ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
14 (1996) 273–280

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

Determination of proteins in the presence of imidazole buffers

F. Molina[a,*], A. Rueda[a], J.M. Bosque-Sendra[a], L. Megías[b]

[a]*Department of Analytical Chemistry, University of Granada, 18071 Granada, Spain*
[b]*Department of Morphological Sciences, University of Granada, 18071 Granada, Spain*

Received for review 20 March 1995; revised manuscript received 15 June 1995

Abstract

Imidazole buffers were found to interfere with the determination of soluble proteins using Lowry's classical method. The influence of the constituent elements of the buffer on the calibration line was studied statistically. By combining the data corresponding to different experimental sequences, interserial calibration curves for different concentrations of imidazole buffer (10–30 mM) were obtained. The absorbance–buffer volume dependence curves produced a good fit to second-order polynomials. The accuracy of protein determination in a medium with imidazole buffer, using appropriate calibration curves, were tested by comparison with the technique of multiple standard addition and by means of recovery studies. These experiments were performed on chick brain homogenate samples. Other important aspects of validation, such as sensitivity and accuracy, were also studied.

Keywords: Protein determination; Lowry assay; Imidazole buffer interference; Chick brain homogenate samples

1. Introduction

The determination of protein must be performed regularly in many biological studies, including the purification of enzymes or the measurement of their specific activity [1], and there is a constant requirement for accuracy and precision and, depending on the type of study, sensitivity and/or simplicity.

Among the analytical methods currently in use, the principal ones are those based on the principle of protein–dye binding [2–4], those that use bicinchoninic acid [5,6], those based on ultraviolet spectrophotometric measurements [7–9] and those measuring the amino acids obtained from protein hydrolysis [10–12]. However, the most widely employed is still Lowry's method [13] owing to its sensitivity, simplicity and reproducibility [14]. This method, however, has received considerable attention in the literature owing to its disadvantages in terms of lack of specificity [15–17], slow reaction rates, the instability of some reagents [18] and the non-linearity of the calibration curve [19,20]. Moreover, various modifications have been suggested to permit the determination of hydrophobic proteins [16,21,22] or to automate the process of determination [23]. The list of substances that interfere in Lowry's method is long [14], although no other protein assay method has been so thoroughly explored.

* Corresponding author.

SSDI 0731-7085(95)01615-5

In this laboratory, in the course of a series of experiments intended to measure the specific activities of enolase isoenzymes (EC 4.2.1.11), after their separation with imidazole buffers with different KCl contents [24], it was found that those buffers interfered with the determination of soluble proteins using Lowry's method.

The effects of other buffers such as citrate, phosphate, Tris, bicine, Hepes and Pipes on Lowry's protein assay method have been reported [14,25–27], but no information could be found regarding the compatibility of an imidazole buffer with the analytical system.

Imidazole–HCl buffer [28] is currently one of the most widely used in biochemical research, owing to its neutrality, good buffering capacity in the physiologically important pH range 6–8 (the pK_a of imidazole is 6.95) and its stability against temperature changes ($dpK_a/dT = -0.020$) [29]. Consequently, it was considered of interest to study the influence of imidazole buffers on the application of Lowry's method, with the aim of characterizing its interference. This paper relates the main results obtained from this study.

2. Materials and methods

2.1. Chemicals

Bovine serum albumin (BSA) was purchased from Sigma and imidazole and Folin–Ciocalteu phenol reagent from Merck; all other reagent used were of analytical grade. Water was of reverse osmosis quality and the experiments were carried out at room temperature unless stated otherwise.

2.1.1. Standard BSA solution, 1 mg ml^{-1}

This was prepared in reverse osmosis water.

2.1.2. Working standard BSA solutions, 400 and 200 $\mu g\ ml^{-1}$

These were prepared by appropriate dilution of the stock standard solution with water. They were stored under refrigeration (6–8°C). The 400 $\mu g\ ml^{-1}$ BSA solution was stable for the period under observation (24 h) and the 200 $\mu g\ ml^{-1}$ BSA solution was stable for at least 2.5 days.

