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Physiological HEPES Buffer Proposed as a Calibrator for pH Measurement in Human Blood

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N-(2-hydroxyethyl)-piperazine-N'-2-ethanesulfonic acid, known as HEPES buffer, with p*K* in the physiological range was studied for use as an alternative to conventional phosphate buffer for the calibration of pH in modern clinical analyzers. In different series of aqueous equimolar HEPES buffer, pH was measured at 37 °C with a capillary glass electrode standardized previously using phosphate, and variations due to changes in total HEPES buffer concentration (0.025 to 0.320 mol/l), and NaCl (0 to 0.250 mol/l) were monitored. For 0.05 equimolar HEPES buffer without NaCl, the pH of 7.362 ± 0.003 (n = 15) obtained coincided well with the reference pH (7.364) from the National Institute of Standards and Technology (NIST). In particular, in the preferred 0.05 equimolar HEPES buffer/0.110 mol/l NaCl, which is isotonic to human plasma (0.160 mol/l), and termed physiological HEPES buffer (PHB), the pH of 7.346 ± 0.003 (n = 84) can be related to the calculated corresponding reference pH from NIST without liquid junction (7.374), and is also compatible with the pH measured in normal arterial blood, pH = 7.403 ± 0.003 (n = 20). Hence, in the two-point calibration of clinical analyzers, PHB, which is defined operationally with respect to the glass electrode and to phosphate buffer, may be useful as a calibrator in the range of buffer adjustment control to meet the correct values for pH when measuring in blood.

Whereas Na-HEPES salt is hygroscopic and does not meet the declared purity grade (> 99 %), pure HEPES acid is non-hygroscopic and conforms to the manufacturer's purity grade (≥ 99 %). Therefore, for easy preparation of PHB, HEPES acid is the preferred starting material.

Key words: Physiological HEPES buffer (PHB); Apparent p*K*; Calibrating; Blood pH.

Introduction

pH measurement in blood with modern blood gas analyzers can be easily and rapidly carried out in small sample volumes of the order of 30 to 120 µl, a practice which is well-established in the daily clinical routine. Originally, this measurement became possible when suitable glass electrodes became available for clinical use (1, 2). Initially, all pH reference values for blood, e.g., in the Siggaard-Andersen nomogram (3), normal pH = 7.400 at 37 °C, pCO_2 = 40 mmHg carbon dioxide tension, and full oxygen saturation of hemoglobin (97 %), or in the pathological range, i.e., pH < 7.37 acidosis, and pH > 7.43 alkalosis, were related to the calibrating phosphate buffer of the pH glass electrode system recommended by the International Federation of Clinical Chemistry (IFCC) (4). With further development, clinical analyzers became available which were not restricted to the measurement of blood gas parameters, i.e., pH, pCO_2, pO_2 (oxygen partial pressure), but also allowed measurement of the electrolyte status (Na^+, K^+, Ca^{2+}, Cl^-), lactate or glucose, thus additionally necessitating the development of new calibrating solutions for pH and combined electrolytes. In this respect, phosphate buffer was unsuitable for combination with electrolytes, especially considering its incompatibility with calcium ions. As a result, in the two-point calibration of pH measuring systems, N-(2-hydroxyethyl)-piperazine-N'-2-ethanesulfonic acid, known as HEPES buffer (5, 6) with p*K* in the physiological pH range, and 3-(N-morpholino)-2-hydroxypropanesulfonic acid, MOPSO, both of which show no such interactions with electrolytes, have been proposed and recommended by the National Institute of Standards & Technology (NIST) as pH standards for physiological fluids (7, 8): 0.08 equimolal HEPES/Na-HEPES salt/NaCl, and 0.08 equimolal MOPSO/Na-MOPSO salt/NaCl with pH_{NIST} = 7.373, and 6.694, respectively, measured at 37 °C in a cell without liquid junction (Harned cell). However, if the setting of the pH at the two calibration points of the glass electrode system is done in the same way as with phosphate, the pH values obtained for blood are not correct, but erroneously biased by an amount which is related to the residual liquid junction potential of HEPES buffer with respect to physiological 1:4 phosphate buffer (1:4 PPB). To the clinician, the system of established normal values of pH in blood is not a matter of discussion and, therefore, must be preserved with respect to introduction of a new pH reference standard. As a consequence, the calibrating solutions which are used in practice by the manufacturers are modified, termed "stabilized" or "adjusted" with respect to their reference pH, e.g., Radiometer S 1575: 6.800 ± 0.005, or S 1565: 7.400 ± 0.005 (9).

The following measurements and proposals are restricted to HEPES buffer only and are designed for development of a physiological calibrating buffer in the normal pH range of human blood:

1. Proposed Physiological HEPES buffer (PHB) is composed of 0.050 mol/l HEPES acid, 0.050 mol/l Na-HEPES salt, and 0.110 mol/l NaCl with reference pH at 37 °C predicted from experimental data from NIST

for the platinum/hydrogen electrode in a cell without liquid junction (Harned cell), pH_{NIST} = 7.374, i.e., the concentration of buffer base and total ionic strength is similar to that of human plasma at normal pH.

2. If the platinum/hydrogen electrode in a cell without liquid junction is replaced by a glass electrode and the same reference electrode with liquid junction, the measured pH will change due to the liquid junction potential at the interface of the junction. Therefore, from measurement of pH in PHB at 37 °C with the glass electrode standardized previously by phosphate (IFCC procedure), the residual liquid junction potential (δE_J) can be approximately evaluated with respect to physiological 1:4 phosphate buffer (1:4 PPB), pH = 7.383 at 37 °C by comparison with the corresponding reference values of pH from NIST without liquid junction, pH_{NIST}, and, with the same liquid junction of saturated KCl salt bridge, pH_{NIST}(l.j.).
3. pH in equimolar HEPES buffer solution should be precisely measured with the glass electrode system and thoroughly investigated to assess how it varies with changes in NaCl concentration or ionic strength, total HEPES buffer concentration, or instrument variation.
4. A simple procedure should be established for preparing PHB from suitable starting materials, taking advantage of the available high quality products from different commercial sources, high purity grade crystalline HEPES acid (≥ 99.5 %), and Na-HEPES salt (≥ 99 %), should be tested in comparison to the corresponding standard reference material (SRM) from NIST with respect to: costs, purity, stability, hygroscopic properties, drying procedure, handling, and reliability.

