Practical Laboratory Medicine 21 (2020) e00166

Contents lists available at ScienceDirect

Practical Laboratory Medicine

journal homepage: www.elsevier.com/locate/plabm

Selection of appropriate protein assay method for a paper microfluidics platform

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ARTICLE INFO

Keywords:
Bromocresol green
Tetrabromophenol blue
Serum protein
Urine protein
Paper device

ABSTRACT

Background: Paper-analytical devices (PADs) have gained popularity as a simple and low-cost alternative for determining a wide range of analytes including proteins. Even though several colorimetric PADs methods for protein estimation are reported in literature, they lack justification for the chosen method and parameters therein.
Aim: Major aim of this work was to thoroughly evaluate the most commonly used colorimetric protein assays and recommend the most appropriate method for PADs platform.
Method: We performed following six colorimetric protein assays on PADs: biuret, lowry, bicinchoninic acid, bradford, bromocresol green, and tetrabromophenol blue. We obtained assay signal by analyzing images of the PADs and then assessed analytical figures of merit.
Result: Precision, accuracy, LOD, and LOQ of PADs protein assay methods ranged from 1.2 to 6.4%, 73.3–102.4%, 0.3–3.8 mg/mL, and 1.2–12.8 mg/mL, respectively. Out of six methods, we determined bromocresol green and tetrabromophenol blue as the best methods for serum and urine samples, respectively based on their optimized parameters and analytical figures of merit. The total average serum and urine protein in human samples were found to be 94.6 ± 16.2 mg/mL and 2.1 ± 1.5 mg/mL, respectively using PADs methods. The PADs result on human samples moderately correlated with the results from spectrophotometric determination ($r^2 > 0.6$).
Conclusion: Paper-based protein assays were easy to perform and were completed with thousand-fold less volume of samples/reagents without a spectrophotometer compared to conventional assay methods. After testing human samples, we found one protein assay method may not be appropriate for all types of samples.

1. Introduction

Proteins are one of the important classes of biomolecules that perform various functions in human body including repair and maintenance of cells, tissues, and organs as a source of energy [1], regulation of several body processes as hormones [2], regulation of metabolic processes as enzymes [3], transport and storage of certain molecules as transport and storage proteins [4], and defense against disease as antibodies [5]. Thus, the level of protein in human serum and urine has clinical significance. Clinically, the serum protein level of 60–80 g/L and urine protein level of <0.2 g/L are considered normal ranges [6]. Amount of proteins outside normal range is associated with several disease conditions such as inflammation [7], nutritional status [7,8], dehydration [1], kidney disorder [2], liver

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https://doi.org/10.1016/j.plabm.2020.e00166
Received 12 May 2019; Received in revised form 5 January 2020; Accepted 27 April 2020

disorder [3] and others.

Protein levels in bodily fluids are, therefore, tested regularly in clinical settings for diagnosis purpose. There are several analytical methods available that use techniques including UV-vis spectrometry, electrophoresis, mass spectrometry, proteomics, immunoblotting etc. [9]. Choice of a protein analysis method depends on the type of information needed and availability of resources. The most widely used methods for total protein analysis are the colorimetric assays including the protein test strips [10]. Currently practiced colorimetric protein assay methods need a spectrophotometer to measure the amount of total protein, and therefore, are not suitable in field settings. Alternative to the conventional protein assay methods are the paper-analytical devices (PADs) or paper microfluidic platforms that have gained popularity for qualitative and quantitative determination of proteins [11], among other analytes. The PADs provide a new system for fluid handling and analysis in various applications including health diagnostics, environmental monitoring as well as food quality testing [12]. Made from paper, these devices take advantage of ubiquity and compatibility of paper in chemical, biological and medical applications. As a result, PADs offer the advantage of being low-cost, easy-to-use (can be used by non-skilled users), portable and disposable analytical platform for several applications. Additionally, they utilize very less volumes of samples and reagents per assay which significantly reduces cost and produces minimal waste [12]. The PADs protein assay methods can function with thousand-fold less volume of samples and reagents compared to spectrophotometric methods. Amount of analyte in the sample is determined based on a color produced or a change in color in the test zone on paper device due to the assay reaction. While qualitative and semi-quantitative results are obtained by comparing the assay color in color chart provided, smartphones or scanners are used for quantitative results [13]. One of most commonly recognized commercial devices is the paper-based pregnancy test strips [14].

Among several colorimetric methods traditionally been used for total protein analysis, PADs have adopted some of them but without providing justification for the chosen method and the parameters. These reports also lack details of analytical performances of the assays. Moreover, most of these studies report protein assays using reference protein standards or artificial samples. Very few works have reported protein assays on PADs with human serum and urine samples [15–17]. Given that each assay method has its unique analytical performances, it is critical to wisely select a method for an application of interest and target sample.