2.1.3. Imidazole–HCl buffers (pH 6.8; 50 mM)

These contained 2 mM $MgCl_2$ and 40, 150 or 240 mM KCl (40, 105 and 240 mM buffers, respectively). Imidazole buffer containing 40 mM KCl and without Mg^{2+} was also prepared.

2.2. Apparatus

The apparatus used included a Bausch & Lomb Spectronic 2000 spectrophotometer with 10 mm cells, a Crison Digit-501 pH meter with a combined glass–calomel electrode and a Beckman XL-70 ultracentrifuge.

2.3. Calibration curves in the absence of imidazole buffer

For this study, the classical Lowry procedure was followed [13]. The calibration standards were prepared by introducing into 10 ml test-tubes a volume of working standard BSA solution sufficient to contain 10–60 μg of protein and adding water to obtain 0.5 ml. Then 3 ml of working alkaline copper reagent (a freshly prepared solution obtained by mixing 100 volumes of 2% Na_2CO_3 in 0.1 N NaOH with one volume of 2% sodium potassium tartrate and with one volume of 1% $CuSO_4 \cdot 5H_2O$ in water) were added. After waiting for 10 min, 150 μl of Folin–Ciocalteu phenol reagent were added and the mixture was immediately shaken for 5–7 s, then the test-tubes were stored for 30 min in darkness. A reading was taken of absorbance at 750 nm against water and net absorbance was obtained by subtracting the absorbance of a reagent blank solution, prepared under identical conditions, but in the absence of proteins.

2.4. Calibration curves with imidazole buffer

The calibration standards were prepared in an analogous fashion to the previous section, except that a suitable volume of test buffer was added to the working BSA solution, which was then made up to 0.5 ml with water.

In those cases where, owing to the presence of the buffer, the concentration of KCl in the final solution was greater than 12 mM (specifically,

when 300 μl of buffer 150 mM in KCl or with 200 μl or greater volumes of buffer 240 mM in KCl, for a final volume of 3.65 ml, were used) the appearance of a white precipitate was observed, a phenomenon which has already been commented upon [30]. To be able to measure the absorbance without interference in these cases, the tube was centrifuged for 2 min at 25*g*, 27 min after adding the Folin–Ciocalteu reagent. Subsequently, the absorbance of the supernatant was measured.

2.5. Interserial calibration curves

These curves were calculated by combining the data on net absorbance obtained from BSA standards of up to 60 μg of protein in different experimental sequences, performed by the same person, with the same spectrophotometer and, usually, with the same Folin–Ciocalteu reagent, but on different days and thus with different working BSA solutions and a different alkaline Cu^{2+} solution. The number of experimental data employed in obtaining each interserial curve varied from 48 to 69.

2.6. Biological samples and treatment

Shaver Star-cross 288 chick embryos were used. Brain samples were treated without delay by homogenization with 40 mM buffer (1:5, w/v) and ultracentrifugation (100000*g*) for 60 min at 4°C. The supernatant was used for enolase isoenzyme separation by DEAE-cellulose chromatography [24] with 40, 150 and 240 mM buffers. The proteins were determined in the supernatant and in each of the three fractions eluted from the column (fractions αα, αγ and γγ). The samples were refrigerated (4–6°C) and analysed within 5 h, during which period their stability was apparent. For protein determination, sample volumes of 100–300 μl were taken.

2.7. Statistical treatment of the data

The Statgraphics statistical program (STATGRAPHICS 6.0, Statistical Graphics Corp., 1993) was used for the statistical treatment of the data. To compare the regression lines, the equality of three statistical parameters was tested: the variance (s^2), the slope (b) and the independent term (a) of the lines [31].

Variance equality was tested by using an F-test [32]. The null hypothesis (equality of variances) was accepted for a level of significance of greater than 5%.

A t-test was used [32] to compare both the slopes and the independent terms. In the case of the slopes, the null hypothesis (homogeneity of slopes) was accepted for a level of significance of greater than 5%. For the independent term, the null hypothesis (the independent term is zero) was accepted for a level of significance of greater than 5%.

