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pH adjustment of human blood plasma prior to bioanalytical sample preparation

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Abstract

pH adjustment in bioanalytical sample preparation concerning ionisable compounds is one of the most common sample treatments. This is often done by mixing an aliquot of the sample with a proper buffer adjusted to the proposed pH. The pH of the resulting mixture however, does not necessarily have to be the same as the pH of the used buffer due to the significant buffer capacity of the sample. Calculation methods from titration technology were adapted and applied to this problem. The acid–base characteristics of human blood plasma and serum samples were determined and used to calculate the pH of buffer–plasma mixtures. Based on these parameters and the characteristics of the used buffers, two alternative methods were described to prepare buffers that lead to the proposed pH when mixed in the right volume ratio with human plasma samples.

The resulting pH of several mixtures of different buffers with human blood plasma were in good accordance with the calculated pH. The proposed calculation methods and recommended buffer preparation methods may lead to more robust bioanalytical methods.
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1. Introduction

In bioanalytical sample preparation, the adjustment of the pH of the sample to be analysed is one of the most common treatments of the sample [1].

The aim of proper pH adjustment is to bring ionisable analytes to an ionisation state at which they can be extracted from the sample matrix or to force unwanted compounds to an ionisation state at which they are not extracted, either by means of liquid–liquid extraction or solid phase extraction.

In liquid–liquid extraction of ionisable compounds the sample pH should be low enough for acids and high enough for bases to achieve a satisfactory recovery [1,2]. In solid phase extraction, SPE, the optimal pH of the sample depends on the extraction mechanism. In hydrophobic interaction SPE, the pH should be adjusted to suppress ionisation of the analytes to achieve retention on the SPE column whereas at ion exchange-SPE the pH should be chosen to enhance the ionisation of the analytes in order to interact with the charged groups on the SPE stationary phase [3].

The sample pH can be adjusted at extreme values to be sure of complete ionisation or complete suppression of ionisation [1]. However, extreme pH may lead to chemical instability [4] and may also lead to co-extraction of unwanted compounds. Proper pH adjustment of the sample to be extracted can be a powerful tool to achieve a different selectivity in liquid–liquid extraction [2].

Adjustment of the sample pH is usually performed by mixing an aliquot of that sample with a certain volume of strong acid or base or a buffer solution with a suitable pH.

We experienced that most workers assume the resulting pH of the mixture to be the same as the pH of the buffer added to the sample. This is not necessarily the case as the buffer capacity of the sample matrix plays a significant role in the pH equilibriums.

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This holds especially for human blood plasma or serum as one of the most used sample matrix in bioanalytical and clinical chemistry.

As far as we know, neither the acid–base behaviour of human plasma over a wide pH range nor a method to predict the pH of plasma buffer mixtures have ever been described before.

In this paper we propose a model to calculate the pH of a mixture of a human plasma sample or serum samples and a buffer solution with known concentration and pH or to calculate the pH of this buffer in order to achieve the proposed pH after mixing it with an aliquot of a plasma sample. These calculations are based upon the acid–base characteristics of human plasma which determination is also described.

2. Theory

In many disciplines of analytical chemistry, pH calculations are used. Also, in chemical education, much effort is put into the calculation of the pH of mixtures of acids or bases. However, in many textbooks concerning analytical chemistry, pH calculations are based upon many assumptions and approximations [5,6]. As a consequence, many different equations are needed to calculate a pH of a relatively simple mixture of a weak acid (or base) and a strong base (or acid) depending on their relative concentrations. Some methods were developed to calculate the pH or titration curves of complex multicomponent mixtures [9,10] but they are all based upon pH calculations of solutions of which the composition and concentration of each acid–base species is known or these methods assume the complete titration of pure acidic or basic substances. We needed to calculate the pH of two buffer mixtures with different, known, compositions in buffer substance and concentration and pH. Such solutions can be explained as partially titrated mixtures of pure acid with a certain amount of acid equivalents (molars of protons) to reach the actual pH. This solution will be mixed with a solution with unknown composition and a certain pH, the plasma sample.

In order to calculate the final pH of a mixture of a buffer and a human plasma sample we divided the problem up in two parts:

(1) Find a method to calculate the final pH of a mixture of buffers with known composition and pH.
(2) Express the plasma sample as a hypothetical buffer composed of a mixture of weak acids and bases.

2.1. Calculating the pH of mixtures of buffers

Straight forward pH calculation taking all the buffer species into account leads to a polynomal equation to be solved with many solutions and is hard to calculate.