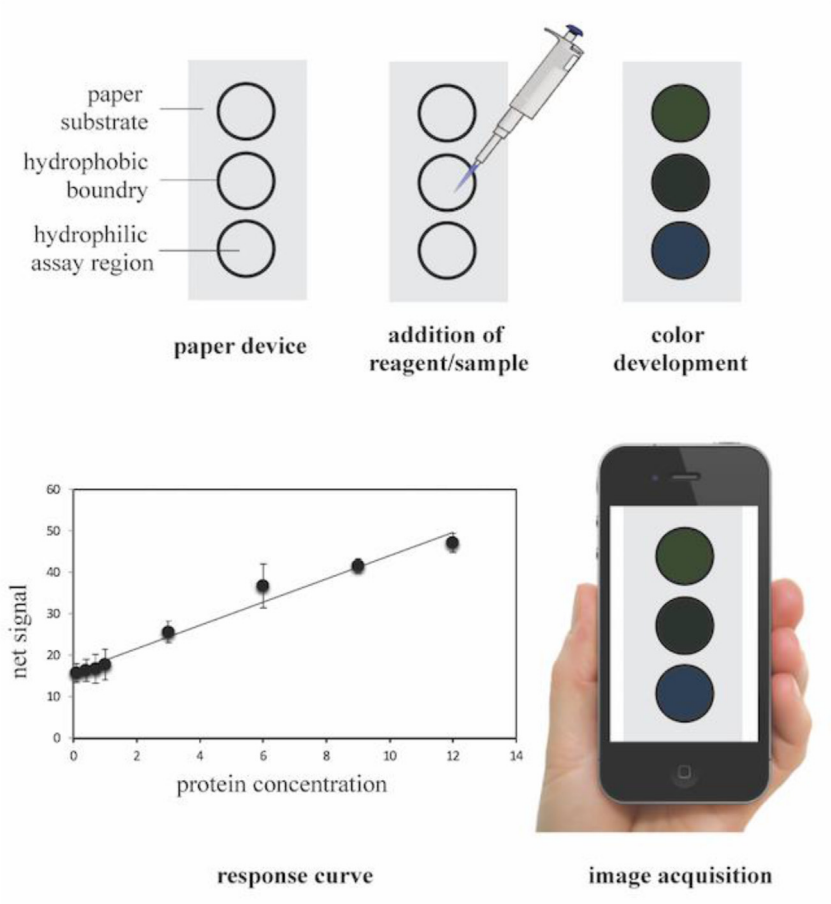

Fig. 1. A general procedure for performing protein assay in paper device. Circular test zones were created on paper substrate and then required reagents and/or samples were added into the test zones using a micropipette. The color developed due to protein assay was then imaged using a camera or phone camera. The color was then quantitated, and data were plotted.

In this paper, we present a thorough evaluation of the most commonly used six colorimetric protein assay methods using PADs and provide a detailed investigation of analytical performance of these methods. We specifically examined three protein-copper chelation methods: Biuret assay [18], Lowry assay [19], and Bicinchoninic acid (BCA) assay [20] and three dye-binding methods: Bradford assay [21], Bromocresol green (BCG) assay [22], and Tetrabromophenol blue (TBPB) assay [23]. We selected the best methods - one each for serum and urine - based on their analytical performances and used those PADs methods to determine the levels of total protein in human serum and urine samples.

2. Experimental

2.1. Materials

Bromocresol green, citric acid, Coomassie brilliant blue (CBB) (G-250), copper sulfate, Folin-Ciocalteau (FC) reagent, O-phosphoric acid, sodium carbonate, sodium hydroxide, sodium potassium tartrate, succinic acid, and tri-sodium citrate were purchased from Thermo Fisher Scientific India Pvt. Ltd., India. Bicinchoninic acid and tetrabromophenol blue were purchased from Carbosynth Limited, Compton, Berkshire, UK. Sodium bicarbonate was purchased from NICE Chemicals Pvt. Ltd., India. Ethanol (>99%) was purchased from Changshu Jangyuan Chemical, China. Bovine serum albumin (BSA) – Fraction V, purchased from Himedia Laboratories Pvt. Ltd., India, was used as standard protein throughout the research. Reagent grade triple deionized distilled water was purchased from Marech Pvt. Ltd., Lalitpur, Nepal. Whatman No. 1 filter paper was purchased from Whatman International Ltd, Maidstone, England, and glass microfiber filter was purchased from VWR International, Pennsylvania, USA. Epoch™ microplate spectrophotometer (Biotek instruments, Inc., USA) was used for spectrophotometric determination of proteins in serum and urine samples.

2.2. Paper-based assays

We chose Whatman No. 1 paper as a substrate for most of our protein assays as recommended by Garcia group [24]. Assay region or test zone on cellulosic paper was created by plotting a permanent Digital Versatile Disc marker (Cello® Permaline) [25] around the reference circle (8.0 mm diameter) resulting in a circle of 5.3 ± 0.2 mm internal diameter. The method of manual cutting using punching machine was utilized to create a test/detection discs of 5 mm diameter in case of glass microfiber-based devices [26,27]. Details of fabrication procedure is given in supplementary information (SI A).

