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Original Article

Production and stability study of a hospital parenteral nutrition solution for neonates

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ABSTRACT

Standard parenteral nutrition solutions are mixtures comprising interacting components that may degrade themselves over time. The objective of this study was to investigate the physicochemical and microbiological stability of a hospital preparation for parenteral nutrition in neonatology. The analyses were performed throughout the storage of the preparations at 2–8 °C (up to 4 months). The extent of stability was based on the determination of amino acids dosage, visual and physicochemical properties (glucose and electrolytes concentrations, pH and osmolality measurements, particle counting) and microbiological analysis (sterility test). A thermal degradation of ascorbic acid was conducted to evaluate the antioxidant properties of the parenteral mixture. Physicochemical and microbiological controls were found to comply with the specifications. Amino acids showed a good stability throughout the 4months storage except for cysteine, which was progressively degraded to cystine, conferring a yellow coloration to parenteral solutions. Parenteral nutrition standards solutions remain stable for 4 months at 2–8 °C, ensuring safe administration in preterm infants.

1. Introduction

The management of premature infants requires specific parenteral nutrition [1]. Some nutrients are essential to their development and the quality of parenteral nutrition can condition their future [2]. The prescription of parenteral nutrition in neonatology is standardized according to the recommendations of the European Society for Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) and the European Society for Clinical Nutrition and Metabolism (ESPEN) [3,4]. Several parenteral nutrition formulations (industrial, standardized or individualized formulations) are available [5]. The standardized formulations might be prepared in-house by hospital pharmacists to rapidly meet the nutritional needs of a large number of premature infants. At hospital, standardized solutions are at a high risk of microbiological contamination and the stability of the preparation must be ensured. After development and production by hospital pharmacists, the preparation is checked before release and must remain stable throughout its period of use [6]. The amino acids present in the parenteral nutrition solutions may degrade over time, in particular by an oxidation phenomenon. Therefore, it is mandatory to verify the stability of the amino acids during the storage of the solutions to respond to protein needs. The ratio between essential and non-essential amino acids must also be preserved to promote nitrogen balance and anabolism of premature infants. In 2016, the Council of Europe specified the quality and safety requirements for hospital preparations [7]. To our knowledge, no data on the long-term stability of amino acids in hospital standardized solutions is available in the literature. Different methods for amino acids assay are currently available, including automatic amino acids analyser [8–11], and high-performance liquid chromatographic (HPLC) coupled with mass spectrometric [12–15] or fluorescence detection [16,17]. However, there is no specific HPLC method coupled with ultraviolet (UV) detection for amino acids in parenteral

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nutrition mixture assay.

After the development and the validation of a stability-indicating reversed phase-HPLC method, the main objective of this study was to evaluate the stability of a hospital standardized formulation for parenteral nutrition. Amino acid composition, physicochemical and microbiological analyses of parenteral nutrition solution were carried out simultaneously to ensure the safety of the final preparation.

2. Methods

2.1. Sterile manufacture of neonatal parenteral nutrition solutions

Parenteral nutrition solutions for neonates were prepared aseptically in a class 100 isolator (ISO 5) by using an automated pumping system (ExactaMix® 2400, Baxter Healthcare Corporation, Deerfield, IL, USA) and in compliance with Good Preparation Practices [18]. The composition of the parenteral nutrition solution is shown in Table 1. A commercial amino acid solution (Primene® 10%, Baxter Healthcare Corporation, Deerfield, IL, USA) was used to bring essential and non-essential amino acids to hospital preparation. Primene® 10% is initially indicated in the parenteral nutrition of the newborn or premature infants when oral or enteral feeding is insufficient or impossible. The parenteral nutrition solution was packaged in 250 mL EVA bags (ExactaMix®, Baxter Healthcare Corporation, Deerfield, IL, USA). The bags were labelled and individually packaged (unique bag number and bar code).

2.2. Stability study

In order to prolong the duration of use initially set at 11 days after preparation, a stability study of the hospital preparation was carried out. Bags prepared and double wrapped were stored at 2–8 °C. The analyses were carried out after 1, 8, 15, 22, 29, 57, 85 and 118 days of storage at 2–8 °C. They were run from a pilot batch of 72 bags divided into 3 random groups. Each group consisted of 24 bags and was tested independently (8 bags for sterility test, 8 bags for the physicochemical test series and 8 emergency bags). For each period of stability, 2 bags were taken from each of the groups:

- a 200 mL bag of parenteral nutrition was used for amino acids, glucose, phosphorus, sodium, potassium, calcium, pH, absorbance and osmolality measurements, and particle counting. Each of these analyses was performed 3 times.
- a 200 mL bag of parenteral nutrition was used for sterility test.

Table 1
Composition of the standard parenteral nutrition solution.

