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1 **Towards harmonization of solutions used for cystic fibrosis diagnosis by nasal**
2 **potential difference measurements: a formulation approach with CHES[®] software**

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24

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26

27 *Reference count: 15*

28 **Abstract**

29 **Background:** One diagnosis of cystic fibrosis involves measuring the nasal transepithelial
30 potential difference (NPD) as a complementary technique in the forms of the disease, where the
31 sweat test is non-discriminating. The NPD is measured using solutions with and without
32 chlorides, containing a variety of substances whose activities on nasal mucus membranes are
33 studied or assessed. Among the solutions described in the literature and used in specialized
34 centers, none seems to be best adapted for industrial production for reasons of stability
35 (formulas of the international consensus of Rowe et al. and formulas of Knowles *et al.*) and/or
36 potential toxicity (formulas of Middleton *et al.*).

37 **Objective(s):** Defining new formulas, according to those of the international consensus, with
38 greater physicochemical and microbiological stability

39 **Methods:** The reformulation tests were conducted on the formulas of Rowe et al., using
40 CHESS® (CHemical Equilibrium of Species and Surfaces) software for modeling aqueous
41 systems that substantially reduced the number of experiments. CHESS® software was first
42 validated using models of ideal and non-ideal solutions. Thereafter, experimentation was carried
43 out for the seek of comparison with theoretical data.

44 **Results:** CHESS® software using models of ideal and non-ideal solutions were validated. The
45 experimentation confirmed the theoretical data, and new formulas were assessed on the basis of
46 their physicochemical (pH, content, Osmolality) and microbiological stability.

47 **Conclusion:** The new formulas defined here guarantee excellent physicochemical and
48 microbiological stability of diagnostic solutions, indispensable criteria for harmonizing and
49 comparing results from different specialized centers using NPD measurements. These new
50 formulas are applicable to the harmonization approach of techniques for measuring nasal
51 transepithelial potential difference.

52

53 **Key Messages**

54 **What is already known on this subject:**

- 55 - Nasal transepithelial potential difference (NPD) measurement is used as a
56 complementary technique for cystic fibrosis diagnosis
57 - Two types of solutions were employed for the test: solutions with and without chlorides
58 - Absence of any harmonization at international level

59 **What this study adds:**

- 60 - Development of new stables formulas
61 - Very important for routine and research results reliability

62

63

64 **1. Introduction**

65 Cystic fibrosis is characterized by an ion transport anomaly in epithelial cell membranes,
66 resulting from a mutation of the CFTR gene (Cystic Fibrosis Transmembrane
67 conductance Regulator). Patients presenting symptoms of the disease exhibit an
68 increase in their nasal transepithelial potential difference (NPD) resulting from increased
69 transport of Na⁺ ions and reduced transport of Cl⁻ ions, both of which depend on CFTR
70 [1, 2]. The technique of measuring NPD is therefore used by specialized centers for the
71 diagnosis and therapeutic monitoring of patients with cystic fibrosis.

72 Potential difference measurements use two types of solutions, one (with chloride) rich in
73 Cl⁻ ions and a second (without chloride) lacking Cl⁻ ions [3, 4]. These solutions are the
74 solvents for substances whose activities on nasal mucus membranes are studied or
75 assessed. The measurement of NPD explores the bioelectric properties of membranes
76 and so the solutions used for the diagnosis comply with a number of properties in terms
77 of composition and safety. The composition of these solutions must be as close as
78 possible to that of the extracellular matrix, in particular concerning osmolarity, the
79 concentration of calcium ions, and pH. The solutions must also be sterile for the purpose
80 of stability and safety.

81 Even though a large number of solutions have been described in the literature, none is
82 ideal:

83 a) The solutions of Knowles *et al.* [5] have the disadvantage of containing glucose that
84 isomerizes to fructose during moist heat sterilization;

85 b) The formulas of Middleton *et al.* [6] contain glucose and HEPES (4-[2-hydroxyethyl]-1-
86 piperazine ethane sulfonic acid), a zwitterion for which there are few data on its
87 pharmacological and toxicological properties [7, 8];

88 c) The formulas of Rowe *et al.*, reference solutions [9], have resulted from an
89 international standardization of procedures used to measure NPD with an entirely
90 inorganic solution containing Cl⁻ and one without Cl⁻ in which the anion is replaced by
91 gluconate or sulfate ions (SO_4^{2-}). The major disadvantage of these solutions is their
92 instability at physiological pH, resulting primarily from the precipitation of calcium
93 phosphate salts.

94 It is clear that pH plays a central role in cellular activity, cellular homeostasis and
95 membrane exchanges, especially CFTR activity and expression.

96 In order to ensure experimental data reliability, standardization efforts of diagnostics
97 solutions based on composition and pH control/stability therefore remain mandatory.

98 This publication deals with the domain of pH stability of the formulas of Rowe *et al.*
99 CHESS[®] modeling software for electrolyte equilibria in aqueous systems was used for
100 comparison with theoretical and experimental data. The stability of the formulas of Rowe
101 *et al.* was then improved by minimal changes to the original formulas.

102

103 **2. Material**

104 **Software**

105 CHESS[®] 2.3. software (CHemical Equilibrium of Species and Surfaces, Ecole des Mines
106 de Paris) [10] was used to simulate the equilibrium state of complex aqueous systems.

