peroxide formed (a
110 factor in toxicity) as a function of temperature and natural light exposure during the
111 storage over time, and sterility of the formulations as assessed immediately after mixing.
112

112 **METHODS**

113 **Preparation of the admixtures studied**

114 Three TPN admixture formulas for neonatal use were prepared aseptically in 300-
115 ml multilayered bags (Halex Istar, Goiânia, Brazil) under a laminar-flow hood in
116 accordance with the National Health Department specifications Nr. 272 (1988), designed
117 for infusion through central access. The TPN admixture formulas were prepared with
118 market products from pharmaceutical industries (**Table 1**) and based on official
119 regulations [15]. The three formulas, PC, P1 and P2, differ in calcium content according
120 to **Table 1**. The calcium/phosphorus ratios for P1 and P2 admixtures are (calcium and
121 phosphorus) 2/1 and 4/1, respectively, and the control parenteral nutrition (PC) is free of
122 calcium. Thus the calcium content of the admixtures was different in each formula, while
123 the organic phosphate content was the same in the concomitant presence of vitamins
124 and oligoelements.

125

126 **Experimental Procedures**

127 Each admixture was prepared in triplicate or quadruplicate (refered to as 3 or 4
128 lots) divided into three groups and stored at different temperatures: 4 °C ± 2 °C, in
129 refrigerator; 25 °C ± 3 °C, simulating room temperature; and in an incubator at 37 °C ± 2
130 °C, simulating an infusion temperature in a worst-case scenario. The experiments were
131 performed on the day of the TPN admixture preparation and also 24 h, 48 h, 72 h and 7
132 days after preparation, time periods indicated as D0, D1, D2, D3 and D7, respectively.

133 Samples for the sterility test and for the physicochemical tests were aseptically
134 collected from each formulation at appropriate intervals using a plastic syringe. The

physicochemical compatibility and stability parameters evaluated were general visual aspect, presence of precipitation, pH, sterility, osmolarity, peroxide formation, and the size of lipid globules.

Sterility test

The maintenance of the TPN admixture sterility was evaluated using a biphasic culture medium, which contains a liquid phase composed of sterile soybean casein broth and a solid phase composed of sterile soybean casein agar (TSA/TSB). This culture medium promotes growth of non-specific aerobic microorganisms and is used in accordance with recommendations of USP 31, chapter 71 [15]. The analysis is based on inoculation of a TPN admixture sample (2 ml) in a flask containing the biphasic culture medium at the end of the manipulation of each bag. This flask was immediately stored after inoculation in an oven at 30 °C ± 2 °C for a 14-day period. At intervals (24 h) during the incubation period, the media were examined for macroscopic evidence of microbial growth in the solid phase. If no evidence of microbial growth was found after 14 days the product complies with the test for sterility. This procedure was carried out in all admixture lots.

Physicochemical assessments

On visual inspection, parameters that evidence instability and incompatibility events were examined. Color change, precipitation, film formation and phase separation were sought. The color change assay was not graduated; instead, a positive response was registered when darkening occurred. Precipitation was evaluated visually by

158 observing formation of rigid crystalline structures. The stability of the emulsion, on a
159 visual basis, was assessed by the observation of a yellow fat layer on the top of the
160 admixture, referred to as film formation (creaming). The film thickness was measured
161 using a ruler with millimeter precision. Phase separation was recorded when two
162 different phases could be observed. The visual inspection was performed in all the
163 admixtures during the seven days of study at all temperatures and was registered
164 through digital photography.

165 For the evaluation of pH, a Mettler Toledo potentiometer calibrated with pH 4 and
166 pH 7 buffers was used. For each measurement, a 10 ml sample was collected and
167 placed in an amber glass flask. The pH was measured by dipping the electrode directly
168 into the emulsion, at room temperature. The visual inspection and pH determination
169 were carried out each day in quadruplicate for each formulation in all the conditions
170 studied.

171 The osmolalities were measured experimentally using a Wescor micro-
172 osmometer. The TPN admixture osmolality is determined through its freezing point.
173 Before each measurement session, the equipment was calibrated with standard
174 solutions of 100, 290 and 1000 mOsm/Kg H_2O, composed of NaCl and preservative in
175 water. The equipment range is from 0 to 3000 mOsm/Kg H_2O. The osmolality (mOsm/Kg
176 H_2O) was converted into osmolarity (mOsm/l H_2O) using the density of the TPN
177 admixtures measured with a pycnometer. The mean density of the admixtures studied
178 was 1.04 g/ml at 25 °C. The osmolarity of the TPN admixtures was estimated by
179 multiplying the measured osmolality by the TPN admixture density and subtracting the
180 concentration (g/ml) of the solutes present in the emulsion, according to Martin *et al.* [16]
181 The concentration of the solutes calculated for the TPN admixture was 0.2 g/ml at the

