Universidade de Lisboa

Faculdade de Farmácia

Control of the physicochemical and microbiological stability of parenteral nutrition produced or manipulated in hospitals

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Mestrado Integrado em Ciências Farmacêuticas

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Resumo

A nutrição parentérica (PN) permite a administração por via intravenosa dos nutrientes necessários aos pacientes que não têm o trato gastrointestinal funcional, sendo essencial para a sua sobrevivência. Em alguns casos é vantajoso que seja produzida nos serviços farmacêuticos hospitalares, no entanto, é necessário ter em atenção a estabilidade das preparações. A PN trata-se de uma preparação farmacêutica bastante complexa, com uma grande propensão a interações entre os seus componentes e instabilidade físico-química. Para além disso, está muitas vezes associada a infeções nos pacientes que a recebem. É por isso necessário encontrar formas de garantir a segurança do doente, tendo a garantia e o controlo de qualidade um papel fundamental.

O objetivo deste trabalho é identificar os fatores que afetam a estabilidade/qualidade da PN e determinar quais os procedimentos ideais para a produção, controlo de qualidade e conservação da PN nos hospitais, de forma a garantir a segurança e a eficácia da terapêutica para o doente e a rentabilização dos recursos dos serviços farmacêuticos.

O período máximo de estabilidade encontrado para as misturas ternárias (com lípidos) foi de 30 dias, e para as binárias 2 meses para a estabilidade físico-química e 6 meses para a microbiológica. Os fatores limitantes da estabilidade parecem ser a peroxidação lipídica e a degradação das vitaminas. No entanto, a primeira é evitada protegendo a mistura da luz e as vitaminas podem ser adicionadas apenas antes da administração. O controlo de qualidade físico-química é um aspeto a melhorar nos hospitais, devendo, para além da análise do aspeto macroscópico, proceder-se a testes de pH, gravimetria, análise do tamanho das gotículas lipídicas e determinação da concentração dos componentes críticos.

As taxas de contaminação obtidas nos controlos de esterilidade logo após a preparação foram no geral baixas (inferiores a 2%) e os estudos de esterilidade ao longo do tempo no geral não encontraram contaminações. No entanto, nos estudos em que se procedeu à contaminação artificial das misturas, a maioria dos microrganismos proliferou, com destaque para a *Candida albicans*, que é uma levedura oportunista. Pelo contrário, a *Pseudomonas aeruginosa* e o *Staphylococcus epidermidis*, que podem facilmente chegar às bolsas de PN, não cresceram bem. A proliferação microbiana demonstrou ser mais elevada nas misturas ternárias. Estes resultados mostram que um dos aspetos mais importantes da produção de PN é a preparação em condições de assepsia segundo

procedimentos validados e realizada por operadores devidamente qualificados e treinados. A PN deve ser preparada numa câmara de fluxo de ar laminar localizada numa sala limpa de Grau B (EN/ISO 14644-1) com pressão positiva em relação às salas exteriores, o que ainda não acontece em todos os hospitais portugueses. Para além disso, deve ser realizado o teste de esterilidade das preparações terminadas.

Em suma, são diversos os fatores que influenciam a estabilidade da PN, nomeadamente a sua composição e as condições de preparação/conservação. Os resultados encontrados reforçam a importância de um correto processo de preparação e do controlo de qualidade das misturas e apontam para que, mediante a realização de mais estudos, seja possível aumentar os prazos de utilização estipulados, ainda que, alguns dos componentes das misturas tenham de ser adicionados pouco tempo antes da administração. Importa também referir que, sendo uma preparação farmacêutica, a PN deve ser controlada como tal e, portanto, a sua estabilidade deve ser avaliada. Seria vantajoso que cada hospital realizasse estudos de estabilidade adaptados às características das suas bolsas e às suas necessidades individuais, de forma a poder atribuir um prazo de utilização às bolsas preparadas que garanta a segurança dos doentes, mas também que permita uma melhor rentabilização dos recursos/custos dos serviços farmacêuticos e a diminuição do desperdício.

Palavras-chave: Nutrição parentérica, estabilidade, controlo de qualidade, infeções nosocomiais

Abstract

Parenteral nutrition (PN) allows the intravenous administration of the necessary nutrients to patients that do not have a functioning gastrointestinal tract, being essential for their survival. In some cases, it is advantageous that PN is produced in hospital pharmacies, however, the stability of the admixtures has to be considered. PN is a quite complex pharmaceutical preparation, highly prone to interactions between its components and physicochemical instability. Furthermore, it is often associated to infections in patients who are receiving it. It is therefore necessary to find ways of ensuring patient safety, and quality assurance and control play a key role.

The aim of this work is to identify the factors which affect the stability/quality of PN and determine the ideal procedures for the compounding, quality control and storage of PN admixtures in hospitals, in order to ensure the safety and efficacy of therapy for patients and a better management of pharmaceutical services' resources.

The maximum stability period found for ternary admixtures (with lipids) was 30 days, and for the binary ones 2 months for physicochemical stability and 6 months for microbiological stability. The limiting factors are lipid peroxidation and vitamin degradation. However, the first is avoided by protection from light and vitamins can be added only before administration. Physicochemical quality control is an aspect to be improved in hospitals, which should, in addition to the analysis of macroscopic aspect, perform pH and gravimetric assays, diameter analysis of lipid droplets and determine the concentration of critical components.

Contamination rates obtained in sterility controls performed right after preparation were generally low (under 2%) and sterility studies performed over time in general did not detect contaminations. Nevertheless, in studies where PN admixtures were artificially contaminated, most microorganisms multiplied, with relevance for *Candida albicans*, which is an opportunistic yeast. On the contrary, *Pseudomonas aeruginosa* and *Staphylococcus epidermidis*, which can easily reach PN bags, did not grow well. Microbial growth was higher in ternary admixtures. These results show that one of the most important aspects of PN preparation is aseptic compounding following validated procedures and performed by adequately qualified and trained operators. PN should be prepared in a laminar flow chamber located in an EN/ISO 14644-1 Grade B clean room with positive pressure in relation to outer rooms, which is not done in all Portuguese

hospitals yet. Furthermore, the test for sterility of the finished preparations should be performed.

In conclusion, there are several factors that influence PN stability, namely its composition and the preparation/storage conditions. The obtained results reinforce the importance of an adequate preparation process and of the quality control of admixtures and suggest that, if more studies are performed, it could be possible to extend the shelf life assigned to admixtures, even if some components have to be added only before administration. It is also important to recall that PN is a pharmaceutical preparation and should therefore be controlled as such and have its stability evaluated. It would be beneficial if hospitals performed their own stability studies adapted to the characteristics of their bags and their individual needs, in order to assign to their PN admixtures a shelf life that would ensure patient safety, but that would also allow a better management of pharmaceutical resources/costs and a decrease in the waste of bags.

Keywords: Parenteral nutrition, stability, quality control, nosocomial infections

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Abbreviations

AQL - Acceptable quality level

ASHP - American Society of Hospital Pharmacists

ASN – Average sample number

ASPEN - American Society of Parenteral and Enteral Nutrition

ATCC - American Type Culture Collection

BHI – Brain heart infusion

CFU – Colony-forming units

EMA - European Medicines Agency

EN – European Norm

EVA - Ethylene Vinyl Acetate

DEHP - Bis(2-ethylhexyl) phthalate

GMP – Good Manufacturing Practices

HEPA - High-efficiency particulate air

ICU – Intensive care unit

ISO - International Organisation for Standardisation

LFC – Laminar flow cabinet

PIC/S - Pharmaceutical Inspection Convention/Pharmaceutical Inspection Co-Operation Scheme

PN – Parenteral nutrition

PUFAs - Polyunsaturated fatty acids

PVC - Polyvinyl chloride

RQL - Rejectable quality level

RT – room temperature

TSA – Tryptone soya agar

TSB – Tryptone soya broth

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1 Introduction

1.1 The importance of nutrition

Nutrition is a central aspect of health, which affects a person's general condition, well-being and response to illness. Malnutrition is associated with immune function weakening and an overall energy deficit, thus leading to an increased risk of infections, prolonged hospitalisation and higher morbidity and mortality. (1, 2) Critically ill patients often present increased catabolism, anorexia and inability to eat normally, all of which contribute to malnutrition. (3, 4) Therefore, providing the appropriate nutritional support is currently considered an essential part of these patients' treatment, as it attenuates the systemic inflammation associated with critical illness, regulates the immune system and generally enhances the course of the disease. (5) Furthermore, it has been shown that providing preoperative nutritional therapy to malnourished patients improves surgical outcomes and decreases operative mortality. (1, 6)

1.2 General considerations on parenteral nutrition

1.2.1 What is parenteral nutrition?

Parenteral nutrition (PN) is a way of delivering water and essential nutrients such as amino acids, glucose, lipids, vitamins, electrolytes and trace elements through the intravenous route with the objective of ensuring a patient's survival and/or adequate nutrition, providing energy for the physiologic and metabolic processes. In neonatal and paediatric cases, it should also maintain appropriate growth. It is an individualised therapy, adapted to each patient's metabolic and energetic needs and considering their comorbidities. (6, 7)