Materials and Methods

Chemicals, reagents and blood specimens

Crystalline HEPES acid and Na-HEPES salt were obtained from the following sources, respectively (purity grade: % acid; % salt): Applichem, Darmstadt, Germany (99.5 %; 99 % calculated for anhydrous product); Fluka, Buchs, Switzerland (≥ 99.5 %; > 99 %, H_2O ≤ 2 %); ICN Biomedicals, Eschwege, Germany (not declared; approx. 99 %); Merck, Darmstadt, Germany (> 99 %; > 99 %); National Institute of Standards & Technology (NIST), Gaithersburg, MD, USA (SRM 2181; and 2182); Serva, Heidelberg, Germany, (analytical grade; research grade); Sigma-Aldrich, Deisenhofen, Germany (> 99.5 %; 99.5 %). NaCl (> 99.5 %) from Merck; Standard solution of 1 mol/l aqueous hydrochloric acid; 1 mol/l aqueous sodium hydroxide; oxalic acid dihydrate (> 99.5 %) from Fluka; H_2O (Ampuwa sterile and apyrogenic with conductivity < 1 µS/cm) from Fresenius, Bad Homburg, Germany.

Anhydrous Na-HEPES salt was prepared from the product from NIST (SRM 2182), and from Fluka (BioChemica 54466) by a rigorous drying procedure, whereby 4.2 g of the respective salt was dried over silica gel in a drying apparatus at 100 °C and under oil vacuum (ca. 3 mmHg) over a period of 20 hours, and then kept in the desiccator over silica gel.

Elemental analysis of C, H, N, S of HEPES acid and Na-HEPES salt was performed in an analyzer of type Vario EL from Heraeus, Hanau, Germany. Each element was analyzed twice from a sample of low weight (1.5 to 3 mg), and once from a sample of high weight (2 to 6 mg).

The titre of 1 mol/l aqueous NaOH used in preparation of the buffer solutions was controlled by titration of weighed samples of oxalic acid dihydrate (volumetric standard) against phenolphthalein, and was defined on a mass scale: 0.964 ± 0.001 mmol NaOH per g of 1 mol/l NaOH, and, vice versa, 1 mol/l aqueous HCl by titration with 1 mol/l NaOH: 0.985 ± 0.003 mmol HCl per g of 1 mol/l HCl. Similarly, for titration of HEPES acid with 0.2 mol/l NaOH against thymolphthalein, and of Na-HEPES salt with 0.1 mol/l HCl against methyl red, 0.2 mol/l NaOH was standardized against oxalic acid, and, vice versa, 0.1 mol/l HCl by 0.2 mol/l NaOH.

The blood specimens for measurement of pH were prepared from freshly obtained heparinized human blood (20 ml from the *vena cubitalis* of three healthy volunteers, mean age 53 ± 4 yrs), in which the concentration of Heparin-sodium salt (Braun 25000 U/5 ml, Melsungen, Germany) was about 25 U/ml blood.

Preparation of equimolar HEPES buffer solutions

The aqueous equimolar HEPES buffer solutions (a volume of 100 ml in each case), consisting of HEPES acid and Na-HEPES salt, both with or without NaCl, were prepared from pure HEPES acid and 1 mol/l aqueous NaOH (absolute density at 22 °C: 1.0405 g/ml) at half of the equivalent amount to produce the corresponding buffer salt. Whenever aqueous NaOH was used, it was added by weight, not by volume. The water used as a solvent was sterile and apyrogenic (Ampuwa) and was not permanently boiled to remove carbon dioxide, since no effect on pH could be observed. In this study, the predominantly used buffer was physiological HEPES buffer (PHB) composed of 0.100 mol/l HEPES acid, 0.050 mol/l NaOH, and 0.110 mol/l NaCl adjusted to a total ionic strength of 0.160 mol/l. Especially, when prepared from standard reference material from NIST (SRM 2181 HEPES acid, and 2182 Na-HEPES salt), it was used as a reference buffer to test the quality of commercial products of HEPES acid and Na-HEPES salt from different sources. In this case, PHB was also prepared from pure Na-HEPES salt (0.100 mol/l) and 1 mol/l aqueous HCl (absolute density at 22 °C:1.0154 g/ml) at half of the equivalent amount by weight (0.050 mol/l) and NaCl (0.060 mol/l), and directly from the components: 0.050 mol/l HEPES acid, 0.050 mol/l Na-HEPES salt and 0.110 mol/l NaCl. The buffer components, HEPES acid and Na-HEPES salt, were used both directly from the container without drying, and after drying over anhydrous $CaCl_2$ in a desiccator at room temperature and slight vacuum (water jet: approx. 20 mmHg) to constant weight over a period of several days (HEPES: 2 days; Na-HEPES salt: 7 days).

Measurement of pH

For measurement of pH in the buffer solutions at 37 °C, a commercial capillary glass electrode system with calomel reference electrode and saturated KCl as a salt bridge (Radiometer BMS 2 Mk 2) was used. The whole set-up was thermostated at 37 ± 0.1 °C and calibrated using two precision buffer solutions recommended by the International Federation of Clinical Chemistry (IFCC): S 1500 equimolar phosphate buffer (1:1), used as a primary standard with pH = 6.841 ± 0.005, and S 1510 Sörensen 1:4 phosphate buffer, used as a secondary standard with pH = 7.383 ± 0.005, both available from Radiometer, Copenhagen, Denmark. Between each measurement or calibration, the pH glass electrode was flushed with an enzyme-containing rinse solution from Radiometer (S 4306). The pH electrode system was allowed to warm up for at least three hours before measurements were carried out, and

the pH in the buffer solution was not measured until at least one day after it had been prepared. The reliability of the glass electrode was monitored by measuring pH in the same buffer solution with three pH instruments of the same type (Radiometer BMS 2 Mk 2), but with pH glass electrodes (Radiometer G299A) varying in their time of usage (3 years, 1.5 years, and 1 month; all with relative sensitivity in the range 59.95 to 60.98 mV/pH-unit, which corresponds to 97.4 to 99.1 % of the theoretical Nernst factor 61.538 at 37 °C).