We therefore chose to use a well-defined calculation method used in modern titration technology [5,7] to solve this problem. This method actually involves calculation of the composition of the buffer solution at a given pH, which is the reverse of the conventional way. The benefit of this method is that it uses no approximations and calculations do not become extremely complex as more acid–base constants are involved, i.e. complex mixtures of acids and based either strong or weak.

For our work this method can be explained as the calculation of the titration status, T, of a mixture of acids and/or bases. The titration status refers to the number of equivalents of strong acid or basis equivalents added to a mixture of the pure acids and based to reach the actual pH of that mixture.

We derived the titration status from Refs. [5,7].

The main equation in these papers to describe a titration of an acid, a, with a base, b, is given by the next proportion:

$$\frac{V_b}{V_a} = \frac{\sum F_a C_a - \Delta}{\sum F_b C_b + \Delta} \tag{1a}$$

Here V represents the volume of the acid and the base and F is a function that describes the proton dissociation status of the acids involved and will be explained later in the text. C is the corresponding molar concentration of the acid or base involved.

Δ Expresses the difference between $[H^+]$ and $[OH^-]$ ($[H^+] - [OH^-]$) and is used to incorporate the effect of the dissociation of water. The $[OH^-]$ can also be expressed as $K_w/[H^+]$, where K_w is the dissociation constant of water at 20 °C: 1×10^{-14} at 20 °C.

Cross-multiplication of Eq. (1a) shows the proton transfer.

$$V_a \left(\sum F_a C_a - \Delta \right) = V_b \left(\sum F_b C_b + \Delta \right) \tag{1b}$$

The left hand side of this equation counts the moles of protons removed from an acid and the right hand side counts the moles of protons added to a base.

Assigning only the acidic part of the titration equation to the variable T, we obtained the equation of the titration status for a mixture consisting of i acidic components, Eq. (2):

$$T = V \left(\sum_i F_i C_i - \Delta \right) \tag{2}$$

Since all our equations are based on acidic species, all acid–base equilibriums should be written in the acidic form. For this reason we only speak of acidic equivalents. This means that basic equivalents are mathematically expressed as negative concentrations of acid equivalents.

The F-function actually represents the relative amount of protons released by the acidic components involved as a function of the actual H^+ concentration in the acid mixture and can be expressed by Eq. (3) [5] for a monoprotic acid where K_a is the dissociation constant of the acid:

$$F_a = \frac{K_a}{[H^+] + K_a} \tag{3}$$

Based on the above-mentioned known and well-described theory used in titration technology we developed the methodology to solve our problem as described in the remainder of this paper. The process of mixing of two different buffer solutions was considered as a transfer of protons from the buffer with the lowest pH to the buffer with the highest pH until the pH of both buffer solutions is equal.

In other words, the amount of equivalents of acid (i.e. molars of protons) lost by the low pH buffer is equal to the amounts of equivalents of acid gained by he high pH buffer as the final

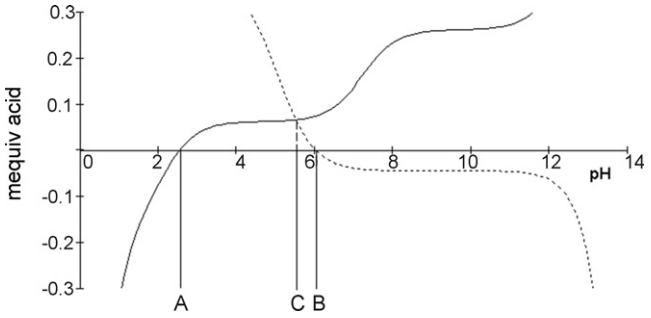

Fig. 1. Determination of the pH of a mixture of a phosphate buffer (—) and a citrate buffer (- - -).

pH after mixing is achieved. This is also known as the proton balance [7].

We can now define a proton balance equation for the mixing of buffers with known concentration, C, and pH. We can therefore define the titration status, T, of both buffers A and B of which only buffer A is represented in Eqs. (4) and (5). Here V_A represent the volume of buffer A at the initial situation before mixing, init. The final volume, V_{final}, is the sum of V_A and V_B, after mixing. The difference in T represents the number of acidic equivalents that is transferred during the mixing process.

$$T_{A,init} = V_A(F_A C_{A,init} - \Delta) \quad \text{or} \quad T_{A,init} = V_A\left(F_A C_{A,init} - \left(10^{-pH_A} - \frac{K_w}{10^{-pH_A}}\right)\right) \tag{4}$$