Pharmaceutically, PN is a solution or emulsion with various components, which should be sterile and physically and chemically stable. (6)

1.2.2 When is PN used?

PN is costly and has risks associated, so it should be used only in high-risk clinical situations, when the oral or enteral routes cannot be used or are insufficient. This includes patients with gastrointestinal tract dysfunction, intestinal failure,

gastrointestinal fistulas, short bowel syndrome, absorption disorders and patients who are going to be or have been submitted to surgery. (8, 9) Studies have shown that there are also benefits to PN being initiated at an early stage in preterm and low birth weight children. (10)

1.2.3 PN composition

PN should satisfy each patient's nutritional requirements, which are influenced by age, sex, weight, physical activity, biochemical parameters and clinical situation. Admixtures should contain enough macronutrients to maintain enzymatic and structural protein reserves (amino acids), and to meet daily energy requirements (glucose and lipids). They should also take into account the patient's need for liquids. Moreover, PN should provide the necessary micronutrients (vitamins, electrolytes and trace elements). (8)

1.2.4 Types of PN admixtures

1.2.4.1 Binary and ternary admixtures

Regarding their composition, there are binary and ternary admixtures. Binary or 2-in-1 formulations do not include lipids, consisting of amino acids, glucose, electrolytes, vitamins and trace elements. Ternary, 3-in-1 or all-in-one admixtures include all macro- and micronutrients, in other words, the ones present in binary admixtures plus lipids, and they are the most used nowadays. (9, 11)

1.2.4.2 Hospital compounded and commercial preparations

PN admixtures can be compounded in hospital pharmacies or industrially produced. The first allow a more individualised therapy, being advantageous in patients with special nutritional needs, children and neonates, but present stability and contamination risks. Industrial formulations are usually commercialised in multi-chamber or multicompartment bags with amino acids, glucose and lipids separated in different compartments, which separation seal has to be broken before administration to the patient. Vitamins and trace elements are added to the mixture also only before administration. This allows an improvement in stability and prolonged shelf life. (12, 13) This work will focus on PN admixtures compounded in hospital pharmacies.

1.3 Stability problems and risks of PN

PN admixtures are complex pharmaceutical preparations, with a high number of components (often more than 50) and therefore very prone to physicochemical interactions and incompatibilities between them and their surroundings (the bag material, oxygen, light and temperature). In addition, they are administered through the intravenous route, which increases the risk of infections/sepsis. These problems may lead to serious complications for patients and even put their life in danger. (11, 13, 14)

1.3.1 Physicochemical instability/interactions

1.3.1.1 Lipid emulsion instability

The lipid emulsion is one of the most critical components of all-in-one PN admixtures.

Particle diameter of intravenous formulations should be between 0.4 and 1 µm (the size of chylomicrons), and should not be larger than 5 µm, to prevent being trapped in capillary beds and causing embolism. (15)

Changes in pH, temperature and especially zeta potential can destabilise lipid emulsions, causing oil droplets to aggregate (flocculation and creaming), which is reversible through shaking, and later to coalesce into bigger droplets, which is irreversible (Figure 1). (13)

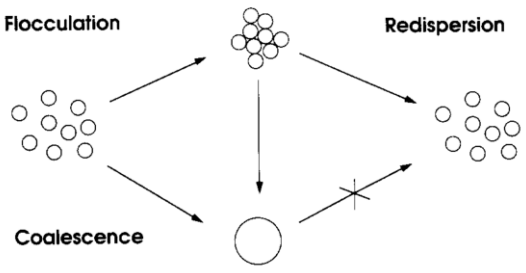

Figure 1 Flocculation and coalescence. (16)

This is normally prevented by the negative surface charge (zeta potential) of the fat particles provided by the phospholipid layer, which causes them to repel each other. However, factors as electrolytes, mixture pH and trace elements might reduce these

repulsive forces and originate particles bigger than 5 µm. (17) Cationic electrolytes neutralise the negative charge, being that divalent (calcium and magnesium) and trivalent (iron) cations have a bigger influence. For that reason, the total cation amount expressed in monovalent cations should be less than 600 mmol/L. (13, 17) Admixture pH lower than 5.5, which may be originated by acidic amino acids and glucose, can also decrease surface potential and destabilise the lipid emulsion. (17) On the other hand, neutral and basic amino acids help to stabilise 3-in-1 admixtures, since they form complexes with divalent cations, weakening their activity, they are adsorbed to the surface of oil droplets, stabilising them, and they have a buffering effect, preventing the excessive lowering of pH. (13)

Lipid peroxidation is another critical aspect of lipid emulsions. It occurs in the presence of oxygen and light and originates free radicals, which react with various body molecules, causing cell damage, and other toxic compounds such as aldehydes. (13, 15) Polyunsaturated fatty acids (PUFAs) are the type of lipids more easily peroxidised. (15) Conventional lipid formulations, composed of soybean and/or safflower oil, were rich in PUFAs. Nowadays, admixtures include lipids from more sources, namely fish, coconut and olive oil, which contain both medium-chain and long-chain fatty acids. This mixture proved to be more stable than emulsions composed of long-chain fatty acids alone. (17, 18) Moreover, vitamins C and E protect the lipid emulsion from peroxidation. (18, 19) Temperature and ion concentration, especially iron and copper, also influence this reaction. (13)

1.3.1.2 Calcium phosphate precipitation

Calcium phosphate precipitation can be fatal, since it might originate particles bigger than 5 µm, which are dangerous, as explained before. The risk of precipitation is affected by the amount of monovalent ($Ca(H_2PO_4)_2$) and divalent ($CaHPO_4$) calcium phosphate in the mixture. The first one is more soluble (18 g/L) and therefore less prone to precipitation, while the second one is almost insoluble (0.3 g/L) and easily precipitates. Calcium phosphate precipitation is influenced by pH, amino acid concentration, temperature, type of calcium and phosphate sources and presence of other ions. (13)

At typical PN pH (less than 6.4), there is a higher concentration of monovalent phosphate, which originates the most soluble form of calcium phosphate. However,

when pH increases, the concentration of divalent phosphate also increases, originating more divalent calcium phosphate and consequent precipitation. pH is affected by glucose concentration (it acidifies the mixture) and amino acid concentration (buffering effect). (13)

Amino acids can also form complexes with calcium and phosphate, preventing them from precipitating. (13)

Higher temperatures increase the dissociation of calcium and phosphate salts, leaving these ions available to form more divalent calcium phosphate, which precipitates more easily. (13)

As for ion sources, organic phosphate salts, such as sodium glycerophosphate, are less likely to precipitate and glycerophosphate contributes to the buffering effect. Inorganic monovalent phosphate is less likely than the divalent form. Calcium chloride dissociates more easily than, for example, calcium gluconate or glubionate. (13)

Magnesium increases calcium phosphate solubility when pH increases and Mg/Ca molar relation is less than 2. (13)

Calcium phosphate precipitation is much more likely to occur in PN mixtures with very low amino acid concentrations, such as peripheral or paediatric PN. In the majority of other formulations, calcium phosphate concentrations are generally less than what is needed for precipitation. (13)

1.3.1.3 Vitamin degradation

Vitamins are easily affected by light, oxygen, temperature, bag material, trace elements and bisulphites. Vitamin C suffers oxidation because of the residual air inside the bag and its permeability to oxygen. This reaction may be catalysed by copper. Ascorbic acid might also degrade to oxalic acid, which precipitates when it reacts with calcium ions. Vitamin B1 suffers reduction by sodium metabisulphite (an antioxidant), light and temperature. Vitamin A is also degraded by light and adsorbed by plastic bags and administration systems, but lipid emulsions decrease its degradation. Vitamin D loses some of its activity in formulations without lipids. Vitamin B2 also suffers photo-degradation. Vitamins B6, B9, B12 and E are generally stable in PN admixtures. (13, 20, 21)

1.3.1.4 Amino acid degradation

Some amino acids are photosensitive, such as tryptophan, histidine, methionine, tyrosine and cysteine. Riboflavin (vitamin B2) aids their photo-degradation, while ascorbic acid (vitamin C) inhibits it. (13)

Tryptophan is also degraded in the presence of sodium bisulphite. (13, 22)

Furthermore, glucose might react with amino acids through the Maillard reaction. Amino acids most susceptible to it are lysine (especially), arginine, histidine and tryptophan. Temperature, pH, humidity, metallic cations and the dextrose structure influence the Maillard reaction. (13, 22)

1.3.2 Microbiological contamination/infections

PN, being administered intravenously, must be sterile, because even if it does not support the proliferation of microorganisms, it might convey them to the patient. (23)

There is always a risk of contamination of the mixtures, during either compounding or administration to the patient. (24) Infection is the most usual serious complication of PN therapy, and it can be particularly concerning in immunologically compromised patients. Microbial concentrations of 10^3 colony-forming units (CFU)/mL or more can be dangerous to the patient. (25, 26)

The risk of contamination during administration is aggravated by the prolonged administration time (up to 12 or 24 hours) and is mostly related to inadequate handling of the bags and catheters, poor catheter maintenance and/or improper hygiene measures by nurse staff. On the other hand, contamination during manufacture is the pharmacist's responsibility and, although less frequent, can be more dangerous, as it has the potential for affecting several patients. (24, 25)

The probability of infection because of microbial contamination during compounding is influenced by the asepsis of the procedure, the admixture composition and the storing conditions.