Components	Concentrations
Sodium Sodium lactate	34 mM
Potassium Monopotassium phosphate	20 mM
Calcium Calcium gluconate	20 mM
Phosphorus Monopotassium phosphate	20 mM
Glucose	944 mM (170 g/L) 680 kcal/L
Amino acids	436 mM (48 g/L) 192 kcal/L
Nitrogen	7.2 g/L
Total energy	872 kcal/L
Glucose/nitrogen energy	95 kcal/g
Protein-energy ratio	5.5 g/100 kcal

Before analysis, the bags were removed from the cold room and maintained at 18–22 °C for 24 h to simulate the time of administration of a parenteral nutrition solution in the medical wards. The stability tests were conducted in accordance with the recommendations of the International Conference of Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) [19,20] and the European Pharmacopoeia [21]. The evaluation of stability was based on the determination of amino acids concentration, visual inspection, physicochemical parameters (glucose, phosphorus, sodium, potassium, calcium, pH, osmolality, absorbance and count of non-visible particles), and microbiological analysis (sterility test). The number of bags and the analysis (physicochemical and microbiological) were determined according to the recommendations of the European Pharmacopoeia [21]. The specifications were defined according to the initial composition of the solution with an acceptance interval of ± 15%.

2.2.1. Amino acid stability

A stability-indicating reversed phase-HPLC method was developed to determine the concentration of the amino acids contained in the parenteral nutrition solution (i.e. L-aspartic acid, L-glutamic acid, L-serine, L-histidine, L-glycine, L-threonine, L-arginine, L-alanine, L-taurine, L-tyrosine, L-cystine, L-valine, L-methionine, L-tryptophan, L-phenylalanine, L-isoleucine, L-ornithine, L-leucine, L-lysine and L-proline).

Concentrations of two amino acids (L-cysteine, L-taurine) were not or partially determined during the study. L-cysteine was not detected in this study because its assay required a different derivatization method. However, the concentration of cystine, the degradation product of L-cysteine, was monitored during the study. Specifically, an additional cystine assay was performed after 330 days of storage to model the rate of cystine formation in standard parenteral nutrition solution.

2.2.1.1. Chemicals and reagents. Amino acids standards (> 99% purity) (Sigma Aldrich®, Saint-Louis, Missouri, USA) were used for identification and quantification by HPLC. Chemicals for buffer solution were all of analytical grade: disodium hydrogen phosphate (Na_2HPO_4 anhydrous), sodium tetraborate decahydrate ($Na_2B_4O_7$; $10H_2O$), sodium azide (NaN_3), hydrochloric acid 37% (HCl), and phosphoric acid 85% (H_3PO_4) (Thermo Fisher Scientific®, Waltham, Massachusetts, USA). The reagents for derivatization of amino acids (3-mercaptopropionic acids (3-MPA), ortho-phthalaldehyde (OPA), and 9-fluorenylmethylchloroformate (FMOC)), and borate buffer (0.4 M in water, pH 10.2) were obtained from Agilent® technologies (Santa Clara, California, USA) and were stored at 4 °C. Acetonitrile (ACN) and methanol (MeOH) were of HPLC grade and received from Thermo Fisher Scientific® (Waltham, Massachusetts, USA). Water was purified through a Purelab® Option Q7 purifying system (Veolia® water technologies, Saint-Maurice, France). The buffer solution was filtered through polyvinylidene fluoride (PVDF) at 0.22 μm (Thermo Fisher Scientific®, Waltham, Massachusets, USA) and all samples were filtered through regenerated cellulose (RC) at 0.20 μm (Thermo Fisher Scientific®, Waltham, Massachusetts, USA).

2.2.1.2. Instrumentation. An HPLC system (Agilent® 1290 Infinity Quaternary LC System, Santa Clara, California, USA) was equipped with a quaternary pump with integrated vacuum degasser, a thermostated column compartment, an autosampler and a

multiple wavelength detector. The results were collected and evaluated statistically using HP ChemStation® software (Agilent®, Santa Clara, California, USA).

2.2.1.3. Stock solution and standard solutions for chromatographic analysis. A stock solution containing all of twenty amino acids was prepared (Table S1) with dissolution of amino acids in 0.1 M HCl (100 mL) submitted to magnetic agitation at 4 °C over 12 h. From the stock solution, standard solutions of amino acids were prepared in 20–1000 μM range (Table S2).

2.2.1.4. Preparation of sample solution. To quantify amino acids in parenteral nutrition bags, samples were diluted 1:50 (v:v) in 0.1 M HCl, then filtered through a 0.20 μm RC membrane (Dutscher®, Brumath, France) and injected to the column.