After mixing, the concentration of the pH active components is diluted according to the ratio of the volumes of both buffers:

$$T_{A,final} = V_{final}\left[F_A C_A \frac{V_A}{V_{final}} - \Delta\right] = F_A C_A V_A - \Delta V_{final} \tag{5}$$

with

$$\Delta = 10^{-pH_{final}} - \frac{K_w}{10^{-pH_{final}}}$$

The proton balance can now be defined as a combination of Eqs. (4) and (5) and is in fact a balance of the titration status of both the acid and the basic solution before and after mixing them together:

$$T_{A,final} - T_{A,init} = -(T_{B,final} - T_{B,init}) \tag{6}$$

The function to be solved will then be:

$$T_{A,final} - T_{A,init} + T_{B,final} - T_{B,init} = 0 \tag{7}$$

The initial minus sign in the right part of Eq. (6) indicates the difference between uptake and release of protons. The final pH can now be determined by solving Eq. (7) for pH_{final} by a numerical optimisation algorithm such as an iterative Newton-Rhapson method. An example of such an optimisation algorithm is the Microsoft Excel-solver option [5]. In this way, each of the 7 parameters (C_A, V_A, pH_A, C_B, V_B, pH_B and pH_{final}) can be calculated, provided the other 6 are given.

An example is given in Fig. 1. Here 1 ml of a 0.2 M phosphate buffer with a pH of 2.5 is mixed with 1 ml of a 0.2 M citrate buffer with a pH of 6.0. The differences of the titration status, ΔT (i.e. the number of transferred acidic equivalents) of both buffer solutions are presented at a range of pH values according to the following equation:

$$\Delta T = T_{final} - T_{init}$$

The intersections with the X-axis, A and B, represent the initial pH of the phosphate buffer and the citrate buffer, respectively, corrected for the mixing volume before proton transfer. The X-axis value of the intersection of the both curves, indicated as C, represents the final pH after proton transfer. The Y-value of point C, represents the number of transferred protons (mmol). It is the job of the numerical optimisation algorithm to find this intersection point.

Analogous to this method we can also calculate the initial pH of one of the two buffers to achieve a desired final pH after mixing, provided the pH of the second buffer and the concentrations of both buffers are known.

2.2. Expressing human plasma as a buffer solution

The second issue is to express a plasma sample as a buffer solution composed of multiple pH active compounds, each with their own specific dissociation constant and concentration. Therefore a plasma sample can be titrated with a solution of a strong acid and with a base at well known concentration, and record the pH after each addition of titration solvent.

Each addition adds an amount of acid equivalents, equal to the difference between the titration status of the plasma sample after the addition and the initial situation, ΔT. This number of acid equivalents which is the product of the concentration of the titrant and the volume of the titrant, is plotted against the corresponding measured pH. The number of fictitious buffer components can be visually determined from the resulting (inverted) titration curve by the number of relatively steeper regions. Each steep region indicates a buffering region where the pH change is relatively small after addition of acid equivalents compared to the rest of the curve, i.e. a higher buffer capacity. The pH at the centre of such a region is a measure of the corresponding pK_a (i.e. negative logarithm of K_a) value and the width of the region is a measure of the corresponding concentration. These pK_a values and corresponding concentrations can now be used in the proton balance for the plasma sample in Eq. (8).

$$\Delta T = T_{plasma,final} - T_{plasma,init} = -V_{titrant} C_{titrant} \tag{8}$$

where $V_{titrant}$ and $C_{titrant}$ represent the volume and the molar concentration of the titration solution, respectively. Since our equations are based on acidic species, C has a negative sign when a strong base is used in titration experiments as a base is mathematically the opposite of an acid.

For a plasma sample with a volume V_{plasma} and i different pH active components we can write Eq. (9) as the equation for ΔT

after each addition of titrant.

$$\Delta T = V_{\text{plasma}} \sum_i F_{\text{final},i} C_{\text{init},i} - \Delta_{\text{final}} V_{\text{final}} - V_{\text{plasma}} \left(\sum_i F_{\text{init},i} C_{\text{init},i} - \Delta_{\text{final}} \right) \quad (9)$$

with

$$\Delta = \left(10^{-\text{pH}} - \frac{K_w}{10^{-\text{pH}}} \right)$$

The different K_a values of the plasma buffer components and their respective concentrations can now be determined by calculating ΔT after each addition of titration solvent. For pH_{final}, the measured pH is used at the corresponding addition.

Of each ΔT the actual amount of added acid equivalents ($V_{\text{titrant}} C_{\text{titrant}}$) is subtracted and the results are squared. The sum of these squared differences is the parameter to be minimised by varying the K_a values used in the F-functions and their corresponding concentrations also by means of a numerical solver algorithm as can be found in many calculation software such as the Microsoft Excel solver option.