182 highest concentration of calcium. The measurements were carried out on three different
183 lots on day 0 for each formulation at each temperature condition.
184
185 **Globule size measurement**
186 The size of the lipid globules was measured with an Axioplan 2 optical
187 microscope (Carl Zeiss, Germany) using a digital camera connected to a computer and
188 a television set, at a total magnification of 1000x. This combination makes it possible to
189 measure globules as small as 0.2 µm. A 5-ml sample of TPN admixture was collected
190 from the bag with a sterile syringe and placed in a sterile vacutainer. From this
191 vacutainer, a 20-µl sample was collected and diluted 1:4 using its own admixture but
192 without lipids. From this dilution, a 6-µl sample was transferred to a slide with an
193 automatic pipette and then an 18 x 18 mm cover slip was laid over it. Initially different
194 fields were examined to investigate incompatibility based on the presence of dibasic
195 calcium phosphate crystals in the sample. When crystals were not found, evaluation of
196 the lipid globule stability was initiated. Between 1000 and 1500 lipid globules per field
197 were observed and counted in each of two fields in each admixture sample. The
198 measurement was carried out with two different lots of each formulation for each of the
199 studied conditions. For each lot, images of three randomly chosen fields were registered
200 for posterior analysis by the software. Results are presented in **Figure 1** as a
201 representative field from a set of three collected from each of two different lots, on
202 different days. The globules are grouped according to size range: 0-1 µm; 1-2 µm; 2-3
203 µm and > 3 µm, and the percentage in each range is calculated in relation to the total

204 number of measured globules (**Figure 2**). Maximum globule diameter was also recorded
205 (**Table 5**).

206 The images were collected and kept for further analysis. The images were treated
207 with the *AnalySIS* software (Soft Imaging System, Münster, Germany), which converts
208 the images into gray scales and then into a black and white binary system, allowing the
209 identification and counting of the lipid globules.

210

211 **Peroxide formation**

212 Peroxide formation in lipids was quantified by evaluating the oxidation of iron in a
213 xylenol orange solution (Spectrum, New York, USA), modified for use with lipids
214 according to the FOX 2 method previously described [17, 18]. The method is based on
215 the reaction of 2-thiobarbituric acid (TBA) with aldehydes formed by oxidized lipids in
216 acid medium. The reaction is processed after 30 min, followed by centrifugation. This
217 reaction forms a red-colored product, which is read at a wavelength of 560 nm in a
218 Shimadzu model UV 2401PC spectrophotometer. With this methodology we tested all
219 the admixtures during seven days at all temperatures, as well as the admixtures
220 contained in bags with photo protection at 25 ℃.

221 The peroxide concentration in the admixture samples was calculated from a
222 hydrogen peroxide standard curve containing five known concentrations (0, 1, 2, 3, 5
223 and 7 μM). The hydrogen peroxide standard curve was linear and reproducible (n= 3),
224 with deviations as follows: slope (b) = 0.2926 $\pm$ 0.07910; intercept (a) = 0.1678 $\pm$
225 0.01164 and r^2 = 0.9985 $\pm$ 0.0006897. The experiments were carried out with 3 different
226 lots (i.e. in triplicate).

227

228 **Statistical treatment**

229 The results are expressed as mean and standard deviation. The graphical

230 displays were created on SigmaPlot 10.0 (SPSS, Erkrath, Germany). The experimental

231 results were analyzed for significance using Student's *t* test at $P < 0.05$.

232

232 **RESULTS**

233 The following parameters were evaluated through visual inspection: color
234 alterations, precipitation, film formation, and phase separation, for the three admixtures
235 studied over a period of seven days at temperatures of 25 °C, 4 °C and 37 °C, as
236 demonstrated in **Table 2**. After 24 h at temperatures of 25 °C and 37 °C, color alteration
237 was observed in all the formulations. On the other hand, there was no color alteration at
238 any time for admixtures at 4 °C. No precipitation due to incompatibility was observed in
239 any of the admixtures. The formation of a film layer, the first step in a phase separation
240 process, was observed only after 72 h and only for admixtures maintained at 25 °C and
241 37 °C. It is worth emphasizing that formation of this film was easily reversed with a
242 smooth shake. No phase separation was observed in any of the admixtures.

243 There were no colonies forming units (CFUs) on the solid phase in the culture
244 medium after fourteen days of incubation in an oven at 30 °C. The absence of CFUs in
245 the biphasic culture medium demonstrates the maintenance of the admixtures' sterility.

246 **Table 3** shows the mean and standard deviation of pH values for TPN admixtures
247 during the seven days of study at the three temperatures selected. In all cases the pH
248 remained around 5.5 throughout the study period, demonstrating no statistically
249 significant alteration between the values of D7 and D0 in each condition tested ($P < 0.05$,
250 $n = 3$).

251 The measured osmolalities were converted into osmolarity as described in
252 Methods. The osmolarity values for TPN admixtures are presented in **Table 4**. The
253 results are presented separately according to temperature, since each TPN admixture
254 corresponds to a different lot. The mean values showed no significant differences with
255 temperature, also demonstrating homogeneity among the lots ($P < 0.05$, $n = 3$).

256 The presence of particles in the admixtures was evaluated by optical microscopy
257 (OM). Particles are easily recognized due to the rigid crystalline structure of the
258 precipitates. No evidence of precipitation was observed in the admixtures under any of
259 the conditions studied.

260 Among parameters obtained through OM, the one selected to evaluate the
261 admixtures' stability was the diameter of the lipid globules. This parameter provides an
262 index of the size of the lipid globules, making it possible to monitor size changes due to
263 temperature and type of admixture over time. **Figure 1** represents the mean diameter of
264 the lipid globules over the seven days; maximum values are shown in **Table 5**,
265 demonstrating that no globules greater than 4 μm were formed.

266 **Table 5** serves to emphasize that occasionally a larger lipid globule could be
267 found, regardless of the day of analysis, as for example in D0, where there is a 4 μm
268 lipid globule in admixture P2.

269 **Figure 2** shows the distribution of globule size range in percent of total number of
270 globules measured under each condition on days 0 and 7. None of the lipid globules
271 exceeded 4 μm.