3. Results and discussion

3.1. Effects of imidazole buffer on protein determination using Lowry's method

To identify the possible interference produced by the constituent elements of the imidazole buffers, the effects that these produced both on the reagent blank and on the protein standards for different concentrations were studied. For this purpose, calibration curves were obtained, in triplicate, in the range 0–60 μg of BSA in the presence of various volumes of the imidazole buffers (pH 6.8; 50 mM) 2 mM in $MgCl_2$ and differing KCl content (40, 150 and 240 mM). Table 1 shows the characteristic regression parameters for two different buffer volumes (100 and 300 μl). In addition for comparison, an interserial calibration was obtained for the same range of BSA concentrations, but with no buffer.

The results obtained (Table 1 only shows a small proportion) seem to indicate that the presence of the buffer leads to an increase in the blank reagent absorbance and a decrease in the slope of the calibration line. These effects depend on the volume of buffer but not on the KCl content. Indeed, when a statistical comparison is made between the slopes of the calibration lines when a buffer is present and that of the interserial without buffer, it is seen that they are significantly differ-

Table 1
Calibration curves with imidazole buffers at different concentrations in KCl[a]

Buffer volume (μl)	Parameter[b]	40 mM	150 mM	240 mM
100	$(a \pm s_a) \times 10^{-3}$	1 ± 2	2 ± 2	3 ± 4
	$(b \pm s_b) \times 10^{-3}$	3.38 ± 0.07	3.43 ± 0.06	3.31 ± 0.10
	r	0.998	0.998	0.995
	s^c	5.09×10^{-3}	4.65×10^{-3}	7.86×10^{-3}
	$A_{blank} \pm s_{bl}$	0.050 ± 0.005	0.049 ± 0.002	0.055 ± 0.002
300	$(a \pm s_a) \times 10^{-3}$	-1 ± 3	-1 ± 2	0 ± 3
	$(b \pm s_b) \times 10^{-3}$	2.88 ± 0.08	2.96 ± 0.06	3.06 ± 0.08
	r	0.996	0.998	0.996
	s^c	6.01×10^{-3}	4.57×10^{-3}	6.92×10^{-3}
	$A_{blank} \pm s_{bl}$	0.074 ± 0.003	0.078 ± 0.005	0.075 ± 0.003

Interserial calibration in the absence of buffer ($n=48$):
$(a \pm ts_a) \times 10^{-3} = 2 \pm 3$; $(b \pm ts_b) \times 10^{-3} = 4.03 \pm 0.08$; $r = 0.998$; $s^c = 6.20 \times 10^{-3}$; $A_{blank} \pm s_{bl} = 0.015 \pm 0.003$

[a]Calibration curves for 0–60 μg of BSA in 0.5 ml of solution ($n=12$; four standards in triplicate).
[b]Linear model: absorbance $= a + bC$ (C = μg BSA); t = Student's t, for 95% confidence and $n-2$ degrees of freedom.
[c]Standard error of regression.

ent ($P < 0.01\%$ in all cases). Second, the slopes of the calibration lines for the same buffer volume are significantly equal ($P > 20\%$ in all cases), irrespective of the KCl concentration of the buffers. This corroborates the above and indicates that the KCl in the buffers is not responsible for the interference observed in the application of Lowry's method.

A calibration was also obtained, in triplicate, in the presence of 300 μl of imidazole buffer 40 mM in KCl but without Mg^{2+}, in order to test the possible influence of this ion (cofactor of the enolase) on the previous effects. The absorbance (A) versus amount of BSA (μg) dependence fitted the equation $A = 1 \times 10^{-4} + 3.12 \times 10^{-3} C$ ($r = 0.999$), and the reagent blank had an absorbance of 0.085 ± 0.003. These parameters more closely approach those of the calibrations obtained in the presence of buffer with Mg^{2+} than those obtained only in water (Tables 1 and 2). Thus it is seen that, first, the blank absorbance lies within the confidence interval of the blank obtained in the presence of the same volume of buffer with Mg^{2+} (Table 2), second, the statistical comparison of the slope without Mg^{2+} with that corresponding to the interserial calibration in the absence of buffer indicates that they are significantly different ($P < 0.01\%$) and finally, the statistical comparison of the slopes obtained in presence of 300 μl of buffer with and without Mg^{2+} (Table 1) indicates that they are significantly equal ($P > 47\%$). It is believed, therefore, that the Mg^{2+} ion is not responsible for the interference of the buffers tested. Furthermore, at no time was there any indication the precipitation of the phenol reagent due to the presence of the Mg^{2+} ion remarked upon by Kuno and Kihara [33], although it is true that the concentration of Mg^{2+} in the last solution, after adding the Folin–Ciocalteu reagent, was never greater than the relatively low 0.16 mM (Kuno and Kihara did not specify the concentration at which they observed this phenomenon).

