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The pH of chemistry assays plays an important role in monoclonal immunoglobulin interferences

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ABSTRACT

Objectives: Immunoglobulin paraproteins can interfere with multiple chemistry assays. We want to investigate the mechanisms of immunoglobulin interference.
Design and methods: Serum samples containing paraproteins from the index patient and eight additional patients were used to investigate the interference with the creatinine and total protein assays on the Beckman Coulter AU5400/2700 analyzer, and to determine the effects of pH and ionic strength on the precipitation of different immunoglobulins in these patient samples.
Results: The paraprotein interference with the creatinine and total protein assays was caused by the precipitation of IgM paraprotein in the index patient's samples under alkaline assay conditions. At extremely high pH (12–13) and extremely low pH (1–2) and low ionic strength, paraprotein formed large aggregates in samples from the index patient but not from other patients.
Conclusions: The pH and ionic strength are the key factors that contribute to protein aggregation and precipitation which interfere with the creatinine and total protein measurements on AU5400/2700. The different amino acid sequence of each monoclonal paraprotein will determine the pH and ionic strength at which the paraprotein will precipitate.

1. Introduction

Cases of monoclonal immunoglobulin (or 'paraprotein') interference with a wide variety of chemistry and immunochemistry tests on various automated chemistry or nephelometry (including turbidimetry) analyzers have been reported. Besides a few systematic studies—such as paraprotein interference with the measurement of total and direct bilirubin and high density lipoprotein cholesterol (HDL-C) on two automated chemistry analyzers using 88 samples with paraproteins [1], and the study of interference with a direct bilirubin assay using 117 samples containing paraproteins

Abbreviations: ; AMR, analytical measurement range; AU2700, Beckman Coulter AU2700 analyzer; AU5400, Beckman Coulter AU5400 analyzer; CV, coefficient of variation; HCl, hydrochloric acid; HDL-C, high density lipoprotein cholesterol; IFE, immunofixation electrophoresis; IRB, Institutional Review Board; LPL, lymphoplasmacytic lymphoma; NaCl, sodium chloride; NaOH, sodium hydroxide; Cobas 8000, Roche Cobas 8000; SPEP, serum protein electrophoresis; WM, Waldenström's macroglobulinemia

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[2]—the majority of these reports described specific cases of either IgM, IgG, or IgA paraprotein interference with various laboratory tests. There have been several reports of IgM paraprotein interference with albumin [3], glucose [4,5], C-reactive protein [6], uric acid [7], HDL-C [8–10], total bilirubin [8,11], phosphate [5], creatinine [12–15], calcium [16], and total protein [17–19]. Interestingly, each of these cases described individual IgM paraprotein interferences with a particular assay on a particular platform. When other samples containing IgM paraproteins were tested with the same assay, the interferences were typically not observed. When the original samples were tested on different platforms, the interference may or may not be observed [8,11,13–15]. In some reports, the interference was believed to be caused by the precipitation of paraproteins under assay conditions [1,2,5,11]. We have encountered a case of a unique IgMκ paraprotein that exhibited interferences with the creatinine, total protein, and direct bilirubin assays on the Beckman Coulter AU5400 (AU5400) and AU2700 (AU2700) analyzers (Beckman Coulter, Brea, CA).