272 Other optical microscopy parameters related to stability such as Ferret diameter,
273 area, sphericity, perimeter, convex perimeter, format factor and stretching were
274 measured. There was no significant alteration in any of these parameters for any of the
275 admixtures with time or temperature.

276 The peroxide level described as safe for lipid emulsions in TPN is 500 μM [8]. PC,
277 P1 and P2 admixtures were tested for peroxide at days D0, D1, D2, D3 and D7, at each
278 temperature, and at 25 °C with and without photo protection.

279 The concentrations of peroxide formed in each admixture during seven days at
280 each temperature are presented in **Figure 3**. None of the values approached the
281 permitted limit of 500 µM. Statistical analysis of values obtained at D0 and D7 revealed
282 a significant increase ($P < 0.05$) in all formulations stored at 25 °C (**Figure 3 A**).
283 However, at 37 °C, only PC showed a significant alteration (**Figure 3 C**) and at 4 °C,
284 none of the admixtures showed a significant alteration (**Figure 3 B**). Peroxide was not
285 detected in admixtures with photo protection studied at 25°C on days D0 and D1. The
286 peroxide values on day D2 were: 0.72, 0.02 and 0 µM, on day D3: 1.20, 0.40 and 0.62
287 µM, on day D7: 0.32, 0.30 and 0 µM, respectively for PC, P1 and P2 admixtures. The
288 values obtained with photo protection are significantly lower than those observed at the
289 same temperature, under light exposure (**Figure 3 A**); with photo protection, peroxide
290 levels observed at 25 °C were of the same order of magnitude as those in admixtures
291 kept at 4 °C in absence of the light (cf. **Figure 3 B**).
292

292 **DISCUSSION**

293 A search for more effective means of supplying calcium and phosphate in TPN
294 has led in the last decade to the publication of many clinical papers demonstrating the
295 advantages of using organic phosphorus [19-22]. Among these publications, the work of
296 Devlieger and cols [4], stands out; a group of 28 preterm patients was studied, of whom
297 15 received TPN admixture with a high dose of organic calcium and phosphorus, while a
298 second group of 13 patients received lower amounts of organic calcium and inorganic
299 phosphorus. The calcium retention of the group that received high organic calcium and
300 phosphorus was much greater, significantly improving bone mineralization.

301 In addition to the concern of offering a nutritionally efficient TPN admixture, the
302 physicochemical stability of these formulations has been discussed. Lipid emulsions are
303 heterogeneous dispersions composed of two immiscible phases (oil-in-water) and
304 sensitive to many instability processes such as aggregation, flotation, coalescence and
305 phase separation, according to the second law of thermodynamics. However, their
306 physical stability can be improved by using surfactants that form a coalescence energy
307 barrier that carries electric charges around the dispersed liquid, aiming at decreasing the
308 surface tension and increasing the repulsion force between the dispersed liquids [23]. In
309 the case of TPN admixtures containing egg lecithin as anionic emulsifier, the lecithin
310 produces a negative charge around the lipid globules through the ionization of the
311 phosphate groups. Any positively charged ion, such as calcium, could cause instability
312 in this system, neutralizing the negative phospholipid droplets and promoting
313 coalescence. This process is irreversible [8].

314 Therefore, the pH of the final admixture is important as a factor that directly
315 influences the ionization of the lipid globule phospholipids, and also interferes in the

316 dissociation of ions in solution. Very low pH values influence the surface charge of the
317 globules, increasing the surface tension between them and consequently threatening
318 the stability of the emulsion. Many factors determine the final pH of the TPN admixture
319 [9]. These factors are mentioned here in order of relevance: composition, final
320 concentration of the amino acids, type and final concentration of the phosphate, cysteine
321 addition, and final glucose concentration. The pH for pediatric TPN admixtures ranges
322 from 5.0 to 6.0, and the admixtures contain high concentration of amino acids such as
323 cysteine, histidine and arginine, as well as a high concentration of glucose [13, 26]. The
324 pH of the admixtures studied remained within this range with no significant alterations
325 over time, showing that the high calcium/phosphorus ratio in the admixture and the
326 temperature of storage had no adverse effect on pH.

327 All the admixtures tested were free of crystal formation, such as dibasic calcium
328 phosphate, an insoluble salt. Thus no evidence of incompatibility between calcium and
329 organic phosphorus was observed, even at highest calcium concentration tested.

330 The main parameter currently used to evaluate LE stability is the percentage of
331 globules larger than 5 μm. This value should not exceed 0.05% of the total number of
332 lipid globules in the admixture [9]. An increase in diameter of the lipid globules implies a
333 progression toward of the LE. No lipid globule larger than 4 μm was detected by OM in
334 any of the admixtures during the seven days of analysis at any temperature, even in the
335 presence of high amounts of calcium. The maximum globule size observed, shown in
336 **Table 5**, represents a vanishingly small percentage when compared to the total
337 numbers of globules measured, which is the reason that a percentage of this range
338 cannot be visualized in **Figure 2**. The values observed were well below the

339 recommended limit; however, the could occur sporadically, such that it would be
340 important to connect filters in line with the infusion equipment in order to ensure
341 uniformity in the size of the lipid globules infused in these preterm infants. The other size
342 parameters quantified through OM showed a profile similar to the mean diameter, with
343 no significant alteration during the study. OM is an adequate technique for the qualitative
344 evaluation of TPN admixture stability and its profile, since many randomly selected fields
345 can be considered in the analysis. However, it fail to predict the quantitative evaluation
346 of the lipid stability ($PFAT_5$) of the admixtures overall due the small amount of sample
347 employed in the OM analyses. So, these results need be considered within the
348 limitations of the technique and require confirmation by light obscuration.