A general procedure for performing assays on PADs is outlined in Fig. 1 and step-by-step procedure for protein assay is given in supplementary information (SI A). In brief, after the test zone was created, reagent and/or sample were added to the test zone using a micropipette. The assay reaction then produced a characteristic color. The image of the device was then captured using a camera or a phone camera which was then analyzed using an image analysis software ImageJ [28] to measure the intensity of color produced. The intensity was plotted against the concentration of standard protein solution to obtain a response curve which was used to study the analytical performance of the assay.

We adopted all six colorimetric reactions on paper following their standard spectrometric assay procedures with some modifications where deemed necessary. Generally, modifications were made on reagent concentration, sample/reagent volume, and assay time. Details of modified procedure of paper-device methods can be found in supplementary material (SI A).

2.3. Image analysis

We used Coolpad CP8298-100 smartphone (13 MP, ~267 ppi, autofocus) and Samsung ES95 (16 MP) digital camera as imaging devices and ImageJ (version 1.50I) for image analysis [28]. We employed the ImageJ software to obtain digital color information from the digital images in RGB color space. A color channel that gave a higher net signal was chosen for each assay [29]. A step-by-step procedure for image analysis using ImageJ is given in supplementary information (SI B). A circular region of interest was drawn inside the detection zone on the digital image to measure the signal. To determine the most suitable color space for net signal, image was split into three different color spaces (R, G, and B) using ImageJ. We measured the mean signal of the selected area in the image and subtracted with blank signal to get net signal of the assay.

2.4. Sample collection

Nagarik Community and Teaching Hospital, Bhaktapur, Nepal provided human serum ($n = 30$) and human urine ($n = 30$) samples. The samples (0.5 mL in a 2 mL vial) were collected and stored in refrigerator until analysis. Ethical approval (Ref. no. 1820, Reg. no. 14/2018) for these samples was obtained from Nepal Health Research Council, Kathmandu, Nepal.

2.5. Data analysis

The data reported was the measure of mean intensity of color developed on paper discs. All tests were performed in triplicate and reported values are mean ± standard deviation (SD). Statistical analyses and the graphs were made in R-language using R-studio (version 1.1.463), a software for statistical analysis [30].

3. Results

We began our experiments by simply testing the colorimetric reactions on cellulosic paper platform. All reactions except for Bradford assay showed characteristic assay color in the presence of protein on cellulosic filter paper. The device substrate plays an important role in such assays as it affects color development, intensity, and uniformity [24]. Evans et al. reported that Whatman No 1 filter paper and Whatman No 1 chromatography grade paper had better color uniformity and intensity than other substrates they tested. However, for Bradford assay, the assay color on filter paper was no different than the color of blank. This implies that the assay may have been interfered by the cellulose in the paper. We used glass microfiber to solve this problem. The glass microfibers have very fine pores, hence are also applicable in assays involving reagent mixing and storing as well [31]. The Bradford assay performed on glass microfiber filter produced a characteristic color which was distinct from the blank. Visual inspection confirmed all six reactions took place. We performed image acquisition and analysis for quantitative analysis of protein assays.

3.1. Image processing and assay optimization

A number of parameters such as image acquisition settings, type of camera, and focal distance impacts reproducibility, sensitivity, and analytical figures of merit of the assay. The selected area on assay region should be as representative as possible without uneven shadows. We studied the optimum distance between camera and paper device by fixing other parameters [32] such as volume and concentrations of fluids used in the assays. The focal distance tested ranged from 10 to 30 cm and 18 cm gave highest net signal (net signal = sample signal – blank signal) (*see* Fig. SI 1). This distance was maintained while taking image of assay in all experiments. Images were taken in ambient light condition in the laboratory. Among the three different color spaces, the Biuret and BCA assays showed higher net signal in green color space while remaining four assays gave higher net signal in red color space.