The resulting K_a and concentration values are treated as constants. We can now define the F-function for plasma or serum (F_p) as a function of the pH for a mixture of k components, each with their own K_a and concentration, C:

$$F_p = \sum_{i=1}^{k} \frac{K_{a_i}}{10^{-\text{pH}} + K_{a_i}} C_i \quad (10)$$

Here "i" represents each of the k components.

2.3. Calculation of the pH of a plasma–buffer mixture

Analogous to Eq. (5) the proton balance for a mixture of plasma with a buffer, A, will be:

$$T_{\text{A,final}} - T_{\text{A,init}} = -(T_{\text{P,final}} - T_{\text{P,init}}) \quad (11)$$

where the right hand side of the equal sign now represents the difference in titration status of the plasma:

$$-\left(F_{p_{\text{final}}} V_p - \left(10^{-\text{pH}_{\text{final}}} - \frac{K_w}{10^{-\text{pH}_{\text{final}}}} \right) V_{\text{final}} - V_p \left(F_{p_{\text{init}}} - \left(10^{-\text{pH}_p} - \frac{K_w}{10^{-\text{pH}_p}} \right) \right) \right)$$

in which pH_p represents the initial pH of the plasma sample.

The function to be solved for the plasma–buffer mixture will be again a balance of the titration status of both the plasma sample and the buffer solution as a function of the pH before and after mixing:

$$T_{\text{A,final}} - T_{\text{A,init}} + T_{\text{P,final}} - T_{\text{P,init}} = 0 \quad (12)$$

The aim in bioanalytical sample preparation will be to obtain a predefined pH of the plasma–buffer mixture prior to further analysis. Also, in order to achieve some buffer capacity, the pH of the resulting buffer–plasma mixture should be within 1 pH unit away from one of the pK_a values of the buffer used [6]. However, this does not necessarily mean that the pH of the buffer should be adjusted to this pH.

The pH of the buffer will be overestimated by an amount of acidic equivalents necessary to adjust the initial pH of the sample to the desired final pH. For example, to adjust a plasma sample to pH of 4 by means of a formate buffer, the pH of that buffer will be adjusted to a pH lower than pH 4. How much lower depends on the buffer concentration and the intended volumes to be used.

By solving Eq. (12) for $\text{pH}_{\text{A,init}}$, this overestimation is incorporated in the result.

The higher the concentration of the buffer species or the larger the volume used in mixing, the less overestimation of the pH is needed. This indicates that care must be taken to adjust the pH in a region of high buffering capacity (i.e. in the vicinity of a pK_a value or at extreme low or high pH values). A small change in pH in such a pH region may result in a relative high transfer of acidic equivalents (molars of protons). Mixing such a buffer with plasma may, therefore, not result in the desired pH.

Another option is to calculate the amount of acidic equivalents needed to adjust a fixed volume of plasma to the desired pH. Adding this amount of acid equivalents to a buffer adjusted to the desired final pH, should also result in the desired pH when the proper volume of this buffer is mixed with the plasma sample.

Using an overestimated buffer restricts the volume to be used to the intended volume, as the overestimation is calculated for fixed volumes of buffer and plasma. The pH of a buffer mixed with a volume of sample converges to the pH of the buffer, when increasing the buffer volume.

2.3.1. Uncertainties

Since pH measurements are based on activities rather than on molar concentrations, problems could arise when calculations are based on literature values of pK_a and molar concentrations [8]. Many pH calculation models dealing with titration curves are based on the molar concentrations and theoretical values of acid–base constants [5,7,9,10]. It is known that activities of dissolved compounds can change as pH changes due to changes in ionic strength. The same holds for the acid–base constants [6,8] which can shift to either higher or lower values as ionic strength changes.

Also possible fluctuations in environmental temperature, plasma sample consistency and experimental errors in the preparation of buffers as well as the mixing volumes of the buffer with the plasma sample will affect the final pH of the resulting mixture.

The plasma acid–base values in our proposed method are based on pH measurements in the actual matrix over a wide pH range. Since pH measurements are based on measurements of activity rather than on actual molar concentrations, this method takes (at least part of) the ionic strength into account. The pK_a of the used buffer substances are rather theoretical literature values and the concentration of these buffer substances are molar concentrations rather than activities. For this reason some discrepancy between the calculated values and measured values can be expected.

2.3.2. Planning of buffer preparation

Buffers are usually prepared by mixing the appropriate amounts of acids and conjugated bases in water and, if necessary, adjusting the pH to the desired value by adding more of the acid or conjugated base. Another way to prepare buffers is to dissolve an amount of acid or its conjugated salt and adjust the pH to the desired value by adding strong acid or base resulting in a partial titration of the acid to achieve the desired ratio of acids and their conjugated bases.