The first is strongly related to the aseptic compounding technique used, including PN preparation in the hospital pharmacy in clean rooms and laminar air-flow chambers and the training in aseptic technique of operators who prepare it. (27, 28)

Candida albicans, an opportunistic yeast, is the microorganism responsible for the majority of sepsis occurrences in patients receiving PN. However, bacteria as *Serratia*, *Escherichia coli*, *Enterobacter cloacae*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Staphylococcus epidermis* and *Streptococcus faecalis* are also a common cause of clinical infections due to PN administration. (24)

1.4 Finding ways to improve patient safety and optimise hospital resources and costs

According to the American Society of Hospital Pharmacists (ASHP), the American Society of Parenteral and Enteral Nutrition (ASPEN) and the 2008 Spanish Consensus on the Preparation of Parenteral Nutrition Admixtures, the hospital pharmacist is responsible for ensuring the quality of PN admixtures compounded or manipulated in the hospital. (13) Quality control of the finished PN bags does not ensure by itself the quality of the whole batch, as not all units are usually tested. Therefore, other measures should be employed to assure the quality of the product. The assurance of quality consists of maximising the quality of a product by using the right raw materials and procedures.

PN, being such a complex formulation, requires very careful handling and monitoring for it to fully benefit the patient.

Even though aseptic preparation techniques are used, there is always a risk for patients if a technical error occurs. In addition, they have a high risk of physicochemical instability. For this reason, microbiological and physicochemical control plays a central role in assuring patient safety. Nevertheless, a 2009 study concluded that the quality control of the final product is one of the weakest points in the preparation process of neonatal PN in Portuguese hospitals. (29)

Furthermore, most hospitals assign short shelf lives to their admixtures, sometimes as short as 48 hours, because they have not performed stability studies. The study mentioned above observed that, in 2009, only two Portuguese hospitals had performed stability studies for their standard neonatal PN bags compounded in the pharmacy. (29)

Thus, bags are often prepared on a daily basis in hospital pharmacies. Therefore, PN preparation is time-consuming and costly, requiring skilled labour and special

equipment and facilities. In addition, if standard bags are prepared in batches, some of them might not be used before their expiration date and have to be disposed of, regardless of the fact that they could still be stable.

In these work, sterility evaluations, stability studies and microbial challenge studies on PN that have been published are analysed, together with the main guidelines and recommendations on the matter. However, even though they provide useful information for pharmacy hospitals, they should not be directly applicable to admixtures with a different composition and should always be adapted to each hospital's necessities.

2 Aim

The aim of this work is to identify the factors which affect the stability/quality of PN and determine the ideal procedures for the compounding, quality control and storage of PN admixtures in hospitals, in order to ensure the safety and efficacy of therapy for patients and a better management of pharmaceutical services' resources. The contamination rate and the microbiological and physicochemical stability of PN admixtures over time is analysed, including their ability to support microbial growth. The guidelines and regulations on this matter and what is currently done in Portuguese hospitals is also presented.

3 Methods

Published literature in English and Spanish about parenteral nutrition practices, the physicochemical and microbiological stability of parenteral nutrition, physicochemical and microbiological stability and microbial challenge studies on parenteral nutrition and guidelines and recommendations from the European Pharmacopoeia 9.0 and the United States Pharmacopeia 39 (USP 39) on the preparation and control of parenteral nutrition/sterile pharmaceutical preparations were reviewed.

A search was made in PubMed and Web of Science databases, with different combinations of the terms “parenteral nutrition”, “stability”, “physicochemical”, “microbiological”, “microbial contamination”, “quality control” and “sterility”.

No limit of publication date was imposed. The oldest publication analysed is from 1973 and the most recent from 2017. Articles which tested the sterility of parenteral nutrition bags after administration were not considered.

A comparison between published studies and guidelines/recommendations was made.

4 Results

4.1 Quality assurance

4.1.1 Ingredients, devices and equipment

All materials involved in the compounding process should be obtained from certified suppliers. The USP 39 recommends checking the labelling and certificates of analysis of ingredients used in sterile preparations and having and following written procedures for equipment calibration, maintenance, monitoring and operation. (30)

4.1.2 Aseptic preparation

4.1.2.1 Validated compounding procedures

The European and the United States Pharmacopoeias and the "PIC/S Guide to good practices for the preparation of medicinal products in healthcare establishments" establish that sterile products should be compounded using an aseptic procedure in accordance with Good Manufacturing Practices (GMP). (30-32) This is essential in the case of PN, since it cannot go through a terminal sterilisation process in the final container because of its labile components and sterilising filtration cannot be used because of particle size.

All steps in the sterile processing should follow validated Standard Operating Procedures, including clothing and washing of personnel, material disinfection, mixture compounding and sanitation of premises. Aseptic procedures should be validated through a media-fill test, where all the aseptic compounding procedure is simulated using a liquid culture medium, such as Soybean–Casein Digest Medium. Media-fill bags are then incubated at 20° to 25°C or at 30° to 35°C for at least 14 days in order to evaluate their sterility. (32, 33)

In 2009, 72.7% of Portuguese hospitals had written and updated procedures about the operators' aseptic technique, 86.4% about environmental control (air and surfaces), another 86.4% about the neonatal PN production process and also 86.4% about the quality control of these bags. However, only 54.5% of the hospitals had all of these written procedures and 36.4% had three of them. (29)

4.1.2.2 Quality of compounding environment

The compounding should take place in a laminar flow cabinet (LFC) (European Norm (EN) International Organisation for Standardisation (ISO) 14644-1 Grade A) placed in a Grade B clean room (see Table 1 and Table 2). It should be preceded by a Grade C buffer area, which in turn is preceded by a Grade D ante area (see Table 1 and Table 2) (Figure 2). These rooms should be supplied with high-efficiency particulate air (HEPA)-filtered airflow and a positive pressure differential of 10 Pascals to the adjacent lower-class room, so that the air always flows outwards, avoiding contamination of the product. Rooms should have airlocks to allow the passing of personnel, materials and equipment. (30, 32)

Table 1 Classification of clean rooms and clean air devices in accordance with EN/ISO 14644-1. (32)

Grade	Maximum permitted number of particles/m³ equal to or greater than the tabulated size			
	At rest		In operation	
	0,5 µm	5 µm	0,5 µm	5 µm
A	3 520	20	3 520	20
B	3 520	29	352 000	2 900
C	352 000	2 900	3 520 000	29 000
D	3 520 000	29 000	not defined	not defined

Table 2 Recommended limits for microbiological contamination of clean areas during operation. (32)

Grade	Air sample (CFU/m³)	Settle plates - diam. 90 mm (CFU/4 hours)	Contact plates - diam. 55 mm (CFU/plate)	Glove print 5 fingers (CFU/glove)
A	< 1	< 1	< 1	< 1
B	10	5	5	5
C	100	50	25	-
D	200	100	50	-

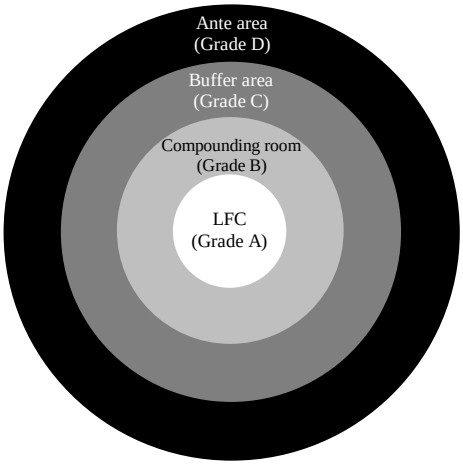

Figure 2 Conceptual representation of PN compounding facilities (adapted from USP 39). (30)

Clean areas should not have corners and recesses that easily accumulate particles and microorganisms and are difficult to clean. All surfaces should be smooth and impervious and easy to clean and disinfect. Furniture and equipment should be reduced to the minimum required. (33)

In addition, only sterile materials should be taken into Grade A and B areas. If not sterile, they should be adequately disinfected before entry, for example, by spraying with 70% ethanol or isopropanol and wiping. (32)

The microbiological quality of air and compounding areas should be regularly monitored, using particle counting, settle plates, volumetric air and surface sampling - contact plates and swabs (see Table 3). The results obtained should be within the mentioned limits. If this is not the case, measures should be taken to achieve those limits. (32)

According to Neves *et al.*, in 2009, 54.5% of Portuguese hospital pharmacies prepared neonatal PN in EN/ISO 14644-1 Grade B clean rooms (see Table 1), 9% in rooms classified between Grade B and C, 31.8% did not know their compounding room air quality grade and 4.5% prepared PN in a non-controlled environment. 38.1% compounded PN in class II A1 biosafety chambers, 14.3% in class II A2 and 47.6% did not know what kind of equipment they used. (29)