2.2.1.5. Amino acids chromatographic assay. Amino acids might be assayed with pre-column derivatization and UV detection. The primary amino groups reacted with OPA in the presence of 3-MPA (pH 10) to form an isoindole derivative (Fig. S1A). The secondary amino groups reacted with FMOC (pH 10) to form a secondary amide (Fig. S1B). The products of OPA derivatization were detected at 338 nm while products of FMOC derivatization were detected at 262 nm. The autosampler allowed online derivatization by a program injection below: 2.5 μL of borate buffer and 1 μL of sample were drawn successively, and then mixed five times. After 0.2 min, 0.5 μL of 3-MPA/OPA reagents was added and mixed ten times; then 0.4 μL of FMOC reagent was taken and mixed ten times. Finally, 32 μL of diluent was drawn, and then 12 μL of the mixture was injected. The chromatographic separation of amino acids was obtained by using a C_{18} (3.0 mm × 150 mm, 2.7 μm) column (Poroshell® HPH, Agilent® Technologies, Santa Clara, California, USA) for high pH thermostated at 40 °C. Mobile phase flow rate was 0.64 mL/min. The separation was achieved with gradient using two eluents. Eluent A contained 10 mM Na_2HPO_4, 10 mM $Na_2B_4O_7$, and 5 mM NaN_3 adjusted to pH 8.2 with concentrated HCl and eluent B included 45% of ACN, 45% of MeOH and 10% of ultra-pure water. The initial mobile phase composition was 98% A and 2% B, which changed linearly from 0.53 to 20.10 min to the composition of 43% A and 57% B. The second linear gradient started from 20.10 to 20.25 min, where composition was 100% B and this composition was maintained for 3.3 min. Subsequently, the mobile phase returned at the initial composition in 0.15 min and was remained during 3.3 min.

The range of calibration was adapted to theoretical concentration of twenty amino acids in parenteral nutrition bag. The validation was realized applying in the first instance standards solutions of amino acids and then dosage from parenteral nutrition bags. The analytical validation was performed according to the recommendations of the ICH Q2B [20] including the assessment of system suitability, specificity, linearity, accuracy, precision (repeatability, intermediate precision), limits of detection (LOD) and quantification (LOQ) (see more details in Supplementary material) and capability. The system suitability was determined from replicate injections of the system suitability standard before and during sample analysis. Retention time, the number of theoretical plates, and capacity factor were the chromatography parameters selected for the system suitability test. The capability allowed to assess the risk of false negatives that can be generated by a validated analytical method and was calculated as follows: Cp = [difference between the highest and lowest limits of confidence interval]/6σ. A simple calculation of the capability of the method allowed to estimate the risk of false negative results and performance of the method [22].

2.2.2. Visual controls

Before any analysis, a macroscopic examination of the bags was carried out. Visual inspection of the bags included the colour and clarity of the solution, as well as the absence of visible particles and precipitates.

2.2.3. Physicochemical stability

The glucose concentration was determined using a UV photometric enzymatic method (DiaSys® Dia-gnostic Systems, Glucose GOD FS, Holzheim, Germany). The glucose reacting with oxygen was converted into gluconic acid and hydrogen peroxide by the action of glucose oxidase dehydrogenase. Hydrogen peroxide was then converted by peroxidase to quinone imine after reaction with 4-aminoantipyrine and phenol. Following the enzymatic cascade, quinone imine was measured at 500 nm with a UV/Visible spectrophotometer (UV-2101 PC®, Shimadzu®, Kyoto, Japan). The technical characteristics of this assay were as follows: the detection limit was 0.06 mM, the linearity was satisfactory between 0.06 and 22.2 mM, and the maximum coefficient of variation of the fidelity did not exceed 0.87%. Glucose assays were performed on parenteral nutrition solutions diluted 1:200 (v:v) in distilled water. Phosphorus was measured using a UV photometric method with end-point measurement (DiaSys® Diagnostic Systems, Glucose GOD FS, Holzheim, Germany). The phosphate reacted with sulfuric acid and ammonium molybdate to form a long inorganic phosphorus molybdate complex. The maximum absorption of the complex was determined at 340 nm. The detection limit was 0.065 mM, the linearity was satisfactory between 0.06 and 9.69 mM, and the maximum coefficient of variation of the fidelity did not exceed 2.22%. Phosphorus assays were performed on parenteral nutrition solutions diluted 1:10 (v:v) in distilled water. The absorbance between 200 and 800 nm of the parenteral nutrition solution was measured with a UV/visible spectrophotometer (UV-2101 PC®, Shimadzu®, Kyoto, Japan) after 1:20 (v:v) dilution in distilled water. The pH was measured using an HQ30d multi-parameter meter (Hach Lange®, Marne-la-Vallée, France) thermostated and calibrated before each measurement with buffer solutions (buffer pH 4.01 and 7.00). Osmolality (mOsm/kg) was measured by freezing point depression using an osmometer (Model 2020®, Advanced Instruments®, Norwood, Massachusetts). Since the expected specifications were given in osmolarity (mOsm/L), a conversion of the measurements was carried out. Particle counting was carried out in accordance with the European Pharmacopoeia (monograph 2.6.1 "Sterility") [21] using an automatic optical counter (Hiac/Royco 9103®, Beckman Coulter®, Brea, California, USA). Electrolytes (sodium, calcium, potassium) were assayed by atomic absorption spectrophotometry (SAA240FS Agilent Varian®, Santa Clara, California, USA). The quantification limits of sodium, calcium and potassium were respectively 0.03 μM, 0.1 μM and 0.06 μM.