The patient was a 72 year-old male (index patient) who was referred to UCLA Medical Center for workup of his renal insufficiency indicated by elevated serum creatinine levels which fluctuated between 1.5 and 3.2 mg/dL over a six month period with an unrevealing renal ultrasound study. The initial laboratory evaluation at UCLA revealed an M-spike protein (paraprotein) with a concentration of 3.4 g/dL by serum protein electrophoresis (SPEP) which was identified as IgMκ by immunofixation electrophoresis (IFE). The index patient was diagnosed with Waldenström's macroglobulinemia (WM) supported by immunohistochemical and flow cytometric studies on a bone marrow biopsy sample. Thereafter, serum total protein, creatinine, quantitative IgM, viscosity, and M-spike by SPEP were tested on a regular basis to follow the patient's disease course. About three months after the presentation to our institution, a discrepancy in the patient's total protein results was recognized by the clinical pathologist who was interpreting the patient's SPEP. A total protein result of 7.1 g/dL was reported on the sample which had an albumin result of 3.0 g/dL and IgM concentration of >6.4 g/dL. This finding triggered a laboratory investigation of the total protein and other chemistry tests performed on this sample which revealed that the incorrect low total protein result of 7.1 g/dL was generated by the on-board dilution protocol. When the sample (Sample 1A) was initially tested with AU5400, a total protein result of 10.2 g/dL was obtained which exceeded the upper limit of the validated AMR of 10.0 g/dL. The instrument initiated the rerun with on-board dilution and obtained a result of 7.1 g/dL. This problem was recapitulated when the same sample was repeated on the AU2700 (the AU5400 and AU2700 use the same chemistry assays) with an initial result of 10.2 g/dL and a rerun result of 7.2 g/dL obtained with the on-board dilution. We also found the index patient's creatinine results puzzling, which in contrast to his previous elevated results (both the method and platform are unknown to the authors), became undetectable (below the lower limit of detection) on three occasions after being referred to our institution without having any treatment for renal infufficiency. The creatinine result on Sample 1A was cancelled due to technical difficulties to obtain a consistent result. Therefore, this study investigates the causes of the interference with the creatinine and total protein assays on the AU5400/2700 and identifies the factors that cause the IgM paraprotein to precipitate.

2. Materials and methods

2.1. Patient specimens

Serum samples from the index patient (Subject 1) and randomly selected thirteen other patients (Subjects 2–14) were used in this study. Nine samples from the index patient (Samples 1A–1I) collected from different times, and eleven samples from eight additional patients who had elevated monoclonal IgM (Samples 2A, 2B, 3A, 3B, 3C, 5A–10A), three samples from a patient with elevated biclonal IgMλ and IgMκ (Samples 4A, 4B, and 4C), a sample with elevated monoclonal IgG (Sample 11A), and a sample with polyclonal hypergammaglobulinemia (Sample 12A) were used in the studies of creatinine interference as well as the causes for protein precipitation. In addition, two samples (Samples 13A, and 14A) without elevated immunoglobulins were used as controls for the studies. Note that both numbers and letters are used to name a sample with the numbers denoting subjects and the letters denoting samples collected at different times. This study was performed using de-identified and discarded samples that were obtained for patient care and not for the purpose of research; therefore, Institutional Review Board (IRB) approval was not required.

2.2. Creatinine and total protein assays

Creatinine and total protein were measured on the AU5400/2700 analyzers and the Roche Cobas 8000 (Cobas 8000) (Roche Diagnostics, Indianapolis, IN) respectively. The creatinine assay on the AU5400/2700 uses a modified kinetic Jaffé method under alkaline conditions (pH around 12.7–12.9) and the result is determined by the rate of change of the absorbance at 520 nm. The creatinine reagent contains 120 mmol/L sodium hydroxide (NaOH), 2.9 mmol/L picric acid and some unspecified preservatives in the final reaction. The order of addition for samples and reagents to the reaction cuvette is as follows: 8 μL of the sample, 48 μL of the R1 reagent (containing NaOH), 36 μL of water, and 48 μL of the R2 reagent. The analytical measurement range (AMR) of the creatinine assay is 0.2–25.0 mg/dL. The coefficient of variation (CV) is 0.8% at the creatinine level of 0.93. The creatinine assay on the Cobas 8000 uses an enzymatic method performed under a less alkaline condition (pH around 8.1) with an AMR of 0.06 to 30.5 mg/dL, and CV of 1.3% at the creatinine level of 1.0 mg/dL. Both the AU5400/2700 and the Cobas 8000 use a biuret method for testing total proteins at alkaline conditions (pH around 12–13).

The protein reagent of the AU5400 contains 200 mmol/L NaOH, 32 mmol/L potassium sodium tartrate, 18.8 mmol/L copper sulfate, and 30 mmol/L potassium iodide in the final reaction. The order of addition for samples and reagents to the reaction cuvette for the total protein assay on AU5400 is as follows: 5 μL of the sample, 33 μL of the R1 reagent (containing NaOH), 90 μL of water, and 33 μL of the R2 reagent. Our validated AMR is 0.2–10.0 g/dL. The CV is 0.5% at the protein level of 9.5 g/dL.