107 All modeling studies with CHESS[®] were conducted at 25°C, with pCO₂ equal to zero.

108 The calculations complied with electric neutrality and included solvent activity. The

109 advantage of CHESS[®] is to provide the concentrations of all chemical species in solution

110 at equilibrium. Theoretical equations were resolved with Mathematica® v9 software
111 (Woldfram Research, Boston, MA).

112

113 **Reagents and solutions**

114 Sodium chloride, sodium gluconate, potassium gluconate monohydrate and magnesium
115 gluconate monohydrate were purchased from Sigma-Aldrich (Steinheim, Germany);
116 calcium gluconate monohydrate from VWR (Geldenaaksebaan, Belgium); anhydrous
117 dibasic potassium phosphate, monobasic potassium phosphate, magnesium sulfate
118 heptahydrate, potassium chloride and magnesium chloride hexahydrate were obtained
119 from Merck (Darmstadt, Germany).

120 In view of the pH values of the solutions and to avoid container-content interactions, all
121 experiments were performed using type I glass containers (borosilicate glass) (Cooper,
122 France), characterized by a high hydrolytic resistance due to the chemical composition
123 of the glass itself.

124 Stock solutions of 2.8 mM mono- and dibasic potassium phosphate (KH_2PO_4 , K_2HPO_4)
125 were used to prepare diluted solutions.

126 The composition of the original solutions according to Rowe formulation were 148 mM
127 NaCl, 4.05 mM KCl, 2.4 mM K_2HPO_4 , 0.4 mM KH_2PO_4 , 2.25 mM CaCl_2 and 1.2 mM
128 MgCl_2 for the solution with chloride (solution A) and 148 mM $\text{C}_6\text{H}_{11}\text{NaO}_7$, 4.05 mM
129 $\text{C}_6\text{H}_{11}\text{KO}_7 \cdot \text{H}_2\text{O}$, 2.4 mM K_2HPO_4 , 0.4 mM KH_2PO_4 , 2.25 mM $\text{C}_{12}\text{H}_{22}\text{CaO}_{14} \cdot \text{H}_2\text{O}$ and 1.2
130 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ for the solution without chloride (solution B) [9]. D-gluconic acid was
131 assayed in the solutions with the R-Biopharm (formerly Boehringer Mannheim)
132 enzymatic test kit (UV-method).

133 For sterility test, typticase Soy agar (TSA) and Sabouraud media were purchased from
134 Biomérieux (Paris, France), and a 0.45 µm pore size membrane (reference
135 HAWG047S6) obtained from Millipore (Molsheim, France) was used for the membrane
136 filtration. Sterile saline (0.9% NaCl) was supplied by Fresenius Kabi France SA (Sevres,
137 France). The reference bacterial (*Bacillus subtilis* (ATCC 6633)) and fungal (*Aspergillus*
138 *Niger* (ATCC 16404)) strains were provided by the BioReference Laboratory, Eurofins
139 (Lille, France).

140

141 **Instrumentation**

142 A Varian SpectrAA 220Z Spectrometer (Varian, Australia) with Zeeman background
143 correction was used for analyses of electrolytes. The concentrations of Mg, K and Ca,
144 were determined by flame atomic absorption spectrometry at the wavelengths of 285.2,
145 766.5 and 422.7 nm respectively, and the concentration of Na was obtained with flame
146 atomic emission spectrometry at 589 nm.

147 The pH values of the solutions were obtained using a Metrohm 713 pH meter (Metrohm,
148 France) at 25°C (precision ± 0.05 unit). Solutions were sterilized by filtration (0.22 µm
149 pore size membrane) or by autoclaving in a Stericlav® autoclave (Cominox, France) at
150 121°C. An Advanced™ Model 3250 Single-Sample Osmometer (Fisher Scientific,
151 France) was used to determine osmolality. Before all studies, the osmometer was
152 calibrated using the commercial Clinitrol™ 290 mOsm/kg standard solution.

153

154

155

156

157 **3. Methods**

158 **Evaluation of the stability of solutions with CHESS®**

159 Prior to using CHESS® software as a modeling tool to study the stability of solutions, it
160 was validated by comparing its results to both theoretical equations and experimental
161 results. CHESS® was validated with two models adapted from the work of Yoonjee *et al.*
162 [11]:

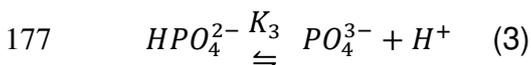
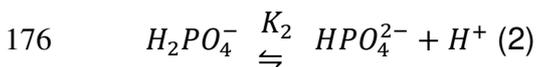
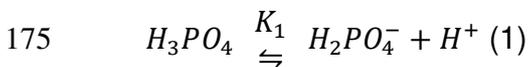
- 163 - First, a model of an "ideal" solution with low ionic strength, composed of 2.4 mM
164 K_2HPO_4 and 0.4 mM KH_2PO_4
- 165 - Secondly, a model of a "non-ideal" solution obtained by adding electrolytes to the
166 above ideal phosphate solution: 148 mM NaCl, 4.05 mM KCl and 1.2 mM $MgCl_2$.