With respect to the absorbance of the reagent blank, this basically depends on the Folin–Ciocalteu reagent: on whether or not it is freshly prepared [20], on the manufacturer and even on the batch number. Thus, when the reagent from the same manufacturer but of different batch numbers was used, blank absorbance values of 0.021 ± 0.005, 0.024 ± 0.006 and 0.015 ± 0.003 were obtained (mean $\pm s_{bl}$ of 15 data obtained on different days). The last value (0.015 ± 0.003) is that corresponding to the reagent used for most

Table 2
Interserial calibration curves with different concentrations of imidazole–HCl buffer[a]

Parameter[b]	Volume of buffer (μl)			
	100	150	200	300
$(a \pm ts_a) \times 10^{-3}$	2 ± 3	1 ± 4	2 ± 2	-1 ± 4
$(b \pm ts_b) \times 10^{-3}$	3.35 ± 0.08	3.24 ± 0.10	3.07 ± 0.06	2.95 ± 0.11
r	0.997	0.993	0.997	0.990
s[c]	6.0×10^{-3}	9.2×10^{-3}	5.4×10^{-3}	11×10^{-3}
Linearity (%)[d]	98.9	98.5	99.0	98.0
n	50	60	60	69
$A_{blank} \pm s_{bl}$	0.051 ± 0.004	0.060 ± 0.007	0.068 ± 0.007	0.089 ± 0.017

[a]Interserial calibration curves for 0–60 μg of BSA in 0.5 ml of solution, containing different volumes of 50 mM imidazole buffer (pH 6.8), 2 mM $MgCl_2$ and a variable concentration of KCl (8–144 mM).
[b]Linear model: absorbance $= a + bC$ ($C = \mu$g BSA); t = Student's t, for 95% confidence and $n-2$ degree of freedom.
[c]Standard error of regression.
[d]Using the statistical model proposed in Ref. [34].

of the experiments described in this paper. The presence of the imidazole buffer led to a significant increase in absorbance over the previous values, depending on the buffer volume (Table 2, Fig. 1), reaching values as high as 0.1 for 300 μl of buffer.

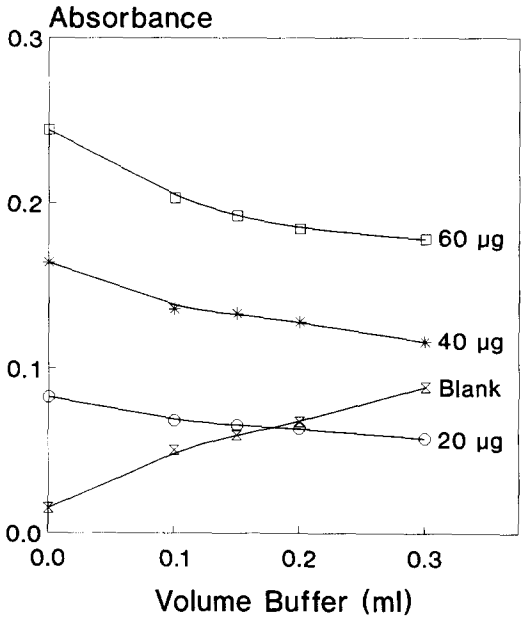

Fig. 1. Influence of the volume of imidazole buffer on the net absorbance of different concentrations of BSA and on blank absorbance. Values are means ± SD of 15 determinations.