2.3. Other tests

IgM, IgG and IgA levels were determined by immunonephelometric methods on Siemens Dimension Vista 1500 (Siemens Healthcare Diagnostics, Tarrytown, NY). SPEP and IFE were performed using the Sebia HYDRASIS 2 system (Sebia, Norcross, GA). The concentration of M-spike was determined from the product of the relative intensity of the band of the protein in the SPEP electrophoretogram and the concentration of total protein obtained with AU5400.

2.4. Removing IgM paraproteins

Three samples from the index patient (Samples 1B, 1F, and 1H), one sample with IgMλ paraprotein (Sample 2A), and one sample with normal immunoglobulins (Sample 13A) were used in this study. The average molecular weight of an IgM molecule is 900 kDa [20]. Ultrafiltration using Amicon Ultra 100 K (100 kDa Molecular weight cutoff) Centrifugal Filters (EMD Millipore, Merck KGaA, Darmstadt, Germany) at 5000 *g* for 20 min can remove IgM proteins and other proteins with higher molecular weight from patient samples. Creatinine and total protein were measured with AU5400/2700 on these samples before and after ultrafiltration.

2.5. Experiments with pH and ionic strength

Solutions with pH ranging from 1 to 13 were prepared using either NaOH or hydrochloric acid (HCl) in deionized water. Knowing that the first step of the creatinine assay on the AU5400 is the mixing of 8 μL of patient sample with 48 μL of the R1 reagent, we manually performed this step in a 10-fold enlargement in test tubes by mixing 80 μL of patient sample with either 480 μL of the R1 reagent or 480 μL of each of the pH solutions in order to observe the reaction. The R1 reagent of creatinine assay contains NaOH which determines the pH of the reaction. We observed protein precipitates and/or aggregates after addition of the R1 reagent, which did not re-dissolve in solution when water or the R2 reagent was added to the mixture. Thus, the ratio of sample to pH solution (1:6) in these experiments was identical to the ratio of sample to R1 reagent (1:6) in the creatinine assay.

Solutions containing 0, 20, 40, 100, and 140 mM of sodium chloride (NaCl) at neutral pH (pH 7) were prepared. One of the index patient's samples (Sample 1I), one sample with biclonal IgMλ and IgMκ (Sample 4C), and one sample without paraprotein (Sample 13A) were used in this study. Eighty μL of patient sample was mixed with 480 μL of the NaCl solutions (the same sample to reagent ratio of 1:6) in test tubes. The tubes were similarly examined to look for precipitates and/or aggregates.

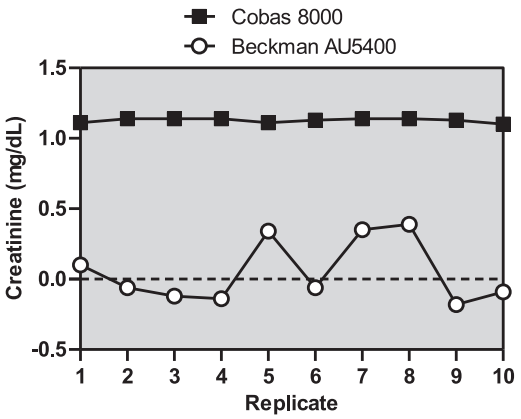

Fig. 1. Ten replicate creatinine results for Sample 1B from the index patient obtained with the Beckman Coulter AU5400 (open circles) and the Roche Cobas 8000 (filled squares).

Table 1
Creatinine results obtained with the Beckman Coulter AU5400 in samples from the index patient and nine other patients.

Subject[a]	Sample ID[b]	Time interval between collections[c]	Type of paraprotein	IgM concentration (mg/dL)	Replicates of creatinine (mg/dL)		
1	1A	First	IgMκ	7120	1.31	1.35	−1.85
	1B	199 days		4150	0.14	0.08	0.09
	1C	34 days		4040	0.63	0.77	0.35
	1D	0 day		*n.d.*	0.41	0.72	0.73
	1E	23 days		*n.d.*	0.42	0.41	0.03
	1F	46 days		4130	0.53	−0.15	−0.25
	1G	0 day		*n.d.*	−0.01	−2.00	0.56
	1H[d]	30 days		> 6400	−0.09	−2.61	−5.35
					−0.34	2.50	−0.02
					−2.38	−0.78	−0.91
2	2A	First	IgMλ	2150	0.70	0.67	0.68
	2B	27 days		2410	0.77	0.75	0.75
3	3A	First	IgMκ	> 6400	1.35	1.38	1.34
	3B	457 days		> 6400	1.51	1.45	1.45
	3C	41 days		> 6400	1.75	1.84	1.80
4	4A	First	IgMκ and IgMλ	> 6400	1.85	1.67	1.70
	4B	35 days		> 6400	1.62	1.83	1.79
	4C	3 days		3890	1.67	1.60	1.57
5	5A	First	IgMκ	2250	1.51	1.45	1.40
6	6A	First	IgMκ	3320	1.26	1.28	1.45
7	7A	First	IgMκ	2180	1.21	1.21	1.20
8	8A	First	IgMκ	2340	0.89	0.91	0.92
9	9A	First	IgMλ	2030	0.84	0.79	0.78
12	12A[e]	First	Polyclonal	170	10.50	10.20	10.40