167 Considering the effect of pH on transmembrane ion transport, pH is a parameter that
168 must be controlled in order to ensure the quality of the diagnostic test. In addition, pH is
169 a reliable marker of the stability of solutions, explaining why it is the parameter
170 monitored in the different models used.

171

172 **Chemical equilibria involving K_2HPO_4 and KH_2PO_4 [11-13]**

173 Phosphate salts (K_2HPO_4 and KH_2PO_4) in aqueous solution dissociate into HPO_4^{2-} and
174 $H_2PO_4^-$ ions, resulting in three equilibrium reactions:



178

179 where K_1 , K_2 and K_3 are equilibrium constants. The values of equilibrium constants, $pK_i =$
180 $-\log_{10}K_i$ ($i=1, 2$ or 3) are 2.16, 7.21 and 12.32 at 25°C [12].

181 The concentrations C_1 , C_2 , C_3 and C_4 and activity coefficients γ_1 , γ_2 , γ_3 and γ_4 of the four
182 phosphate species in solution (H_3PO_4 , $H_2PO_4^-$, HPO_4^{2-} and PO_4^{3-}) were calculated from
183 these dissociation equilibria.

184 The expression of equilibrium constants for activities $\alpha_i = \gamma_i C_i$, are:

185
$$K_1 = \frac{\alpha_{H^+} \cdot \alpha_2}{\alpha_1} = \frac{C_{H^+} \cdot C_2}{C_1} \frac{\gamma_{H^+} \cdot \gamma_2}{\gamma_1} \quad (4)$$

186
$$K_2 = \frac{\alpha_{H^+} \cdot \alpha_3}{\alpha_2} = \frac{C_{H^+} \cdot C_3}{C_2} \frac{\gamma_{H^+} \cdot \gamma_3}{\gamma_2} \quad (5)$$

187
$$K_3 = \frac{\alpha_{H^+} \cdot \alpha_4}{\alpha_3} = \frac{C_{H^+} \cdot C_4}{C_3} \frac{\gamma_{H^+} \cdot \gamma_4}{\gamma_3} \quad (6)$$

188 The total phosphate concentration is $C_T^0 = C_2^0 + C_3^0 = 2.8$ mM, where C_2^0 and C_3^0 are the
189 initial concentrations of $H_2PO_4^-$ and HPO_4^{2-} .

190

191 **Validation of CHESS®**

192 Validation involved comparing the pH profiles obtained with CHESS®, theoretical
193 approaches and experimental results obtained with the two models used.

194

195 **Theoretical equations**

196 - **Ideal model (IM)**

197 The activity coefficients with this model are close to unity. It is considered that:

198
$$\gamma_1 = \gamma_2 = \gamma_3 = \gamma_4 = \gamma_{H^+} = 1 \quad (7)$$

199 The majority of H_2PO_4^- and HPO_4^{2-} ions are in solution in the pH range between $\text{p}K_1 + 2$
200 ($\text{pH} = 4.12$) and $\text{p}K_3 - 2$ ($\text{pH} = 10.67$)

201 Equation (5) can be reduced to: $K_2 = \frac{C_3^0 \times C_{\text{H}^+}}{C_2^0}$

202 In the expression $R = \frac{C_3^0}{C_2^0}$ (8), R correspond to the concentration ratio of H_2PO_4^- and
203 HPO_4^{2-} ions.

204 We have the Henderson-Hasselbach equation: $\text{pH} = \text{p}K_2 - \log R$ (9)

205
206 The pH of diagnostic solutions must be close to physiological values and so all models
207 were used for R values between 0.125 and 4. This interval covers the pH range from 6
208 to 8.

209
210 - **Non-ideal model (NIM)**

211 A "non-ideal" model ($\gamma \neq 1$) was obtained by adding electrolytes to the phosphate buffer.
212 The Debye-Hückel theory enabled the theoretical approach of this model by providing a
213 simple relationship linking the activity coefficient (γ_i) and ionic strength (μ) (mol.L^{-1}):

214 $\log \gamma_i(z, \mu, a) = -\frac{A(T)z_i^2 \sqrt{\mu}}{1 + B(T)a\sqrt{\mu}}$ (10), where A and B are constants ($A = 0.51 \text{ mol}^{-1/2}$ and B
215 $= 0.33 \cdot 10^8 \text{ \AA}^{-1} \cdot \text{mol}^{-1/2}$ at 25°C), z_i is the number of charges and a is the effective mean
216 diameter of the ion [14].

217 When this expression replaces the dissociation equations of the acid, we obtain the
218 following for the three concentrations C_1 , C_2 and C_3 :

219 $C_1 = C_4 \times 10^{\frac{-9A\sqrt{\mu}}{1+4B\sqrt{\mu}} - 3pH + pK_1 + pK_2 + pK_3}$ (11)

220 $C_2 = C_4 \times 10^{\frac{-8A\sqrt{\mu}}{1+4B\sqrt{\mu}} - 2pH + pK_2 + pK_3}$ (12)

221 $C_3 = C_4 \times 10^{\frac{-5A\sqrt{\mu}}{1+4B\sqrt{\mu}} - pH + pK_3}$ (13)

222

223 Ionic strength (μ) is calculated as follows:

224 $\mu = \frac{1}{2} \sum_i C_i z_i^2$ (14)