Table 3 Recommended frequencies of microbiological monitoring for clean areas. (32)

	Direct working environment (Grade A zone)	**Background environment**
Settle plates	Every working session	Weekly
Glove finger dabs	At the end of each working session	At the end of each working session
Surface samples (swabs or contact plates)	Weekly	Monthly
Active air samples	Quarterly	Quarterly

4.1.2.3 Personnel training and performance monitoring

According to USP 39 and PIC/S, all personnel involved in PN preparation should receive regular training in PN and microbiology basics, in the specificities of compounding facilities and equipment and in the adequate aseptic procedures, including hygienic procedures, and they should be aware of the risks and responsibilities involved in their tasks. Technicians should be qualified and trained in sterile preparation and their aseptic technique should be validated on a regular basis. This can be done through frequently supervising the compounding procedure, and through the media-fill challenge test, as explained above (see 4.1.2.1 Validated compounding procedures). (30, 32)

Only qualified and authorised personnel should have access to clean rooms and their number should be limited to the minimum necessary in each moment. They should wash their hands before entering, wear sterile protective clothes and items (facemask, footwear, gloves, and headgear) and not wear wristwatches, make-up or jewellery. (32, 33)

4.1.3 Order of addition of PN components

According to the 2008 Spanish Consensus on the Preparation of Parenteral Nutrition Admixtures, amino acids and glucose should be mixed first, and only afterwards should the lipid emulsion be added. Electrolytes and trace elements should not be directly

added to the lipid emulsion. The monovalent ions (such as sodium and potassium) should be added first, then phosphate and magnesium and finally calcium. Vitamins and the lipid emulsion should be the last to be added. (13, 14)

4.1.4 Storage conditions

According to the 2008 Spanish Consensus on the Preparation of Parenteral Nutrition Admixtures, after compounding, PN bags should be protected from light with an overwrap and refrigerated at 2-8 ºC. (13) They should not be left at room temperature for more than one hour. (34)

In 2009, 45,5% of Portuguese hospital pharmacies who participated in the study by Neves *et al.* reported to store neonatal PN bags in the fridge (2-8ºC). Binary admixtures were protected from light in 78.9% of the hospitals and lipid emulsions in 89.5%. (29)

4.1.5 Traceability/documentation

All steps of the compounding process should be documented, from starting material entry to distribution of the final product, including records of all the personnel, materials and equipment involved. (35)

Neves *et al.* concluded that 72.7% of the studied Portuguese hospitals register the batch numbers of the products used in the preparation of neonatal PN and 54.5% assign a batch number to the compounded neonatal PN bags. (29)

4.2 Quality control

As mentioned above, the quality control of the finished product should only be one of several measures employed to assure quality. (33)

The first step to be controlled is the visual aspect of all starting materials, to ensure that they match the required specifications. (32)

4.2.1 Physicochemical control

According to the 2008 Spanish Consensus on the Preparation of Parenteral Nutrition Admixtures, PN physicochemical control should involve (a) inspecting the macroscopic aspect of the admixtures (colour, presence of particles, phase separation on the lipid emulsion and bag integrity); (b) checking compounding accuracy (verification of components packages and syringes used to measure them); (c) gravimetric control (confirming that the final weight of the bags is similar to the theoretical one); (d) physicochemical analysis (determining the glucose, amino acid and ion concentration and osmolarity). Regarding gravimetric control, the gravimetric error should be less than 5%, according to the European Medicines Agency (EMA). (13)

The USP 39 also recommends compounding accuracy checks through double-checking the containers of ingredients used and the volumes measured, preferably by someone other than the compounder. In addition, each finished unit should be visually examined for particles or other foreign matter, using good lighting and against white or black background. (30)

Another important aspect in ternary admixtures is lipid globule size: droplets with diameter greater than 5 μm must be less than 0.05% of the total fat content. (17) Particle size can be measured by optical microscopy, laser diffraction or dynamic light scattering. (6)

Furthermore, admixture pH should be between 5.5 and 6.4. (17)

A study about neonatal PN preparation practices that involved 22 Portuguese hospitals concluded that, in 2009, 95.2% of the hospitals evaluated the macroscopic aspect of neonatal binary admixtures and only 14.3% of the lipid emulsion. None of the studied hospitals performed other physicochemical analysis. (29)

4.2.2 Microbiological control (test for sterility)

According to both the European Pharmacopoeia 9.0 and the USP 39, the test for sterility of pharmaceutical preparations required to be sterile, which is the case of PN, should be carried out through the membrane filtration technique or the direct inoculation technique. In the first, a certain volume of the contents of a PN bag (see Table 4) is filtered through a membrane filter, normally of cellulose nitrate, capable of retaining

microorganisms, with a pore size not greater than 0.45 µm. The membrane filter is then transferred to a culture medium or the medium transferred onto the membrane in the apparatus. In the direct inoculation technique, the same volume of admixture (see Table 4) is transferred directly to a culture medium so that the volume of the product is not more than 10% of the volume of the medium. In both methods, the culture media are incubated for at least 14 days. The turbidity should be checked regularly during that period. If there is no turbidity, the admixture complies with the test for sterility. In the case of all-in-one admixtures, which turn the media turbid because of the lipid emulsion, after 14 days of incubation, portions of at least 1 mL have to be transferred to new vessels of the same medium and incubated for at least 4 days. The media used are fluid thioglycollate medium (thioglycollate broth), which is ideal for anaerobic bacteria and should be incubated at 30-35 ºC, and soya-bean casein digest medium (tryptone soya broth – TSB), in which fungi and aerobic bacteria grow and that should be incubated at 20-25 ºC. (36, 37)

Table 4 Minimum quantity of product to be used for each medium according to the European Pharmacopoeia 9.0 and the USP 39. (36, 37)

Quantity per container	Minimum quantity to be used for each medium
Greater than 40 mL and not greater than 100 mL*	20 mL
Greater than 100 mL**	10% of the contents of the container, but not less than 20 mL

* Usually neonatal PN bags

** Usually adult PN bags

According to both Pharmacopoeias, a minimum number of units in each batch must be subjected to the sterility test, as described in Table 5. (36, 37)

Table 5 Minimum number of items to be tested for sterility in each batch according to the European Pharmacopoeia 9.0 and the USP 39. (36, 37)

Number of items in the batch	Minimum number of items to be tested
Not more than 100 containers	10% or 4 containers, whichever is the greater
More than 100 but not more than 500 containers	10 containers
More than 500 containers	2% or 20 containers (10 containers for large-volume parenterals), whichever is less

On the other hand, the Pharmaceutical Inspection Convention/Pharmaceutical Inspection Co-Operation Scheme (PIC/S) states it is not necessary to perform microbiological control on every batch produced in the hospital, but instead have a regular analysis programme or even just validate the sterility of the process/operators' technique on a regular basis. (32) When testing a batch, samples should be representative of the whole and cover parts with higher risk of contamination, including containers filled at the beginning and at the end of the process and after significant interventions. (33)

The 2008 Spanish Consensus on the Preparation of Parenteral Nutrition Admixtures recommends a randomisation sampling method, the Cumulative Sum Control Chart, which compares the accumulated number of observed defective units against specified limits, allowing the detection of worsening or improvement in flaw rates. The acceptable quality level (AQL) is the maximum rate of defective units which is accepted, while the rejectable quality level (RQL) is the minimum rate of defective units that determines the initiation of a corrective action. The recommended levels are AQL=5%, RQL=12% and error probability α=0.05. With these values, the decision point *H* is calculated in 3.15 units and the average sample number (ASN) in 74.46 units. The ASN is the number of samples needed to detect the transition from the AQL to the RQL. As long as the sum of defective units remains under the *H* value, the process is under control. (13)

Previous studies have found that contamination rates in sterility tests of PN bags after compounding are generally low, most ranging from 0 to 2%. Table 6 shows the results obtained in various studies.

Table 6 Overview of studies on the microbiological control of PN admixtures.