n.d., not determined.
[a] Subject numbers denote different patients; Subject 1 is the index patient.
[b] Letters denote samples collected at different times from the same patient.
[c] Time interval between the collection of the sample and the collection of the previous sample; 'First' indicates the first sample encountered for each patient by this study.
[d] Creatinine results were measured for a total of nine replicates for this sample.
[e] Patient had polyclonal hypergammaglobulinemia, IgG = 2200 mg/dL.

3. Results

3.1. IgM interference with the creatinine and total protein assays on the AU5400/2700

As a first approach to our investigation, we repeated creatinine measurement in duplicate with the AU2700 on Sample 1A and obtained values of 3.78 and 2.08 mg/dL, which was strikingly different than the previously reported undetectable results obtained with the same instrument in our laboratory. Due to sample quantity constraints of Sample 1A, we used another sample from the index patient (Sample 1B; IgM, 4150 mg/dL; viscosity, 2.19 cP; M-spike, 2.8 g/dL) collected 199 days after Sample 1A and measured creatinine and total protein 10 times with both the AU2700 and the Cobas 8000, respectively.

The mean of the ten replicates of the total protein results on Sample 1B obtained with the initial protocol (undiluted) on the AU2700 was 8.4 g/dL with a CV of 0.6%, similar to the mean of 8.86 g/dL and a CV of 2.5% obtained with the Cobas 8000. The concordant total protein results between the AU2700 and the Cobas 8000 showed that the measurements of total

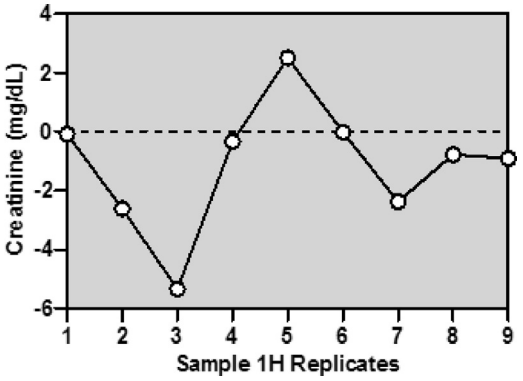

Fig. 2. Nine replicate creatinine results obtained with AU5400 on sample 1H from the index patient.

protein with AU5400/2700 using the initial protocol (undiluted) were not affected by the IgM paraprotein in the index patient's sample. Therefore the interference was constrained to the on-board dilution for the total protein assay on the AU5400/2700 analyzers.

The 10 replicates of creatinine results obtained with the AU5400 and Cobas 8000 are plotted in Fig. 1. The results with AU5400 ranged from −0.18 to 0.39 mg/dL with a mean of 0.065 mg/dL and a CV of 340% which greatly exceeded the assay's imprecision (CV of 16% at creatinine level of 0.05 mg/dL) and was in sharp contrast to the more consistent results obtained with the Cobas 8000 (mean = 1.13 mg/dL, CV = 1.4%).

To determine if other IgM paraproteins would interfere with the Jaffé creatinine assay on the AU5400, we measured creatinine in triplicate on 13 samples from 8 patients who had elevated monoclonal IgM immunoglobulins (Samples 2A–9A in Table 1), one sample from a control patient who had polyclonal hypergammaglobulinemia (Sample 12A in Table 1), along with 8 samples from the index patient (Samples 1A–1H in Table 1). Results are listed in Table 1 and the random fluctuating creatinine results of Sample 1H from the index patient are plotted in Fig. 2. In contrast to the 8 samples from the index patient, none of the other patient samples demonstrated fluctuating or spurious creatinine results. Therefore, the particular IgM paraprotein in the index patient's sample appears unique in that it was the only one that interfered with the kinetic Jaffé method on the AU5400/2700.