Choice of concentration scale: molal or molar units

Normally, the preferred concentration unit for describing the composition of a calibrating buffer solution is molality (m, mol/kg H_2O), since it is independent of temperature. However, when medical or clinical chemistry laboratory results are documented, molarity (mol/l) is generally used. To demonstrate the order of pH difference measured with the glass electrode in relation to the concentration scale, two series of four HEPES buffer solutions, each composed of m_1 HEPES acid, m_2 Na-HEPES salt, m_3 NaCl: (0.050; 0.050; 0), (0.050; 0.050; 0.050), (0.050; 0.050; 0.100), and (0.050; 0.050; 0.110), were prepared from HEPES acid, NaOH and NaCl, both on a molal and on a molar scale.

pH of equimolar HEPES buffer measured with the glass electrode upon variation of NaCl and of total HEPES buffer concentration

Effect of total ionic strength on the pH of equimolar HEPES buffer solutions at 37 °C was studied upon variation of NaCl concentration at constant total HEPES buffer concentration, e.g., in 0.05 equimolar HEPES buffer with NaCl concentration ranging from 0 to 0.25 mol/l, and measuring pH with the glass electrode. Similarly, the effect of total HEPES concentration (0.025 to 0.320 mol/l) on pH was measured in equimolar HEPES buffer solutions without NaCl, and, respectively, at 0.025 and 0.160 mol/l with NaCl concentration 0.1425, and 0.08 mol/l.

Comparison of measured pH in PHB prepared from standard reference material (SRM) from NIST and from different commercial sources

For comparison of different commercial products of HEPES, i.e., HEPES acid, and Na-HEPES salt, with corresponding standard reference material from NIST (SRM), measurement of pH in PHB at 37 °C was performed. To avoid possible interference from impurities either from HEPES acid and/or Na-HEPES salt, PHB was prepared separately from its basic buffer components. One series was prepared only from HEPES acid (0.100 mol/l); NaOH (0.050 mol/l); NaCl (0.110 mol/l), and the other only from Na-HEPES salt (0.100 mol/l); HCl (0.050 mol/l); NaCl (0.060 mol/l).

pH in normal arterial human blood

The blood specimens for measurement of pH under normal physiological conditions (pO_2 = 90 mmHg; pCO_2 = 40 mmHg; BE = 0 mmol/l) were prepared from freshly obtained heparinized human blood. In a tonometer (IL 237, Instumentation Laboratory, Kirchheim, Germany) thermostated at 37 °C, about 4 ml of blood were equilibrated with the appropriate humidified gas mixture continuously produced by a precision gas mixer (192, Corning, Medfield, USA) from the pure gases oxygen (99.99 %), carbon dioxide (99.9 %) and nitrogen (99.995 %). Usually, equilibrium was reached after 30 minutes: this was controlled by measurement of pH, pCO_2 and pO_2 in a blood gas analyzer (Radiometer ABL 500, Copenhagen, Denmark). Only after this time were samples of about 100 µl of equilibrated normal blood in glass capillaries taken for accurate measurement of pH with a standardized pH glass electrode (Radiometer BMS 2 Mk 2).

Calculated values of NIST reference pH in aqueous HEPES buffer solutions at 37 °C without liquid junction

Reference pH at 37 °C for aqueous HEPES buffer solutions used in this investigation were calculated from the equation by Feng *et al.* (6) derived from extended electromotive force (emf) measurements of cells without liquid junction (Harned cell) and with liquid junction for the HEPES system with and without NaCl at the National Institute of Standards & Technology (NIST). However, the original equation by Feng *et al.* was slightly modified with respect to the specific effect of HEPES on the activity coefficient of NaCl, experimentally determined and published by NIST in a later paper (10). With the constants at 37 °C for dissociation of zwitterionic HEPES acid ($HZ^{\pm} = H^+ + Z^-$): thermodynamic pK = 7.427, and two empirical parameters, $\alpha = 0.15$; $\beta = 0.45$, pH without liquid junction can be calculated in any HEPES buffer solution with respect to concentration of HEPES acid (m_1), Na-HEPES salt (m_2), and NaCl (m_3) within the experimental range ($m_1 = m_2$: 0.02 to 0.08; m_3: 0 to 0.1) by the equation:

$$\mathrm{pH} = 7.427 + \log_{10}(m_2/m_1) + 0.15\, m_1 + 0.45\, I - 0.5215\, \sqrt{I}/(1+1.38\sqrt{I}) + 0.0382\, I - (0.11 - 0.42\, m_3)m_1 - (0.45 - 0.44\, m_3)m_1^2 \qquad \text{(Eq. 1)}$$