Study - year	Number of PN bags involved in the study	Type of PN admixture	Technique	Volume or % of bag volume tested	Contamination rate
Turmezei *et al.* (38) - 2015	Unknown	Ternary	Direct inoculation; culture media: Columbia agar with 5% sheep blood, chocolate agar with polyviteX and Sabouraud medium	10 mL	0%
Montejo *et al.* (39) - 2000	25 000	Ternary	Membrane filtration; culture medium: blood-agar	50 mL	0.008%
Rubió *et al.* (40) - 1993	11 023	Unknown	Direct inoculation; culture medium: unknown	10 mL	Simple culture technique – 1.27% Double sample technique – 0.34%
Martí-Bernal *et al.* (41) - 1989	1920	Unknown	Direct inoculation; culture media: blood-agar and thioglycollate broth	1 mL	1.5%
Spiliotis *et al.* (42) - 1989	80	Ternary	Direct inoculation; culture medium: unknown	2 mL	0%
Llop *et al.* (43) - 1989	28501	Unknown	Direct inoculation; culture medium: BHI	3 mL	0.65% (0.21% in verification culture)
Cardona *et al.* (44) - 1986	50	Ternary	Membrane filtration; culture medium: blood-agar	10%	10% (2% considered true positives)

			Direct inoculation; culture medium: TSB	10 mL	4% (2% considered true positives)
Nordfjeld *et al.* (45) - 1984	20	Binary	Membrane filtration; culture medium: BHI	50 mL 100 mL 500 mL	0%
Herruzo-Cabrera *et al.* (46) - 1984	1074 in the Pharmacy 41 in the ICU	Unknown	Direct inoculation; culture medium: Mueller-Hinton agar	1 mL	Pharmacy – 18.8% ICU – 17.1%
Dévaux-Goglin *et al.* (47) - 1980	45	Unknown	Membrane filtration; culture medium: unknown	10%	2.2%
Bernick *et al.* (48) - 1979	600	Binary	Membrane filtration; culture medium: TSA	50 mL	8%
			Direct inoculation; culture medium: BHI	50 mL	8%

TSA – tryptone soya agar

TSB – tryptone soya broth

BHI – brain heart infusion

ICU – intensive care unit

Regarding Portuguese hospitals, according to Neves *et al.*, in 2009, 72.7% of them performed microbiological control of neonatal binary admixtures (22.7% controlled all units, 18.2% randomly selected one unit per day and 31.8% did a systematic sampling). On the other hand, only 35% controlled lipid emulsions. (29)

4.3 Stability

According to the 2008 Spanish Consensus on the Preparation of Parenteral Nutrition Admixtures, PN in multilayer bags with photoprotection can be stored up to 4 days at

2-8 ºC. They should not be more than 24 hours at room temperature and never at temperatures higher than 28 ºC. (13)

The USP 39 advises a maximum storage time of 30 hours at room temperature and 9 days when refrigerated for preparations with medium-risk of contamination, which is the case of PN. (30)

In 2009, 10% of Portuguese hospital pharmacies assigned a shelf life of 48 hours to neonatal PN bags stored in the fridge (2-8ºC) and 90% assigned them 72 hours. For neonatal standard bags compounded in the hospital pharmacy, 2 hospitals defined their shelf life based on literature and other 2 based on stability studies. These shelf lives varied between 48 hours and 30 days. (29)

4.3.1 Physicochemical stability

The main aspects that should be monitored when evaluating the physicochemical stability of PN admixtures are the concentrations of the various components (glucose, amino acids, electrolytes, vitamins, trace elements) over time, identifying their degradation products if any occur. In the case of ternary admixtures, the most critical aspect is the stability of the lipid emulsion: changes in particle size, lipid peroxidation and factors that might affect them should be evaluated. (6)

Published stability studies have found that 3-in-one PN admixtures can remain physicochemically stable for up to 7, 14 or 30 days regarding most parameters (macroscopic aspect, particle diameter, pH and ion content, among others), and 2-in-one admixtures for up to 8 days or even 2 months (Table 7). Paediatric/neonatal admixtures were shown to stay stable for at least 1 or 2 weeks. The limiting factors are lipid peroxidation (in case of ternary admixtures), which has been shown to start in the first 24 hours after compounding, and vitamin degradation, which usually starts after 48 or 72 hours. Table 7 summarises published studies on PN physicochemical stability.

Table 7 Overview of published studies on the physicochemical stability of PN admixtures.

Study - year	Type of PN admixture	Bag material/type	Analysed parameters (results)	Storage temperature	Period of stability
Turmezei *et al.* (38) - 2015	Ternary (paediatric)	Unknown	Particle size (≤ 5 µm)	2-8 ºC 25 ºC 30 ºC	At least 14 days
			Zeta potential (negative)		
			L-alanyl-L-glutamine concentration (variation < 10%)		
			Vitamin C concentration (variation < 10%)	2-8 ºC	2 days
				25 ºC 30 ºC	Less than 24 hours
Janu *et al.* (17) - 2011	Ternary	EVA	Macroscopic aspect (absence of creaming - dense white layer at the surface of a mixture, free oil, and precipitation after rigorous agitation)	2-8 ºC (24 hours before analysis at 23-25 ºC)	At least 30 days
			Particle size (≤ 5 µm)		
			pH (between 5.48 and 6.08)		
			Osmolarity (stable)		
			Ion Content (variation < 10%)		

Tuan *et al.* (49) - 2011	Ternary (neonatal)	EVA	Particle size (≤ 5 µm; average diameter: 3 µm)	2-8 ºC (24 hours before analysis at 25 ºC)	At least 5 days
				2-8 ºC for 3 days and 25 ºC for 18 days	At least 21 days
Skouroliakou *et al.* (15) - 2008	Ternary (neonatal)	EVA	Macroscopic aspect (redispersible cream layer, absence of oil globules or yellow droplets)	4 ºC 25 ºC	At least 7 days
			pH (between 4.8 and 5.6)		
			Particle size (≤ 5 µm)		
			Peroxide concentration (increase of 7- or 13-fold in 24 hours)*		Less than 24 hours
			α-tocopherol (vitamin E) concentration (loss < 10%)	4 ºC	24 or 48 hours**
				25 ºC	Less than 24 hours or 48 hours**
Mirkovic *et al.* (50) - 2008	Ternary	Multicompartment EVA	Macroscopic aspect	Unknown	At least 60 hours
			pH		
			Particle size (≤ 5 µm)		

Celma *et al.* (51) - 2016	Neonatal	Unknown	Concentration of glucose, potassium, sodium, magnesium and calcium (stable)	2-8 ºC	At least 8 days
Ribeiro *et al.* (21) - 2011	Binary (neonatal)	Multilayer (3 layers)	pH (around 5.5)	4 ºC 25 ºC	At least 3 days
			Vitamin B_1 (variation < 10%)		
			Vitamin B_2 (variation < 10%)		
			Vitamin B_6 (variation < 10%)		
			Vitamin C (variation < 10%)	4 ºC	At least 3 days
				25 ºC	2 days
Nordfjeld *et al.* (22) - 1983	Binary	PVC	pH (between 5.5 and 6.4)	4 ºC	At least 6 months
			Colour of the solution (unchanged – slightly yellow)		
			Precipitation (absence)		
			Dextrose concentration (variation < 10%)		
			Electrolytes (variation < 10%)		
			Trace elements (variation < 10%)		
			Amino acids (variation < 10%)		

			• Tyrosine, lysine, histidine		At least 6 months
			• Leucine		5 months
			• Valine, isoleucine, arginine, tryptophan, phenylalanine		4 months
			• Glycine, threonine, alanine		3 months
			• Methionine, proline		2 months

* With or without vitamin E, respectively

** Two different admixtures were analysed.

EVA - Ethylene Vinyl Acetate

PVC - Polyvinyl chloride

The main factors that have been found to influence physicochemical stability of PN admixtures are the storage temperature, the bag material/structure (including its permeability to oxygen) and the residual oxygen in the bag.

Storage temperature does not seem to significantly affect pH, lipid peroxidation, globule diameter or zeta potential. (15, 38, 49) It does not seem to influence the degradation of vitamins B1, B2 or B6 either. (21) However, storage at 2-8 ºC reduces/delays the degradation of α-tocopherol (vitamin E) and ascorbic acid (vitamin C). (15, 21, 38)

Multilayer bags have been observed to help decreasing the oxidation of compounds such as vitamin C when compared to simple Ethylene Vinyl Acetate (EVA) bags. (20, 21) Dupertuis *et al.* concluded that 6-layer bags are more effective at preventing ascorbic acid degradation than 3-layer bags. (52) Moreover, a higher quantity of oxygen present in the bag during storage also increases oxidation. (20, 21)

Some authors observed that vitamins have a protective effect against lipid peroxidation, namely vitamin E (α-tocopherol) alone, as well as multivitamin preparations. (15, 19)

Regarding the effect of photoprotection on vitamins, results are controversial. Silvers *et al.* found that the degradation of vitamin B2 decreases with photoprotection, while vitamin C does not seem to be affected by light. (19) On the other hand, Ribeiro *et al.* found that photoprotection does not seem to influence the degradation of vitamins C, B1, B2 and B6 at least during 72 hours at 25 ºC. (21)

4.3.2 Microbiological stability

Published studies have reported that all-in-one PN admixtures can remain sterile for up to 14 or 30 days and binary ones for 25 days and even 6 months, when compounded in aseptic conditions and stored at 2-8 ºC (Table 8). Paediatric and neonatal formulations remained stable for 14 or 25 days. In addition, Turmezei *et al.* reported that a ternary admixture remained sterile during at least 14 days stored at both 2-8, 25 and 30 ºC. (38)

Table 8 Overview of published studies on the microbiological stability of PN admixtures.