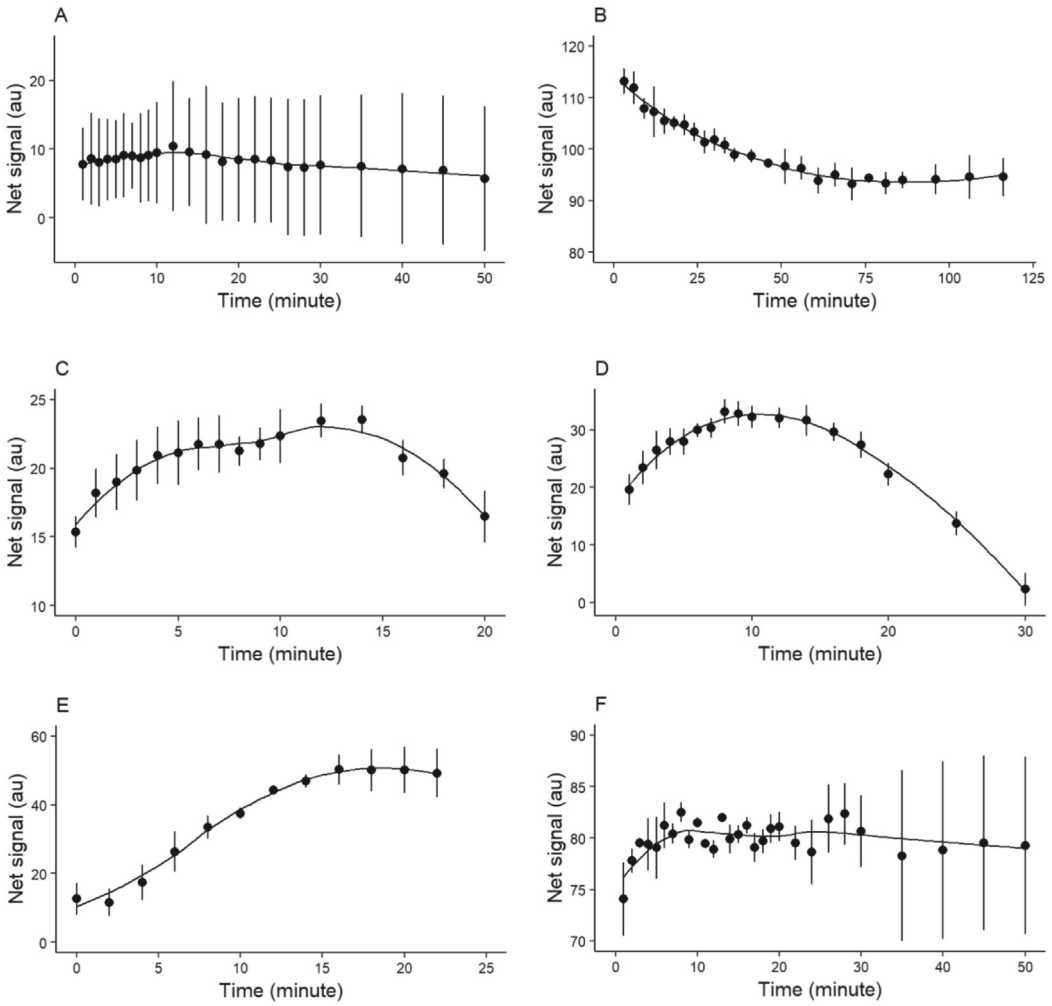

Fig. 2. Variation of protein assay signal with reaction time on paper device (a) Biuret, (b) Lowry, (c) Bradford, (d) BCA, (e) BCG, and (f) TBPB assay. Each data point in all assays are mean ± SD.

We next assessed the appropriate time for capturing an image. This time also tells us the time required for color development or protein reaction and the stability of the color complex. Fig. 2 shows the variation of assay signal with time. The signal was monitored from 20 min up to 120 min. In most of the assays, except Lowry, the signal increased in the beginning, remained stable for some time and decreased later or remained the same (e.g., BCG and TBPB). The decrease in the signal, mostly in protein-dye complex, has been referred to aggregation of the complex [21]. Based on these observations, the optimum time for signal collection were chosen to be 10–20 min depending on the assay type. Optimized imaging time of each assay is given in Table 1.

Details of reagent volumes and their concentrations are given in Table 1. To optimize the volume of colorimetric reagents for the assays, we considered the volume appropriate to completely wet the detection zones and ratios of reagent and sample for higher signal. A smaller volume could not completely fill the detection/reaction zone and a larger volume could drip from the bottom of the device. The volume of reagents tested ranged from 2 to 10 μL and chosen volume of reagents ranged from 3 to 10 μL. The Biuret, Bradford and BCA methods needed higher volume of reagents (9 or 10 μL) while Lowry, BCG, and TBPB assay methods required less volume of respective reagents (only 3 μL). We also determined the appropriate concentration of reagents required for each assay on paper platform. The concentration of reagents has effects on the intensity of assay signal. Range of reagent concentrations tested, and the chosen optimum concentrations are shown in Table 1. For example, the concentration of copper sulfate solution tested for Biuret assay was from 0.15% to 3% and the best concentration was found to be 0.75%. Same copper sulfate solution concentration varied from 1 to 35% and 2% solution gave best result for Lowry assay. The CBB dye used in Bradford assay ranged from 0.01 to 0.5% and the lowest concentration gave best signal. The BCA concentration tested for BCA assay ranged from 1 to 2.5% and middle range concentration was chosen for optimized concentration. Similarly, the chosen concentration of bromocresol green dye was 120 mM in BCG assay, which was based on testing its concentration from 30 to 150 mM. The concentration of TBPB chosen was 3 mM based on its tested concentration range of 0.5–10 mM.