pH corresponds to $-\log a_{H+}$ where a_{H+} is the conventional activity of the hydrogen ion referred to the standard state ($p°H_2$ = 101.325 kPa or 1 atm) on a molal scale without liquid junction. I, total ionic strength is defined as $I = {}^1/_2\, \Sigma\, m_i\, z_i^2$, with charge number z_i , and molality m_i of the ith ion, and is equal to the sum of molality of Na-HEPES salt and NaCl, i.e., $m_2 + m_3$. The last two terms describe the experimental effect of HEPES on the activity coefficient of NaCl at 25 °C, $\log \gamma_{Cl-} = \log \gamma°_{Cl-} - (0.11 - 0.42\, m_3)m_1 - (0.45 - 0.44\, m_3)m_1^2$, and were not corrected to 37 °C because the difference to 25 °C depending on HEPES concentration is in the order of the fourth decimal and, therefore, negligible, e.g., at constant 0.08 mol/kg NaCl and variable HEPES concentration (in the range from 0.050; 0.080; 0.100 to 0.120 mol/kg), this difference is only –0.0002; –0.0004; –0.0005 to –0.0015, respectively (10). The term $\log \gamma°_{Cl-}$ is the activity coefficient of NaCl in the absence of HEPES ($m_1 = 0$), and is approximated by use of the extended Debye-Hückel equation adjusted for the chloride ion, terms 5 and 6 on the right side of (Eq. 1). According to (Eq. 1), pH greatly depends upon pK and on the concentration ratio of HEPES-base to corresponding HEPES-acid in the second term, which disappears at equimolal concentrations, $m_1 = m_2$; on total ionic strength, and on the concentration of HEPES acid and NaCl. It is also evident that thermodynamic pK is the pH value of an equimolal HEPES buffer solution extrapolated to total zero ionic strength. For a liquid medium of constant ionic strength, such as human plasma (I = 0.160 mol/kg), thermodynamic pK and the concentration dependent terms can be combined to apparent pK', and Eq. 1 simplifies to the well-known Henderson-Hasselbalch (HH) equation: $\mathrm{pH} = pK' + \log_{10}(m_2/m_1)$. Because (Eq. 1) is derived on molality scale, all other concentrations, e.g., molar concentrations, must be converted into molal units by the density of the solution. However, the effect is only small. Even an increase of all buffer components (HEPES/Na-HEPES/NaCl) by 5 % or a conversion factor 1.05, e.g., in a buffer solution of 0.050/ 0.050/0.100 to 0.0525/0.0525/0.105, calculated pH would only change from 7.372 to 7.374. Therefore, since in the used buffer solutions the factor for conversion

into molality is approximately 1.04, (Eq. 1) can also be applied if using molarity. The validity of Eq. 1 is verified by comparison of pH obtained directly from NIST (8) without liquid junction for 0.05 equimolal HEPES without NaCl, 7.364, and for 0.08 equimolal HEPES/Na-HEPES salt/NaCl, 7.373, with those calculated (Eq. 1): 7.363, and 7.374, respectively.

Calculated residual liquid junction potential (δE_J) between aqueous HEPES buffer and 1:4 physiological phosphate buffer (1:4 PPB) at the interface to saturated KCl

The residual liquid junction potential at 37 °C between aqueous HEPES buffer and 1:4 PPB at the interface to saturated KCl as liquid junction can be obtained from the pH in the same buffer solution measured operationally with the used glass electrode system (pH_{GE}), and from pH_{NIST} reference values calculated from (Eq. 1) in a cell without liquid junction. With respect to 1:4 PPB, pH = 7.383 at 37 °C , the pH difference for HEPES buffer solutions is $\delta pH_{GE} = pH_{NIST} - pH_{GE}$. This was preferred as the calibrating buffer for pH measurements in blood, since its total ionic strength (0.173 mol/kg) is closer to that of plasma (0.160 mol/kg) than of 1:3.5 PPB (0.100 mol/kg). By multiplying δpH_{GE} by S, the relative sensitivity factor of the glass electrode, and g_o = 61.538 mV, the theoretical Nernst factor (11) at 37 °C, one obtains an idea of the residual liquid junction potential for HEPES buffer with respect to 1:4 PPB in mV, $\delta E_{J,GE} = S \cdot g_o \cdot \delta pH_{GE}$. On the other hand, from the data of extended emf measurements by Feng *et al.* (6) residual liquid junction potentials are available for HEPES buffer of various composed solutions with and without NaCl with respect to primary standard reference phosphate buffer (1:3.5 PPB) in a cell with flowing liquid junction:

Pt, (H_2) | 1:3.5 PPB or HEPES buffer/NaCl | | KCl (sat.)| calomel reference electrode

The same authors also give an empirical formula in the experimental range ($m_1 = m_2$: 0.02 to 0.08; m_3: 0 to 0.1) for calculation of δE_J at 25 °C, which was used after extrapolation to 37 °C and replacing the liquid junction potential of standard buffer 1:3.5 PPB (2.9 mV) by that of 1:4 PPB (3.1 mV) (3):

$$\delta E_J = 17\ m_{NaCl} + 0.7 \qquad \text{(Eq. 2)}$$

With δE_J in mV converted into δpH_J by division of g_o, pH predicted for the above cell with a Pt/H_2-electrode with liquid junction, pH_{NIST}(l.j.), was obtained from pH_{NIST} without liquid junction by subtraction of δpH_J, and compared with measured pH with the glass electrode (pH_{GE}).

Results and Discussion

pH difference in equimolal and equimolar HEPES buffer solutions

pH was measured at 37 °C with the glass electrode in two series of HEPES buffer solutions differing only in concentration scale, four equimolal HEPES buffer solutions (mol/kg H_2O), and four equimolar HEPES buffer solutions (mol/l). From the results in Table 1, it is evident that the difference in pH measured with the glass electrode is negligible and independent of the used concentration scale. Therefore, Eq. 1 can also be applied if using molar units.

The effect of NaCl and of total HEPES buffer concentration on the pH of equimolar HEPES buffer measured with the glass electrode