225 where the sum involves all species in solution. After all species in solution are included,

226 the following expression is obtained:

227

228 $\mu = \frac{C_T^0}{2} + \frac{C_2}{2} + 2C_3 + \frac{9C_4}{2} + 3C_{CaCl_2} + C_{KCl} + 3C_{MgCl_2} + C_{NaCl} + \frac{C_T^0}{2(1+R)}$ (15)

229

230 When the last four equations are combined with the charge and mass balances, a

231 system of three equations is deduced:

232

233 $C_0^T = C_4 + C_4 10^{\frac{-5A\sqrt{\mu}}{1+4B\sqrt{\mu}} - pH + pK_3} + C_4 10^{\frac{-8A\sqrt{\mu}}{1+4B\sqrt{\mu}} - 2pH + pK_2 + pK_3} + C_4 10^{\frac{-9A\sqrt{\mu}}{1+4B\sqrt{\mu}} - 3pH + pK_1 + pK_2 + pK_3}$ (16)

234 $3C_4 + C_4 2^{\frac{1-5A\sqrt{\mu}}{1+4B\sqrt{\mu}} - pH + pK_3} 5^{\frac{5A\sqrt{\mu}}{1+4B\sqrt{\mu}} - pH + pK_3} + C_4 10^{\frac{-8A\sqrt{\mu}}{1+4B\sqrt{\mu}} - 2pH + pK_2 + pK_3} + 3C_4 R + C_4 R 2^{\frac{1-5A\sqrt{\mu}}{1+4B\sqrt{\mu}} - pH + pK_3} 5^{\frac{5A\sqrt{\mu}}{1+4B\sqrt{\mu}} - pH + pK_3} + C_4 R 10^{\frac{-8A\sqrt{\mu}}{1+4B\sqrt{\mu}} - 2pH + pK_2 + pK_3} = C_0^T (2+R)$ (17)

235
$$\mu = \frac{C_T^0}{2} + \frac{9C_4}{2} + C_4 2^{\frac{1 - \frac{5A\sqrt{\mu}}{1+4B\sqrt{\mu}} - pH + pK_3}{5} - \frac{5A\sqrt{\mu}}{1+4B\sqrt{\mu}} - pH + pK_3} + C_4 2^{\frac{1 - \frac{8A\sqrt{\mu}}{1+4B\sqrt{\mu}} - 2pH + pK_2 + pK_3}{5} - \frac{8A\sqrt{\mu}}{1+4B\sqrt{\mu}} - 2pH + pK_2 + pK_3} +$$

236
$$3C_{CaCl_2} + C_{KCl} + 3C_{MgCl_2} + C_{NaCl} + \frac{C_T^0}{2(1+R)} \quad (18)$$

236 This system of equations was resolved using commercial Mathematica® software. By
 237 setting the composition of the solution and the total concentration C_T^0 , for each value of
 238 R, the values of C_4 is obtained, μ and pH, enabling curves of pH vs. R to be plotted.

239

240 **Experimental measurements**

241 Stock solutions for each model were prepared with R values from 0.125 to 4, and
 242 $C_T^0 = 2.8mM$.

243

244 **CHESS® calculations**

245 Once the concentrations values of ions correspondent to the 2 models were entered,
 246 CHESS® was run and calculations were rapidly made (< 2 s). In contrast to theoretical
 247 equations based on hypotheses leading to a certain number of approximations, the
 248 advantage of CHESS® is to provide the concentrations of all chemical species in solution
 249 at equilibrium.

250 CHESS® was used to study the stability of cystic fibrosis diagnosis solutions only for the
 251 solution containing chloride.

252

253 **Real-time study of stability of solutions of Rowe *et al.* and reformulation**

254 In parallel to validating the CHESS® approach ($0.125 < R \leq 4$), a stability study was
 255 carried out on the formulas of Rowe *et al.* ($R = 0.167$) at 4°C and 25°C. One batch of

256 each formulation of the solution with chloride (148 mM NaCl, 4.05 mM KCl, 2.4 mM
257 K₂HPO₄, 0.4 mM KH₂PO₄, 2.25 mM CaCl₂ and 1.2 mM MgCl₂) and the solution without
258 chloride (148 mM C₆H₁₁NaO₇, 4.05 mM C₆H₁₁KO₇.H₂O, 2.4 mM K₂HPO₄, 0.4 mM
259 KH₂PO₄, 2.25 mM C₁₂H₂₂CaO₁₄.H₂O and 1.2 mM MgSO₄.7H₂O) was prepared and
260 sterilized by sterilizing filtration (n = 30 bottles). The physical stability of the solutions
261 was monitored in triplicate on days 0, 5, 15 and 30 by visual examination of the
262 appearance of the solutions: clearness, color, presence of particles (precipitates,
263 crystals). Simultaneously, the chemical behavior of the solutions was determined by
264 atomic absorption measurements (K⁺, Ca²⁺ and Mg²⁺), emission atomic measurements
265 (Na⁺) and enzymatic determination (gluconic acid). In addition, the osmolality and pH
266 were determined.