3.2. Analytical performance

After optimizing various parameters described above, we generated protein standard response curves for all six assays using a range of BSA protein concentrations (*see* Fig. 3). The protein concentration tested ranged from 0.05 to 120 mg/mL (*see* Table 2). To determine the linear range of the paper-based protein assays, least-squares linear regression equations were calculated using the experimental data. The determination coefficient (r^2) ranged from 0.954 to 0.980. The sensitivity of the assays was measured as a slope of the regression lines and ranged from 0.04 (Biuret) to 12.07 (TBPB). The Biuret method was the least sensitive [33] (0.04 unit signal/mg protein) among other colorimetric paper protein assay methods examined. This method is considered to be independent of protein composition and is not applicable to single amino acids and dipeptides. The TBPB method was the most sensitive paper-based protein assay producing 12.07 units signal for a given mg protein standard as estimated from the slope of the linear regression best fit line. Here, the sensitivity of the assay is defined as the amount of signal generated per mg change in protein concentration. This analytical parameter measures the ability of an assay to distinguish close concentrations of an analyte with high signal difference. The more the difference the more sensitive the assay is and vice versa.

We also assessed the repeatability of all six paper-based protein assays. The intra-assay precision ranged from 1.2 to 6.4% ($n > 10$) (*see* Table 2). The precision has been reported as relative standard deviations in the measurement. Accuracy was assessed by the spike recovery test. We measured the difference between the determined concentration of the sample and a known concentration of the analyte added. This spike recovery was developed at three known concentrations within the standard curve representing both low and high quantification range. Average recovery values are shown in Table 2 and individual percentage recovery is given in Table 3. The average accuracy ranged from 73.3 ± 28.4% (TBPB) to 102.4 ± 18.9% (BCA). The variability within an assay also varied on the concentration of standard protein sample spiked. For example, for TBPB assay, the accuracy was 53.2% for 1 mg/mL standard and 93.4% for 6 mg/mL standard.

The limit of detection (LOD) and limit of quantitation (LOQ) of the assays were estimated using 3 s/m and 10 s/m formula, respectively where 's' is the standard deviation of the lowest detectable concentration and 'm' is the slope of the calibration curve. The LOD of protein assays ranged from 0.3 mg/mL for BCA to 3.8 mg/mL for BCG. As the Biuret assay was less sensitive and had relatively higher standard deviation in its measurement, this assay was not quantifiable on paper platform. Similarly, the LOQs ranged from 1.2 mg/mL for BCA to 12.8 mg/mL for BCG. Based on the optimization parameters (Table 1) and analytical figures of merit (Table 2), we chose two paper-based protein assays for human samples. The BCG method was selected for samples containing higher amount of

Table 1
Range of parameters tested for six protein assays on paper platform.

Parameters		Biuret	Lowry	Bradford	BCA	BCG	TBPB
Substrate type		Cellulose	Cellulose	Microfiber	Cellulose	Cellulose	Cellulose
Reagent volume (μL)	range tested	3–9	3–9	5–10	6–10	3	2–3
	selected	9	3	10	10	3	3
Reagent concentration	range tested	0.15%–3.0%	1%–35%	0.01%–0.5%	1%–2.5%	30–150 mM	0.5–10 mM
	selected	0.75%	2%	0.01%	1.5%	120 mM	3 mM
Assay time (minute)	range	0–50	0–126	0–20	0–30	0–22	0–50
	selected	12	5	14	8	16	5
RGB color space		Green	Red	Red	Green	Red	Red
Order of fluid addition		Sample first	Reagent first	Reagent first	Sample first	Reagent first	Reagent first