Study - year	Type of PN admixture	Compounding procedure	Sterility testing technique	Storage temperature	Times of testing (time after compounding)	Period of stability
Turmezei *et al.* (38) - 2015	Ternary (paediatric)	Unknown	10 mL of tested TPN samples were inoculated onto Columbia agar containing 5% sheep blood, chocolate agar	2-8 ºC 25 ºC 30 ºC	Every day during 14 days	At least 14 days

			containing polyviteX and Sabouraud medium incubated at 37 ºC for 24 hours and at RT for another 24 hours			
Janu *et al.* (17) - 2011	Ternary	Prepared aseptically under clean room conditions using an automated admixing device	According to the Ph. Eur. Ed. 6, chap. 2.6.1.	2-8 ºC	30 days	At least 30 days
Mirkovic *et al.* (50) - 2008	Ternary	Prepared aseptically in laminar air flow environment	According to Ph. Yug. V	Unknown	Unknown	At least 60 hours
Celma *et al.* (51) - 2016	Neonatal	Unknown	Membrane filtration through a 0.22 µm filter cultured afterwards in blood-agar	2-8 ºC	8, 11, 13 and 25 days	At least 25 days
Nordfjeld *et al.*	Binary	Prepared in a horizontal laminar air	Unknown	4 ºC	6 months	At least 6 months

(22) - 1983		flow bench in a clean room				
Takagi *et al.* (53) - 1989	Unknown	Unknown	Aseptic filtration using an inline 0.22 µm filter afterwards suspended in BHI broth and incubated at 35 ºC	RT	1 day 3 days 7 days	At least 7 days (but 2% MG on Day 1 and 1% MG on Day 3)

Ph. Eur. Ed. 6, chap. 2.6.1. – European Pharmacopoeia, Edition 6, Chapter 2.6.1.

Ph. Yug. V – Yugoslav Pharmacopoeia V

MG – microbial growth

Of the sterility studies that were found, microbial growth was detected only by Takagi *et al.*. They obtained interesting results: 2% of the PN admixtures tested 24 hours after compounding and 1% of the ones tested 72 hours after compounding presented microbial growth, while no contamination was detected in the group tested 7 days after preparation. The bags were stored at room temperature (RT). The microbial strains identified were coagulase-negative *Staphylococcus* species and gram-positive bacilli. (53)

It is also relevant to evaluate the capacity of PN admixtures to support microbial growth when contamination occurs. This can be done through the Efficacy of Antimicrobial Preservation assay described in the European Pharmacopoeia 9.0. This test consists of inoculating the preparation, preferably in its final container, with adequate microorganisms, storing it at the suitable conditions and quantifying the microbial load at specified intervals of time. The indicated microorganisms are *Pseudomonas aeruginosa* American Type Culture Collection (ATCC) 9027, National Collection of Industrial Food and Marine Bacteria (NCIMB) 8626 or Collection of Institut Pasteur (CIP) 82.118, *Staphylococcus aureus* ATCC 6538, National Collection of Type Cultures (NCTC) 10788, NCIMB 9518 or CIP 4.83, *Candida albicans* ATCC 10231,

National Collection of Pathogenic Fungi (NCPF) 3179 or Institut Pasteur (IP) 48.72 and *Aspergillus brasiliensis* ATCC 16404, International Mycological Institute (IMI) 149007 or IP 1431.83. Other appropriate strains might be used. Microbial suspensions should be prepared immediately before use and have a concentration of about 10^7-10^8 microorganisms/mL. When inoculated to the product, it should give an inoculum of 10^5-10^6 microorganisms/mL. The volume of the inoculum suspension should not exceed 1% of the volume of the product. The inoculated product is then maintained at 20-25 ºC protected from light and samples, normally 1 mL, are removed at time zero and at appropriate intervals and the number of viable microorganisms is determined by plate count or membrane filtration. The medium used is TSA for bacteria and Sabouraud-dextrose agar for fungi. The acceptance criteria for parenteral preparations (such as PN) are described in Table 9. (54)

Table 9 Acceptance criteria for the efficacy of antimicrobial preservation test according to the European Pharmacopoeia 9.0. (54)

		Log_{10} reduction in the number of viable microorganisms against the value obtained for the inoculum				
		6 hours	**24 hours**	**7 days**	**14 days**	**28 days**
Bacteria	A	2	3	-	-	NR
	B	-	1	3	-	NI
Fungi	A	-	-	2	-	NI
	B	-	-	-	1	NI

A – recommended efficacy

B – for justified cases where the A criteria cannot be attained, for example for reasons of an increased risk of adverse reactions

NR – no recovery

NI – no increase in number of viable microorganisms compared to the previous reading

Some studies have been published on this matter regarding PN (Table 10). Generally, the authors used bacterial and fungal strains with documented association to clinical infections and/or sepsis caused by PN administration. (24, 26)

Table 10 Overview of published studies on the response of PN admixtures to microbial contamination.

Study - year	Type of PN admixture	Tested strains	Method	Results
Didier *et al.* (55) - 1998	Ternary	*S. aureus* *S. epidermidis* *S. saprophyticus* *P. aeruginosa* *E. cloacae* *K. oxytoca* *S. marcescens* *A. calcoaceticus* *S. maltophilia* *B. cepacia* *Flavobacterium spp* *C. albicans*	Inoculum introduced into 3 mL of the test solution (initial inoculum: 10^1-10^2 CFU/mL); storage at 4, 25 or 35 ºC; samples were obtained at 0, 12, 24, 48, 72 hours and 5 days (25 and 35 ºC) or at 0, 48 hours and 7 days (4 ºC); serial 10-fold dilutions subcultured onto TSA, which was incubated at 35 ºC	None of the species tested grew at 4°C; at 25 and 35°C, only *C. albicans* and *S. saprophyticus* grew, and only after 24 hours of incubation
Fossum *et al.* (56) - 1988	Ternary	*S. aureus* Oxf. 209 *S. epidermidis* l-1478 *B. cereus* ATCC 11778 *S. marcescens* NCTC 1377 *K. pneumoniae* NCTC 4633 *A. calcoaceticus* No 8, Bøvre *P. aeruginosa* ATCC 27853	1 mL microbial suspension inoculated into 49 mL PN admixture (initial inoculum: 10^1 CFU/mL); stored at 6 or 22 ºC; 1 mL subcultured after 0 hours, 1, 5 and 12 days (6 ºC) or after 0, 5, 24 and 48 hours (22 ºC) onto blood-agar, which was incubated for 20 hours at 37 ºC	Gram-positive bacteria did not multiply at either 22 ºC or 6 ºC; *S. marcescens* and *F. marinotyphicum* multiplied at 6 ºC; at 22 ºC, gram-negative bacteria grew rapidly, except for *A. calcoaceticus* and *P. aeruginosa*; *C. albicans* grew moderately at 22 °C

		E. aerogenes NCTC 10006 *E. coli* MI3 3804 *F. marinotyphi-cum* ATCC 19260 *C. albicans* ATCC 24433		
Bronson *et al.* (57) - 1988	Binary	*E. coli* *E. cloacae* *K. pneumoniae* *P. vulgaris* *P. aeruginosa* *S. aureus* *S. pyogenes* Group A *S. epidermidis* *C. albicans*	0,1 mL microbial suspension ($5x10^1$-$3x10^2$ CFU/mL) to PN bag; contents of the bag filtered through a 0.22 µm filter over 8-12 hours; 2 mL TSB introduced into the filter set; incubated at 22±2 ºC for 10 days; turbidity determined at time 0, 12 and 24 hours and then daily	All samples showed visual turbidity after 4 days, except for the sample contaminated with *S. epidermidis*, in which turbidity was visible only after 6 days
Goldmann *et al.* (58) - 1973	Binary	*P. mirabilis* *E. coli* *S. aureus* *P. aeruginosa* *E. cloacae* *K. pneumoniae* *S. marcescens* *T. glabrata* *C. albicans*	Two types of admixture tested: with casein hydrolysate or synthetic amino acids; 0,1 mL of a 10^{-6} dilution of each bacterial strain and 0.5 mL of a 10^{-6} dilution of each fungal strain added to 100 mL aliquots of the PN admixtures (inoculum: 1 CFU/mL); storage at 25 and 4 ºC; samples obtained for plate counts at 12, 24, 48 hours and 7 days	Admixture with casein hydrolysate at 25 ºC: *C. albicans*, *T. glabrata*, *K. pneumoniae*, *S. marcescens* and *S. aureus* increased > 1 log at 12h and > 3 log at 24h, other bacteria tested multiplied more slowly, and *P. aeruginosa* was inhibited; admixture with synthetic amino acids at 25 ºC: fungi grew more