2.2.4. Microbiological stability

The sterility test was carried out on the parenteral nutrition bags by the membrane filtration technique recommended by the European Pharmacopoeia (monograph 2.6.1 "Sterility") [21]. This test was carried out in a closed system under a horizontal laminar flow hood (ISO 5) with a Steritest Equinox® pump (Millipore®, Molsheim, France). The filtered volume was 200 mL. The sterility test required two liquid culture media (Trypcase-Soja and Thioglycolate, Biomérieux®, Lyon, France). The culture media were observed after 24 h, 48 h, 7 and 14 days of incubation.

2.3. Anti-oxidant properties

The antioxidant properties of parenteral nutrition solution were evaluated by studying the thermal degradation of ascorbic

Table 2
Composition of aqueous solutions of glucose, amino acids, glucose and amino acids, supplemented or not with ascorbic acid.

Solutions	Components (g/L (mM))		
	Ascorbic acid	Glucose	Amino acids[a]
A	87 (494)	–	–
B	87 (494)	74 (411)	–
C	87 (494)	–	42 (382)
D	87 (494)	74 (411)	42 (382)
E	–	74 (411)	43 (390)

[a] Cysteine concentration was about 0.79 g/L (6.5 mM).

acid. Ascorbic acid oxidizes to dehydroascorbic acid under the effect of an increase in temperature according to first-order kinetics [23]. Glucose and some amino acids are likely to reduce the thermal oxidation of ascorbic acid [24,25]. Four ascorbic acid solutions were prepared by diluting a commercial injectable solution of ascorbic acid (Laroscorbine® injectable, Bayer Healthcare, Lyon, France; ascorbic acid 1 g – 5 mL, excipients: water for injection, *p*-methylhydroxybenzoate, propyl-*p*-hydroxybenzoate, sodium hydroxide) in (A) water for injection, (B) glucose solution, (C) an amino acid solution (Baxter Healthcare®, Deerfield, IL, USA), and (D) a binary glucose and amino acid solution (Primene® 10%, Baxter Healthcare®, Deerfield, IL, USA). A binary solution of glucose and amino acids (Primene® 10%, Baxter Healthcare®, Deerfield, IL, USA) was prepared without the addition of ascorbic acid (E). The qualitative and quantitative composition of the A–E solutions is shown in Table 2. Each solution was maintained at 40 °C for 150 min and then at 55 °C for 30 min. After the heat treatment, the absorption spectrum of the A–E solutions was determined between 200 and 800 nm. Finally, a visual comparison of the solutions was carried out using photographs.

2.4. Statistical analysis

Statistical tests for the validation of the amino acid assay by HPLC method were performed by the Excel® software (Microsoft Office®, Redmond, Washington, USA, 2007) with a risk α of 5%. The linearity was evaluated by a linear regression model that was estimated by the method of least squares. The verification of the homogeneity of variance was performed by *Cochran's* test. The test for determining the existence of a significant slope was made by a *Fischer-Snedecor's* test. To validate the study of the accuracy, a *Cochran's* and an *ANOVA* tests were performed. The confidence interval of the average recovery was determined by *Student's* test. Finally, the precision was validated by *Cochran's* test. The coefficients of variation were calculated for the intermediate precision and the repeatability.

Statistical analysis of the stability results was carried out using KaleidaGraph® software (Synergy® Software, Reading, PA, USA) with a risk α of 5%. A *Wilcoxon's* test was performed to test the significance of the results between day 1 and day 118 of storage for the analysis of cystine concentration.

3. Results

3.1. Amino acids chromatographic assay

3.1.1. Analytical development and optimization

In the first condition, the mobile phase was composed of eluent A (phosphate buffer pH 7.82) and eluent B including 45% of ACN, 45% of MeOH and 10% of ultra-pure water through a column Zorbax Eclipse®-AAA (4.6 mm × 150 mm, 3.5 μm). In the second condition, the mobile phase was composed of eluent A (10 mM Na_2HPO_4, 10 mM $Na_2B_4O_7$, and 0.5 mM NaN_3 adjusted to pH 8.2) and eluent B (45% of ACN, 45% of MeOH and 10% of ultra-pure

Table 3
Qualitative and quantitative amino acid (g/L) composition of standard parenteral nutrition solution during storage at 2–8 °C.