267 These studies resulted in experimental reformulations with the goals of developing
268 stable solutions at room temperature as well as reduced production costs. This involved
269 giving preference to moist heat sterilization rather than sterilization by filtration and the
270 composition of solutions as close as possible to the reference solutions of Rowe *et al.*
271 As a result, a pilot batch (n = 30 bottles) made of the new formulas was produced and
272 distributed in 100 mL type I glass containers, then heat-sterilized before sealing. No As
273 moist heat sterilization was performed at 121 °C for 20 min, it was also used to assess
274 the sensitivity of the solutions under such heat stress conditions. For the stability
275 assessment, the samples were placed in a calibrated hot-air oven at 25 °C for 3 years,
276 without any specific protection from light. In addition to the physicochemical analysis
277 mentioned above, additional sterility tests were performed under aseptic conditions
278 according to the recommendations of the European Pharmacopoeia (Sterility test: 2.6.1.)
279 [15] to evaluate the reformulated solutions. Trypticase Soy agar (TSA) and Sabouraud

280 media were used respectively for the growth of bacteria and fungi. For each type of
281 solution, the test was realized on 6 samples of 100 mL (3 samples per media). Each
282 sample was filtered, rinsed with 250 mL of sterile saline (0.9%NaCl). The membrane
283 was placed on a media culture then incubated during a fourteen-day period at 37 °C and
284 30 °C respectively for the bacteria and the fungi. The method was validated using by
285 means of the sterility test of the culture media, the fertility test of the culture media with
286 the recommended micro-organisms strains (*Bacillus subtilis* and *Aspergillus Niger*) and
287 the fertility test of strains with the solutions (5 days incubation time at 30 °C and 37°C
288 respectively for the fungi and the bacteria).

289

290 **4. Results and discussion**

291 **Stability studies**

292 **Validation of CHESS®: Ideal solution model (phosphate buffer alone)**

293 In general, the highest pH results were obtained with the theoretical equation while the
294 lowest resulted from experimentation. As R increased, pH decreased, tending towards a
295 limit value of 6.50.

296 There was considerable similarity between experimental and theoretical values
297 (CHESS® and equation 9) (Figure 1). The pH prediction by CHESS® and the theoretical
298 equation were in good agreement and the relative errors were less than 2%.

299

300 Figure 1: Comparison of typical profiles of pH vs. R in the ideal model (Black lines): (IM
301 from equation (9) = Small dash; IM from CHESS® = Big dash; and Experimentation =
302 Solid line); with non-ideal models (NIM) (Blue lines) obtained from theoretical equations
303 (Mathematica®) (Small dash), from CHESS® (Big dash); and Experimentation (Solid line)

304

305 **Validation of CHESS®: non-ideal solution model**

306 As in the case of the ideal model, an increase of R with the non-ideal model resulted in a
307 decrease of pH. For R values between 0.125 and 4, the pH varied from 7.6 to 6.0.

308 Increasing the ionic strength lowered pH by about 0.4 unit compared to the ideal model
309 (Figure 1).

310 The non-ideal model also provided good prediction of pH from experimental data
311 provided by CHESS® and Mathematica®, with relative errors less than 1% and 3%,
312 respectively.

313 The results of comparisons of the models of ideal and non-ideal solutions of CHESS®
314 with theoretical equations and experimental results were well correlated. This
315 demonstrates the considerable value of using this software as a modeling tool for
316 complex mixtures of inorganic ions in solution.

317 As a result, the next step involved CHESS® modeling of non-ideal solutions to which
318 calcium was added.

319

320 **Real-time stability studies and modeling by CHESS®**

321 The instability of solutions was observed for all solutions stored at 25°C relatively early,
322 on day 5 (D5). At 4°C, precipitates were seen on D5 in the solution with Cl⁻ and on D15
323 in the solution without Cl⁻. This instability was correlated with a pH decrease that was
324 greater with increasing temperature (Tables 1, 2). The relative stability of solution B
325 compared to solution A could be explained by the presence of gluconate salts in the
326 former, which chelated a portion of calcium ions, rendering them unavailable for
327 precipitation reactions.

328 Similar results were obtained with CHESS® by examining the behavior of solution A with
329 variable values of R (Table 1). At equilibrium, the software predicted the formation of two
330 precipitates: hydroxyapatite ($\text{Ca}_5(\text{PO}_4)_3(\text{OH})$) and monobasic calcium phosphate
331 ($\text{CaHPO}_4, 2\text{H}_2\text{O}$) (8:1).

332

333 Table 1: Variations of percentages of Ca^{2+} precipitated as hydroxyapatite (HA) and pH
334 vs. R calculated by CHESS®.

335

336 Table 2: Changes of appearance and pH of solutions.

337

338 The quantity of precipitate increased with increasing pH, since precipitation reactions
339 consumed primarily HPO_4^{2-} ions. In addition to reducing the pH, precipitation also
340 caused a reduction of the concentration of Ca^{2+} ions in solution. In the case of the
341 formula of Rowe *et al.* with chloride ($R = 0.167$), more than 50% of calcium added
342 participated in precipitation reactions at equilibrium, accompanied by a decrease of one
343 pH unit; this calcium decrease was unacceptable in light of the importance of calcium
344 ions and pH in transmembrane exchanges.

345 Experimental results confirmed by theoretical studies showed that solutions based on
346 the formula of Rowe *et al.* could not all be used as is. This is why reformulation was
347 considered, by modifying the components of solutions.