349 Visual inspection is a simple method to evaluate the LE physicochemical
350 compatibility, but extremely important. Color alteration, presence of precipitate and
351 instability phases was evaluated in this step. Color alteration in TPN is well known due
352 to the Maillard reaction and oxidation of some vitamins [6, 12]. It is undesirable because
353 it may represent a bioavailability, loss for some nutrient [5]. In the present study the color
354 alteration that occurred was unrelated to the concentration of calcium, since the PC
355 admixture (without calcium) showed the same change as P2. Color alteration was
356 observed in admixtures stored at 25 °C and 37 °C, but no alteration was observed when
357 stored at 4 °C. Color alteration is related not only to temperature, but also to light, which
358 is the reason for the great difference in color between bags kept at 25 °C, in room
359 temperature, and at 4 °C, in the refrigerator. In the bag stored at 25 °C with photo
360 protection, no color alteration was observed during the days of study (data not shown).
361 These results indicate that the ideal storage condition is 4 °C, with photoprotetion,
362 regardless of calcium. The data suggest that the admixtures should not be kept for a

long period at 25 °C before use, and that equipment with photo protection should be used.

The maintenance of the sterility of the admixtures studied in this paper was fundamental for us to attribute the alterations to possible incompatibility or lipid emulsion instability events due to the high calcium concentration, and not to changes caused by microbiological contamination.

The measurements of osmolarity provided an initial characterization of the admixtures that determines the type of suitable intravenous access for the TPN admixture; this access may be either peripheral or central. For peripheral access, smaller veins are used and glucose concentrations were limited to below 12.5%. For this reason, high caloric values cannot be provided through this type of access [24]. For central access permits the administration of high glucose concentrations, i.e., hyperosmolar solutions, with only minor inconvenience. The solution infused is diluted by the intense blood flow in that area, occasionally reaching adult osmolarity levels up to 3000 mOsm/L. In neonatology, the osmolarity level must be kept around 800 mOsm/L, not exceeding twice the regular serum osmolarity [25]. High osmolarity arises from high ion concentrations as well as glucose, and the ionic components may also affect the lipid globule stabilization. In this study, the experimental osmolarities were kept within the recommended range for the central access administration of the admixtures studied; and as expected, no significant differences in osmolarities were observed at different temperatures.

The peroxide formation caused by exposing TPN admixtures to phototherapy is well-known in the neonatology field [18]. The infusion of oxidized lipids and secondary peroxidation products can be extremely cytotoxic, and may cause undesirable effects

387 such as an increase in vascular resistance in the lung, among others [20, 27]. The
388 recommended limit for LEs is 500 μM [7]. The peroxide quantification was performed
389 with photo protection at 25 °C due to the high values obtained (similar to 37 °C) when
390 stored at 25 °C with incident room light, with no sun exposure. Despite the fact that all
391 peroxide values measured were much lower than the recommended limit (**Figure 3**), the
392 photo- protected admixtures presented, even lower peroxide values, close to zero,
393 different from observed in **Figure 3**. This observation reinforces the necessity of photo
394 protection for the TPN admixture infusion equipment.
395

CONCLUSION

In conclusion, we have demonstrated that, according to the parameters evaluated, the high calcium/phosphorus concentration was compatible. The physicochemical stability of the AIO admixtures studied appeared to be stable. None of the three admixtures presented a peroxide level greater than 500 µM. There was no significant pH alteration during the study period. The preliminary results suggest that the admixtures studied did not have abnormal particle or globule counts, and thus, were compatible and stable by OM, but the fact that visual changes were noted in the color and the extent of the lipid film formation in the admixtures stored at 25 °C or 37 °C may suggest otherwise. As our data are preliminary with regard to particle or globule size analysis in neonatal AIOs, they will require confirmation that by light obscuration to confirm these admixtures are indeed compatible and stable, and thus safe for parenteral administration.

LIST OF ABBREVIATIONS

TPN - total parenteral nutrition;

LE - lipid emulsion;

PC - control parenteral nutrition;

P1 – parenteral nutrition 1;

P2 – parenteral nutrition 2;

TSA/TSB - liquid phase composed of sterile soybean casein broth and a solid phase composed of sterile soybean casein agar;

CFUs - colonies forming units;

OM - optical microscopy;

AIO – all-in-one.

421 **COMPETING INTERESTS**

422 The authors declare that they have no competing interests in relation to this study.

423

424 **AUTHORS' CONTRIBUTION**

425

426 DRO developed the study design under the supervision of VPS and NMV. BWL and
427 VFV performed the microscopic measurements. LMC contributed to the data
428 interpretation and review of the manuscript. VPS and DRO had primary responsibility for
429 writing the manuscript, but all the others authors provided comments on the draft.

430

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501

501 **FIGURES**

502 **Figure 1 - Number of globules counted for each bar, use of OM.**

503 Bar graphs represent the mean and standard deviation of the diameter of lipid globules

504 present in admixtures PC, P1 and P2 stored at 25 °C (A), 4 °C (B) and 37 °C (C), during

505 seven days of study D0 – D7 (n= 2 lots).

506

507 **Figure 2 - Percentage of lipid globules in relation to total number of globules**
508 **examined, distributed by size range.**
509
510 Bar graphs represent the mean percentage of lipid globules in each range present in

511 admixtures PC, P1 and P2 stored at 25 °C, 4 °C and 37 °C after 0 and seven days of

512 study (n= 2 lots).

