Long-term stability of gentamicin sulfate-ethylenediaminetetraacetic acid disodium salt (EDTA-Na2) solution for catheter locks

Anne-Sophie Fiolet, Elise Jandot, Pauline Doucey, Coralie Crétet, Célia Brunel, Christine Pivot, Jean-Marc Ghigo, Christophe Beloin, David Lebeaux, Fabrice Pirot

To cite this version:
Anne-Sophie Fiolet, Elise Jandot, Pauline Doucey, Coralie Crétet, Célia Brunel, et al.. Long-term stability of gentamicin sulfate-ethylenediaminetetraacetic acid disodium salt (EDTA-Na2) solution for catheter locks. Journal of Pharmaceutical Analysis, Elsevier B.V. on behalf of Xi’an Jiaotong University, 2018, 8 (6), pp.386-393. 10.1016/j.jpha.2017.09.004. pasteur-02015660

HAL Id: pasteur-02015660
https://hal-pasteur.archives-ouvertes.fr/pasteur-02015660
Submitted on 12 Feb 2019

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L’archive ouverte pluridisciplinaire HAL, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d’enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Distributed under a Creative Commons Attribution 4.0 International License
Original Research Article

Long-term stability of gentamicin sulfate-ethylenediaminetetraacetic acid disodium salt (EDTA-Na₂) solution for catheter locks

Anne-Sophie Fiolet a,1, Elise Jandot a,1, Pauline Doucey a,1, Coralie Crénet a, Célia Brunel a, Christine Pivot a, Jean-Marc Ghigo b, Christophe Beloin b, David Lebeaux b,c,d, Fabrice Pirot a,e

a Service Pharmaceutique, Plateforme FRIPHARM, Groupe Hospitalier Centre Edouard Herriot, Hospices Civils de Lyon, 5, Place d'Arsonval, F-69437 Lyon Cedex 03, France
b Unité de Génétique des Biofilms, Département de Microbiologie, Institut Pasteur, 28 rue du docteur Roux, F-75724 Paris Cedex 15, France
c Service de Microbiologie, Unité Mobile de Microbiologie Clinique, Assistance Publique-Hôpitaux de Paris, Hôpital Européen Georges Pompidou, 20 rue Leblanc, 75015 Paris, France
d Université Paris Descartes, 12 rue de l’École de Médecine, 75270 Paris Cedex 06, France
e Laboratoire de Recherche et Développement de Pharmacie Galénique Industrielle, UMR 5305, Plateforme FRIPHARM, F-69373, Faculté de Pharmacie, Université Claude Bernard Lyon 1, 8, avenue Rockefeller, F-69373 Lyon Cedex 08, France

A R T I C L E  I N F O

Article history:
Received 4 July 2017
Received in revised form 21 September 2017
Accepted 22 September 2017
Available online 23 September 2017

Keywords:
Gentamicin-EDTA-Na₂ loaded antimicrobial lock solution
Pharmaceutical compounding
Stability indicating HPLC assay method
Totally implantable venous access ports

A B S T R A C T

A lock solution composed of gentamicin sulfate (5 mg/mL) and ethylenediaminetetraacetic acid disodium salt (EDTA-Na₂, 30 mg/mL) could fully eradicate in vivo bacterial biofilms in totally implantable venous access ports (TIVAP). In this study, fabrication, conditioning and sterilization processes of antimicrobial lock solution (ALS) were detailed and completed by a stability study. Stability of ALS was conducted for 12 months in vials (25 °C ± 2 °C, 60% ± 5% relative humidity (RH), and at 40 °C ± 2 °C, RH 75% ± 5%) and for 24 h and 72 h in TIVAP (40 °C ± 2 °C, RH 75% ± 5%). A stability indicating HPLC assay with UV detection for simultaneous quantification of gentamicin sulfate and EDTA-Na₂ was developed. ALS was assayed by ion-pairing high performance liquid chromatography (HPLC) needing gentamicin derivatization, EDTA-Na₂, metallocomplexation of samples and gradient mobile phase. HPLC methods to separate four gentamicin components and EDTA-Na₂ were validated. Efficiency of sterility procedure and conditioning of ALS was confirmed by bacterial endotoxins and sterility tests. Physicochemical stability of ALS was determined by visual inspection, osmolality, pH, and sub-visible particle counting. Results confirmed that the stability of ALS in vials was maintained for 12 months and 24 h and 72 h in TIVAP.

© 2018 Xi’an Jiaotong University. Production and hosting by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

1. Introduction

Central venous catheters (CVC) are frequently used in oncology, nephrology and intensive care units to administer medication or fluids to patients [1]. The use of CVC is complicated by the risk of colonization by pathogenic microorganisms on their surface, causing infectious complications such as catheter-related bloodstream infections (CRBSI) [2]. Optimal management of CRBSI involves systemic antimicrobial therapy and CVC removal, which might be questionable in specific clinical situations (e.g., limited vascular access or bleeding disorders) [3]. Therefore, direct instillation of a small volume of highly-concentrated antimicrobial solution into the lumen of the catheter, known as antimicrobial lock therapy (ALT), might be favored as an alternative strategy for both prevention and treatment of CRBSI [4,5]. Several studies reported the use of antibiotics such as aminoglycosides, e.g., gentamicin, [3], beta-lactams, fluoroquinolones, folate antagonists (sulfamethoxazole/trimethoprim), glycopeptides, glycyclines, lipopeptides, oxazolidinones, polymyxins, and tetracyclines in ALT [6]. However, surface of medical device might be colonized by a reservoir of pathogenic microorganisms forming a biofilm, a complex microbial consortium surrounded by an heterogeneous matrix composed of water, polysaccharides, proteins and deoxyribonucleic acid [6], decreasing the antibiotic efficacy [7]. Potential additives (e.g., ethylenediaminetetraacetic acid disodium salt, EDTA-Na₂, and sodium citrate) have shown their capacity to disrupt biofilm and to enhance bactericidal effect of current antibiotics [8,9–11]. Recent in vitro and in vivo study demonstrated the
beneficial effect of EDTA-Na₂ against Pseudomonas aeruginosa biofilm and the concomitant decrease of minimal inhibitory concentration of ciprofloxacin and ampicillin (30-fold) and gentamicin (two-fold) [12].