3.2. Precipitation of IgM paraprotein caused the interference

To better understand the mechanisms of the interference with the total protein and creatinine assays for the AU5400/2700 in the index patient's sample (Sample 1A), both assays were performed in test tubes using 10-times more volume of the sample and the respective reagents as that required by the assay reactions on AU5400. When 40 μL of Sample 1A was mixed with 330 μL of the R1 reagent for the total protein assay, a large bulky protein aggregate was observed. Similarly, large protein aggregates and tiny precipitates were observed when 80 μL of Sample 1A was mixed with 480 μL of the R1 reagent for the creatinine assay. These aggregates did not re-dissolve in solution when water and the R2 reagent of the creatinine assay were added thereafter. Similar precipitates and aggregates were also observed when additional samples from the index patient were tested. However, precipitates were not observed in control samples that had normal immunoglobulin levels. This indicates that the total protein and the creatinine assays of the AU5400 do not cause protein precipitation in samples with normal immunoglobulin levels. It also provided evidence that the IgM paraprotein in the index patient's samples aggregated and precipitated under the assay conditions.

Next, we determined if removal of IgM paraproteins from the index patient's sample using ultrafiltration would resolve the interference with the AU5400/2700 creatinine assay. Table 2 lists creatinine and total protein results before and after ultrafiltration measured with AU5400 on three samples from the index patient collected on different dates (Samples 1B, 1F, and 1H), one sample from a patient with IgMλ paraprotein which did not interfere with the AU5400/2700 creatinine assay (Sample 2A), and one control sample from a patient who did not have paraproteinemia (Sample 13A). In all of the samples, total protein after ultrafiltration was reduced to 0.5 g/dL or less, indicating successful removal of IgM and other big proteins. In the original samples of the index patient, the creatinine results were low but with marked random variation (−0.81 to 0.18 mg/dL). The creatinine results after ultrafiltration were more consistent (0.92 to 1.01 mg/dL; Table 2) and similar to the results obtained with the enzymatic method with Cobas 8000 (around 1.1 mg/dL). Creatinine results in the other two patient samples were not significantly affected by the removal of proteins (Table 2).

3.3. Effect of pH and ionic strength on the precipitation of paraproteins

The effect of pH on the formation of protein aggregates and/or precipitates was tested in one of the index patient's samples (Sample 1I), one sample with biclonal IgMλ and IgMκ paraproteins (Sample 4C), one sample with a monoclonal IgMκ (Sample 10A), one sample with a monoclonal IgGκ (Sample 11A), and two samples without paraprotein (Samples 13A

Table 2
Creatinine and total protein results obtained with the Beckman Coulter AU2700 on samples before and after ultrafiltration.

Sample ID[a]	IgM (mg/dL)		Creatinine (mg/dL)		Protein (g/dL)	
	Original	Filtrate	Original	Filtrate	Original	Filtrate
1B[b]	4150	176	−0.81	0.92	8.2	0.3
1F	4130	182	0.18	1.01	8.4	0.5
1H	> 6400	217	−0.71	0.95	9.1	0.5
2A[c]	2150	73	0.66	0.52	6.6	0.3
13A[d]	308	< 5	1.01	0.90	7.2	0.3

[a] Numbers in Sample ID denote individual patients and letters denote samples collected on different dates.
[b] Samples 1B, 1F and 1H were from the index patient.
[c] Patient had IgMλ paraprotein.
[d] Patient did not have paraproteinemia.

Table 3
Effect of pH on the precipitation of paraproteins.