513

514 **Figure 3 - Peroxide formation in the admixtures.**

515 Bar graphs represent the mean and standard deviation of the peroxide (TBARS) formed

516 in admixtures PC, P1 and P2 stored at 25 °C, 4 °C and 37 °C during seven days of

517 study D0 – D7. (n= 3 lots; *P< 0.05, D7 compared with D0).

518

519

520

520

521 **Table 1 - Formulations used in the study.**

Insumes	Daily allowance	PC	P1	P2
Pediatric amino acid with taurine 10%	1–3 g/kg/d	3 g	3 g	3 g
Glucose 50%	4–12 mg/kg/min	8.64 g	8.64 mg	8.64 mg
Lipid emulsion 20% MCT/LCT*	1-3 g/kg/d	2 g	2 g	2 g
NaCl* 20%	3-5 mEq/kg/d	4 mEq	4 mEq	4 mEq
KCl* 10%	2-3 mEq/kg/d	2 mEq	2 mEq	2 mEq
Calcium gluconate	200-800 mg/kg/d	0	500 mg	1000 mg
Calcium	18.6-37.2 mg/kg/d Ca	0	46.5 mg	93.0 mg
Sodium glycerophosphate	1-2 mEq/kg/d	1.1 mEq	1.1 mEq	1.1 mEq
Magnesium sulphate	0.25-0.5 mEq/kg/d	0.25 mEq	0.25 mEq	0.25 mEq
Pediatric element + zinc acetate	Zn- 400 μg/kg/d	350 μg	350 μg	350 μg
	Cu- 20 μg/kg/d	20 μg	20 μg	20 μg
	Cr- 0.2 μg/kg/d	0.2 μg	0.2 μg	0.2 μg
	Mn-1.0 μg/kg/d	2.0 μg	2.0 μg	2.0 μg
Multivitamin	A– 2500 IU/d	0.128 mg	0.128 mg	0.128 mg
	D- 250 IU/d	0.5 μg	0.5 μg	0.5 μg
	E- 1.67 mg/d	1.0 mg	1.0 mg	1.0 mg
	B_1- 11.25 mg/d	0.3 mg	0.3 mg	0.3 mg
	B_2- 2.5 mg/d	0.36 mg	0.36 mg	0.36 mg

	B_3- 25 mg/d	4.0 mg	4.0 mg	4.0 mg
	B_5- 6.5 mg/d	1.5 mg	1.5 mg	1.5 mg
	B_6- 3.0 mg/d	0.4 mg	0.4 mg	0.4 mg
	B_7- --	6.0 μg	6.0 μg	6.0 μg
	B_9- 200 μg/d	40 μg	40 μg	40 μg
	B_{12}- 3.0 μg/d	0.5 μg	0.5 μg	0.5 μg
	C- 250 mg/d	10 mg	10 mg	10 mg
Total volume	100-150 mL/kg/d	100 ml	100 ml	100 ml

522 MCT/LCT- medium- and long-chain triglycerides; NaCl – sodium chloride; KCl
523 –potassium chloride; Zn–Zinc; Cu–Copper; Cr–Chromium; Mn–Manganese; A–
524 Retinol; D–Cholecalciferol; E–Tocopherol; B_1–Thiamine; B_2-Riboflavin; B_3–
525 Nicotinamide; B_5–Pantothenic acid; B_6–Pyridoxine; B_7–Biotin; B_9–Folic acid;
526 B_{12}–Cyanocobalamin; C–Ascorbic acid.
527

Table 2 - Visual inspection of TPN admixtures.

Parameters	PC					P1					P2				
	25 °C														
	D0*	**D1**	**D2**	**D3**	**D7**	**D0**	**D1**	**D2**	**D3**	**D7**	**D0**	**D1**	**D2**	**D3**	**D7**
Color alteration	A	**P**	**P**	**P**	**P**	A	**P**	**P**	**P**	**P**	A	**P**	**P**	**P**	**P**
Precipitation	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
Film formation	A	A	A	**P**	**P**	A	A	**P**	**P**	**P**	A	A	A	**P**	**P**
Phase separation	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
	4 °C														
	D0	**D1**	**D2**	**D3**	**D7**	**D0**	**D1**	**D2**	**D3**	**D7**	**D0**	**D1**	**D2**	**D3**	**D7**
Color alteration	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
Precipitation	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
Film formation	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
Phase separation	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
	37 °C														
	D0	**D1**	**D2**	**D3**	**D7**	**D0**	**D1**	**D2**	**D3**	**D7**	**D0**	**D1**	**D2**	**D3**	**D7**
Color alteration	A	**P**	**P**	**P**	**P**	A	**P**	**P**	**P**	**P**	A	**P**	**P**	**P**	**P**
Precipitation	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
Film formation	A	A	A	**P**	**P**	A	A	A	**P**	**P**	A	A	A	**P**	**P**
Phase separation	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A

*D: day, A: absence, P: presence; n= 4 lots.