For the preparation of overestimated buffers to achieve the desired pH after mixing it with a predefined volume of sample, there are two approaches which are both based on the final buffer concentration and the intended volume mixing ratio with a plasma sample.

2.3.2.1. Buffer preparation method 1. The first preparation method involves the definition of the mixing ratio, the target pH and the intended concentration of the buffer substance in the final mixture. A suitable buffer substance is selected with a pK_a value not more than 1 pH unit away from the target pH and the concentration of this substance in the buffer solution will be derived from the desired end concentration, corrected for the intended volume ratio. Finally the pH of this buffer is calculated by solving Eq. (12) for $pH_{A,init}$. The disadvantage of this method can be the over- or underestimation of the amount of acidic equivalents (molars of protons) when the buffer pH is adjusted to a pH in region of high buffer capacity.

2.3.2.2. Buffer preparation method 2. The second method is more suitable for buffers to be adjusted in a region of high buffer capacity.

The planning of the preparation involves in fact three stages of mixing:

(1) The adjustment of the pH of a fixed volume of plasma sample with a pre-defined volume of strong acid or base with a concentration to be calculated based on Eq. (8) where $V_{titrant}$ is now the proposed volume of strong acid to be used. This equation is solved for C, the molar concentration of the strong acid.
(2) The preparation of a suitable buffer at the target pH at a concentration corrected for the mixing volumes including the volume of strong acid or base.
(3) Pre-mixing of the proper volumes of buffer and the strong acid or base.

3. Experimental

In order to express human plasma as a buffer solution we performed several titration experiments on different batches of human plasma and serum. From the results of the titration experiments the acid–base constants and the belonging concentrations were calculated.

Finally, a series of experiments was performed to check the validity of the proposed procedure to adjust plasma samples to a desired pH.

3.1. Chemicals and reagents

All titrations were performed on a Metrohm 794 basic titrino automatic titrator (Metrohm AG, Herisau, Switzerland) equipped with a combined glass electrode.

The titrator was controlled by the software program Metro data TiNet 2.5 (Metrohm AG, Herisau, Switzerland) running on a personal Windows-based computer. This software was also used for data acquisition.

The pH measurements were performed with a Metrohm model 713 pH meter (Metrohm Herisau, Switzerland). The 0.100 M hydrochloric acid and 0.100 M sodium hydroxide (Merck, Darmstadt, Germany) solutions were used in the titration experiments. Sodium di-hydrogen phosphate monohydrate, tri-sodium hydrogen phosphate dodecahydrate, sodium citrate, sodium hydroxide and hydrochloric acid 37% used to prepare the buffer solutions were all of analytical grade from Merck (Merck, Darmstadt, Germany). Ammonium formate used to prepare a buffer solution was also of analytical grade (Fluka, Buchs, Switzerland).

3.2. Titration experiments

Eight different batches of human plasma from which four batches contained solid K2-EDTA as anticoagulant (batch 1–4), four batches contained sodium heparin (batch 5–8) and two batches of serum (batch 9–10) were used to determine the acid–base parameters by titration experiments.

Of each batch, 5.00 ml was titrated with 0.100 M of hydrochloric acid and another 5.00 ml of the same batch was titrated with 0.100 M of sodium hydroxide. The titration equipment operated in monotonic endpoint titration (MET) mode which means that the volume increments are equal throughout the titration curve. The data was exported to a Microsoft Excel spreadsheet and the titration data of each plasma batch were merged and sorted in ascending pH order. The number of equivalents of acid (mmol protons) was plotted against the measured pH. We mention again that the sodium hydroxide titration was expressed as negative acidic equivalents.

From the resulting titration curves the acid–base parameters were derived.

3.3. Plasma pH adjustment experiments

To demonstrate the use of the proposed equations, a set of experiments was set up to adjust the pH of six different batches of plasma which were independent from the plasma batches used in the titration experiments. A wide pH range was covered with different kinds of buffer species to cover the common pH values needed in bioanalytical chemistry.

All buffers were prepared at a concentration of 0.200 M by dissolving the proper amount of buffer salt and adjust the pH with concentrated hydrochloric acid or with a 10 M sodium hydroxide solution. All buffers were stored at 4 °C until use.

Before use, the pH of each buffer was measured at room temperature and the calculations were based on these measured pH values.