Effect of varying the concentration of NaCl, and hence total ionic strength, on pH of equimolar HEPES buffer solutions at 37 °C is demonstrated in Table 2. For 0.050 equimolar HEPES buffer without NaCl, prepared from pure 0.100 mol/l HEPES acid and 0.050 mol/l NaOH, pH measured with the glass electrode was 7.361 ± 0.004, and combined with the data from Table 1, a mean of 7.362 ± 0.003 (n = 15) was obtained, which was not very different from the NIST reference pH, 7.364, proposed as calibrating buffer (8). In the same buffer with NaCl, pH initially decreased to 7.348 at 0.025 mol/l, and then remained practically constant over the whole range of NaCl concentrations from 0.025 to 0.250 mol/l with a mean of 7.347 ± 0.003 (n_{tot} = 36; 9 buffer solutions). Similar values, 7.345 ± 0.002, were also found at lower total HEPES buffer concentrations (0.025; 0.050 mol/l) with concentration of NaCl (0.1425; 0.130 mol/l) adjusted to total ionic strength of 0.155 mol/l (Table 2). In the presence of NaCl, pH values measured with the glass electrode were decreased compared to pH_{NIST} without liquid junction calculated by (Eq. 1). On the other hand, variation of total buffer concentration in equimolar HEPES buffer solutions without NaCl affected pH strongly in the low concentration range (0.025 to 0.050 mol/l), the values being higher (7.393, 7.381) than pH_{NIST}, but remaining practically the same (7.361, and 7.363), when varied from 0.100 to 0.160 mol/l, which was in excellent agreement with the pH_{NIST} without liquid junction as obtained from Eq. 1 (7.363, and 7.360). Even at the highest total buffer concentration, 0.320 mol/l, with equimolar HEPES at a total

Tab. 1 Measured pH at 37 °C in aqueous HEPES buffer in relation to choice of the concentration scale: molality versus molarity.

Composition of buffer HEPES/Na-HEPES/NaCl	Molality (mol/kg H_2O) pH ± SD (n;k)[1]	Molarity (mol/l) pH ± SD (n;k)[1]
0.050 / 0.050 / 0	7.363 ± 0.000 (8;1)	7.362 ± 0.000 (8;1)
0.050 / 0.050 / 0.050	7.340 ± 0.001 (8;1)	7.341 ± 0.001 (8;1)
0.050 / 0.050 / 0.100	7.340 ± 0.000 (8;1)	7.340 ± 0.001 (8;1)
0.050 / 0.050 / 0.110	7.342 ± 0.001 (8;1)	7.342 ± 0.001 (8;1)

[1] mean, standard deviation (SD), and number of pH measurements (n), and of buffer solutions (k).

Tab. 2 Effect of total ionic strength (I_{tot}) on pH at 37 °C in aqueous equimolar HEPES buffer upon variation of total HEPES buffer concentration and NaCl.

HEPES/NaOH (mol/l)/(mol/l)	NaCl (mol/l)	I_{tot} (mol/l)	pH_{NIST} from Eq.1	δpH_J from Eq.2	pH_{NIST}(l.j.) predicted	pH ± SD (n;k)[2] measured	ΔpH
1:4 PPB[1]		0.173[1]	7.383	0	7.383	7.383	0
0.025/0.0125	0	0.0125	7.383	0.011	7.372	7.393 ± 0.006 (7;1)	0.021
	0.1425	0.155	7.371			7.345 ± 0.002 (3;1)	
0.050/0.025	0	0.025	7.372	0.011	7.361	7.381 ± 0.001 (5;2)	0.020
	0.130	0.155	7.372			7.345 ± 0.002 (3;1)	
0.100/0.050	0	0.050	7.363	0.011	7.352	7.361 ± 0.004 (7;2)	0.009
	0.025	0.075	7.361	0.018	7.343	7.348 ± 0.004 (6;2)	0.005
	0.050	0.100	7.363	0.026	7.337	7.346 ± 0.002 (8;2)	0.009
	0.110	0.160	7.374	0.042	7.332	7.344 ± 0.001 (8;2)	0.012
	0.150	0.200				7.347 ± 0.002 (7;1)	
	0.200	0.250				7.349 ± 0.001 (5;1)	
	0.250	0.300				7.346 ± 0.001 (2;1)	
0.160/0.080	0	0.080	7.360	0.011	7.349	7.363 ± 0.002 (9;2)	0.014
	0.080	0.160	7.374	0.034	7.340	7.351 ± 0.002 (9;2)	0.011
0.320/0.160	0	0.160		0.011		7.368 ± 0.003 (3;1)	

[1] 1:4 PPB: Physiological 1:4 phosphate buffer S1510 from Radiometer composed of 0.01334 mol/kg KH_2PO_4 and 0.05338 mol/kg Na_2HPO_4 with total ionic strength 0.173 in molal units

[2] mean, standard deviation, and number of pH measurements (n) and of buffer solutions (k)

Comparison of measured pH with the glass electrode with corresponding reference values derived from NIST: without liquid junction, pH_{NIST}, with liquid junction, pH_{NIST}(l.j.), predicted from calculated δpH_J for saturated KCl, and pH difference between the glass electrode and NIST with liquid junction, ΔpH.

ionic strength of 0.160 mol/l, pH was not considerably different (7.368 ± 0.003). The former may be explained by the fact that the residual liquid junction potential of 0.05 or 0.08 equimolar HEPES buffer with respect to 1:4 PPB at the interface of saturated KCl for the capillary glass electrode is practically negligible, i.e., pH measured with the glass electrode is the same as pH_{NIST} without liquid junction. This is in contrast to Eq. 2 with intercept 0.7 mV for the same cell, but a platinum/hydrogen electrode, and is caused by a different geometry of the liquid junction. For 0.05, and 0.08 equimolar HEPES buffer, and concentrations of NaCl in the range 0–0.11 mol/l, where Eq. 1 and 2 can certainly be applied, from Table 2, the pH difference between pH_{NIST} without liquid junction and measured with the glass electrode, δpH_{GE}, as a function of NaCl, can be described by a linear equation (n = 6; R^2 = 0.938) along with slope (l/mol), intercept, and standard deviation (± SD):

$$\delta pH_{GE} = (0.272 \pm 0.035) \cdot c_{NaCl} + (0.002 \pm 0.002) \quad \text{(Eq. 3)}$$

or if converted into mV by the sensitivity factor of the glass electrode (60.5 ± 0.27 mV; n = 10), correspondingly:

$$\delta E_{J,GE} = S \cdot g_0 \cdot \delta pH_{GE} = (16.5 \pm 2.10) \cdot c_{NaCl} + (0.12 \pm 0.12) \quad \text{(Eq. 4)}$$

According to Eq. 4, $\delta E_{J,GE}$ is increased with increasing NaCl concentration with a mean slope slightly lower than predicted by (Eq. 2). From this, it is obvious that in this range all pH values measured with the glass electrode are roughly higher by 0.010 ± 0.003 than pH_{NIST}(l.j.), the corresponding values predicted for a cell with a Pt/H_2-electrode and saturated KCl as liquid junction, see Figure 1. Whereas pH_{NIST} without liquid junction calculated by Eq. 1 increases with increasing NaCl, both pH_{GE} measured and pH_{NIST}(l.j.) with liquid junction predicted by use of Eq. 2 decrease, and pH_{GE} is essentially constant over a wide concentration range of NaCl.