530

531 **Table 3 - pH values for TPN admixtures during the seven days of study at the three**
532 **temperatures selected.**

PC					
Temp. (ºC)	**D0**	**D1**	**D2**	**D3**	**D7**
25	5.57 ± 0.33*	5.54 ± 0.32	5.52 ± 0.30	5.52 ± 0.32	5.52 ± 0.30
4	5.53 ± 0.38	5.51 ± 0.39	5.50 ± 0.38	5.50 ± 0.38	5.50 ± 0.38
37	5.58 ± 0.32	5.53 ± 0.31	5.51 ± 0.31	5.50 ± 0.33	5.50 ± 0.32
P1					
25	5.57 ± 0.32	5.54 ± 0.33	5.52 ± 0.31	5.50 ± 0.31	5.52 ± 0.30
4	5.54 ± 0.36	5.52 ± 0.37	5.49 ± 0.39	5.50 ± 0.38	5.51 ± 0.36
37	5.59 ± 0.31	5.54 ± 0.31	5.68 ± 0.01	5.75 ± 0.16	5.77 ± 0.23
P2					
25	5.57 ± 0.32	5.56 ± 0.31	5.55 ± 0.29	5.54 ± 0.29	5.53 ± 0.28
4	5.53 ± 0.38	5.52 ± 0.35	5.52 ± 0.34	5.52 ± 0.35	5.51 ± 0.36
37	5.57 ± 0.33	5.55 ± 0.32	5.54 ± 0.30	5.54 ± 0.30	5.52 ± 0.30

533 * Mean ± standard deviation, n= 4 lots.

534

535

535 **Table 4 - Values of the experimental osmolarity for TPN admixtures at the three**
536 **temperatures selected.**

	Experimental Osmolarity (mOsm/L)		
	25℃	**4℃**	**37℃**
PC	848 ± 87*	791 ± 2.4	792 ± 8.0
P1	791 ± 7.0	772 ± 1.8	805 ± 17
P2	831 ± 13	865 ± 14	836 ± 54

537 Mean ± standard deviation, n= 3 lots, measured on D0.

538

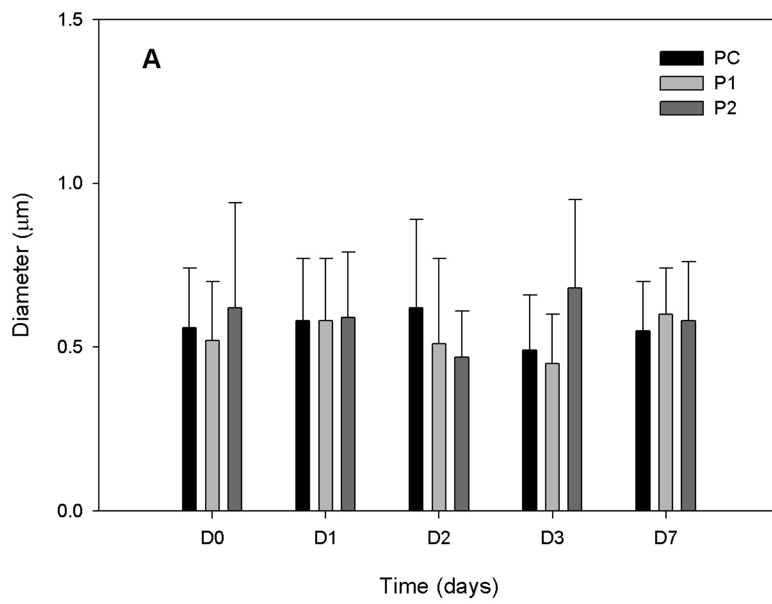
538

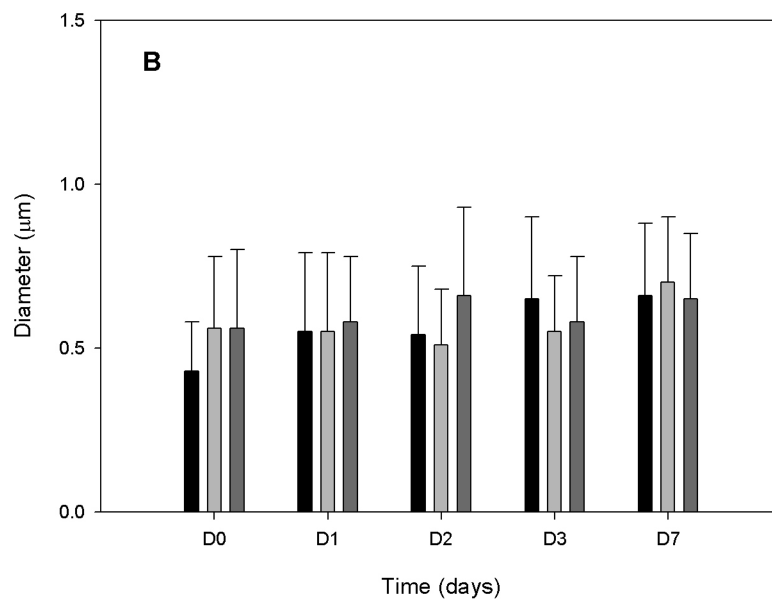
Table 5 - Maximum lipid globule size diameter.

Temperature (ºC)	Maximum diameter (μm)				
	D0	D1	D2	D3	D7
			PC		
25	2.2	2.7	3.5	2.2	1.3
4	2.6	2.1	1.9	2.1	2.4
37	1.9	2.4	2.7	2.9	2.6
			P1		
25	2.3	1.9	2.3	1.6	3.8
4	2.4	2.9	2	1.5	1.8
37	2.1	1.6	2.2	1.8	3.1
			P2		
25	3.7	3	1.6	2.3	1.8
4	3.1	2.4	2.8	1.9	2
37	4	2.8	2.6	2.5	2.2

539

n= 2 lots for each condition at each time.