348

349 **Reformulation tests**

350 It is known that the instability of these solutions results from the coexistence of
351 phosphate and alkaline earth ions in the same solution, although the kinetics of

352 precipitate formation are slow at room temperature. This is important for the stability of
353 the solution during the test that lasts only several dozen minutes. This implies that
354 freshly prepared solutions should be used for the test because precipitation occurs over
355 time, precluding the preparation of solutions in advance. This finding is consistent with
356 the preparation of two solutions for each formula in which the ions responsible for
357 precipitation are separated and that are reconstituted just before use in the test.

358 In order to reduce production costs, we considered the possibility of developing three
359 types of solutions: solution A' with chloride, solution B' without chloride and the addition
360 of alkaline earth ions in solution C'. Several formulas were prepared and studied and
361 only one was selected (Table 3) as a result of its physicochemical stability (> 1 year) and
362 the possibility of moist heat sterilization of the solutions. In the new formula, calcium and
363 magnesium were added as the gluconate salts.

364
365 Table 3: Composition of solutions proposed for the new formula. The solution with
366 chloride is composed of solutions A' and C' (19:1, v/v) and the solution without chloride
367 is composed of solutions B' and C' (19:1, v/v).

368
369 The comparison of formulas with and without Cl⁻ revealed minimal differences between
370 the formulas of Rowe *et al.* and our new formulas (Table 4). The concentrations of
371 calcium ions and mono- and dibasic potassium phosphate are identical in the two
372 formulas, as a result of the major importance of these electrolytes in transmembrane
373 exchanges. Subsequently, no substantial variation of pH of the solutions was observed
374 over time.

375 The appropriate monitoring of the compositions of solutions and pH stability are
376 therefore defined as the main quality attributes for the purposes of manufacturing
377 strategy and process validation, relevant for the standardization of CF diagnosis tests.
378 The major role of microbiological decontamination provided by moist heat sterilization
379 has also been added.

380

381 Table 4: Comparison of the ionic compositions of the solutions of Rowe et al. and our
382 new formulas after reconstitution.

383

384 Furthermore, in order to address any stability issue of the new solutions, real time
385 quantitative analysis of the constituents (Na^+ , K^+ , Mg^{2+} , Ca^{2+} and gluconic acid) and a
386 microbial contamination test were performed (Table 5), and confirmed stability over 1
387 year. Subsequently, relevant specifications were defined to ensure the reproducibility
388 and reliability of the manufacturing process.

389

390 Table 5 : Analytical and microbiological data and specifications of the new formulas.

391

392 **5. Conclusion**

393 The new formulas defined here guarantee excellent physicochemical and
394 microbiological stability of diagnostic solutions, indispensable criteria for harmonizing
395 and comparing results from different specialized centers using NPD measurements. The
396 use of three solutions with the new formulas rather than two with the reference formulas
397 lead to higher production costs. Potential errors resulting from the reconstitution of

398 solutions before use can be attenuated or eliminated by training users to comply with
399 recommendations for the reconstitution of solutions. Reformulation and the definition of
400 new formulas were facilitated by the modeling results provided by CHESS[®] that reduced
401 the volume of experimental tests. Initially used in geochemistry, this software may have
402 an important place in pharmaceutical research and development where the issue of
403 stability conditions the development of new drugs.

404

405 **6. References**

406 [1] Castellani C, Linnane B, Pranke I, Cresta F, Sermet-Gaudelus I, Peckham D. Cystic
407 Fibrosis Diagnosis in Newborns, Children, and Adults. *Semin Respir Crit Care Med.*
408 2019 Dec;40(6):701-714.

409 [2] Solomon GM, Bronsveld I, Hayes K, Wilschanski M, Melotti P, Rowe SM, Sermet-
410 Gaudelus I. Standardized Measurement of Nasal Membrane Transepithelial Potential
411 Difference (NPD). *J Vis Exp.* 2018 Sep 13;(139).

412 [3] Fedick AM, Zhang J, Edelmann L, Kornreich R. Prenatal Diagnosis of Cystic
413 Fibrosis. *Methods Mol Biol.* 2019;1885:221-231.

414 [4] Schüler D, Sermet-Gaudelus I, Wilschanski M, Ballmann M, Dechaux M, Edelman A
415 *et al.* Basic protocol for transepithelial nasal potential difference measurements. *J Cyst*
416 *Fibros.* 2004 Aug;3 Suppl 2:151-5.

417 [5] Knowles MR, Carson JL, Collier AM, Gatzky JT, Boucher RC. Measurements of nasal
418 transepithelial electric potential differences in normal human subjects in vivo. *Am Rev*
419 *Respir Dis.* 1981 Oct;124(4):484-90.