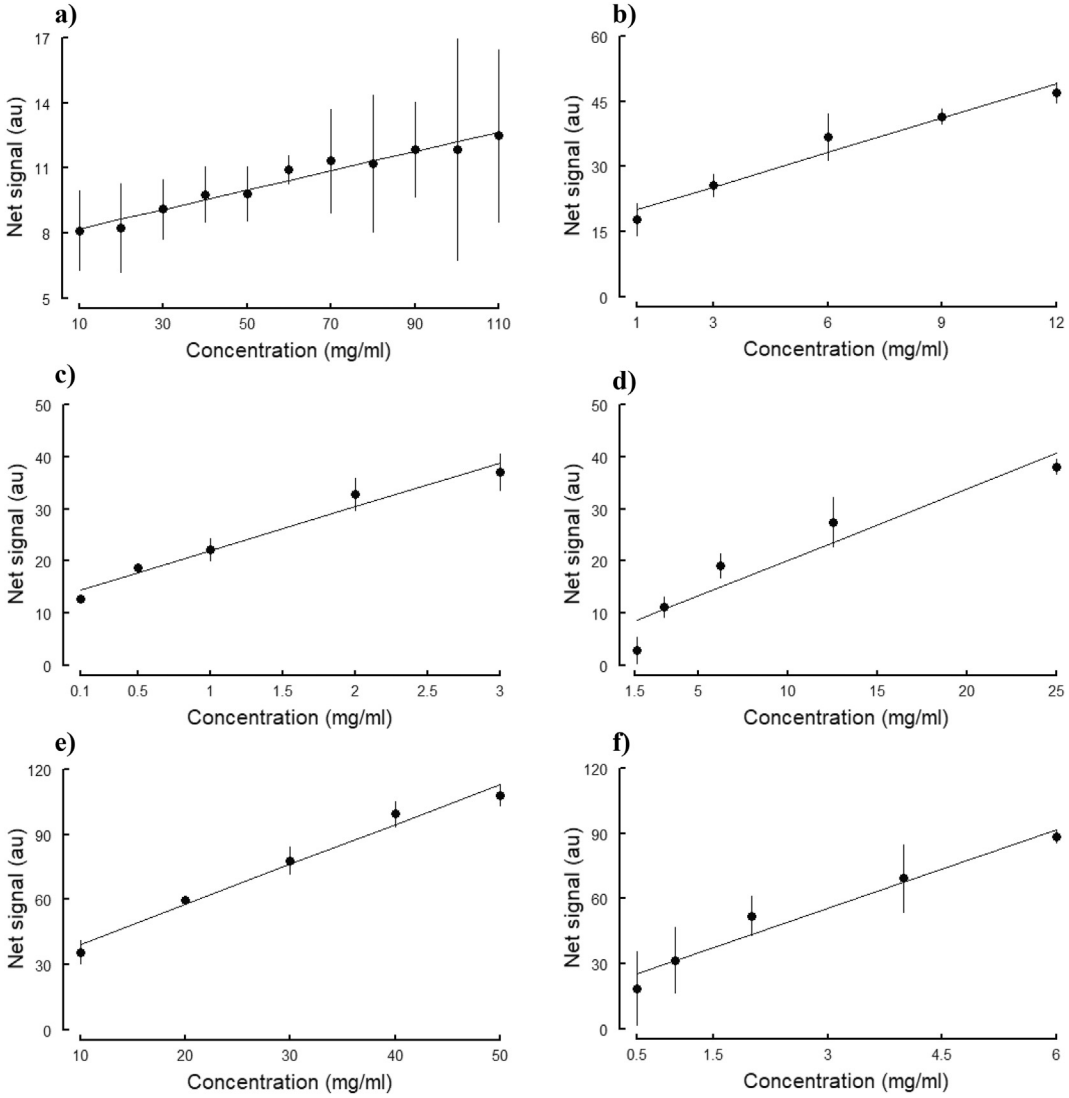

Fig. 3. Response curves using paper device for a) Biuret, b) Lowry, c) BCA, d) Bradford, e) BCG, and f) TBPB method.

Table 2
Analytical figures of merits of six protein assays on paper platform.

	Biuret	Lowry	BCA	Bradford	BCG	TBPB
Protein tested (mg/mL)	5.0–120.0	0.05–15.0	0.097–100.0	0.05–4.0	10.0–100.0	0.5–16.0
Linear range (mg/mL)	10.0–110.0	0.1–12.0	3.1–25.0	0.1–3.0	10–50	0.5–6
r^2	0.960	0.976	0.954	0.967	0.980	0.958
Slope	0.04	2.81	1.16	8.43	1.84	12.07
Precision (%)	3.7	1.6	1.2	3.1	2.9	6.4
Accuracy (%)	92.2 ± 11.9	91.4 ± 15.0	102.4 ± 18.9	94.1 ± 6.4	91.5 ± 8.8	73.3 ± 28.4
LOD (mg/mL)	NA	2.2	0.3	0.5	3.8	0.9
LOQ (mg/mL)	NA	7.3	1.2	1.5	12.8	2.9

protein such as serum samples and TBPB method was selected for samples containing lower amount of protein such as urine samples as the best methods. These findings provided clear justification on the use of BCG and TBPB protein methods as reported in previous literatures [16,34].

3.3. Analysis of serum and urine samples

To verify the applicability of the paper-based protein assays, two best methods were used to determine the protein levels in human

Table 3
Percentage recovery from spike recovery experiments.