Amino acids (g/L)	Specifications (g/L)	Storage time (day)							
		1	8	15	22	29	57	85	118
Essentials									
L-histidine	1.55–2.09	1.93 ± 0.04	1.94 ± 0.03	1.92 ± 0.08	1.88 ± 0.04	1.89 ± 0.15	1.96 ± 0.05	1.86 ± 0.03	2.01 ± 0.03
L-isoleucine	2.74–3.69	3.34 ± 0.07	3.36 ± 0.05	3.32 ± 0.11	3.24 ± 0.05	3.24 ± 0.24	3.29 ± 0.09	3.22 ± 0.06	3.33 ± 0.04
L-leucine	4.08–5.52	5.12 ± 0.12	5.14 ± 0.08	5.07 ± 0.17	4.93 ± 0.08	4.96 ± 0.37	5.05 ± 0.14	4.93 ± 0.09	5.10 ± 0.07
L-lysine	4.49–6.07	6.08 ± 0.17	5.99 ± 0.12	5.93 ± 0.22	5.80 ± 0.10	5.80 ± 0.44	6.02 ± 0.17	5.69 ± 0.23	6.06 ± 0.12
L-methionine	0.98–1.32	1.16 ± 0.03	1.14 ± 0.05	1.08 ± 0.05	1.03 ± 0.03	1.09 ± 0.12	1.05 ± 0.07	1.05 ± 0.03	1.07 ± 0.07
L-phenylalanine	1.72–2.31	2.13 ± 0.04	2.11 ± 0.03	2.09 ± 0.07	2.02 ± 0.03	2.04 ± 0.16	2.09 ± 0.06	2.01 ± 0.04	2.13 ± 0.03
L-threonine	1.51–2.04	1.84 ± 0.04	1.85 ± 0.03	1.82 ± 0.05	1.77 ± 0.03	1.79 ± 0.13	1.83 ± 0.05	1.78 ± 0.03	1.85 ± 0.02
L-tryptophan	0.82–1.10	1.10 ± 0.02	1.06 ± 0.03	1.05 ± 0.06	1.00 ± 0.03	0.99 ± 0.09	1.02 ± 0.04	0.93 ± 0.03	1.03 ± 0.05
L-valine	3.11–4.19	3.90 ± 0.10	3.91 ± 0.05	3.85 ± 0.13	3.70 ± 0.06	3.78 ± 0.28	3.80 ± 0.13	3.75 ± 0.07	3.87 ± 0.04
Total [1]	21.00–28.33	26.72 ± 0.63	26.62 ± 0.47	26.29 ± 0.94	25.48 ± 0.45	25.73 ± 1.88	26.28 ± 0.80	25.40 ± 0.61	26.67 ± 0.44
Non-essentials									
L-alanine	3.27–4.41	4.29 ± 0.10	4.30 ± 0.06	4.25 ± 0.13	4.12 ± 0.06	4.24 ± 0.35	4.01 ± 0.11	3.96 ± 0.07	4.06 ± 0.06
L-arginine	3.43–4.63	4.30 ± 0.09	4.22 ± 0.31	4.22 ± 0.11	4.10 ± 0.06	4.10 ± 0.33	4.19 ± 0.12	4.07 ± 0.08	4.22 ± 0.05
L-aspartic acid	2.45–3.31	2.99 ± 0.07	3.01 ± 0.07	2.98 ± 0.08	2.92 ± 0.04	2.93 ± 0.22	3.00 ± 0.08	2.94 ± 0.05	3.02 ± 0.04
L-glutamic acid	4.08–5.52	5.21 ± 0.12	5.29 ± 0.10	5.19 ± 0.16	5.05 ± 0.08	5.11 ± 0.39	5.18 ± 0.14	5.09 ± 0.09	5.25 ± 0.07
glycine	1.63–2.20	2.02 ± 0.06	2.01 ± 0.04	2.00 ± 0.07	1.92 ± 0.03	1.97 ± 0.15	2.02 ± 0.06	1.95 ± 0.04	2.02 ± 0.04
L-ornithine	1.29–1.76	1.55 ± 0.06	1.50 ± 0.05	1.48 ± 0.05	1.47 ± 0.03	1.41 ± 0.12	1.56 ± 0.05	1.45 ± 0.05	1.55 ± 0.05
L-proline	1.23–1.65	1.12 ± 0.16	1.26 ± 0.16	1.34 ± 0.18	1.27 ± 0.08	1.43 ± 0.09	1.27 ± 0.11	1.59 ± 0.04	1.29 ± 0.05
L-serine	1.64–2.20	2.01 ± 0.05	2.03 ± 0.04	2.01 ± 0.06	1.94 ± 0.03	1.97 ± 0.14	2.02 ± 0.06	1.97 ± 0.04	2.03 ± 0.03
L-taurine	0.24–0.34	0.34 ± 0.02	0.33 ± 0.01	0.34 ± 0.02	0.33 ± 0.01	0.25 ± 0.05	0.27 ± 0.01	0.24 ± 0.01	0.28 ± < 2%
L-tyrosine	0.18–0.25	0.22 ± 0.01	0.22 ± 0.01	0.21 ± 0.01	0.21 ± < 2%	0.21 ± 0.02	0.22 ± 0.01	0.21 ± < 2%	0.22 ± < 2%
Total [2]	19.44–26.26	24.05 ± 0.74	24.17 ± 0.85	24.02 ± 0.87	23.33 ± 0.46	23.62 ± 1.86	23.74 ± 0.97	23.47 ± 0.51	23.94 ± 0.39
Total [1 + 2]	44.44–54.59	50.77 ± 1.37	50.79 ± 1.32	50.31 ± 1.81	48.48 ± 0.91	49.35 ± 3.74	50.02 ± 1.77	48.87 ± 1.12	50.61 ± 0.83
Ratio [1/2]	0.80–1.46	1.13 ± 0.06	1.10 ± 0.06	1.11 ± 0.08	1.11 ± 0.04	1.09 ± 0.17	1.11 ± 0.06	1.08 ± 0.05	1.11 ± 0.04

Values are expressed as: mean concentration ± standard deviation or ± < coefficient of variation (%).