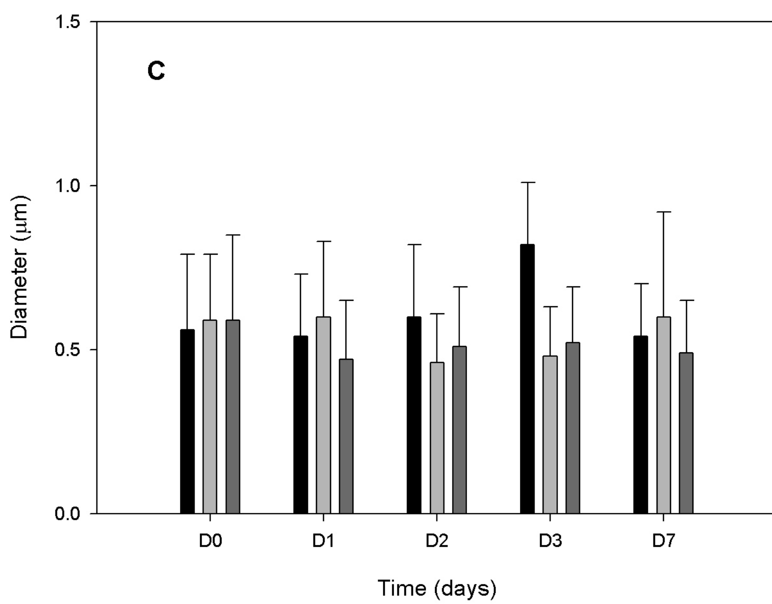
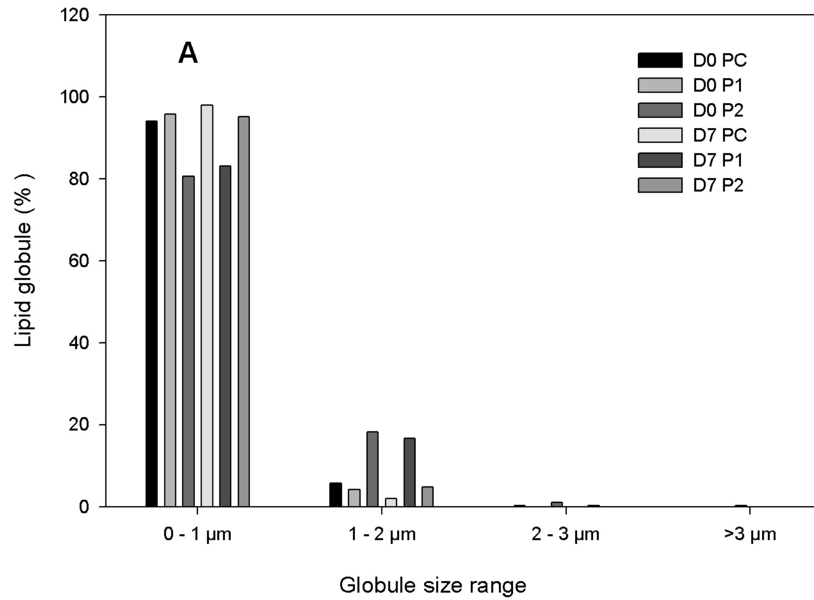
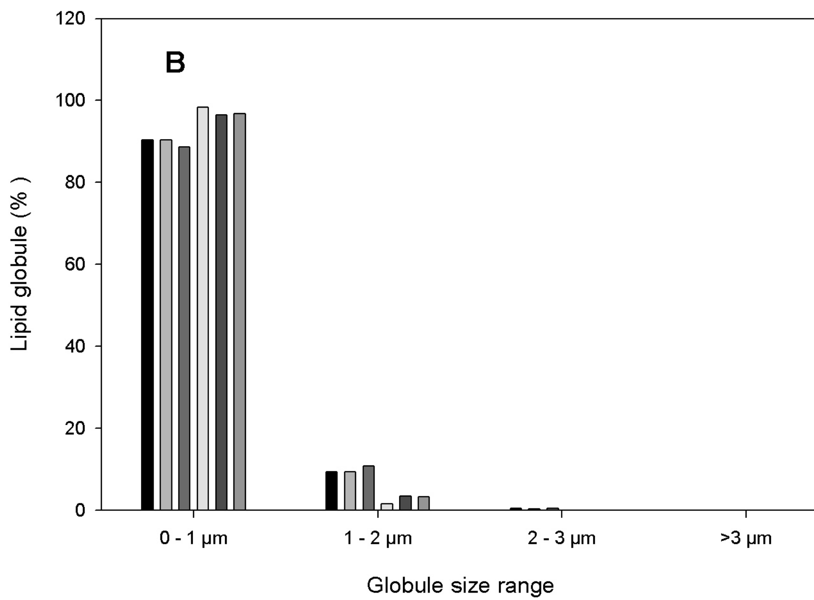