- 420 [6] Middleton PG, Geddes DM, Alton EW. Protocols for in vivo measurement of the ion
421 transport defects in cystic fibrosis nasal epithelium. *Eur Respir J.* 1994 Nov;7(11):2050-
422 6.
- 423 [7] Lepe-Zuniga JL, Zigler JS Jr, Gery I. Toxicity of light-exposed HEPES media. *J*
424 *Immunol Methods.* 1987 Oct 23;103(1):145.
- 425 [8] Zigler JS Jr, Lepe-Zuniga JL, Vistica B, Gery I. Analysis of the cytotoxic effects of
426 light-exposed HEPES-containing culture medium. *In Vitro Cell Dev Biol.* 1985
427 May;21(5):282-7.
- 428 [9] Rowe SM, Clancy JP, Wilschanski M. Nasal potential difference measurements to
429 assess CFTR ion channel activity. *Methods Mol Biol.* 2011;741:69-86.
- 430 [10] van der Lee J. Thermodynamic and mathematical concepts of CHESS. *Ecole des*
431 *Mines de Paris ;* 1998
- 432 [11] Yoonjee P, Sook Heun K, Sadis M, Nien-Hwa LW, Elias IF. Effect of phosphate
433 salts concentrations, supporting electrolytes, and calcium phosphate salt precipitation on
434 the pH of phosphate buffer solutions. *Fluid Phase Equilibria.* 2009;278, 76–84
- 435 [12] *Handbook of Chemistry and Physics.* 90th Edition; 2009 – 2010.
- 436 [13] Migneault DR, Forcé RK. Dissociation Constants of Phosphoric Acid at 25°C and
437 the Ion Pairing of Sodium with Orthophosphate Ligands at 25°C. *J Solution Chem.* Vol
438 17, pages 987–997(1988)
- 439 [14] Atkins PW. *Physical Chemistry,* Oxford University Press; 2006
- 440 [15] *European Pharmacopoeia,* 10th Ed; Sterility test: 2.6.1., 2020

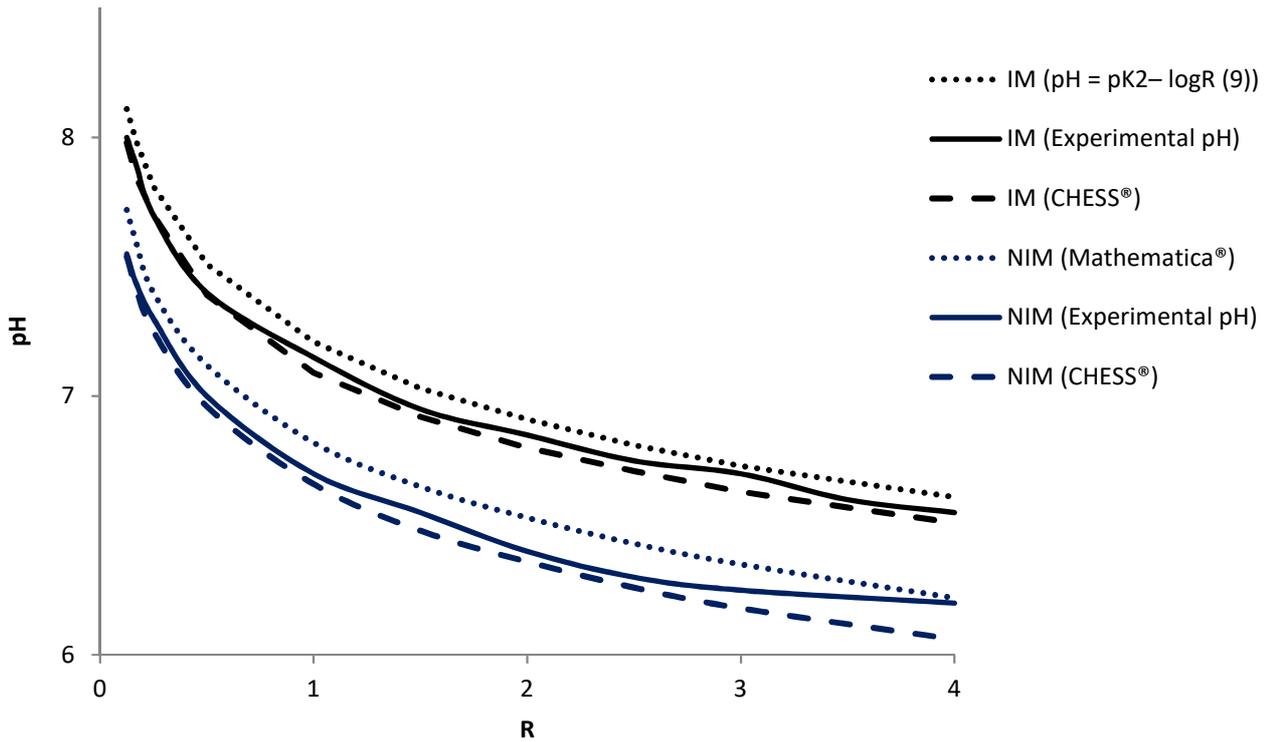


Figure 1: Comparison of typical profiles of pH vs. R in the ideal model (Black lines): (IM from equation (9) = Small dash; IM from CHESS® = Big dash; and Experimentation = Solid line); with non-ideal models (NIM) (Blue lines) obtained from theoretical equations (Mathematica®) (Small dash), from CHESS® (Big dash); and Experimentation (Solid line)

Figure 1: Comparaison des profils types de pH par rapport à R dans le modèle idéal (lignes noires): (IM de l'équation (9) = petit tiret; IM de CHESS® = grand tiret; et expérimentation = trait plein); avec des modèles non idéaux (NIM) (lignes bleues) obtenus à partir d'équations théoriques (Mathematica®) (petit tiret), de CHESS® (grand tiret); et expérimentation (trait plein)

Table 1: Variations of percentages of Ca²⁺ precipitated as hydroxyapatite (HA) and pH vs. R calculated by CHES[®].