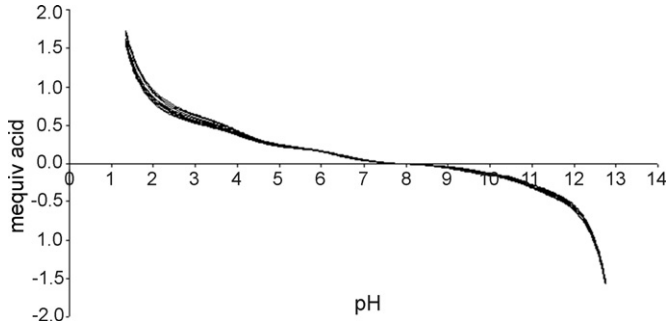

Fig. 2. Reciprocal titration curves of 10 batches of human plasma and serum.

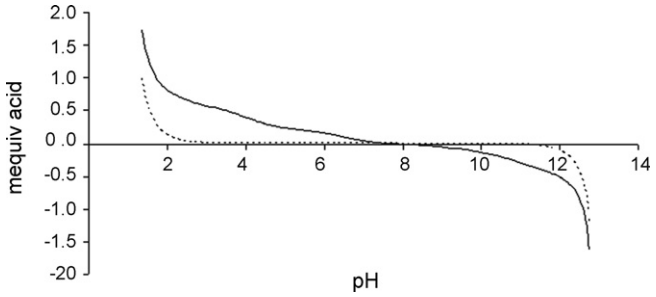

Fig. 3. Resulting titration curve of plasma (solid line) constructed with the mean values of Table 1 and the theoretical curve of pure water (dotted line).

The experiments were set up to mix the buffer and the plasma at an 1:1 ratio and to reach a final pH of the plasma–buffer mixture, the target pH, which should be at last 1 pH unit away from the pK_a of one of the corresponding buffer species. For phosphate the target pH was 2.5, 7.0 and 12.0, for formic acid the target pH was 4.0, for citrate the target pH was 3.0 and 3.8.

First, the pH of the buffer was adjusted to the target pH and the pH of the plasma–buffer mixture was measured and also calculated by solving Eq. (12) for pH_{final}.

Secondly the pH of the buffer solutions was calculated by solving Eq. (12) for $pH_{A,init}$ to reach the target pH, after mixing. The pH of the resulting plasma–buffer mixture was also measured.

Two additional buffers, a phosphate buffer with target pH of 2.5 and a citrate buffer with target pH of 3.0 were prepared according to procedure 2, as described in Section 2.3.2.2.

The buffer preparation methods as described in Section 2.3.2 are demonstrated in the next sections.

The results of the plasma–buffer mixtures are represented in Section 4.2.

3.3.1. Example of buffer preparation method 1

To demonstrate the use of buffer preparation method 1, 1.00 ml of a plasma sample will be adjusted to a target pH of 3.8, with 1.00 ml of a citrate buffer. This pH is between two pK_a values of citrate which are both involved in the pH adjustment of the plasma sample. The pH of the citrate buffer should be adjusted to a lower value than the target pH to overcome the pH gap of the plasma from its original pH of 8.0, to the final pH of 3.8.

To achieve a final buffer concentration of 0.100 M of citrate in the resulting buffer–plasma mixture, the initial buffer concentration should be prepared at a concentration of 0.200 M.

Solving Eq. (12) for $pH_{A,init}$ results in a buffer pH of 3.07.

A citrate buffer was prepared at a concentration of 0.200 M and the pH was adjusted to 3.07. At the time of use, the pH was measured again and was found to be 3.14. Solving Eq. (12) for pH_{final} using the measured buffer pH, results in a predicted pH of the mixture of 3.86. (See Section 4.2, experiment #11).

3.3.2. Example of buffer preparation method 2

To demonstrate the use of buffer preparation method 2 we chose to adjust 1.00 ml of a plasma sample to pH 2.5 with 1.00 ml of a phosphate buffer.

(1) The intention was to adjust the pH of 1.00 ml of plasma sample with 0.100 ml of a hydrochloric acid solution. The concentration of that solution to reach pH 2.5 after mixing was calculated using Eq. (8) where C, the concentration of the hydrochloric acid, is the parameter to be solved. The amount of acidic equivalents was calculated to be 0.129 mmol of protons, corresponding to 0.100 ml of a 1.29 M hydrochloric acid solution, which was prepared by carefully diluting hydrochloric acid (37%) with water.
(2) The plasma sample was planned to be adjusted to the target pH, by adding 1.00 ml of the buffer to be prepared.