N	Spiked (mg/mL)	Estimate (mg/mL)	Recovery (%)
Biuret	30.0	24.7	82.2
	50.0	44.5	89.0
	70.0	73.7	105.3
Lowry	0.8	0.6	76.2
	1.6	1.4	91.9
	6.3	6.6	106.3
Bradford	3.0	3.0	98.5
	1.5	1.5	97.2
	0.8	0.7	86.8
BCA	0.8	1.0	123.4
	1.6	1.4	86.7
	6.3	6.1	97.2
BCG	10.0	8.1	81.5
	30.0	28.4	94.7
	50.0	49.1	98.2
TBPB	1.0	0.6	57.1
	6.0	5.5	91.9

serum and urine samples. We analyzed total protein in 30 human serum samples and 30 human urine samples using BCG method and TBPB method, respectively. The average amount of protein in serum samples was 94.6 ± 16.2 mg/mL (range: 67.8–123.1 mg/mL), which is higher to the normal range of 60–80 mg/mL. However, 13 samples (43%) had protein concentration above 100 mg/mL. The average protein in urine samples was 2.1 ± 1.5 mg/mL (range: not detectable to 5.6 mg/mL). We also ran the same samples of serum and urine on spectrophotometer using same method as a reference. We followed a method described by Doumas et al. [22] for BCG assay and by Flores [23] for TBPB assay in spectrophotometric measurements (*see* calibration curves in Fig. SI 2). The serum samples when tested with spectrophotometric method gave average of 85.9 ± 12.2 mg/mL (range: 66.1–108.8 mg/mL) protein in human serum samples. The results from paper device and spectrophotometer were moderately correlated (Fig. 4a) with $r^2 = 0.76$. However, the paper device method overestimated the protein concentration in serum samples by 10.0 ± 9.7%.

The urine samples when tested with spectrophotometric method gave average of 1.05 ± 0.5 mg/mL protein (range: 0.67–3.15 mg/mL). Even though the results from paper device and spectrophotometer correlate moderately (Fig. 4b) with $r^2 = 0.64$, the paper device method estimated higher protein concentration as determined by paper based TBPB assay compared to spectrophotometric TBPB

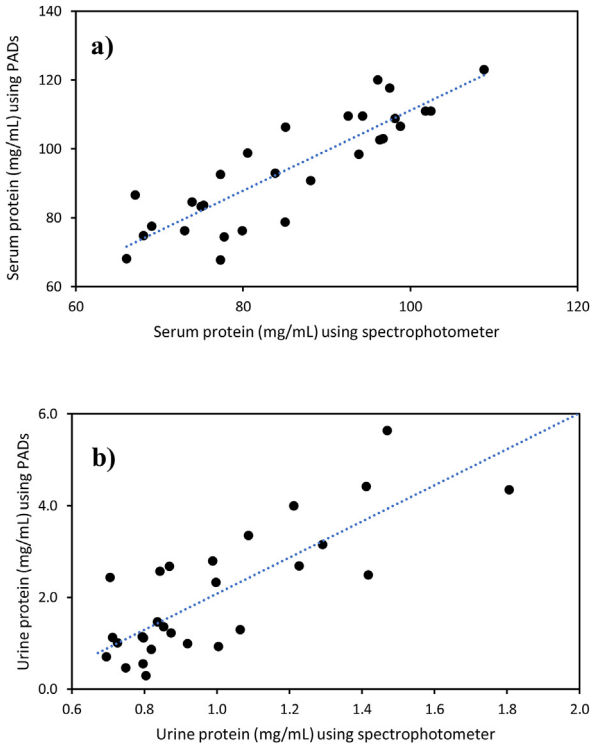

Fig. 4. Comparison of results obtained from paper device and spectrophotometric measurements for a) serum samples and b) urine samples.

method. The overestimation of protein concentration in urine samples by PADs method was on average 81%.

4. Discussion

Several types of papers have been used to fabricate paper-devices, mostly made from cellulosic fibers and few from glass microfibers. The most commonly used papers are the ones that are used for qualitative filtration and chromatographic applications in the laboratories. Filter papers that differ in porosity, thickness, particle retention, filtration speed, and wicking speed [24] are available in the market. Interestingly, property of paper substrate determines the color intensity and uniformity in detection zone of paper device. Among six different papers tested, Garcia group found that Whatman No. 1 filter of chromatographic paper provided faster transfer of solutions and better analytical performances [24]. Glass microfibers have been used as alternative platform in several PADs applications where cellulosic paper was not appropriate. We performed five protein assays on cellulosic paper and remaining one (Bradford) on glass microfiber since the Bradford assay did not produce measurable signal on cellulosic substrate.

Choosing an appropriate color space during image analysis is critical. The color in a photograph is represented in different color spaces such as RGB, CIE L*a*b and HSV. The most commonly used color model is the RGB model. This model assumes every color as a combination of three components: Red (R), Green (G), and Blue (B). The numerical value of color in this model ranges from 0 to 255 [29]. Based on the higher net signal obtained, two types of color spaces were chosen for six protein assays. The signal from assay varies with image taking settings. To minimize the error from lighting conditions, batch-to-batch variation, and camera-to-camera variation, we subtracted the blank signal from the sample signal for each measurement and we have used the term 'net signal = sample – blank'.