An earlier in vivo study reported that an antimicrobial lock solution (ALS) composed of gentamicin (5 mg/mL) and EDTA-Na₂ (30 mg/mL) in association with systemic antibiotics completely eradicated biofilms formed by Escherichia coli, Pseudomonas aeruginosa, Staphylococcus epidermidis or Staphylococcus aureus on the surface of totally implantable venous access ports (TIVAP) [13].

Gentamicin produced by Micromonospora purpurea is used as a lock solution which is a complex mixture of five structurally related components: C₁, C₁ₐ, C₂ and C₂₀a and a minor component C₂₇h [14]. Previous basic stability study showed that gentamicin sulfate-EDTA-Na₂ antibiotic lock solution stored at 25 °C or 37 °C was visually stable for at least 72 h [3].

In the present study, a process of pharmaceutical compounding and conditioning of gentamicin sulfate-EDTA-Na₂ lock solution is detailed and then completed by a study of stability according to the technical requirements of International Conference on Harmonization (ICH) for registration of pharmaceuticals for human use. The physicochemical stability of ALS conditioned in glass vial was analyzed (i) over 12 months of storage at 25 °C ± 2 °C (relative humidity (RH) 60% ± 5%) and 40 °C ± 2 °C (RH 75% ± 5%), and (ii) after 24 h and 72 h of contact time in TIVAP at 40 °C ± 2 °C (RH 75% ± 5%), by high performance liquid chromatography (HPLC) evidencing the impurities and degradation products of gentamicin sulfate and EDTA-Na₂.

2. Materials and methods

2.1. Reagents

Gentamicin sulfate and EDTA-Na₂ dihydrate physicochemical properties are reported in Table 1. Ready-to-use gentamicin sulfate sterile solutions (40 mg – 2 mL and 80 mg–2 mL), and EDTA- Na₂ dihydrate (pharmaceutical grade) were purchased from Panpharma (Beignon, France) and Imresa (Bartenheim, France) laboratories, respectively. Nitrilotriacetic acid and trifluoroacetic acid (HPLC grade, TFA) were purchased from Carl Roth (Lutterbourg, France) and Rhône Poulenc (Lyon, France), respectively. EDTA-Na₂ solution (40 mg/mL) was prepared by dissolution of 22.5 g of EDTA-Na₂ in 562.5 mL of water for injection. Then, EDTA- Na₂ solution (40 mg/mL) was mixed to 187.5 mL of gentamicin sulfate solution for injection (40 mg/mL) and EDTA-Na₂ solution (40 mg/mL) was mixed to 187.5 mL of gentamicin sulfate solution for injection (40 mg–2 mL). Finally, the pH value of the ALS was adjusted to 8.5 by adding few drops of 5 M NaOH solution. ALS was filtered through 0.22 μm sterile filter (Millipore®, Molsheim, France), and then sampled (5 mL) in 150 individual amber type 1 glass injection vials (15 mL) hermetically sealed by bromobutyl stoppers in cleanroom. Finally, ALS vials were autoclaved at 121 °C for 20 min [15,16]. Final concentrations of gentamicin (5 mg/mL) and EDTA-Na₂ (30 mg/mL) in ALS were assayed by HPLC (see Section 2.6).

2.3. Storage stability testing

The environmental factors (temperature, RH) and time of storage upon degradation of gentamicin sulfate and EDTA-Na₂ in ALS conditioned in vials and then stored at 25 °C ± 2 °C, RH 60% ± 5% and 40 °C ± 2 °C, RH 75% ± 5% in climatic test chambers (Froilabo, Meyzieu, France) for 12 months were investigated using HPLC (see Section 2.6) [17]. Complementary physicochemical and hydrochloric acid (HCl) and sodium hydroxide (NaOH) were provided by Carlo Erba Reagents (Peypin, France). Hydrogen peroxide solution (3%) was purchased from Gifrer (Décines, France). Gentamicin for identification (CRS, European Pharmacopoeia reference standard), gentamicin USP standard, sisomicin sulfate, EDTA disodium salt standard, ortho-phthalaldehyde (OPA), thioglycolic acid, boric acid and potassium hydroxide (KOH) were obtained from Sigma-Aldrich (Saint Quentin Fallavier, France). Acetonitrile and methanol (HPLC grade) were purchased from Merck Millipore (Molsheim, France). Copper sulfate was obtained from Cooper (Melun, France). Peptone water (0.1%) was purchased from Oxoid (Basingstoke, United Kingdom). Culture media for anaerobic bacteria (fluid thioglycollate medium), aerobic bacteria and fungi (soya bean casein digest medium), and neutralizing pharmacopoeia diluent used for the gentamicin neutralization were obtained from Biomérieux (Lyon, France). Water for injection was delivered by Lavoisier (Paris, France).