Table 1
Calculated acid–base parameters from the titration experiments of 10 different batches of human plasma and serum expressed as pK_a values and corresponding concentrations

Batch	pKa 1	Conc. 1 (M)	pKa 2	Conc. 2 (M)	pKa 3	Conc. 3 (M)	pKa 4	Conc. 4 (M)	pKa 5	Conc. 5 (M)
1	2.25	0.0505	4.01	0.0713	6.32	0.0431	9.27	0.0252	10.85	0.0592
2	2.21	0.0481	3.98	0.0760	6.35	0.0468	9.42	0.0320	10.98	0.0597
3	2.32	0.0465	4.00	0.0789	6.29	0.0450	9.18	0.0288	10.95	0.0643
4	2.37	0.0389	4.02	0.0781	6.37	0.0446	9.25	0.0278	10.92	0.0646
5	2.42	0.0291	4.05	0.0602	6.50	0.0400	9.30	0.0230	10.92	0.0563
6	2.39	0.0302	3.97	0.0653	6.55	0.0456	9.54	0.0253	10.95	0.0576
7	2.38	0.0286	4.00	0.0640	6.55	0.0410	9.35	0.0239	10.92	0.0567
8	2.39	0.0294	3.96	0.0642	6.38	0.0430	9.38	0.0322	11.01	0.0602
9	2.38	0.0273	3.92	0.0704	6.34	0.0371	9.19	0.0272	10.96	0.0577
10	2.30	0.0291	3.92	0.0719	6.42	0.0421	9.31	0.0278	11.01	0.0600
Mean	2.34	0.0358	3.98	0.0700	6.41	0.0428	9.32	0.0273	10.95	0.0596
S.D.	0.07	0.0093	0.04	0.0065	0.10	0.0029	0.11	0.0031	0.05	0.0029

Table 2
Results of the plasma–buffer mixing experiments

Experiment #	pH target	Buffer			pH after mixing							Statistics			
		Concentration and type	pH theoretical	pH measured	Calculated	P1	P2	P3	P4	P5	P6	Mean	S.D.	Dev. from calc.	Dev. from target
1	2.5	0.2 M phosphate	2.50	2.55	4.57	5.07	5.06	4.98	4.92	4.96	4.92	4.99	0.07	0.42	2.49
2	2.5	0.2 M phosphate	1.47	1.49	2.52	2.19	2.15	2.06	2.05	2.11	2.03	2.10	0.06	−0.42	−0.40
3	7.0	0.2 M phosphate	7.00	6.99	7.06	7.17	7.15	7.14	7.15	7.14	7.17	7.15	0.01	0.09	0.15
4	12.0	0.2 M phosphate	12.00	12.01	11.08	11.31	11.31	11.35	11.35	11.39	11.36	11.35	0.03	0.26	−0.66
5	12.0	0.2 M phosphate	12.75	12.75	12.07	12.09	12.07	12.08	12.09	12.09	12.09	12.09	0.01	0.02	0.09
6	4.0	0.2 M formate	4.00	4.02	4.76	4.98	5.06	5.12	4.98	4.92	5.02	5.01	0.07	0.25	1.01
7	4.0	0.2 M formate	3.27	3.27	4.00	4.01	4.04	3.96	3.94	4.00	3.98	3.99	0.04	−0.01	−0.01
8	3.0	0.2 M citrate	3.00	3.05	3.79	3.70	3.71	3.63	3.64	3.65	3.63	3.66	0.04	−0.13	0.66
9	3.0	0.2 M citrate	1.56	1.76	3.09	2.92	2.94	2.82	2.80	2.87	2.84	2.87	0.06	−0.23	−0.14
10	3.8	0.2 M citrate	3.80	3.85	4.37	4.32	4.33	4.28	4.26	4.30	4.27	4.29	0.03	−0.08	0.49
11	3.8	0.2 M citrate	3.07	3.14	3.86	3.80	3.81	3.72	3.71	3.77	3.74	3.76	0.04	−0.10	−0.04
12	2.5	0.18 M phosphate[a]	1.41	2.53/1.43[b]	2.54	2.54	2.57	2.38	2.38	2.45	2.42	2.46	0.08	−0.08	−0.04
13	3.0	0.18 M citrate[a]	1.43	3.03/1.66[b]	2.99	3.01	3.01	2.91	3.06	2.94	2.92	2.98	0.06	−0.02	−0.02

[a] Buffer prepared with preparation method 2.
[b] pH of buffer before addition of extra equivalents of acid/pH after addition of acid.

This 1.00 ml should also contain the 0.100 ml of 1.29 M hydrochloric acid solution. Therefore a 0.200 M phosphate buffer was prepared which was adjusted to pH 2.5. Of this buffer, 90.0 ml was mixed with 10.0 ml of the hydrochloric acid solution. The buffer concentration is now diluted to 1.80 M. Although the resulting pH of the buffer is of less importance, it can be calculated analogous to Eq. (9), by Eq. (13).

$$\Delta T = T_{\text{buffer,final}} - T_{\text{buffer,init}} = -V_{\text{titrant}} C \tag{13}$$

This theoretical pH was calculated to be 1.41.