				slowly and all bacteria tested failed to multiply; no microbial growth in either admixture at 4 ºC
D'Angio *et al.* (26) - 1987	Binary and ternary	*S. epidermidis* ATCC 14892 *K. pneumoniae* ATCC 13883 *E. coli* ATCC 25922 *P. aeruginosa* ATCC 10145 *C. albicans* ATCC 24433	Each 100-mL admixtures inoculated with 1 ml of the microbial suspension (initial inoculum: $5x10^1$ CFU/mL); stored at RT (25 ºC); 1-ml samples were obtained at 0, 6, 12, 24, 48, 72 and 96 hours and dilutions 10^{-3} and 10^{-4} were plated onto blood agar; plates incubated at 37 ºC for 24 hours for bacteria and 48 hours for *C. albicans*	After 24 or 48 hours, all strains presented significant growth in ternary admixtures, with the exception of *S. epidermidis*; in binary admixtures, growth was inhibited or retarded
Gilbert *et al.* (59) - 1986	Binary and ternary	*S. aureus* ATCC 25923 *E. coli* ATCC 29194 *P. aeruginosa* ATCC 25619 *C. albicans*	1 mL inoculum added to 9 ml of each test solution (initial inoculum: 10^4 CFU/mL); samples taken after 24 hours or every 3 hours in the first day (*E. coli* and *C. albicans*); *C. albicans*: 1 mL inoculum added to 9 ml of each test solution (initial inoculum: 10^2 CFU/mL); samples taken after 24, 48 and 72 hours; incubation at 37 ºC	At an initial inoculum of 10^4 CFU/mL, all strains died in the binary solution and grew minimally or died in the ternary formulation; at initial inoculum of 10^2 CFU/mL, *C. albicans* did not grow well in the PN admixtures
Scheckelhoff *et al.* (60) - 1986	Binary and ternary	*P. aeruginosa* *S. aureus*	Serial dilutions of each admixture inoculated with $5x10^5$ bacteria/mL	*E. coli*, *C. tropicalis* and *C. albicans* grew well in both

	(adult and neonatal)	*S. epidermidis* *S. faecalis* Group JK *Corynebacterium* *E. coli* *C. tropicalis* *C. albicans* *T. glabrata*	or $5x10^3$ fungi/mL and incubated	binary and ternary admixtures; *T. glabrata* multiplied better in binary admixtures; the other strains tested grew better in the ternary ones; no significant difference in microbial growth between adult and neonatal (more dilute) admixtures
Crocker *et al.* (24) - 1984	Lipid emulsions (soybean and safflower oil)	*Serratia* *E. coli* *P. mirabilis* *E. cloacae* *K. pneumoniae* *P. aeruginosa* *S. aureus* *S. epidermis* *S. faecalis* *C. albicans*	Aliquots of each emulsion inoculated with cultures of each microorganism (initial inoculum: $1x10^4$ - $2x10^4$); inoculated aliquots incubated for 48 hours at RT; at 0, 6, 12, 24, and 48 hours, aliquots subcultured to blood agar using a calibrated urine loop method; subcultures then incubated for 48 hours at 37 °C	All strains multiplied well, with the exception of *Serratia*; the gram-positive organisms grew more slowly; microbial growth often reached 10^6 CFU/mL

A. calcoaceticus - Acinetobacter calcoaceticus, B. cepacia - Burkholderia cepacia, B. cereus - Bacillus cereus, C. albicans - Candida albicans, C. tropicalis - Candida tropicalis, E. cloacae - Enterobacter cloacae, E. coli - Escherichia coli, E. aerogenes - Erzterobacter aerogenes, F. marinotyphicum - Flavobacterium marinotyphicum, K. oxytoca - Klebsiella oxytoca, K. pneumoniae – Klebsiella pneumonia, P. aeruginosa - Pseudomonas aeruginosa, P. mirabilis - Proteus mirabilis, P. vulgaris - Proteus vulgaris, S. aureus - Staphylococcus aureus, S. epidermidis - Staphylococcus epidermidis, S. saprophyticus - Staphylococcus saprophyticus, S. faecalis - Streptococcus faecalis, S. pyogenes - Streptococcus pyogenes, S. maltophilia -

Stenotrophomonas maltophilia, *S. marcescens* - *Serratia marcescens*, *T. glabrata* - *Candida glabrata*

Ternary PN admixtures supported the growth of microorganisms significantly better than the binary ones. Strains such as *Escherichia coli*, *Klebsiella pneumoniae* and *Candida albicans* had their growth delayed in the last and *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Streptococcus faecalis* did not multiply at all. (24, 26, 39, 60) *Candida glabrata* seems to be an exception, as it has been shown to multiply better in 2-in-1 admixtures. (60) In binary admixtures, most microorganisms have their growth delayed for at least four days even when stored at room temperature. (26, 57)

However, binary formulations that contain protein hydrolysates have been shown to promote greater bacterial and yeast growth than the ones with synthetic amino acid solutions. Fungi like *C. albicans* and *T. glabrata* multiply more slowly and various bacterial strains fail to grow in the last. (24, 58)

In general, Gram-negative bacteria such as *K. pneumoniae* and *E. coli* presented better growth in ternary admixtures than Gram-positive ones. The exception seems to be *P. aeruginosa*, which in general was not able to multiply. *Staphylococcus epidermidis* (Gram-positive) failed to grow in several studies. (26, 55, 56)

C. albicans seems to be the microorganism with the most consistent growth in PN admixtures, having multiplied in practically all studies analysed, in all types of admixtures (Table 10).

Storage of the PN bags at 2-8 ºC has been reported to inhibit the multiplication of several bacteria and yeast both on binary and ternary admixtures. (55, 56, 58) Fossum *et al.* reported that *Serratia marcescens* was the only strain that multiplied, but slowly and only after 5 days, however, *C. albicans* also survived during 12 days, even though its number did not increase. (56)

5 Discussion

PN is a pharmaceutical preparation quite prone to instability, partly because of its complexity and consequent interactions between components, but also due to the nature of some of those components, the most critical of them being the lipid emulsion, and of the administration to the patient itself.

The hospital pharmacist is responsible for ensuring the quality of PN. This involves not only the quality control of the finished product, but also a series of measures which ensure that quality, and requires knowledge about the stability of the admixtures.

The aseptic preparation is one of the most important aspects in the quality assurance of PN. PN cannot go through a terminal sterilisation process, therefore aseptic compounding is the only way of ensuring the product's sterility and is recommended by guidelines and Pharmacopoeias. (30-32)

These documents advise having and following written validated procedures for all steps in the sterile processing. However, in 2009, only about half of the Portuguese hospitals that prepare neonatal PN had all the recommended procedures. (29) Therefore, some work could be done in this area.

Regarding the compounding environment, only about half the Portuguese hospitals prepared PN in a laminar flow chamber and/or in a EN/ISO 14644-1 Grade B clean room, and about a third of the hospitals did not know the air quality level of their compounding rooms. (29) This is worrisome, since admixtures can easily be contaminated by particles or microorganisms present in air of lower quality.

PN components should be introduced in the bags by a specific order, to reduce the risk of interactions between them and consequent destabilisation of the mixture. Glucose, electrolytes and trace elements should not be directly added to the lipid emulsion, since they can destabilise it. Phosphate and calcium should not be added sequentially, in order to avoid precipitation. Phosphate and magnesium should be added together, because they form soluble and stable complexes, leaving less phosphate free to interact with calcium, which should only be added afterwards. Vitamins (coloured) and the lipid emulsion (opaque) should only be added at the end, in order to facilitate visual examination for particles. (13, 14) In Portuguese hospitals, practices in this regard were

quite heterogeneous and Neves *et al.* does not clarify if they complied with the recommendations. (29)

Recording and documenting all the compounding process facilitates the detection of any errors that might happen and the traceability of a product, should a problem occur, and ensures process and product quality at all moments. (35) Most Portuguese hospitals keep record of the products used in each batch of neonatal PN they produce. (29)

In regard of quality control of the final product, the most important physicochemical tests that should be performed are assessment of the macroscopic aspect of the admixtures, verification of component containers, gravimetry, pH measurement, droplet diameter and quantification of certain components.

Visual evaluation is the easiest, fastest and less dispendious test, yet it can provide important information. Separation of phases in the lipid emulsion and change in colour can mean degradation, and precipitates are often detectable. (6) An admixture pH which is too acidic destabilises the lipid emulsion (by decreasing surface potential) and a higher one promotes calcium phosphate precipitation. (17) Optical microscopy is a less accurate method than laser diffraction or dynamic light scattering for measuring particle diameter, however, it is the most accessible and practical method for hospitals.

Despite the importance and relative simplicity of PN physicochemical quality control, in 2009, the only analysis performed by Portuguese hospitals was visual assessment of the admixtures, at least concerning neonatal PN. In addition, even though almost all hospitals evaluated binary admixtures, only about 14% evaluated the lipid emulsion. (29) This is an aspect that could clearly be improved with ease, especially since hospital pharmacies almost certainly already own a pH measurer and scales and perhaps a microscope.