2.2. Pharmaceutical compounding and conditioning of ALS

EDTA-Na₂ solution (40 mg/mL) was prepared by dissolution of 22.5 g of EDTA-Na₂ in 562.5 mL of water for injection. Then, EDTA- Na₂ solution (40 mg/mL) was mixed to 187.5 mL of gentamicin sulfate solution for injection (40 mg–2 mL). Finally, the pH value of the ALS was adjusted to 8.5 by adding few drops of 5 M NaOH solution. ALS was filtered through 0.22 μm sterile filter (Millipore®, Molsheim, France), and then sampled (5 mL) in 150 individual amber type 1 glass injection vials (15 mL) hermetically sealed by bromobutyl stoppers in cleanroom. Finally, ALS vials were autoclaved at 121 °C for 20 min [15,16]. Final concentrations of gentamicin (5 mg/mL) and EDTA-Na₂ (30 mg/mL) in ALS were assayed by HPLC (see Section 2.6).

### Table 1

| Physicochemical parameters | Gentamicin sulfate | EDTA-Na₂ dihydrate |
|---------------------------|-------------------|--------------------|
| **Chemical structure**    | ![Chemical Structure](image) | ![Chemical Structure](image) |
| Molecular mass (g/mol)    | C₁ = 477.6 (R₁ = R₂ = CH₃; R₃ = H) | 372.2 |
|                          | C₁ₐ = 449.5 (R₁ = R₂ = R₃ = H) |  |
|                          | C₂ₐ = 463.6 (R₁ = R₂ = H; R₃ = CH₃) |  |
|                          | C₂ₐ = 463.6 (R₁ = CH₃; R₂ = R₃ = H) |  |
|                          | C₂ = 463.6 (R₁ = H; R₂ = CH₃; R₃ = H) |  |
| pKa                      | 10.10, 12.55       | 2.0, 2.7, 6.2, 10.3 |
| Log P (octanol-water)     | ~ 3.1              | ~ 11.70            |
| Melting point (°C)        | 102–108            | 250                |
| Water solubility          | 100 mg/mL          | 1 000 mg/mL        |
| Solubility                | Soluble in pyridine, dimethylformamide, in acidic media with salt formation, moderately soluble in methanol, ethanol, acetone, insoluble in benzene, halogenated hydrocarbons |  |
| Case number               | 1405–41–0          | 6381–92–6          |
microbiological controls of ALS were conducted over the period of storage (see Section 2.4). TIVAP, supplied by Perouse Medical (polyisite 4000, 4008 IPS, Ivry le Temple, France), was used to test compatibility between ALS and implantable port. This medical device is made up of a chamber in titanium / polyoxymethylene and a silicone catheter. Total dead space of implantable port was 1.38 mL and internal surface of silicone catheter was 6.6 cm². One mL of ALS was injected into the device and left for 24 h and 72 h at 40 °C ± 2 °C, RH 75% ± 5%. To detect a potential water loss through polymeric wall, implantable port was weighted before and after filling throughout storage. Gentamicin and EDTA-Na₂ contents after 24 h and 72 h of contact time in chambers (n=3) were assessed by HPLC (see Section 2.6).

### 2.4. Physicochemical and microbiological controls of ALS

Physicochemical controls of ALS included pH measurement (EcopHtest2, Thermo Fisher Scientific, Massachusetts, USA) and determination of osmolality (Fiske™, Norwood, MA, USA). Controls of visual aspect, particulate contamination of ALS by visible particles (2.9.20 European Pharmacopoeia Monograph, 2016) and sub-visible particles (2.9.19 European Pharmacopoeia Monograph, 2016, method 1: light obscuration particle count test, Hiac/Royco 9703 +, Pacific Scientific Instruments, IL, USA) were carried out after autoclaving. Microbiological assays of ALS were carried out by membrane filtration using Steritest™ closed filtration device [18] with special low-binding Durapore™ membranes for products with inhibitory properties, e.g. antibiotics (Millipore, Merck KGAA, Darmstadt, Germany), peptone water (0.1%) as rinse fluid, neutralizing pharmacopoeia diluent and both soya-bean casein digest medium and fluid thioglycollate medium (2.6.1 European Pharmacopoeia Monograph, 2016).

#### 2.5. Preparation of gentamicin sulfate and EDTA-Na₂ standard and ALS assay solutions

A derivatization reaction of gentamicin sulfate with OPA allowing a detection gentamicin OPA derivative was adapted from previous reports [19–21]. A stock solution of derivatizing agent was prepared by dissolving 250 mg of OPA in 1.25 mL of methanol, then addition of 23.75 mL of boric acid solution (2.47%, m/v; pH adjusted to 10.4 by 8 M KOH solution) and 0.5 mL of thioglycolic acid. Finally, the pH value of derivatizing agent solution was adjusted to 10.4 by 8 M KOH solution. The pH value was adjusted to 10.4 using 8 M KOH solution, for pre-column derivatization reaction of gentamicin sulfate with OPA alditation excipients, impurities, and potential degradation products by HPLC (see Section 2.6). Therefore, 2.5 mL of EDTA-Na₂ standard solution or ALS were then mixed with 2.5 mL of copper sulfate solution (10 mg/mL) and 0.75 mL of water for injection (final volume: 5.75 mL). Although usually derivatization was carried out at 60 °C constant temperature [20,22], no heating was applied to gentamicin – OPA based solution, taking into account the lack of stability of such chemical reaction and the short half-life of reaction products, as reported elsewhere [23].

The determination of EDTA-Na₂ in ALS was allowed by conversion to Cu(II)/EDTA complex [24] using copper sulfate containing solution and subsequent separation from gentamicin, formulation excipients, impurities, and potential degradation products by HPLC (see Section 2.6). Therefore, 2.5 mL of EDTA-Na₂ standard solution or ALS were then mixed with 2.5 mL of copper sulfate solution (10 mg/mL) and 0.75 mL of water for injection (final volume: 5.75 mL).