Patient with	IgMκ (index patient)	Biclonal IgMκ, IgMλ	IgMκ	IgGκ	No M-protein	No M-protein
Sample ID[a]	**1I**	**4C**	**10A**	**11A**	**13A**[b]	**14A**[b]
IgM (mg/dL)	3940	3890	4130	132	308	179
IgG (mg/dL)	*n.d.*	*n.d.*	360	2840	521	944
pH 1	Aggregate	Clear	Clear	Clear	Clear	Clear
pH 2	Aggregate	Clear	Clear	Clear	Clear	Clear
pH 3	Tiny precipitates	Tiny and large precipitates	Clear	Hazy	Hazy	Clear
pH 4	Tiny precipitates	Clear	Clear	Clear	Clear	Hazy
pH 5	Tiny precipitates	Clear	Clear	Clear	Clear	Clear
pH 6	Tiny precipitates	Clear	Clear	Clear	Clear	Clear
pH 7	Tiny precipitates	Clear	Clear	Clear	Clear	Clear
pH 8	Tiny precipitates	Clear	Clear	Clear	Clear	Clear
pH 9	Tiny precipitates	Clear	Clear	Clear	Clear	Clear
pH 10	Clear	Clear	Clear	Clear	Clear	Clear
pH 11	Clear	Clear	Clear	Clear	Clear	Clear
pH 12	Aggregate	Clear	Clear	Clear	Clear	Clear
pH 13	Aggregate	Clear	Clear	Clear	Clear	Clear

n.d., not determined.
[a] Numbers in Sample ID denote individual patients and letters denote samples collected on different dates.
[b] Patients did not have paraproteinemia.

and 14A). The results of the pH experiment are shown in Table 3. In the index patient's sample (Sample 1I), large protein aggregates were observed at extremely alkaline pH (12–13) and extremely acidic pH (1–2) and tiny precipitates were observed at pH 3–9. In Sample 4C, large and tiny protein precipitates were observed only at pH 3. In Sample 10A, no precipitate was observed at any pH. In Sample 11A, except for a hazy appearance at pH 3, no precipitate was observed at any other pH. Similarly, in normal control samples (Samples 13A and 14A), except for a hazy appearance at pH 3–4, no precipitate was observed at any other pH. The results showed that the IgMκ paraprotein in the index patient's sample is unique and different immunoglobulin paraproteins react to pH differently.

The effect of ionic strength on the precipitation of IgM paraproteins was studied using solutions with various concentrations of NaCl at neutral pH. The results of the ionic strength experiment are listed in Table 4. Tiny precipitates were observed in the index patient's sample (Sample 1I) when added to solutions containing low concentrations of NaCl (0–20 mM), but not in solutions containing higher concentrations of NaCl (higher than 40 mM). No precipitates were observed in Sample 4C and Sample 13A at any of the concentrations of NaCl tested.

4. Discussion

Herein, we present a case of a unique IgM paraprotein which interfered with the modified kinetic Jaffé creatinine and the biuret total protein methods on the AU5400/2700 by forming large protein aggregates under assay conditions. The protein aggregates/precipitates suspended in a solution can scatter light and interfere with the measurements of the absorbance. Because the concentration of immunoglobulin paraprotein is generally high (7–65% of the total protein in serum), the aggregation or precipitation of monoclonal immunoglobulins can change the physical–chemical properties such as turbidity of the solution and cause significant alterations of the measurements of the transmitted or scattered light. If the precipitates are small and evenly suspended in the solution, the errant light scattering may be corrected by background subtraction. However, larger aggregates may not be corrected by background subtraction. The aggregates can randomly move in and out of the light path causing a fluctuation pattern in the measured absorbance. This can be seen in the marked random fluctuating pattern of the creatinine results obtained with the AU5400, as illustrated in Fig. 1. Fluctuating patterns in direct bilirubin results obtained with the AU5400 associated with immunoglobulin paraprotein interference was also reported by

Table 4
Effect of ionic strength on the precipitation of paraproteins.

Sample ID NaCl (mM)	1I IgMκ (Index Patient)	4C Biclonal IgMκ, IgMλ	13A No M-spike
0	Aggregated	Clear	Clear
20	Aggregated	Clear	Clear
40	Clear	Clear	Clear
100	Clear	Clear	Clear
140	Clear	Clear	Clear