All protein assay reactions produced characteristic color when protein molecules reacted with the assay reagent. In Biuret assay, a purplish-violet color complex is formed between the cupric ions in the reagent and the peptide bonds in the protein solution in alkaline medium [33]. Lowry method is a modification of Biuret method. The FC reagent is added after Biuret reaction to get Molybdenum blue-colored product in Lowry method [33] with higher signal [19]. The BCA assay utilizes bicinchoninic acid in addition to the Biuret reagent in alkaline medium producing purple-colored chelation complex that is proportional to the amount of protein in sample [33]. In Bradford assay [21] Coomassie brilliant blue G250 dye binds with protein to form a blue-colored dye-protein complex in acidic condition. The development of blue color is associated with the presence of basic amino acids primarily arginine, lysine and histidine in the protein molecules. The number of Coomassie dye ligands bound to each protein molecule is approximately proportional to the number of positive charges found on the protein. However, free amino acids, peptides, and low molecular weight proteins do not produce color with Coomassie dye. The BCG assay utilizes bromocresol green dye that binds with the protein to produce greenish blue color in acidic medium [22]. Similarly, in TBPB assay, TBPB binds to proteins through a combination of electrostatic and hydrophobic interactions and produce blue-colored complex [23].

Emphasis was given to have the optimized protocol requiring the lowest possible amount of reagents necessary and to obtain the highest possible color intensity. It is obvious that the volume of reagent and sample or standard affects the intensity of the color produced. However, selection of optimum volume also depends on the size of the detection spot/test zone, porosity of paper, and viscosity of fluid. Such volume should be enough to wet the assay zone but should not overflow. One must also consider the fact that high volume of fluid requires longer time to dry, which may significantly increase the assay time.

The paper-based assays overestimated the protein levels in both serum and urine samples when we considered the spectrophotometric method as a reference. The paper assay resulted in $10.0 \pm 9.7\%$ and 81.0% higher protein amount in the serum and urine samples respectively. The difference between paper-device assay method that utilizes image analysis and spectrophotometric method that uses wavelength specific color information may have been contributed from smartphone as signal transducer. Even though smartphones have made significant progress towards their use in chemical and biosensing as optical sensor, the smartphone detection poses substantial limitations. One of the existing limitations is from environmental lighting interference. We minimized such interference by creating similar ambient lighting condition in the lab for every imaging event but that may not have been enough. In addition, as the signal is separated into only three colors, it does not provide wavelength specific information, thereby limiting the sensitivity of the detection method [35]. Moreover, the matrix in urine may have contributed in the higher error while using PADs method. Therefore, the TBPB method that measures low concentration of proteins is not suitable for urine analysis using PADs.

5. Conclusion

In this paper, we presented a thorough investigation of six colorimetric protein assay methods on paper platform using smartphone as signal transducer. Paper-based protein assay methods are easy to perform, require thousand-fold less volume of sample and reagents and do not require expensive spectrophotometer. Out of the six methods, we selected BCG and TBPB methods as the best quantitative methods for total protein in serum and urine samples, respectively based on their analytical performances. Both of these methods provided good precision (2.9% for BCG and 6.4% for TBPB) and accuracy ($91.5 \pm 8.8\%$ for BCG and $73.3 \pm 28.4\%$ for TBPB) in spike recovery experiments. The lower accuracy of TBPB assay was attributed to higher standard deviation in the measurement. The estimated LOD of BCG method was 3.8 mg/mL and linear range of this method was 10–50 mg/mL. Similarly, the estimated LOD of TBPB was 0.9 mg/mL and linear range for this method was 0.5–6 mg/mL. Therefore, the BCG method has been recommended for serum protein analysis and the TBPB method has been recommended for urine protein analysis. When human samples were tested using these paper-based methods, there was a moderate correlation between the PADs method and spectrophotometric methods using same reagents ($r^2 = 0.76$ for BCG method and $r^2 = 0.64$ for TBPB method). Setting the spectrophotometric methods as a reference method, the PADs method for serum samples using BCG was able to give results within $10.0 \pm 9.7\%$ error. However, the TBPB method resulted high error when compared to spectrophotometric methods. The PADs method in its current form, for lower concentration of proteins, may not be

appropriate for testing urine samples.

Declaration of competing interest

There are no conflicts to declare.

CRediT authorship contribution statement

Pravin Pokhrel: Formal analysis, Writing - original draft. **Shashank Jha:** Formal analysis, Writing - original draft. **Basant Giri:** Conceptualization, Supervision, Formal analysis, Writing - review & editing.

Acknowledgements

The work reported in this paper was supported by The World Academy of Science (TWAS), Italy, Grant Number: 15-209 RG/CHE/AS_I- FR3240287080; International Foundation for Science (IFS), Sweden, Grant/Award Number: F/5777-1; and Organization for the Prohibition of Chemical Weapons (OPCW), The Hague.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plabm.2020.e00166.

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