Gentamicin sulfate-standard EDTA-Na₂ solutions were prepared daily by dissolving the appropriate amount of EDTA-Na₂ in water for injection, and then mixing with gentamicin sulfate sterile solution for injection (40 mg – 2 mL or 80 mg – 2 mL) to obtain, after gentamicin sulfate derivatization or EDTA-Na₂ complexation, final concentrations of gentamicin sulfate and EDTA-Na₂ in the range of 87 – 260 µg/mL and 0.52 – 1.56 mg/mL, respectively.

#### 2.6. Stability indicating HPLC assay

An HPLC Agilent™ (Agilent® 1200 Infinity Quaternary LC System, Les Ulis, France), equipped with a binary pump with integrated vacuum degasser, a thermostatted column compartment (40 °C), an autosampler and a diode array detector, was used for gentamicin and EDTA dosages. The separation of gentamicin and EDTA-Na₂ was accomplished using SecurityGuard™ cartridge and column Kinetex™ (C₁₈ 100 Å, 100 mm × 4.6 mm, 2.6 µm) Core-SHELL Technology (Phenomenex®, Le Pecq, France). The detection wavelength was set at 330 nm and injection volume was 10 µL. The mobile phase was a binary mixture of 100 mM TFA, used as pairing reagent in water (phase A, the pH value was adjusted to 3 by adding 5 M NaOH solution) and acetonitrile (phase B) in a gradient elution mode at a flow-rate of 1 mL/min [25,26]. The mobile phase A was filtered and degassed through nylon membranes (0.20-µm pore size) under vacuum before use. A linear gradient elution was programed as 95% A – 5% B (v/v; 0 min), 95% A – 5% B (v/v; 2 min), 50% A – 50% B (v/v; 10 min), and 40% A – 60% B (v/v; 15 min). Therefore, the resolved peaks of gentamicin sulfate OPA derivatization, C₁, C₁₄, C₂₆ and C₁₂, and EDTA-Na₂ were identified from gentamicin for peaks identification (CRS, European Pharmacopoeia reference standard), gentamicin (216 µg/mL) USP standard solutions, and EDTA (1.3 mg/mL) standard solution. Sisomicin and nitrotriatric acid, as gentamicin sulfate and EDTA-Na₂ respective impurities, were identified from sisomicin (4.65 mg/mL) and nitrotriatric acid (43.5 µg/mL) standard solutions.

#### 2.7. HPLC assay validation criteria

The HPLC-UV analytical methods for gentamicin sulfate and EDTA-Na₂ were validated for specificity, precision (repeatability, intermediate precision), linearity, limits of detection (LOD) and quantification (LOQ), accuracy/recovery and robustness to include the essential requirements of ICH guidelines [27,28]. The validation protocol was conducted on three consecutive days by the same operator and fresh solutions were prepared daily. Five standard gentamicin sulfate and EDTA-Na₂ solutions were prepared to enable the determination of drugs concentrations in ALS. Furthermore, forced degradation of ALS was investigated (i) by acidic (0.1–1 M HCl) and alkaline (0.1–1 M NaOH), (ii) heating (80 °C for 1 h) treatments, (iii) under oxidative conditions using 3% H₂O₂ then heating at 80 °C for 3 h, and (iv) from UVA (320–400 nm) irradiation for 6 h [29].

#### 3. Results and discussion

##### 3.1. Physicochemical and microbiological controls of ALS

Results of visual inspections for color, clarity, and particles completed by sub-visible particles counting, pH and osmolality assessments of ALS throughout the 12 months storage at 25 °C ± 2 °C, RH 60% ± 5% and 40 °C ± 2 °C, RH 75% ± 5% are summarized in Table 2. No significant macroscopic and/or microscopic alterations of ALS were evidenced. Efficiency of sterility procedure and conditioning of ALS was confirmed by the absence of microbial growth and endotoxin. No impact of autoclaving either upon particle quality or colligative property of ALS was observed. Interestingly, ALS was isosmotic to serum compared to hyposmotic commercial gentamicin sulfate solution (Panpharma®) in favor of further easy handling in TIVAP filling and blood compatibility.
Table 2  
Characterization of ALS stability over 12 months of storage at 25 °C ± 2 °C, RH 60% ± 5% (at 40 °C ± 2 °C, RH 75% ± 5%). Each data is the mean ± standard deviation of three experimental determinations. nd: Not determined.

| Test                          | Acceptance criteria | Time of storage |
|-------------------------------|---------------------|-----------------|
|                               |                     | Day 1 | Month 1 | Month 3 | Month 6 | Month 12 |
| Appearance                    | Colorless (C), yellowish (Y), limpid (L) | C, L  | C, L    | C, L    | Y, L    | Y, L     |
| pH                            |                      | 8.2 ± 0.2      | 8.3 ± 0.3 | 8.3 ± 0.3 | 8.1 ± 0.1 |
| Osmolality (mOsmol/kg)        | 290 – 310            | 301 ± 1.0      | 308 ± 0.8 | 308 ± 0.8 | 309 ± 0.1 |
| ≥ 10-µm particle count < 6000/mL | nd                  | 0 ± 0.0        | 0 ± 0.0   | 0 ± 0.0   | 1 ± 0.0   |
| ≥ 25-µm particle count < 6000/mL | nd                  | 5.20 ± 0.10    | 5.20 ± 0.02 | 5.10 ± 0.02 | 5.10 ± 0.04 |
| EDTA-Na₂ conc. (mg/mL)        | 27 – 33              | 29.00 ± 0.10   | 29.00 ± 0.36 | 29.00 ± 0.06 | 31.00 ± 0.28 |
| Gentamicin conc. (mg/mL)      | 4.50 – 5.50          | 5.20 ± 0.10    | 5.20 ± 0.02 | 5.10 ± 0.02 | 5.10 ± 0.04 |
| Sterility                     | No bacterial growth  | No bacterial growth | nd | No bacterial growth | No bacterial growth | No bacterial growth |