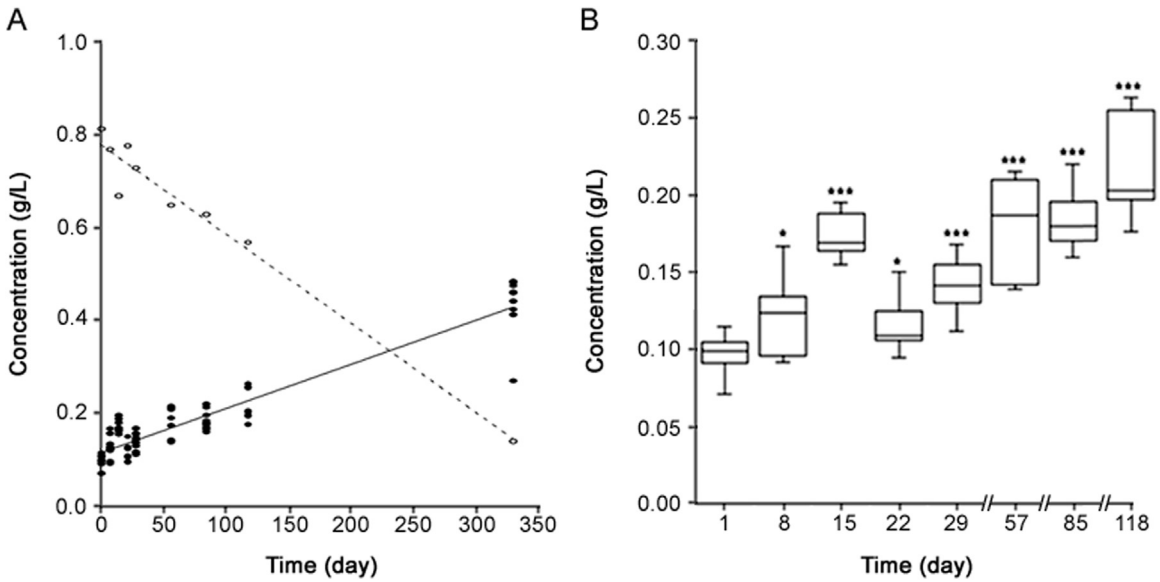

Fig. 1. (A) Linear profiles of (•) cystine ($r = 0.936$, $p < 0.0001$) and (○) cysteine ($r = 0.984$, $p < 0.0001$) concentrations in parenteral nutrition solution as a function of time of storage at 2–8 °C ($n = 9$ experimental determinations). Profile of cysteine concentration was calculated as the difference of initial concentration of cysteine in the parenteral nutrition solution and the experimental cystine concentrations determined throughout the time of storage. (B) Box plot: Cystine concentration for 118 days of storage at 2–8 °C ($n = 9$ experimental determinations). * $p < 0.05$; *** $p < 0.01$ as compared to day 1 group (Wilcoxon's test).

water) through a column Poroshell® HPH C_{18} (3.0 mm × 150 mm, 2.7 μm) The most accurate separation was obtained in the second condition (Fig. S2).

3.1.2. Method validation

After developing the method, a validation was conducted in accordance with ICH guidelines (Q2B) before practical use [20]. The system suitability tests checking the performance of analysis systems before and during routine analysis, were performed automatically for each analysis. Three types of tests performed automatically for each analysis met the acceptance criteria (Table S3). Table S4 reports that retention time and area under the curve remained unchanged even in the presence of glucose. Fig. S3 shows similar retention time and a decrease of amino acids peak area in normal and forced degradation conditions, respectively.

The validation parameters of amino acid assay by HPLC are reported in Table S5. A significant correlation between amino acid peak areas and concentrations was found for each amino acid ($r \geq 0.991$). The precision, determined by repeatability and intermediate fidelity, was performed from solutions ranged between 80 and 600 μM according to the amino acids and was found satisfactory (Table S5; CV < 5.0%). The LOD and LOQ demonstrated that the method was highly sensitive. The capability of each amino acid was greater than 1.33. Therefore, the performance of the method was considered satisfactory and the risk of false negative results was estimated < 0.01%. The validation of the chromatographic amino acids assay according to the ICH guidelines allowed ensuring the reliability, accuracy and precision of amino acids assay for daily analysis. The mean recovery of each amino acid was obtained with a confidence interval ranging from 95% to 105%, demonstrating the high accuracy of the method.

3.1.3. Amino acids stability

The results of amino acids assays in parenteral nutrition solution are reported in Table 3. Fig. S4 shows the chromatogram of the amino acids contained in parenteral nutrition solutions. The concentrations of the amino acids were considered to be consistent throughout the stability study for all the amino acids. The ratio of essential to non-essential amino acids was maintained throughout the stability study. Cystine was found in increasing concentrations during the study while this amino acid was absent or in small quantities initially. Fig. 1 shows cystine concentrations over time and the modelling of the cysteine degradation. Cysteine formation from L-cysteine degradation followed a zero order kinetic.