Table 1: Variations du Ca²⁺ précipité sous forme d'hydroxyapatite (HA) et du pH en fonction de R calculé par CHES[®].

R	[Ca ²⁺] _{initial} (mM)	[Ca ²⁺] _{final} (mM)	HA formed (mM)	% Ca ²⁺ precipitated	pH
0.001	2.25	0.81	0.282	63%	6.51
0.125	2.25	0.99	0.244	54%	6.42
0.167	2.25	1.10	0.233	52%	6.40
0.25	2.25	1.14	0.213	47%	6.36
0.5	2.25	1.37	0.166	37%	6.28
1	2.25	1.67	0.104	23%	6.2
1.5	2.25	1.85	0.067	15%	6.15
2	2.25	1.97	0.042	9%	6.12
2.5	2.25	2.06	0.024	5%	6.11
3	2.25	2.13	0.010	2%	6.09
3.5	2.25	2.17	1	-	6.08
4	2.25	2.18	1	-	6.02

1: Saturation indices < -3, solutions unsaturated in hydroxyapatite (CHES[®])

Table 2: Changes of appearance and pH of solutions.

Table 2: Changement d'apparence et pH des solutions.

	Solution with Cl ⁻ (A)				Solution without Cl ⁻ (B)			
	4 °C		25 °C		4 °C		25 °C	
	Appearance	pH	Appearance	pH	Appearance	pH	Appearance	pH
D0	Cl	7.42±0.01	Cl	7.42±0.01	Cl	7.47±0.02	Cl	7.47±0.02
D5	P	7.35±0.06	P	7.30±0.04	Cl	7.41±0.05	P	7.35±0.03
D15	P	7.23±0.07	P	7.25±0.03	P	7.38±0.03	P	7.35±0.05
D30	P	7.12±0.06	C	7.02±0.05	P	7.20±0.03	P	7.26±0.06

Cl: clear; C: presence of crystals; P: particles in suspension

Table 3: Composition of solutions proposed for the new formula. The solution with chloride is composed of solutions A' and C' (19:1, v/v) and the solution without chloride is composed of solutions B' and C' (19:1, v/v).

Table 3: Composition des nouvelles solutions proposées. La solution avec chlorure est composée des solutions A' and C' (19:1, v/v) et la solution sans chlorure est composée des solutions B' and C' (19:1, v/v).

Solution A'		Solution B'		Solution C'	
	mM		mM		mM
NaCl	155.79	Sodium gluconate	155.79	Calcium gluconate	45
KCl	4.26	Potassium gluconate	4.26	Magnesium gluconate	24
K ₂ HPO ₄	2.53	K ₂ HPO ₄	2.53		
KH ₂ PO ₄	0.42	KH ₂ PO ₄	0.42		

Table 4: Comparison of the ionic compositions of the solutions of Rowe et al. and our new formulas after reconstitution.

Table 4: Comparaison des compositions ioniques des solutions de Rowe et al. Avec nos nouvelles formules après reconstitution.

	Solutions with Cl ⁻			Solutions without Cl ⁻		
	Formula of Rowe <i>et al.</i> (mM)	New formula (mOsmol/kg)	Difference (%)	Formula of Rowe <i>et al.</i> (mM)	New formula (mOsmol/kg)	Difference (%)
Na ⁺	148	148	0	148	148	0
K ⁺	9.25	9.25	0	9.25	9.25	0
Cl⁻	158.95	152.05	4.3	0	0	0
Ca ²⁺	2.25	2.25	0	2.25	2.25	0
Mg ²⁺	1.2	1.2	0	1.2	1.2	0
H ₂ PO ₄ ⁻	0.4	0.4	0	0.4	0.4	0
HPO ₄ ²⁻	2.4	2.4	0	2.4	2.4	0
Gluconate	0	6.9	-	156.55	158.95	1.5
SO ₄ ²⁻	-	-	-	1,2	0	-
Osmolality	-	288	-	-	285	-

Table 5 : Analytical and microbiological data and specifications of the new formulas.

Table 5 : Données analytiques et microbiologiques et spécifications des nouvelles formules.

Characteristics	Test method	Units	Specifications	Results
pH	Glass electrode		7.20-7.50	7.30
Identification of Cl ⁻	Eur. Phar 9.0		Positive	Pass
Na ⁺	Flame AES ¹	mM	148.1 – 163.6	149.3 ± 2.1
K ⁺	Flame AAS ²	mM	9.3 – 10.2	9.4±1.2
Ca ²⁺	Flame AAS ²	mM	42.8 – 47.3	44.7±3.2
Mg ²⁺	Flame AAS ²	mM	21.1 – 23.3	21.4±4.1
Gluconic acid	Enzymatic essay	mM	131.1 – 144.9	132.4±2.8
Osmolality	Freezing point	mOsmol/kg	280.2 – 292.5	286.8±3.8
Sterility test	Eur. Phar 9.0		Absence	Pass

1: Atomic Emission Spectrometry; 2: Atomic Absorption Spectrometry