By combining the absorbance data obained from different experiments, interserial calibration curves were constructed for different volumes of imidazole buffer (100–300 μl in 0.5 ml, equivalent to imidazole concentrations of between 10 and 30 mM). The linear regression analysis data are shown in Table 2. In all cases, the independent term is significantly equal to zero ($P > 10\%$) and a good linearity fit is obtained. Furthermore, the standard deviation values indicate good agreement of the experimental data with those predicted by the regression line.

Fig. 1 shows the curves of the influence of the imidazole buffer on the net absorbance of the different concentrations of BSA and on the reagent blank absorbance. The fit of both curves to second- and fourth-order polynomials was tested and a good fit was observed in each case, with standard deviations of approximately 10^{-3} and 10^{-6}, respectively (Table 3).

3.2. Protein determination in chick brain homogenate samples

The above results demonstrate the interference of imidazole buffers in the application of Lowry's method for the determination of proteins in samples that contain them. This makes it necessary to perform this determination using calibrations made in the presence of the buffer, to use stan-

Table 3
Model fitting results for absorbance (A)–buffer volume dependence

Polynomial fit	Coefficient	Blank	20 μg	40 μg	60 μg
2nd order[a]	a_1	0.016	0.080	0.163	0.244
	a_2	0.349	−0.140	−0.261	−0.476
	a_3	−0.367	0.200	0.362	0.862
	$s \times 10^{-3}$	3	1	3	4
4th order[b]	a_1	0.015	0.008	0.164	0.244
	a_2	0.579	−0.219	−0.676	−0.625
	a_3	−2.970	0.967	6.156	2.929
	a_4	8.579	−1.758	−25.573	−9.020
	a_5	−7.909	0.152	35.954	12.532
	$s \times 10^{-6}$	3	4	10	8

[a] $A = a_1 + a_2V + a_3V^2$.
[b] $A = a_1 + a_2V + a_3V^2 + a_4V^3 + a_5V^4$.

dard addition methods or to separate quantitatively the interfering agent or the analyte itself. These alternatives involve a greater manipulation of the sample, with the consequent risk of human error, and greater consumption of time and reagents.

To test the accuracy of the determination using calibration curves in the presence of imidazole buffers, the total proteins in brain homogenates and in the chromatographic fractions that contained the enolase isoenzymes, $\alpha\alpha$, $\alpha\gamma$ and $\gamma\gamma$, in imidazole buffers (pH 6.8; 50 mM) of different KCl content (see Materials and methods) were determined. The determinations were carried out by multiple standard addition techniques and by using calibration curves constructed both in the presence of the buffer (concentration of buffer in the calibration standards = concentration of buffer in the sample) and in its absence. The results obtained are shown in Table 4. It can be seen that there is close agreement between the results of the application of the first two procedures. The use of calibrations of BSA simply dissolved in water led to negative errors of up to 50%.

Furthermore, a recovery study was carried out on these samples. A percentage recovery was determined for the quantities of BSA added (in the range 17–40 μg) to 100 μl of sample. Table 5 shows the results obtained for the different samples analysed. The recovery was between 95 and 112%.

The influence of protein concentration on the determination was also studied. Table 6 shows the results of experiments carried out using the same volume (100 μl) of different dilutions of brain homogenate with imidazole buffer. Table 6 also shows the results from the linear regression study between the experimental values and those expected. The confidence intervals of the slope and intercept do not differ significantly from 1 and 0, respectively, indicating no significant difference between the values found and the values expected.

Table 4
Determination of protein in brain homogenate samples[a]

Sample	Proteins found (μg)		
	Standard addition method	Calibration with buffer[b]	Calibration without buffer
Homogenate supernatant[c]	13.8	14.4	10.2
$\alpha\alpha$ fraction[c]	5.1[d]	5.3[d]	2.5[d]
$\alpha\gamma$ fraction	28.5	27.3	21.0
$\gamma\gamma$ fraction	15.2	15.0	10.7

[a] Sample volume taken = 100 μl. Each result is the mean of two determinations.
[b] The calibration standards contained 100 μl of imidazole–HCl buffer.
[c] Diluted 1:25 (v/v) with buffer.
[d] Value lower than quantification limit.