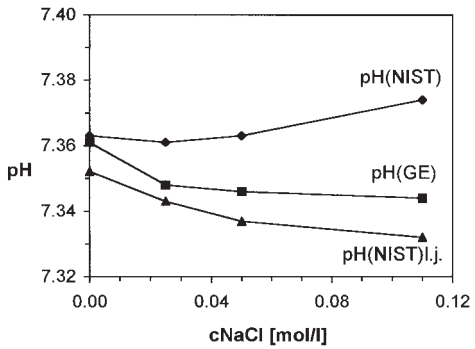

Fig. 1 Effect of NaCl concentration (0 - 0.11 mol/l) on pH at 37 °C in aqueous 0.05 equimolar HEPES buffer: Relation between pH_{NIST} without liquid junction calculated by Eq. 1, pH_{NIST}(l.j.) with liquid junction predicted by Eq. 2, and pH_{GE} measured with the glass electrode (data from Table 2).

Comparison of measured pH in PHB prepared from standard reference material (SRM) from NIST and from different commercial sources

The resulting pH in PHB (0.050 mol/l HEPES, 0.050 mol/l Na-HEPES salt, 0.110 mol/l NaCl) depends upon the starting material of its basic buffer components, HEPES acid and Na-HEPES salt, when used as obtained from the manufacturer without further manipulations. In the case of HEPES acid from seven different commercial sources with purity grade ≥ 99 % (Table 3), the measured pH at 37 °C was consistent within all of the tested products irrespective of whether they were directly used, pH = 7.348 ± 0.003 (total mean of n_{tot} = 28 pH measurements; 9 buffer solutions) or used after drying at room temperature in a desiccator, pH = 7.346 ± 0.002 (n_{tot} = 15; 7 buffer solutions). The weight loss by drying was negligible for all products, indicating that HEPES acid is non-hygroscopic.

However, if PHB was prepared from Na-HEPES salt, the measured pH was decreased in all of the investigated products. If used untreated, the salts differed considerably from each other in measured pH (6.929 to 7.336) and in weight loss after drying to constant weight (0.6 to 5.3 %) despite a purity grade of better than 99 % (Table 3). When used as dried salts, the pH in PHB increased in all products, except for the ICN salt, and converged to a common value of 7.335 ± 0.003 (n_{tot} = 20; 5 buffer solutions) which, however, was not consistent with that of 7.346 obtained after preparation from HEPES acid.

The cause for this discrepancy was explained by an additional amount of residual water still present in the sodium HEPES salt that was not removed by simple drying, as could be demonstrated by elemental analysis. For HEPES acid, both as standard reference material (SRM 2181) from NIST and from Fluka (54457 BioChemica MicroSelect ≥ 99.5 %), and irrespective of whether dried or not, the actual content determined by elemental analysis (C, H, N, S) was in accordance with that declared by the manufacturer: 100.1 ± 0.6 % for SRM from NIST, and 99.6 ± 0.6 % for the product from Fluka, the latter also being assayed by titration: 99.7 ± 0.2 % (n = 2). In contrast to HEPES acid, elemental analysis of C, H, N, S of Na-HEPES salt, both from NIST (SRM 2182), and from Fluka (54466 BioChemica > 99 %), did not conform with the theoretical values: H was too high (106.4 to 107.6 %); C, N and S too low (range: 93.2 to 96.5 %), and the content of the Fluka salt by titration with 0.1 mol/l HCl against methyl red was 95.2 %, which increased to 98.4 % after more rigorous drying (6 hours at 110 °C in the drying oven). Upon heating in apparatus for determination of melting point, white crystalline Na-HEPES salt was stable up to 150 °C, at which point it changed its appearance from white to brown and melted with decomposition (ca. 175 °C).

Tab. 3 Measured pH in physiological HEPES buffer (PHB) at 37 °C.

HEPES acid source	Purity grade[1] %	Before drying pH ± SD (n;k)[2]	Weight loss %	After drying pH ± SD (n;k)[2]
NIST	SRM 2181	7.347 ± 0.001 (4;1)	0	7.346 ± 0.001 (3;1)
Applichem	99.5 %	7.345 ± 0.002 (2;1)	0	7.346 ± 0.001 (2;1)
Fluka	≥ 99.5 %	7.346 ± 0.002 (12;3)	0	7.346 ± 0.001 (2;1)
ICN	not declared	7.349 ± 0.000 (2;1)	0	7.344 ± 0.002 (2;1)
Merck	> 99 %	7.353 ± 0.004 (2;1)	0	7.347 ± 0.001 (2;1)
Serva	Analytical	7.348 ± 0.000 (2;1)	0	7.345 ± 0.002 (2;1)
Sigma	> 99.5 %	7.349 ± 0.001 (4;1)	0	7.347 ± 0.000 (2;1)
Total mean: $\bar{x}$ ± SD		7.348 ± 0.003 (28;9)		7.346 ± 0.002 (15;7)
Na-HEPES salt source				
NIST	SRM 2182	7.327 ± 0.001 (4;1)	anhydrous salt	7.353 ± 0.001 (3;1)
Applichem	99 %	7.284 ± 0.003 (7;1)	5.3	7.332 ± 0.002 (4;1)
Fluka	> 99 %	7.336 ± 0.001 (4;1)	0.6	7.339 ± 0.003 (4;1)
	other charge	7.326 ± 0.001 (4;1)	anhydrous salt	7.348 ± 0.001 (4;1)
ICN	~ 99 %	6.929 ± 0.009 (3;1)	1.6	6.930 ± 0.002 (4;1)
Merck	> 99 %	7.282 ± 0.001 (2;1)	5.3	7.335 ± 0.002 (4;1)
Serva	research	7.292 ± 0.003 (3;1)	5.3	7.335 ± 0.003 (4;1)
Sigma	99.5 %	7.331 ± 0.001 (3;1)	0.6	7.334 ± 0.002 (4;1)
Total mean: $\bar{x}$ ± SD			anhydrous salt	7.350 ± 0.003 (7;2)