3.2. Visual controls

Visual inspection of the bags showed neither precipitates nor visible particles. During the storage of the bags, a progressive yellow colouring of the solution was observed.

3.3. Physicochemical and microbiological stability

The physicochemical and microbiological parameters between 1 and 118 days of storage at 2–8 °C conformed to the specifications. The results of the physicochemical parameters are shown in Table 4. Changes in phosphorus and glucose concentration from day 1 ranged 95%–105% for phosphorus and 88%–101% for glucose. The results of the sodium, potassium and osmolality measurements showed results in accordance with the specifications. The pH measurements were in accordance with the acceptance criteria. The calculated measurement error for physicochemical parameters was less than 15%. The maximum absorption of nutrition solutions was determined at 226 nm over time. The results of the non-visible particle counts were in accordance with the recommendations of the European Pharmacopoeia. The sterility tests confirmed the absence of microorganism contamination through the time of storage, in conformity with the European Pharmacopoeia.

3.4. Anti-oxidant properties

After thermal stress of the ascorbic acid, ascorbic acid – glucose – amino acid based solutions showed variable colouring depending on the presence of glucose and/or amino acids. Solution E (glucose-amino acid solution) remained colourless. Fig. 2A shows the UV–visible absorption spectra of these solutions and Fig. 2B corresponds to the photographs of these solutions after thermal stress. The addition of amino acids and/or glucose to ascorbic acid

Table 4
Characterization of the physicochemical properties of the standard parenteral nutrition solution during storage at 2–8 °C.

Parameters and specifications	Storage time (day)							
	1	8	15	22	29	57	85	118
pH: 4.08–5.52	4.99 ± 0.03	5.05 ± 0.01	5.11 ± 0.03	5.02[a] ± 0.02	5.06 ± 0.04	5.06 ± 0.08	4.92 ± 0.02	5.05 ± 0.03
Osmolarity: 1552–2098 mOsm/L	1698 ± 33	1724 ± 21	1723 ± 39	1758[a] ± 30	1715 ± 16	1704 ± 17	1698 ± 17	1708 ± 33
Glucose: 802–1085 mM	992 ± 28	965 ± 35	981 ± 60	873[b] ± 81	961 ± 17	997 ± 58	912 ± 22	974 ± 28
Absorption maximum wavelength (nm): not specified	226	226	226	226	226	226	226	226
Number of particles/mL								
> 10 μm: ≤ 25	3 ± 1	3 ± 2	1 ± 1	3 ± 2	3 ± 1	4 ± 2	1 ± 1	4 ± 5
> 25 μm: ≤ 3	1 ± 1	0	0	0	1 ± 1	1 ± 1	0	1 ± 1
Sodium: 29–39 mM	37 ± 1	37 ± < 1%	ND	39 ± < 1%	39 ± < 2%	38 ± 1	38 ± 1	38 ± 1
Potassium: 17–23 mM	20 ± 1	20 ± < 1%	22 ± 1	21 ± 1	21 ± < 2%	20 ± 1	20 ± < 2%	20 ± 1
Calcium: 17–23 mM	18 ± < 2%	18 ± < 1%	20 ± < 2%	20 ± < 1%	19 ± < 2%	19 ± < 2%	18 ± < 2%	19 ± < 2%
Phosphorus: 17–23 mM	21 ± 1	21 ± 1	20 ± 1	22[b] ± 1	20 ± 1	20 ± 1	21 ± 1	21 ± 1

Values are expressed as mean ± standard deviation or ± < coefficient of variation (%).
ND: Not determined.
[a] 3 experimental measurements.
[b] 6 experimental measurements.