Table 5
Recovery study for BSA added to brain homogenate samples[a]

Sample	Amount added (μg)	Amount found[b] (μg)	Recovery (%)
Homogenate	17.1	19.1	112
supernatant[c]	28.5	32.1	112
	39.9	42.1	105
$\alpha\alpha$ fraction[c]	17.1	18.5	108
	28.5	31.2	109
	39.9	41.5	104
$\alpha\gamma$ fraction	17.1	16.8	98
	28.5	28.5	100
	39.9	37.9	95
$\gamma\gamma$ fraction	17.1	19.1	112
	28.5	31.2	109
	39.9	40.3	101

[a]Sample volume taken = 100 μl. Each result is the mean of two determinations.
[b]The calibration standards contained 100 μl of imidazole–HCl buffer.
[c]Diluted 1:25 (v/v) with buffer.

Table 7 summarizes the quality parameters obtained for Lowry's method, using standard BSA solutions, in the absence and presence of two different buffer volumes, using the statistical model proposed by Cuadros et al. [34]. On the basis of an examination of these data, it is clear that Lowry's method employed in the presence of imidazole buffer possesses good linearity and low relative standard deviations of the concentration,

Table 6
Influence of dilution of sample on the protein determination[a]

Sample dilution	Amount expected (μg)	Amount found[b,c] (μg)
1:25	19.3	20 ± 1
1:20	24.1	24 ± 2
1:15	32.2	32 ± 1
1:10	48.3	48.0 ± 0.5

Linear regression: $y(\text{found}) = 0.751 + 0.976x(\text{expected})$; $r = 0.996$
For $t_{(P=0.05;\ DF=14)} = 2.145$: $a \pm ts_a = 0.751 \pm 1.609$, $b \pm ts_b = 976 \pm 0.049$

[a]Sample volume taken = 100 μl.
[b]The calibration standards contained 100 μl of imidazole–HCl buffer.
[c]Values are means $\pm s$ of four determinations.

Table 7
Lowry's method: quality parameters without and with imidazole buffer[a]

Parameter	Volume of imidazole buffer (μl)		
	0	100	300
Linearity (%)	97.7	98.0	98.0
Analytical sensitivity (μg)	1.8	1.5	1.5
Detection limit (μg)	4.0	3.4	3.5
Quantification limit (μg)	13.5	11.4	11.6
Precision:			
Amount (μg)	RSD(C) (%)		
20	5.3	4.9	5.0
40	2.8	2.4	2.6
60	2.3	1.9	1.9

[a]From three replicates per four concentration values of different standard BSA solutions.

RSD(C), over the whole range of concentrations tested, these parameters being of a similar order to those obtained in the absence of buffer. In fact, the detection and quantification limits obtained, for both 100 and 300 μl of buffer, are even slightly below those of the standard procedure.

Hence it may be concluded that protein determination in samples containing imidazole buffer may be accurately performed, provided that calibration curves are constructed in the presence of the same concentration of this buffer. This process, moreover, does not imply any loss of sensitivity or precision.

In routine analysis, in which different sample volumes must be analyzed, the difficulty of having to prepare various calibrations may be reduced by employing interserial calibration curves that have been previously constructed or based on the absorbance–volume (or concentration) of buffer dependence curves. Table 8 summarizes, as an example, the results of analysis carried out on samples with different protein contents and, therefore, performed with different sample volumes. Note that absolute agreement between the values obtained with the interserial calibration and those calculated using the fourth-order fit, and the good

Table 8
Protein determination in brain homogenete samples

Sample	Volume (μl)	Absorbance	Proteins obtained[a] (μg)		
			I-C[b]	2nd[c]	4th[d]
A	100	0.190±0.006	56±2	55±2	56±2
B	150	0.134±0.003	41±1	41±1	41±1
C	200	0.168±0.006	54±2	55±2	54±2

[a]Values are means±s of five determinations.
[b]By means of interserial calibration with a buffer volume equal to the sample volume taken.
[c]By means of a calibration derived from the 2nd-order absorbance–buffer volume equations.
[d]By means of a calibration derived from the 4th-order absorbance–buffer volume equations.

agreement obtained even with the values obtained from the second-order fit.

Acknowledgements

This paper was prepared with support from the DGICYT, Project PM 091-0111.

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