Fig. 1. (A) A typical chromatogram of gentamicin standard solution (USP) (216 µg/mL) after OPA derivatization. (B) A typical chromatogram of sisomicin after OPA derivatization (4.65 mg/mL). (C) A typical chromatogram of ALS after OPA derivatization (gentamicin 217 µg/mL). (D) Analysis of ALS (gentamicin 217 µg/mL) under oxidative conditions (3% H₂O₂, 80 °C for 3 h) after OPA derivatization.
3.2. HPLC assay validation

Gentamicin sulfate and EDTA-Na₂ contents in ALS conditioned in amber type I glass vials were analyzed during the storage using HPLC method adapted, for a part, from US Pharmacopoeia USP37-NF32 Monograph [22] and earlier studies [20,21,24]. The chemical structure of gentamicin reveals the lack of chromophore in the molecule, making the direct detection of the antibiotic difficult. Furthermore, besides methods based on microbiological assay [30], enzyme immunoassay, polarization fluorimunoassay [31,32], direct detection methods, e.g., electrochemical detection [14,33,34], evaporative light scattering detection [35,36], charged aerosol detection [26,37], direct capillary electrophoresis [38] require specific and costly instrumentation (e.g., tandem mass spectrometer, [39]) are not commonly found in quality control laboratories. In the present study, gentamicin sulfate components were assayed by UV-detection after pre-column derivatization with OPA based reagent which reacts only with three primary amines of gentamicin to form UV-absorbing fluorophores, ion-pairing chromatographic gradient and separation by a combination of electrical (charge-charge between two underivatized secondary amines of gentamicin positively charged at acidic pH of mobile phase) and hydrophobic interactions with the stationary phase and ions of the mobile phase [25]. No pre-heating of gentamicin-OPA based mixture was carried out, as described by Table 3

Table 3

| USP37-NF32 gentamicin component relative content (%) | Gentamicin component relative content (%) |
|-----------------------------------------------------|------------------------------------------|
| USP standard solution                                | ALS                                      |
| C1: 25 – 50                                          | 37                                       |
| C1a: 10 – 35                                         | 12                                       |
| C2 + C2a: 25 – 55                                    | 51                                       |

![Fig. 2](A) A typical chromatogram of EDTA-Na₂ (USP) (1.3 mg/mL) after CuSO₄ complexation. (B) A typical chromatogram of nitrilotriacetic acid (43 mg/mL) after CuSO₄ complexation. (C) A typical chromatogram of ALS (EDTA-Na₂: 1.3 mg/mL) after CuSO₄ complexation. (D) Analysis of ALS (EDTA-Na₂: 1.3 mg/mL) under stress oxidative conditions (3% H₂O₂, 80 °C for 3 h) after CuSO₄ complexation.
previous authors [19], eliminating tedious manual procedures, reducing error, and thereby increasing method reproducibility. The column was maintained at 40 °C, leading likely to more reproducible in situ derivatization of gentamicin components passing through the C18 stationary phase.

Fig. 1A shows typical chromatogram of gentamicin standard solution. The four components of gentamicin (C1, C1a, C2a and C2) were eluted as distinct peaks with retention time of 9.7, 12.6, 13.3 and 13.5 min, respectively in the order reported by US Pharmacopoeia Monograph [22]. Gentamicin C2b component was below LOD. Furthermore, the relative content of each gentamicin component in gentamicin standard was found in agreement with US Pharmacopoeia Monograph requirements, as reported in Table 3. These findings were similar to those reported in previous study [20], in which the chromatogram of gentamicin sulfate solution (2 mg/mL) prepared from the gentamicin sulfate powder met the USP test specifications. The USP method describes the elution order as C1, C1a, C2a and C2 gentamicin with a 5 mm × 10 cm column that contains 5 μm packing L1 (octadecyl silane chemically bonded to porous or nonporous silica or ceramic microparticles, 1.5–10 μm in diameter, or a monolithic silica rod). In the present method, Kinetex™ (C18 100 Å, 100 mm × 4.6 mm, 2.6 μm, Core-Shell Technology) column was preferred to Luna® C18 (2) column used by earlier authors [20]. Therefore, the elution of last gentamicin component (C2) was nearly 2.5 times shorter (13.5 min versus 31 min) in the present method than that reported previously [20], confirming that the combination of the small particle size and narrow particle size distribution coupled with the significantly shorter diffusion path resulted in a material that yielded significantly increased column efficiency and chromatographic resolution. Chromatographic separation was shortened mandatory, considering that isonitrosoles formed during derivatization are generally highly reactive compounds. As a result, it was considered that any derivative breakdown during chromatography would be time-dependent and that component derivatives with long retention time (C2a and C2) would be particularly affected by degradation prior to detection [40]. Previous analysis of gentamicin in raw material used column (150 mm × 4.6 mm) packed with Ultrasphere® ODS (C18) and elution gradient (A: 5% acetic acid – 25% water-70% methanol and B: 100% methanol) for faster elution of C1 (5 min) and similar elution time for C2 (13.7 min) [41]. In the present study, longer elution time for C1 (~10 min, Fig. 1C) was preferred to elute first the most hydrophilic components of ALS (e.g., excipients from gentamicin sulfate sterile solution for injection, Panpharma®). Interestingly, it was noticed that the relative content of gentamicin components was proportional to molecular weight of C1, C1a, C2a and C2 as equimolar amounts, confirming that the sum of the areas of the four gentamicin components was a relevant measure of the gentamicin concentration. Sisomicin chromatogram shows one main peak surrounded by three minor peaks (Fig. 1B). Possible partial derivatization of one, two or three primary amines of sisomicin might explain the presence of numerous peaks.