The rate of microbiological contamination of sterile preparations has been estimated at 2.2 contaminated units per million produced units when prepared aseptically by qualified personnel in an ISO Class 5 environment. (61) Studies that analysed the sterility of PN admixtures in real or reality-simulation situations obtained slightly higher contamination rates, but nevertheless the majority of them was below 2%. The ones with better statistical significance (representative of a higher number of bags and longer time periods) obtained contamination rates lower than 0.5%. (39, 40, 43)

The analysed sterility studies used one or both techniques described in the European and US Pharmacopoeias (membrane filtration and direct inoculation of the culture medium), however, most of them used different media, mainly blood-agar and BHI. Both of these are non-selective media as TSB, but blood-agar is not as good for the growth of fungi. In addition, none of these media promotes the growth of anaerobic bacteria as thioglycollate broth does, so these studies might have failed in detecting them. In addition, most studies analysed quite smaller volumes of product than what is recommended in the Pharmacopoeias, which could have originated false negatives. Out of the analysed studies, there was no significant difference between the contamination rates of binary and ternary PN.

About 70% of Portuguese hospitals performed microbiological control of the neonatal binary admixtures. However, 65% did not control the lipid emulsion. (29) This is worrisome, since the last one promotes the growth of microorganisms to some extent. Even though the contamination rates seem to be low, procedures may have failures and there is always a risk involved for the patients' health. Furthermore, PN formulations are sterile pharmaceutical preparations and therefore, according to Pharmacopoeias and guidelines such as PIC/S, they should be subjected to sterility testing.

The maximum storage time for PN recommended by guidelines/Pharmacopoeias is 4-9 days at 2-8 ºC, or 24-30 hours at room temperature. (13, 30) The time actually practiced by Portuguese hospitals is 2 or 3 days at 2-8 ºC. (29) However, stability studies that have been published have found more extended periods of stability, up to 14 and 30 days for ternary admixtures, and 25 days or even 2-6 months for binary PN. Nevertheless, lipid peroxidation and vitamin degradation are limiting factors, beginning in the first 24 hours and at 48 or 72 hours, respectively.

Surprisingly, storage temperature does not seem to affect stability, either physicochemical or microbiological, except for vitamins E and C (refrigeration reduces/delays their degradation). Nonetheless, previous studies had observed that storage at 2-8ºC inhibits or at least delays multiplying of bacteria and fungi and that higher temperatures favour the dissociation of calcium ions, leaving them more available to unite with phosphate and precipitate. (14, 58) Furthermore, the 2008 Spanish Consensus on the Preparation of Parenteral Nutrition Admixtures recommends refrigeration of the bags. (13)

Concerning photoprotection, results are also controversial, with some authors finding that it decreases the degradation of vitamins and others observing that it does not influence it. The 2008 Spanish Consensus on the Preparation of Parenteral Nutrition Admixtures also recommends protecting the bags from light, since it seems to avoid the degradation of photosensitive vitamins and peroxidation reactions and to reduce the degradation of some amino acids. (13) Protection from light can therefore be a solution to prevent lipid peroxidation as a limiting factor.

EVA or another inert plastic should be preferred over Bis(2-ethylhexyl) phthalate (DEHP) as a material for PN bags, since lipids cause the last to release plasticisers which are toxic for the patient and it increases vitamin degradation and lipid emulsion destabilisation. EVA bags are however somewhat permeable to oxygen, which causes the oxidation of some PN compounds, such as vitamin C. Therefore, multilayer bags should be used. (13) In multilayer bags, the inner layer is made of an inert material such as EVA and outer layers are impermeable to oxygen (100 times more than EVA) and water vapour. (13, 21) It is also important that the bag size is adequate for the volume it contains, in order to reduce the PN surface of contact with oxygen, and the residual oxygen inside the bag should be removed after its preparation. (20, 21) Multi-chamber bags, in which different components are stored in separate compartments and are only mixed before administration, decrease interactions between PN constituents and allow a longer storage time. (13)

Only one sterility study detected microbial contamination in the admixtures. The contamination rate was low and the bacterial strains identified were coagulase-negative *Staphylococcus* species and gram-positive bacilli. (53) Coagulase-negative *Staphylococci* are part of the human skin flora, but they can be a cause of sepsis, especially when risk factors are present, such as catheters and immune compromise, which is often the case in patients receiving PN. In addition, they have a high rate of antibiotic resistance. (62) Gram-positive bacilli are considered non-pathogenic contaminants in skin and soft tissue infections, but they might be a cause of complications. (63)

Neonatal/paediatric admixtures are theoretically more prone to instability than adult formulations, since they have a lower concentration of amino acids, a higher concentration of calcium and phosphate and a lower concentration of lipids when PN is initiated. (64) Nevertheless, no significant differences in stability, either

physicochemical or microbiological, were observed between adult and neonatal/paediatric admixtures in the studies analysed.

In view of the results obtained, storing PN admixtures at 2-8 ºC protected from light seems like the best option. Multilayer and multicompartment bags should preferably be used. The bag size should be adequate for the admixture volume and the residual air should be removed from the bag after compounding, in order to reduce oxidation. In 2009, less than half of Portuguese hospitals stored neonatal PN bags in the fridge, so that is a point which should be improved, although, the rate of photoprotection was good. (29)

Regarding the storage period, stability studies should be performed by each hospital for their particular admixtures. Actually, the results obtained on previous studies suggest that PN shelf lives could be extended beyond the ones that are currently usually assigned, but vitamin solutions would have to be added ideally 24 hours before administration to the patient. A longer storage period would also mean a decrease in the frequency of preparation of standard admixtures and less probability of discarding bags for having reached the end of their shelf life, if adequate planning is made.

The longest microbiological stability period was found by Nordfjeld *et al.* (22), who observed that a binary admixture was sterile after 6 months at 4 ºC, however, care should be taken regarding this result, since this study analysed a single PN bag and no microbiological tests were performed at intermediate times (before 6 months).

Concerning the response of admixtures to microbial contamination, the studies analysed used smaller microbial initial inocula than what is recommended in the Efficacy of Antimicrobial Preservation assay described in the European Pharmacopoeia 9.0. This makes sense, since smaller inocula (10^1-10^2 CFU/mL) simulate better real contamination levels. However, it is hard to obtain such small inocula and the detection of microbial growth was hindered.

Ternary PN admixtures supported the growth of microorganisms significantly better than the binary ones, as expected. Binary admixtures, being hypertonic and acidic, are not ideal media for microbial growth. (55, 57, 58) However, if they contain protein hydrolysates, they will promote greater bacterial and yeast growth than the ones with synthetic amino acid solutions, as some studies have shown. (24, 58)

The only study found that compared adult with neonatal admixtures observed no significant difference in microbial growth between them, even though the last were less concentrated. (60)

The results obtained in the Takagi *et al.* sterility study (no artificial contamination) suggest that microorganisms die in PN after some time, since they detected microbial growth after 24 and 72 hours, but not after 7 days. (53) However, since the bags tested were not the same at each time, it could have been a coincidence that there were contaminated bags in the groups tested on the first and third days and none in the group tested after 7 days. This is most likely the case, since the results of microbial challenge studies did not show this reduction on microbial load.

The fact that PN supports the growth of *Candida albicans* well is worrisome, since this yeast is an opportunistic pathogen that infects debilitated and immunosuppressed patients. This shows the importance of a correct aseptic preparation procedure, microbiological control and careful handling of PN bags, to avoid delivery of contaminated admixtures to the patients.

On the other hand, the poor growth of *Pseudomonas aeruginosa* and *Staphylococcus epidermidis* is a positive point, since they can both easily contaminate PN bags. *P. aeruginosa* is spread through the hands of healthcare staff and devices such as catheters when hygiene measures are poor (according to Centers for Disease Control and Prevention – CDC). *S. epidermidis* is part of the normal skin flora, but can also be an opportunistic pathogen.

Several studies observed that storage of the PN bags at 2-8 ºC inhibits the multiplication of most bacteria and yeast both on binary and ternary admixtures. This reinforces the importance of storing the admixtures under refrigeration.

6 Conclusion

The stability of PN is easily influenced by several factors, namely its constitution and preparation and storage practices. The results obtained reinforced the importance of a good preparation procedure and of quality control. Even though no critical microbial contaminations were generally found, microorganisms showed the ability to grow in PN admixtures, should contamination occur. Aseptic preparation is essential, and should follow written validated procedures, take place in a laminar flow chamber inside a EN/ISO 14644-1 Grade B clean room and be performed by qualified operators, which does not happen in all hospitals yet. It is important to validate the asepsis of the compounding procedure and the operators' technique periodically. The components should be added by an order that minimises destabilisation and interactions, and records of the process should be maintained. Quality control is vital to ensure patients' safety. The tests performed nowadays in Portuguese hospitals consist only of the visual examination of the bags, but they should also include gravimetry, pH measurement and particle size analysis by optical microscopy at least. The test for sterility is performed by many hospitals, but it should be common practice for all. Admixtures should be stored at 2-8º C protected from light, in multilayer and multicompartment EVA bags. Additional care has to be taken with ternary admixtures, since they are more prone to instability, both physicochemical and microbiological. The results suggest that stability studies made by each hospital pharmacy would be of great importance, as they would help to establish the adequate shelf life for its particular formulations, extending it and helping to define the ideal quantity and frequency of preparation. They would thus allow hospitals to optimise pharmacy human resources involved in PN production and production time and reduce wasted bags, consequently reducing costs related to PN, while ensuring the safety and correct treatment of patients.

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