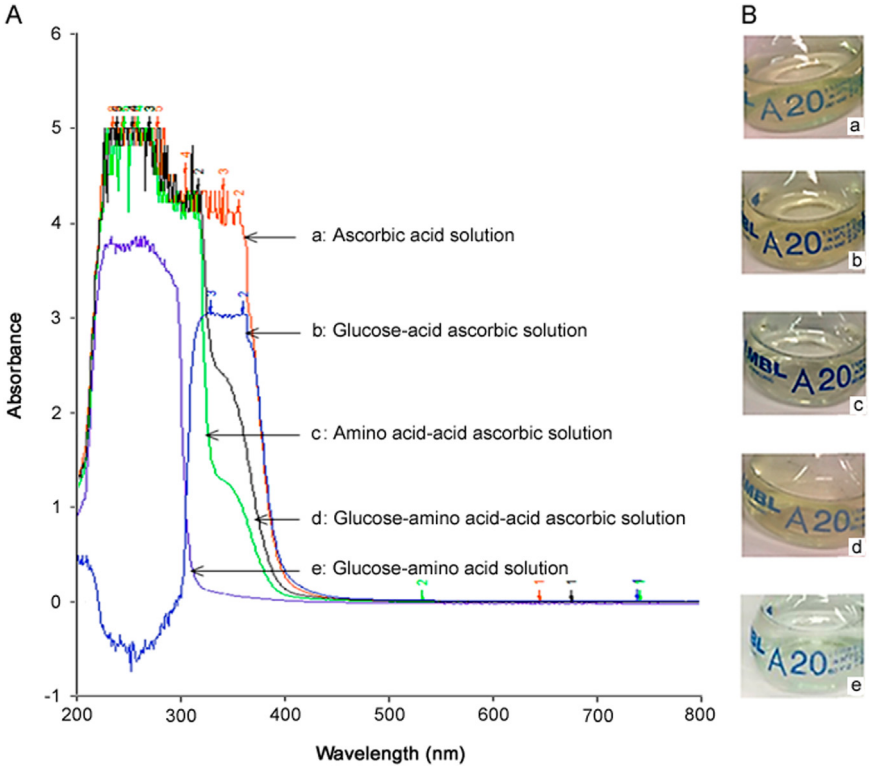

Fig. 2. (A) UV–visible spectra of aqueous solutions of glucose, amino acids, glucose and amino acids, supplemented or not with ascorbic acid. (B) Photographs of aqueous solutions of glucose, amino acids, glucose and amino acids, supplemented or not with ascorbic acid.

resulted in a smaller shift of the absorption spectrum to the visible range. These results were confirmed by a visual inspection showing that solutions C and D were less coloured than solutions A and B.

4. Discussion

Parenteral nutrition solutions are complex mixtures with many components that can interact with each other. A Maillard reaction may develop over time when glucose solution is brought into contact with an amino acid mixture. This reaction is favoured by high temperature, an alkaline pH and high water content [26]. Some amino acids (e.g., lysine, glycine and methionine) are more involved in the Maillard reaction. The low temperature storage and acid pH of the studied parenteral solution prevented potential Maillard reaction [27]. The toxicity of the Maillard reaction products is not known, but recent study showed the antioxidant role of products formed between glucose and cysteine during the Maillard reaction [28–30]. However, in the present parenteral

solution stored at 4–8 °C or heated, no browning of the solution was noted. The yellow colouring would not be due to a Maillard reaction but rather to the cysteine degradation. The amino acids were not degraded and all amino acid concentrations were retained throughout the stability study. The use of synthetic amino acids and not protein hydrolysates might explain the stability of the amino acid content. After storage, the parenteral nutrition solution remains adapted to premature infants. The respective compositions of essential and non-essential amino acids were maintained in order to preserve the ratio of essential amino acids to non-essential amino acids.

The relatively high lysine content was maintained and allowed sufficient neonatal input. Taurine, an essential amino acid in children and not in adults, was preserved during the preservation of the solution in sufficient quantities for premature infants. The oxidation of cysteine, whose concentration was not determined during the study increased over time and led to the formation of cystine by binding two molecules of cysteine. Moreover, in the presence of glucose, the formation of D-glucocysteine was described in the literature [31]. The increasing amounts of cysteine confirmed the oxidation reaction. The cysteine oxidation led to the formation of cystine which is metabolically active but not very soluble in an aqueous medium [6]. The order 0 kinetics of cysteine appearance in the solution imparted a yellow coloration to the preparation and represented a limit for long-term storage. Filling under nitrogen during manufacture, already carried out for commercial solutions, might avoid the degradation of amino acids. The most important losses during the storage of nutrient mixtures were reported for the oxidation-sensitive amino acids (alanine, proline, threonine, methionine, glycine and arginine) contained in polyvinylchloride bags [32]. This observation was partially found in our study with a decrease of methionine, threonine, alanine and arginine concentrations. Some amino acids (glycine, leucine and tryptophan) were sensitive to the intense luminosity which accelerated their degradation [33]. Refrigerated storage protected from light limited this phenomenon.

The energy intake by carbohydrates was retained allowing the preferential use of amino acids for protein synthesis. The bacterial endotoxins were not carried out because the slightly yellow colour of the solution did not allow the colorimetric determination of the endotoxins.

The thermal degradation test showed the protective effect of a glucose-amino acid solution in the ascorbic acid oxidation. The ascorbic acid oxidation in aqueous solution appeared to be limited in the presence of amino acids and glucose. The literature showed that sucrose at pH 5.0 was protective against acid ascorbic oxidation [34]. A supplementation of ascorbic acid in the parenteral nutrition solution would be possible and would stabilize ascorbic acid during the period of administration of the solution. However, further studies will be needed to demonstrate the 24h stability of ascorbic acid supplemented parenteral nutrition mixture.

5. Conclusion

This formulation of standard parenteral nutrition solution remained stable for 4 months at 2–8 °C. The stability of the solution was demonstrated with a validated method to assay amino acids in parenteral nutrition solution. This study presented the first method for the assay of twenty amino acids in complex mixture. The importance of the amino acids assays in hospital standardized solutions and the properties of degradation products should be considered.

Conflicts of interest

The authors declare that there are no conflicts of interest.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jpha.2018.01.002.

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