The determination of EDTA-Na2 in ALS by direct UV detection was challenging since EDTA-Na2 does not contain a significant chromophore. In the present study, EDTA-Na2 in ALS was assayed by ion-pairing HPLC and metallocomplex formation by using TFA in mobile phase and copper sulfate in sample preparation. Typical chromatogram of EDTA-Na2 eluted as EDTA-Cu2+ is shown in Fig. 2A. As reported in previous study assaying EDTA-Na2 in pharmaceutical formulation, EDTA-Cu2+, as ionic analyte, showed minimal retention (Fig. 2C, < 1 min) through reversed-phase by using liquid chromatography with ion-pairing and elution gradient [42], and co-elution of nitrilotriacetic acid-Cu2+ complex (Fig. 2B).

3.3. HPLC validation criteria

3.3.1. Specificity

The specificity of the assay was conducted to evaluate the assay for potential sources of interfering peaks from the matrices used in sample preparation. No interfering peaks were seen from water
used to prepare mobile phase, the mobile phase or a blank filter media. No significant macroscopic and/or microscopic alterations of ALS were evidenced in acidic (0.1–1 M HCl, at 80 °C for 1 h) and basic (0.1–1 M NaOH, at 80 °C for 1 h) conditions. ALS was found stable under heat conditions (at 80 °C) and under UVA (320–400 nm) irradiation for 6 h. Under oxidative stress conditions with 3% H2O2 at 80 °C for 3 h, EDTA-Na2 was degraded to about 60% (Fig. 2D) while all of gentamicin sulfate was degraded (Fig. 1D). Four main degradation products were generated with retention time of 6.4, 7.2, 8.8 and 9.9 min that could correspond to the impurity A (e.g., sisomicin, Fig. 1B).

3.3.2. Precision
Repeatability and intermediate precision of gentamicin sulfate-EDTA-Na2 assays were found to be 1.82% – 1.23%, and 2.16% – 1.47%, respectively, confirming the overall precision of HPLC methods involving pre-column derivatization and metallocomplex procedure.

3.3.3. Linearity
The linearity of assay was determined to have a correlation coefficient (r) for gentamicin sulfate of 0.999 (in the range of 87 – 260 μg/mL) and for EDTA-Na2 of 0.993 (in the range of 0.52 – 1.56 mg/mL) as shown in Figs. 3A and B, respectively.

3.3.4. Accuracy
The recovery of gentamicin sulfate and EDTA-Na2 was found to be in the range of 97.96%–99.81%, and 100.72%–102.72% over all concentration range, respectively.

3.3.5. LOD and LOQ
LOD and LOQ for gentamicin were 8 μg/mL and 24 μg/mL, respectively. The LOD and LOQ for EDTA were found to be 45 μg/mL and 136 μg/mL, respectively.

3.3.6. Robustness
The robustness of the assay in terms of varied injection volume (5–15 μL) and flow rate (0.5 mL/min) did not significantly change the time retention, asymmetry and the percentage of target amounts in the samples of gentamicin sulfate and EDTA-Na2.

3.3.7. Stability study of ALS in glass injection vials and in TIVAP
Stability of gentamicin sulfate and EDTA-Na2 in vials stored for 12 months was confirmed by minimal variation of drug concentration in ALS, as reported in Table 2. A loss of ALS was highlighted by weighing the device throughout contact time in TIVAP. Loss solution per cm² of catheter is represented in Fig. 4. Solution loss was perfectly linear in function of time. After 24 h and 72 h, 124 μL and 363 μL of ALS were evaporated through polymeric wall, respectively. At the same time, HPLC assay of ALS after contact in TIVAP showed that gentamicin and EDTA concentrations increased compared to initial concentrations in ALS (Table 4). After re-calculation considering the extent of water loss (124 μL for 24 h and 363 μL for 72 h), gentamicin and EDTA concentrations were in accordance with initial concentrations in ALS (Table 4).

4. Conclusion
In this study, a process of pharmaceutical compounding and conditioning of ALS was performed. Numerous physicochemical analysis, including the development of validated HPLC methods for dual gentamicin and EDTA-Na2 assays, showed a stability of ALS conditioned in amber type I glass vials for 12 months at 25 °C ± 2 °C (RH 60% ± 5%) and 40 °C ± 2 °C (RH 75% ± 5%). Furthermore, complementary findings reported satisfactory stability of ALS in TIVAP for 24 h and 72 h. At the outset, the present study confirmed the pharmaceutical relevance of gentamicin-EDTA-Na2 combined solution as a new antimicrobial lock therapy.

Conflicts of interest
The authors declare that there are no conflicts of interest.

Acknowledgments
This study was supported by Centre de Recherche Translationale de l’Institut Pasteur, grant Number S-PI15007-02A. JMG, CB and DL are supported by the French Government’s Investissement d’Avenir program: Laboratoire d’Excellence ‘Integrative Biology of Emerging Infectious Diseases’ (grant no. ANR-10-LABX-62-IBEID.), the Fondation pour la Recherche Médicale (grant no. DEQ, 20140329508) and the Center for Translational Science of the Institut Pasteur (S-PI15007-02A).