Yang et al. [1] and Song et al. [2] respectively. The uncontrolled variation in light scattering will generate irreproducible and erroneous results especially when the final result is determined from the difference between two measurement points such as the kinetic Jaffé method for creatinine or between two reaction cuvettes such as the direct bilirubin method. Due to the random light scattering by the large protein aggregates, the absorbance of an earlier measurement or of the blank can be higher than the later measurement or of the reaction. In the kinetic Jaffé method for creatinine assay, the result is calculated from the difference in absorbance measured at two predetermined time point. If the first absorbance exceeds the second absorbance due to random light scattering of protein aggregates, not due to the colorimetric reaction itself, the result can be negative. The direct bilirubin assay on AU5400 uses two cuvettes one for a blank and one for the reaction. If the absorbance of the blank cuvette is higher than that of the reaction cuvette due to random light scattering of protein aggregate, the result calculated from the difference between the two cuvettes can be negative. Therefore, an incorrect result with any value can be obtained. Indeed, this would explain the spurious and fluctuating creatinine results including the negative ones obtained in the index patient's samples in our experiments and the incorrect fluctuating results from his previous laboratory tests.

As for the index patient's IgM paraprotein interference with the total protein assay on AU5400, it appeared to happen only to the on-board dilution protocol in which the instrument pipets 4 μL (instead of 5 μL) of a sample and mixes it with the same amount of reagents as that used for the initial protein assay. The only difference between the initial protein assay and the on-board dilution protocol is in the sample volume. We initially posited that the sample probe may not be capable of accurately pipetting the smaller volume of sample when serum viscosity was highly elevated (4.58 cP, reference interval 1.10–1.80 cP). However this appears unlikely as the instrument can accurately pipet 1.5 μL of the same sample for the albumin assay on the AU5400/2700. The initial total protein result of 10.2 g/dL was corroborated by duplicate measurements with the Cobas 8000 which yielded results of 10.2 and 11.0 g/dL. We cannot explain why the problem was confined to the on-board dilution protocol but speculate that the smaller sample volume happens to either speed up or slow down the aggregation of IgM paraproteins in a way that alters the measurement of absorbance at the time point that is used to determine the result.

Having noted that the pH of both of the total protein and creatinine assays are around 13, we speculated that it was the high pH of these assays that caused the IgM paraprotein in the index patient's sample to precipitate. Thus, we tested and obtained evidence that the extreme alkaline conditions (around pH 13) of both the creatinine and total protein assays of the AU5400 caused the IgMκ paraprotein in the index patient's sample to form large aggregates. By adding the index patient's sample to solutions with different pH at low ionic strength (NaCl was not added) in the same ratio as that of the sample to the R1 reagent of the creatinine assay of the AU5400, we were able to demonstrate the formation of large protein aggregates under extremely alkaline (pH 12–13) and extremely acidic (pH 1–2) conditions, as well as the formation of tiny precipitates at pH 3–9. In addition, low ionic strength (0–20 mM NaCl) at neutral pH also caused this IgMκ paraprotein to precipitate. As noted by Chi et al., pH has a strong influence on the rate of protein aggregation while ionic strength affects the stability of protein conformation and alters protein solubility in aqueous solutions in a complex manner [21]. The index patient's IgM paraprotein appears somewhat unique, as none of the other IgM paraproteins tested in this study demonstrated similar problems with the creatinine or total protein assays on AU5400/2700. Our hypothesis is that this particular IgM protein likely contains multiple opposite charged or polar amino acids such that at extremely high (pH 12–13) or low (pH 1–2) pH, the protein is highly charged, leading to increased repulsions within the folded protein molecules that result in protein unfolding which favors large protein aggregation through hydrophobic interactions. When pH is mildly increased or decreased (e.g. pH 3–9), the dipole-dipole interactions on the surface of the proteins in the absence of salt bridge could cause the protein to aggregate [21]. The pH experiments in this study showed that the index patient's IgM paraprotein was soluble only at pH 10–11 and otherwise insoluble over the remainder of the pH range tested, including pH 7.4. This appeared to be contradictory to the notion that this IgM paraprotein was soluble in plasma at physiologic pH of 7.4. This is because the ionic strength of our pH experiments was low (no NaCl was added), whereas the ionic strength is higher (140 mM NaCl) under the physiologic conditions of plasma which functions as salt bridge to stabilize protein conformations and increase protein solubility. We are unable to get the information related to the ionic strength of the creatinine and total protein assays on the AU5400/2700. In this regard, we assumed the ionic strength is relatively low for both assays, which resulted in the decreased solubility of this particular IgM paraprotein under the assay conditions. The precipitation of monoclonal immunoglobulins under assay conditions is considered one of the mechanisms of paraprotein interference with chemistry assays [22–26]. Our findings support the theory. Furthermore, we believe the precipitation of immunoglobulins is the main cause for immunoglobulin interference with chemistry assays. The results of our study strongly suggest that assay pH and low ionic strength are the two important factors which can induce protein precipitation that ultimately interferes with the assay. Another example of paraprotein interference is the direct bilirubin assay on the AU5400/2700 which has a very low pH (pH 1–2). We also observed interference in the direct bilirubin assay on the AU5400/2700 in the index patient's sample and our pH experiment showed that this particular IgM paraprotein precipitated at pH 1–2. The immunoglobulin paraprotein interference with the direct bilirubin assay on AU5400/2700 has been investigated previously and the interference rate was reported to be 17.1% in samples with monoclonal immunoglobulins [2]. Further review of the chemistry assays on AU5400/AU2700 revealed that the pH for the creatinine (Jaffé method), total protein, and lithium assays are very alkaline (pH 12–13), and for the direct bilirubin, phosphorous, and iron are very acidic (pH 1–2). The extreme pHs of these assays can be used to explain why the majority of the reported cases of immunoglobulin paraprotein interference were associated with these assays. Therefore, our pH experiments and these previous reports together support our conclusion that pH of chemistry assays is a key factor for immunoglobulin interference.