[1] as declared by the manufacturer

[2] mean, standard deviation (SD), and number of pH measurements (n) and of buffer solutions (k)

Buffer was prepared either from HEPES acid (0.100 mol/l, 0.050 mol/l NaOH, 0.110 mol/l NaCl), or from Na-HEPES salt (0.100 mol/l, 0.050 mol/l HCl, 0.060 mol/l NaCl) both from different sources and from NIST (SRM 2181 and 2182).

Tab. 4 Measured pH at 37 °C in physiological HEPES buffer (PHB).

PHB	pH instrument[1]:			pH ± SD (n;k)[2]
prepared from:	1	2	3	
Fluka	7.347	7.344	7.347	7.346 ± 0.002 (3;1)
ICN	7.346	7.346	7.346	7.346 ± 0.000 (3;1)
Merck	7.346	7.347	7.348	7.347 ± 0.001 (3;1)
Sigma	7.346	7.344	7.346	7.345 ± 0.001 (3;1)
pH ± SD (n;k)[2]	7.346 ± 0.001 (4;4)	7.345 ± 0.002 (4;4)	7.347 ± 0.001 (4;4)	7.346 ± 0.001 (12;4)

[1] identical pH instruments, but with glass electrodes different with respect to time of usage: 3 years (1), 1.5 years (2), and 1 month (3)
[2] mean, standard deviation (SD) and number of pH measurements (n), and of buffer solutions (k)

Tab. 5 Summary of mean pH, standard deviation (SD), and number of pH measurements (n) and of buffer solutions (k) from the different series (i) of measurements at 37 °C in physiological HEPES buffer (PHB), and resulting overall representative mean ($\bar{x}$) with overall standard deviation.

PHB prepared from:	$(n;k)_i$	$(pH \pm SD)_i$
0.100 mol/l HEPES acid/0.050 mol/l NaOH/0.110 mol/l NaCl		
Tab. 1: Molarity	(8;1)	7.342 ± 0.001
Tab. 2: NaCl variation	(8;2)	7.344 ± 0.001
Tab. 3: Different products	(28;9)	7.348 ± 0.003
	(15;7)	7.346 ± 0.002
Tab. 4: Instrument and product variation	(12;4)	7.346 ± 0.001
0.100 mol/l Na-HEPES salt/0.050 mol/l HCl/ 0.060 mol/l NaCl		
Tab. 3: Anhydrous salt	(7;2)	7.350 ± 0.003
0.050 mol/l HEPES acid/0.050 mol/l Na-HEPES salt/0.110 mol/l NaCl		
Buffer components: HEPES acid and anhydrous salt	(6;2)	7.348 ± 0.002
Overall mean: $\bar{x} \pm SD$	(84;27)	7.346 ± 0.003

Total mean: $\bar{x} = (1/n_{tot})\sum_i n_i\bar{x}_i$

where $n_{tot} = \Sigma n_i$ with n_i number of pH measurements, and $\bar{x}_i$ mean pH in series i of measurements

Total standard deviation: $SD^2 = \frac{1}{n_{tot} - 1}\sum_i\{(n_i - 1) \cdot SD_i^2 + n_i(\bar{x}_i - \bar{x})^2\}$

In the next step, after the salt had undergone rapid drying (ca. 10 mg of the Fluka salt for 12 hours over silica gel in a drying apparatus at 100 °C under oil vacuum, ca. 3 mmHg, and cooling to room temperature over silica gel in a desiccator), elemental analysis of C, H, N was found to be in good agreement with theoretical values (%): C 36.90 ± 0.10 (36.91 %); H 6.81 ± 0.02 (6.59 %); N 10.78 ± 0.02 (10.76 %). From the anhydrous salt, one portion was then kept in the desiccator, the other directly exposed to room air. After one day, C, H, N analysis of the portion in the desiccator was practically unchanged: 36.77 ± 0.02; 6.71 ± 0.08; 10.66 ± 0.01, whereas the sample exposed to room air showed a significant increase in weight (3.3 %): 35.76 ± 0.01; 6.74 ± 0.04; 10.42 ± 0.04, respectively. The latter values correspond perfectly with those for the hemi-hydrate of Na-HEPES salt, Na-HEPES · 0.5 H_2O, the elemental composition of which is theoretically (%): C 35.68; H 6.74; and N 10.40. On the second day, absorption of water continued to increase: C 35.28 ± 0.01; H 6.79 ± 0.08; N 10.16 ± 0.04, but no simply definable hydrate, such as the monohydrate of Na-HEPES salt was formed.

Therefore, since Na-HEPES salt is hygroscopic, its anhydrous state must be guaranteed before it can be used as a calibrating buffer component. In Table 3, pH measured in PHB prepared from anhydrous Na-HEPES salt (0.100 mol/l), both from NIST and from Fluka, HCl (0.050 mol/l), and NaCl (0.060 mol/l), is shown: NIST material yielded pH 7.353 ± 0.001 (n = 3) and Fluka 7.348 ± 0.001 (n = 4). Upon combination of these values, a mean of 7.350 ± 0.003 (n = 7) was obtained.

In another series of PHB prepared from HEPES acid from four different sources, the influence of instrument variation on pH was studied. Upon measurement of pH

in the same buffer solution with three pH meters of the same type (Radiometer BMS 2 Mk 2), but with different glass electrodes (time in use: 3 years, 1.5 years, and 1 month), mean pH was 7.346 ± 0.001 (n_{tot} = 12; 4 buffer solutions), as shown in Table 4, with only a small overall standard deviation including variation of the instruments (n = 3) and variation of the products (n = 4).