References
[1] I. Raad, H. Hanna, D. Maki, Intravascular catheter-related infections: advances in diagnosis, prevention, and management, Lancet Infect. Dis. 7 (2007) 645–657.
[2] C. Beloin, N. Fernández-Hidalgo, D. Lebeaux, Understanding biofilm formation in intravascular device-related infections, Intensive Care Med. 43 (2017) 443–446.
[3] P.B. Bookstaver, K.E.E. Rokas, L.B. Norris, et al., Stability and compatibility of antimicrobial lock solutions, Am. J. Health-Syst. Pharm. AJHP Off. J. Am. Soc. Health-Syst. Pharm., 70, (2013) 2185–2198.
[4] S. Hogan, M. Zapotoczna, N.T. Stevens, et al., In vitro approach for identification of the most effective agents for antimicrobial lock therapy in the treatment of intravascular catheter-related infections caused by Staphylococcus aureus, Antimicrob. Agents Chemother. 60 (2016) 2923–2931.
[5] L.A. Mermel, M. Allon, E. Bouza, et al., Clinical practice guidelines for the diagnosis and management of intravascular catheter-related infection: 2009 Update by the Infectious Diseases Society of America, Clin. Infect. Dis. Off. Publ. Infect. Dis. Soc. Am. 49 (2009) 1–45.
[6] J.A. Justo, P.B. Bookstaver, Antibiotic lock therapy: review of technique and logistical challenges, Infect. Drug Resist. 7 (2014) 343–363.
[7] D. Lebeaux, J.-M. Ghigo, C. Beloin, Biofilm-related infections: bridging the gap between clinical management and fundamental aspects of recalcitrance toward antibiotics, Microbiol. Mol. Biol. Rev. MMIR 78 (2014) 510–543.

Table 4
Determination of gentamicin and EDTA-Na2 concentrations in ALS after 24 h and 72 h of contact time in TIVAP. Each data is the mean ± standard deviation of three experimental determinations of concentrations in three TIVAP. Relative standard deviation (RSD) (%) of concentrations was calculated as follows: RSD (%) = (initial concentration – experimental concentration) / initial concentration.

| Compounds    | Volumes (μL) | Experimental concentrations (mg/mL) | Recalculated concentrations (mg/mL) |
|--------------|-------------|-----------------------------------|-----------------------------------|
|              | Initial | 24 h | 72 h | Initial | 24 h | 72 h | 24 h × b | 72 h × c |
| Gentamicin   | 1000    | 876  | 637  | 5.00    | 5.40 ± 0.08 (+ 8%) | 7.10 ± 1.28 (+ 43%) | 4.70 ± 0.07 (– 6%) | 4.60 ± 1.18 (– 9%) |
| EDTA-Na2     | 1000    | 876  | 637  | 30.00   | 33.60 ± 0.18 (+12%)| 44.90 ± 3.18 (+49%) | 29.50 ± 0.33 (–2%)  | 28.30 ± 2.00 (–6%)  |
[8] M.G. Berjes, M. van Agteren, Prevention of dialysis catheter-related sepsis with a citrate-taurolidine-containing lock solution, Nephrol. Dial. Transplant. 19 (2004) 1546–1551.

[9] P.B. Bookstaver, J.C. Williamson, B.K. Tucker, et al., Activity of novel antibiotic lock solutions in a model against isolates of catheter-related bloodstream infections, Ann. Pharmacother. 43 (2009) 210–219.

[10] I.J. Raad, R.Y. Hachem, H.A. Hanna, et al., Role of ethylene diamine tetra-acetic acid (EDTA) in catheter lock solutions: EDTA enhances the antifungal activity of amphotericin B lipid complex against Candida embedded in biofilm, Int. J. Antimicrob. Agents 32 (2008) 515–518.

[11] M.C. Weijmer, M.A. van den Dorpel, P.J. Van de Ven, et al., Randomized, clinical trial comparison of trisodium citrate 30% and heparin as catheter-locking solution in hemodialysis patients, J. Am. Soc. Nephrol. 16 (2005) 2769–2777.

[12] Z. Liu, Y. Lin, Q. Lu, et al., In vitro and in vivo activity of EDTA and antibacterial agents against the biofilm of mucoid Pseudomonas aeruginosa, Infection 45 (2017) 23–31.

[13] A. Chauhan, D. Lebeaux, J.-M. Ghigo, et al., Full and broad-spectrum in vivo eradication of catheter-associated biofilms using gentamicin-EDTA antibiotic lock therapy, Antimicrob. Agents Chemother. 56 (2012) 6310–6318.

[14] Council of Europe, Gentamicin sulfate, in: Eur. Pharmacopoeia 88, 8.8, European Directorate for the Quality of Medicines & HealthCare, Strasbourg, France, 2016: 2525–2526.

[15] J.A. Ji, E. Ingham, J.X. Wang, Effect of EDTA and methionine on preventing loss of viscosity of cellulose-based topical gel, AAPS PharmSciTech. 10 (2009) 678–683.

[16] A. Rudin, A. Healey, C.A. Phillips, et al., Antibacterial activity of gentamicin sulfate in tissue culture, Appl. Microbiol. 20 (1970) 989–990.

[17] N.D. Mullins, B.J. Deadman, H.A. Moynihan, et al., The impact of storage conditions upon gentamicin coated antimicrobial implants, J. Pharm. Anal. 6 (2016) 374–381.

[18] G.C. Christiani, T.A. Knoki, A comparison of a disposable membrane filtration system with a direct inoculation system for the sterility testing of veterinary biologics, J. Biol. Stand. 11 (1983) 83–89.

[19] A.I. Al-Arnoud, B.J. Clark, H. Chrystyn, Determination of gentamicin in urine samples after inhalation by reversed-phase high-performance liquid chromatography using pre-column derivatisation with o-phthalaldehyde, J. Chromatogr. B 25 (2002) 89–95.