(3) One ml of the plasma sample and one ml of buffer were mixed. The results are shown in Section 4.2, experiment #12.

4. Results and discussion

4.1. Results of plasma titration experiments

All obtained (reciprocal) titration curves are represented in Fig. 2.

Five fictitious values of K_a values with five corresponding concentrations of acid–base active substances were sufficient to describe the whole titration curve. These fictitious K_a values and concentrations were calculated as described in Section 2.2.

Incorporating more K_a values and concentrations did not lead to a significant better description of the curves whereas less than five leads to an increased sum of squares in the regression as well as a visual discrepancy with respect to mean of the measured curves.

The resulting pK_a values and concentrations are represented in Table 1 and Fig. 3. In Fig. 3, the mean of the calculated pK_a values and corresponding concentrations were used to perform calculations of plasma pH.

Also the theoretical titration curve of pure water is plotted in Fig. 3 to illustrate the buffer capacity of plasma with respect to pure water.

From the data of the titration experiments we can conclude that human plasma has a significant acid–base behaviour which can be accurately described by five pK_a values, five corresponding concentrations and the dissociation constant of water. From Fig. 2 it is obvious that this acid–base behaviour is subjected to some variation, especially in the acidic region. By means of a statistical *t*-test, it can be shown that the mean of conc. 1 of the EDTA plasma sources (batch 1–4) differs significantly from the mean of the other values of conc. 1. pH Calculations based on the mean parameters in Table 1, can therefore lead to some more deviated results compared to the calculated value if no precautions are taken. Although it should be possible to determine the acid–base behaviour of each of the different kinds of plasma and serum sources separately, we decided to treat all these sources together as the same source.

4.2. Results of the plasma pH adjustment experiments

The results of the plasma pH adjustment experiments are represented in Table 2.

The results in Table 2 show that the largest differences between the measured pH values of the mixtures and the target pH are found, as expected, when the buffer pH is equal to the target pH (experiments 1, 4, 6, 8 and 10). This means that the buffer capacity of the used buffer is not sufficient to overcome the pH gap between the original plasma pH and the target pH. However, many sample preparation methods use this method to adjust the pH of the samples prior to further sample preparation. Based on our results, this can be a potential source of error when extracting ionisable compounds.

The largest differences between the measured pH of the mixtures and the calculated pH were found when the pH of the buffer–plasma mixture was not in a pH region of the buffering capacity of the used buffer species and also where the pH of the buffer was adjusted in a high buffering region of the curve (experiments 1 and 2, respectively). Although the titration experiments showed some variation of the acid–base parameters in the acidic range, the S.D. of the pH adjustment experiments in the acidic region do not differ from the other S.D. values. This indicates that the used buffer concentration was sufficient to overcome this variation.

From Fig. 3 it can be seen that the pH buffering effect of water itself plays an important role below pH 2 and above pH 12. At such extreme pH values, an extra amount of acid equivalents added to the buffer solution, results in a minor pH shift. However, this amount of acid equivalents can cause a large pH shift in the final mixture.

This effect is more important when preparing buffers according to preparation method 1 at extreme pH, where the extra amount of acid equivalents to the buffer is added by pH adjustment (experiments 2 and 9). Although the calculated results are well in accordance with the measured values in the high pH region (experiment# 5) the same problems could be expected in this pH region. However this issue is of minor importance when using preparation method 2, where the extra amount of acid equivalents is added volumetrically (experiments 12 and 13). Therefore we believe that method 2 is the most accurate way is to prepare the buffers at the extremes.

The calculated pH values of plasma–buffer mixtures are well in accordance with the measured pH when these resulting pH regions are in a buffering region and the pH of the under or overestimated buffer is away from the buffer region. Table 2 shows, that the sample can be adjusted to the target pH within 0.1 pH units of the calculated pH when carefully selecting the right pH and preparation method (experiments 3, 7, 11, 12 and 13).

5. Conclusions

It is common practice in bioanalytical sample preparation to adjust a plasma sample by mixing it with a buffer with the same pH as the target pH. In this way however, the intended target pH may not be achieved automatically.

The proposed equations can be very useful in understanding the pH behaviour of buffers and human plasma samples mixtures. Proper use of the proposed calculation methods and buffer preparation methods lead to accurate and predictable pH of the resulting plasma–buffer mixture.

Especially, when the pH of the sample is to be adjusted further from the original pH, the resulting pH is more reliable when the buffer pH is adjusted to the proposed, target pH by the given methods.

This proposed calculation method can therefore enhance the robustness of the bioanalytical method.

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