Figure 1





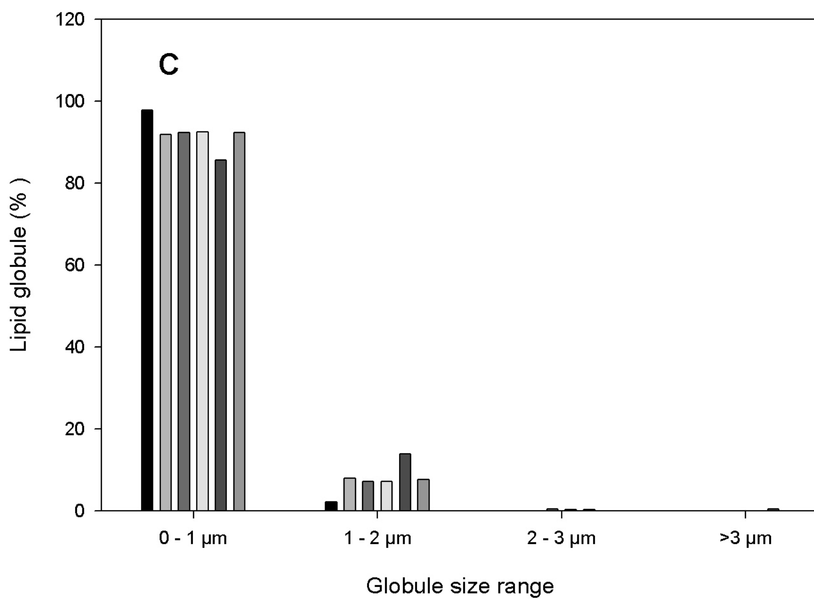
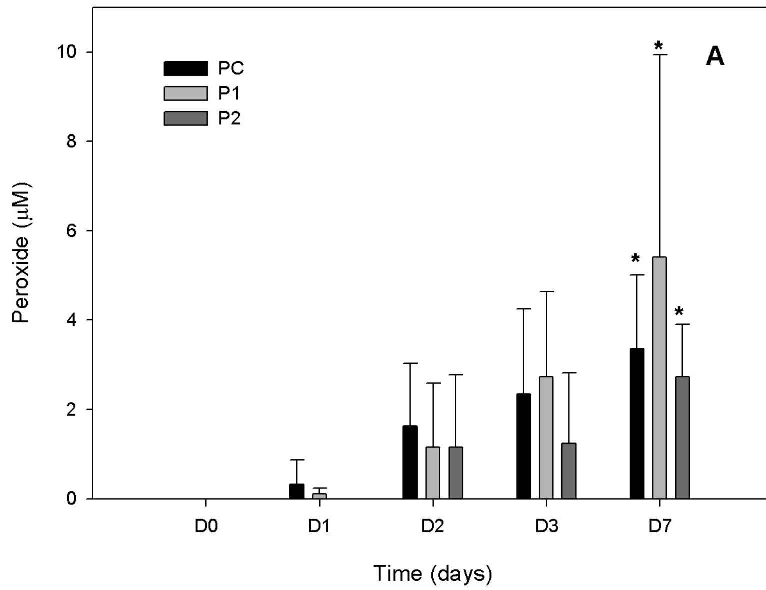
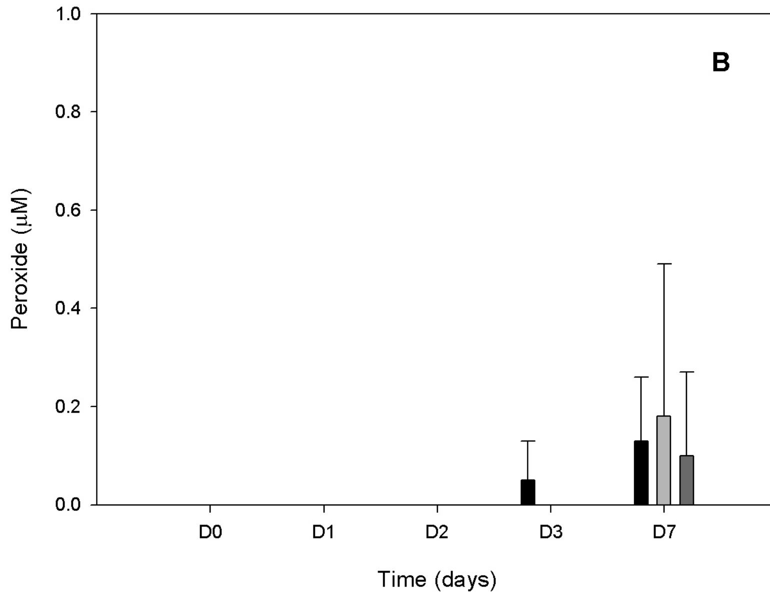


Figure 2





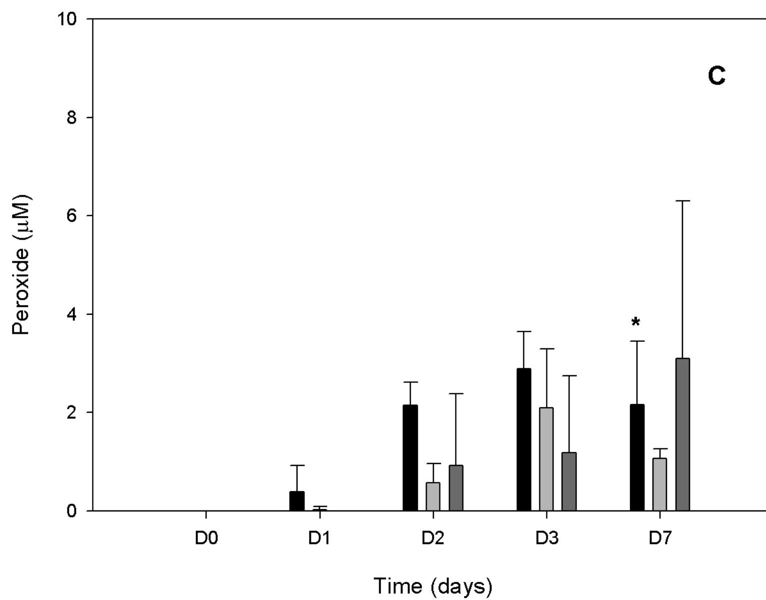


Figure 3