We are able to show that different immunoglobulin paraproteins react to assay pH differently. For example, the sample with biclonal IgMλ and IgMκ paraproteins (Sample 4C) did not precipitate at extremely high or low pH, but did so at pH 3. The amino acid composition of each immunoglobulin determines the isoelectric point of the protein. Individual immunoglobulin paraproteins have unique amino acid sequences and thus different set points for conformational changes in response to changes in pH and ionic strength. Thus, there is always a possibility that a particular immunoglobulin will precipitate under a particular assay condition, whether basic or acidic, or even neutral pH. It would be very difficult to condition an assay that will prevent all paraproteins from precipitation. Manufacturers should be aware of the possibility of immunoglobulin paraprotein precipitation when they develop assays for clinical laboratories. For example, if a reaction must be carried out at extremely high (or extremely low) pH, increasing the ionic strength or adding protein stabilizers or surfactants, provided that they do not significantly affect the test reactions, may help to prevent most paraproteins from precipitating. In this regard, some manufacturers are aware the complex nature of the interference of immunoglobulin and attempted to optimize their assays in order to minimize the problem of paraprotein precipitation [24]. This may partially explain why the IgMκ paraprotein from the index patient presented in this study did not interfere with the total protein assay on the Cobas 8000, which has an assay pH around 13 similar to that of the total protein assay on the AU5400/AU2700. We believe that the majority of the chemistry assays are optimized in order to minimize the problem of paraprotein precipitation to certain degree by most of the manufacturers. That is why only a small percentage of paraprotein immunoglobulins, but not all of them, precipitate under a specific assay condition. Because assay conditions are likely to vary on different platforms, a sample having problems with one method may not have the same problem with a different method or platform.

It is difficult for a clinical laboratory technologist working with an automated chemistry analyzer in a busy clinical laboratory to know the presence of a paraprotein in a sample, unless being specifically alerted to its presence. It would be very difficult for him or her to determine whether or not a result is valid and free from paraprotein interference. However, understanding how paraproteins interfere with an assay will help to identify the problem. For example, a negative result, a part of an analyte (such as direct bilirubin) is higher than the total concentration of the analyte (such as total bilirubin), or a fluctuating pattern upon repeat measurements can serve as a flag. An inconsistent protein result between the direct and diluted measurements can also serve as a flag. If the interference from monoclonal immunoglobulins is suspected, ultrafiltration could be used to remove large proteins prior to certain assays that are not affected by removal of proteins, such as creatinine as demonstrated in this study. Once the interference is identified, a different method or platform can be used and the clinical team should be alerted. Recently, a new “abnormal reaction data-detecting” function on an automated chemistry analyzer, BM2250 (supplied by JEOL Inc. in Japan, and ADVIA 2400 by Siemens Healthcare Diagnostics Inc. in other countries), has been tested and reported to be useful in detecting the abnormal turbidities caused by immunoglobulin precipitations [27]. If this new function does not significantly affect the throughput of the machine, it may help a clinical laboratory to identify samples with paraproteins that interfere with chemistry assays.

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