In Table 5, all mean values of pH in PHB obtained from the different series of measurements are summarized, including pH measured after direct preparation from the buffer components both from NIST and Fluka, 0.050 mol/l HEPES acid, 0.050 mol/l anhydrous Na-HEPES salt, and 0.110 mol/l NaCl. NIST material pH was 7.350 ± 0.000 (n = 3) and Fluka 7.347 ± 0.001 (n = 3) giving a mean of 7.348 ± 0.002 (n = 6). Finally, from all mean values and standard deviations an overall representative mean of pH in PHB with overall standard deviation was derived after weighting for the number of samples measured: this was 7.346 ± 0.003 (n_{tot} = 84; 27 buffer solutions).

pH in normal arterial human blood

In normal arterial blood (pO_2 = 90 mmHg; pCO_2 = 40 mmHg, BE = 0 mmol/l), obtained by tonometry of the blood from each of three healthy adult volunteers, the mean pH measured with the same glass electrode was: 7.404 ± 0.003 (± SD; n = 20). This was in good agreement with the accepted normal value of 7.400 under these conditions (3, 12).

Conclusions

Measurement of pH in blood with the glass electrode system (capillary type with saturated KCl salt bridge, and calomel reference electrode) is defined operationally, and the established normal value, 7.400 at 37 °C, relates to phosphate (IFCC procedure for calibration). If calibration of the glass electrode in the physiological range for pH in blood is based on HEPES buffer, care must be taken to ensure the correct reference pH of the calibrating solution. From pH measurements with the glass electrode (Radiometer BMS Mk2) at 37 °C in aqueous equimolar HEPES buffer (Table 1, 2) with respect to phosphate, those which are proposed from NIST (8), 0.05 equimolar HEPES buffer without NaCl, pH = 7.364, and equimolar HEPES buffer/NaCl, 0.08/0.08/0.08, pH = 7.373, or that used by Radiometer S 1565, pH = 7.400 "stabilized", were compared with physiological HEPES buffer (PHB), 0.05/0.05/0.110, proposed in this work (Table 6). Only pH in 0.05 equimolar HEPES buffer without NaCl, and reference pH from NIST without liquid junction were in good agreement, whereas in equimolar HEPES buffer with NaCl in the physiological range (0.160 mol/l), corresponding pH values differ. In PHB, pH was 7.346 ± 0.003 (n = 84), which was different from the NIST reference pH (7.374) calculated from Eq. 1 by 0.028. And only the former value was also compatible to measured pH in normal arterial blood (pCO_2 = 40 mmHg; pO_2 = 90 mmHg; BE = 0 mmol/l): 7.404 ± 0.003 (n = 20).

At first glance, the calibrating solution S 1565 from Radiometer is not related to the NIST reference pH. However, if pH in PHB is interpreted as apparent p*K'* with respect to composition (total buffer concentration: 0.100 mol/l; total ionic strength: 0.160 mol/l), and to the glass electrode, pH can be calculated using the Henderson-Hasselbalch equation (HH): $pH = 7.346 + \log_{10}$ (*c*HEPES-base/*c*HEPES-acid).

The calibrating solution S 1565 from Radiometer (e.g., for ABL 500) is composed similarly to PHB (in molal units): 0.100 HEPES, 0.055 NaOH, 0.110 NaCl, but not equimolal, and "stabilized" to pH = 7.400. In S 1565, not stabilized, the pH predicted by the above equation is 7.433, and from the composition, reference pH from NIST without liquid junction is 7.462 (Eq. 1), i.e., there is a difference of 0.029 between reference pH and the glass electrode. In S 1565, "stabilized" to 7.400, after recalculation of the new composition using the above equation (0.0469 HEPES acid, 0.0531 Na-HEPES, 0.110 NaCl), the calculated reference pH from NIST by Eq. 1 is 7.429. The difference from pH of the glass electrode is the same and also consistent with the PHB value. Both types of glass electrode systems are compatible. Therefore, PHB with an assigned pH = 7.346 is proposed as a calibrator for pH in different clinical analyzers in the physiological range (buffer adjustment), and where it is not used, the relationship of pH in PHB and in normal blood for that particular measuring system must be verified with respect to phosphate. However, it must be emphasized that before PHB can be accepted as a standard for pH in the strict sense with traceable accuracy, it must be certified using the hydrogen cell without liquid junction, e.g., by the Chemical Reference Laboratory of Radiometer Medical A/S or by NIST.

Tab. 6 HEPES reference buffers proposed and used for pH measurement at 37 °C in the physiological range.

Source:	HEPES/NaHEPES/NaCl Molality (mol/kg H_2O)	I_{tot} (mol/kg)	NIST reference pH	pH glass electrode
NIST	0.050/0.050/0	0.050	7.364 (8)	7.362 ± 0.003
	0.080/0.080/0.080	0.160	7.373 (8)	7.351 ± 0.002
Radiometer	~0.050/~0.050/~0.110	~0.165	7.429 (from Eq. 1)	7.400 ("stabilized")
	0.045/0.055/0.110	0.165	7.462 (from Eq. 1)	7.433 (predicted by HH))
This work[1]	0.050/0.050/0.110	0.160	7.374 (from Eq. 1)	7.346 ± 0.003

[1] Molarity (mol/l)

If PHB is prepared from its components, the recommended procedure is to start from pure HEPES acid, 1 mol/l aqueous NaOH, and NaCl, all by weight, e.g., in preparing 1 l of buffer solution: 23.831 g HEPES acid, 52.025 g 1 mol/l aqueous NaOH (titre: 0.96108 mmol NaOH/g aqueous NaOH), and 6.428 g NaCl. All commercially available products with purity grade ≥ 99 % can be used without further purification. Of these, the very expensive SRM from NIST is not superior. Na-HEPES salt, which is hygroscopic, should not be used without further treatment for preparing calibrating buffer solutions unless it is in the anhydrous form.

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