[20] M.C. Chuong, J. Chin, J.W. Han, et al., High performance liquid chromatography of gentamicin sulfate reference standards and injection USP, Int. J. Pharm. Anal. 4 (2013) 25.

[21] P.J. Kuehl, S. De, B. Eppler, J. Marsters, et al., Development and validation of an HPLC assay for dual detection of gentamicin sulfate and leucine from a novel dry powder for inhalation, J. Anal. Bioanal. Tech. 3 (2012) 1–4.

[22] U.S. Pharmacopeial Convention, Gentamicin sulfate, in: U.S. Pharmacopoeia 37–NF32 US Pharmacopoeial Conv., Rockville, USA, 2014: 3138–3139.

[23] K. Ruckmann, S.Z. Shaikh, P. Khalil, et al., A simple and rapid high-performance liquid chromatographic method for determining tobramycin in pharmaceutical formulations by direct UV detection, Pharm. Methods 2 (2011) 117–123.

[24] A.S. Kord, I. Tumanova, W.L. Matier, A novel HPLC method for determination of EDTA in a cataract inhibiting ophthalmic drug, J. Pharm. Biomed. Anal. 13 (1995) 575–580.

[25] E. Caudron, S. Baghriche, P. Prognon, et al., Simultaneous quantification of gentamicin and colistin sulfate in pharmaceuticals using ion-pairing and polarity gradient chromatography with low-UV detection, Chromatographia 76 (2013) 747–755.

[26] K. Stypulkowska, A. Blazewicz, Z. Fijak, et al., Determination of gentamicin sulphate composition and related substances in pharmaceutical preparations by LC with charged aerosol detection, Chromatographia 72 (2010) 1225–1229.

[27] S.K. Branch, Guidelines from the International Conference on Harmonisation (ICH), J. Pharm. Biomed. Anal. 38 (2005) 798–805.

[28] G.A. Shabir, Validation of high-performance liquid chromatography methods for pharmaceutical analysis: Understanding the differences and similarities between validation requirements of the US Food and Drug Administration, the US Pharmacopeia and the International Conference on Harmonization, J. Chromatogr. A. 987 (2003) 57–66.

[29] M. Blessy, R.D. Patel, P.N. Prajapati, et al., Development of forced degradation and stability indicating studies of drugs—a review, J. Pharm. Anal. 4 (2014) 159–165.

[30] L.D. Sabath, J.I. Casey, P.A. Ruch, et al., Rapid microassay of gentamicin, kanamycin, neomycin, streptomycin, and vancomycin in serum or plasma, J. Lab. Clin. Med. 78 (1971) 457–463.

[31] J.M. Andrews, R. Wise, A comparison of the homogeneous enzyme immunooassay and polarization fluorimunooassay of gentamicin, J. Antimicrob. Chemother. 14 (1984) 509–520.

[32] S.A. Brown, D.R. Newkirk, R.P. Hunter, et al., Extraction methods for quantitation of gentamicin residues from tissues using fluorescence polarization immunooassay, J. Assoc. Off. Anal. Chem. 73 (1990) 479–483.

[33] V. Manyanga, O. Grishina, Z. Yun, et al., Comparison of liquid chromatographic methods with direct detection for the analysis of gentamicin, J. Pharm. Biomed. Anal. 45 (2007) 257–262.

[34] V. Manyanga, K. Kreft, B. Divjak, et al., Improved liquid chromatographic method with pulsed electrochemical detection for the analysis of gentamicin, J. Chromatogr. A 1189 (2008) 347–354.

[35] I. Clarot, P. Chaimbault, F. Hasdenteufel, et al., Determination of gentamicin sulfate and related compounds by high-performance liquid chromatography with evaporative light scattering detection, J. Chromatogr. A 1031 (2004) 281–287.

[36] N.C. Megoula, M.A. Kouparris, Development and validation of a novel LC/ELSD method for the quantitation of gentamicin sulfate components in pharmaceuticals, J. Pharm. Biomed. Anal. 36 (2004) 73–79.

[37] A. Joseph, A. Rustum, Development and validation of a RP-HPLC method for the determination of gentamicin sulfate and its related substances in a pharmaceutical cream using a short pentfluorophenyl column and a charged aerosol detector, J. Pharm. Biomed. Anal. 31 (2010) 521–531.

[38] H. Curiel, W. Vanderzanden, H. Velez, et al., Analysis of underivatized gentamicin by capillary electrophoresis with UV detection, J. Pharm. Biomed. Anal. 44 (2007) 49–56.

[39] K. Vučićević-Pučetić, R. Czevensák, N. Radulović, Development and validation of liquid chromatography tandem mass spectrometry methods for the determination of gentamicin, lincomycin, and spectinomycin in the presence of their impurities in pharmaceutical formulations, J. Pharm. Biomed. Anal. 56 (2011) 736–742.

[40] J.F. Chissell, M. Freeman, J.S. Loram, et al., British pharmacopeial gentamicin sulphate component ratio test by high-performance liquid chromatography. The effect of derivative breakdown on final result, J. Chromatogr. 369 (1986) 213–217.

[41] J.H. Albracht, M.S. de Wit, Analysis of gentamicin in raw material and in pharmaceutical preparations by high-performance liquid chromatography, J. Chromatogr. 389 (1987) 306–311.

[42] G. Wang, F.P. Tomasella, Ion-pairing HPLC methods to determine EDTA and DTPA in small molecule and biological pharmaceutical formulations, J. Pharm. Anal. 6 (2016) 150–156.