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Deproteinization of Serum: Another Best Approach to Eliminate All Forms of Bilirubin Interference on Serum Creatinine by the Kinetic Jaffe Reaction

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The negative interference of conjugated, unconjugated, and delta bilirubin on patient serum creatinine determined by the kinetic Jaffe reaction is the unresolved problem. We compared bilirubin interference on thirty patients' serum creatinine obtained from four analyzers, with and without deprotenization before the Jaffe reaction, to the Vitros dry enzymatic method. We found significant negative interference from bilirubin on serum creatinine in all samples directly applied to four wet chemical methods, except the one incorporated with serum blank rate. The negative interferences linearly related to bilirubin concentration. However, bilirubin did not interfere on serum creatinine obtained from all wet chemical methods incorporated with deproteinization process before the reaction. We conclude that deproteinized serum before the reaction is the best approach to eliminate all forms of bilirubin interference on serum creatinine determined by the kinetic Jaffe reaction. J. Clin. Lab. Anal. 15:116–121, 2001.

Key words: deproteinization; serum creatinine; kinetic Jaffe reaction; bilirubin interference; bilirubin elimination; dry chemistry; wet chemistry

INTRODUCTION

Serum creatinine is a useful index of kidney function. Its concentration reflects glomerular filtration rate (1). At present, most discrete chemical analyzers use the direct kinetic Jaffe reaction to assay creatinine in patient serum because there is no need to remove protein from the sample before the reaction (2). The kinetic method can correct interference from slow-reacting non-creatinine chromogens such as glucose, acetone, and ascorbic acid (2,3). However, fast-reacting substances such as alpha-keto compounds and cephalosporin antibiotic give positive interference (4), while serum bilirubin gives negative interference on creatinine results (1,5).

Bilirubin interference with serum creatinine measurements is still a serious concern for clinical labs. The interference can have clinical significance as we use the creatinine level to monitor the effect of nephrotoxic drugs and adjust the dose of drugs that excrete through the kidney such as aminoglycosides. It is common to see bilirubin of 171–257 μmol/l (10–15 mg/dl) in premature and newborn infants (6). Our previous study (7) showed that bilirubin at 171 μmol/l could significantly depress an abnormal creatinine of 85 μmol/l (1.0 mg/dl) in infants and small children to a normal creatinine value of 42 μmol/l (0.5 mg/dl). This may mislead the clinician in prescribing the same dose of the drugs. The overdose of aminoglycosides may cause permanent deafness in the patient.

There are several techniques to overcome bilirubin interference on serum creatinine by the kinetic method. Oxidation of bilirubin to biliverdin using potassium ferricyanide can minimize bilirubin's interfering effect (7,8). Incorporation of sodium dodecyl sulfate promotes the release of bilirubin from albumin and allows full bilirubin oxidation to biliverdin by alkaline buffer (7,9). Preincubating samples with oxidant, to oxidize bilirubin completely to biliverdin before the Jaffe reaction, is also efficient (7,10). These techniques work well on icteric samples prepared by adding purified unconjugated bilirubin in pooled serum (7). The preincubation technique did not work uniformly on icteric patient sera containing conjugated, unconjugated, and delta bilirubin (7). Because the preincubation technique can correct bilirubin interference in 51% of patient sera (7), removing bilirubin along with albumin by trichloroacetic acid beforehand was suggested as an approach to correct the bilirubin interference (7).

Unconjugated, conjugated, ditaurobilirubin, and delta bilirubin do not interfere on serum creatinine determined by the CRSC Vitros dry chemical analyzer (11). Bilirubin, primarily protein bound, is not allowed to penetrate the spread-

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ing membrane layer to react with enzymatic creatinine reagent layers. There is good correlation of serum creatinine values obtained from the Vitros CRSC to the reference HPLC creatinine in icteric samples (12). Some consider Vitros CRSC the method of choice for creatinine analysis because it is free from bilirubin interference (13). Therefore, the separation of bilirubin along with albumin—either through the membrane layer before the enzymatic reaction (11–13) or by manual acid deproteinization (7,14)—before the kinetic reaction seems to be the best approach to correct bilirubin interference on serum creatinine.

The objective of this study is to prove that use of a deproteinization process before the kinetic reaction by trichloroacetic acid is one excellent approach to correct all forms of bilirubin interferences on serum creatinine. Bilirubin interferences on creatinine in patient sera obtained from five kinetic wet chemical methods, with and without deproteinization, are compared to the Vitros dry enzymatic method. Guidelines to the use of the correcting techniques in clinical labs are also suggested.

MATERIALS AND METHODS

We obtained thirty sera from pediatric and adult patients with low, middle, and high bilirubin and creatinine from our clinical chemistry lab. Total (56–863 µmol/l or 3.2–50.1 mg/dl) and direct (39–714 µmol/l or 2.3–41.4 mg/dl) bilirubin were determined by the Jendrassik & Grof methods (15) on the Beckman Synchron CX5 (Beckman Instrument, Diagnosis System Group, Brea, CA). We kept the samples at –20°C until use.

We precipitated protein in each serum with 0.55 mol/l trichloroacetic acid (Merck, Darmstadt, Germany) at the ratio of 2:1 (sample volume: reagent volume). We mixed and allowed to stand for 10 min, then centrifuged at 1200*g* for 10 min. The supernatant of each sample was removed and subjected to creatinine analysis.

Creatinine measurements in each serum and supernatant were performed on three analyzers: the Synchron CX5, the Technicon AXON (Miles Inc., Diagnostic Division, Tarrytown, NY), and the Merck Vitalab Selectra (E. Merck Diagnostica, Frankfurter, Germany). Creatinine obtained from sera (Nppt) and supernatants (ppt2) from the three analyzers were compared to those obtained from the CRSC method (the Vitros DT60 II with DTSC II module, Ortho Clinical Diagnostics, Rochester, NY). Details of all methods were described in Table 1. To obtain the actual value of creatinine in the supernatant, we multiplied each creatinine result by 1.5 corresponding to the dilution. Data were compared and analyzed by the Friedman two-way ANOVA nonparametric statistical method (21).

We pooled normal-appearing sera to obtain three samples with low, middle, and high serum creatinine levels. We deproteinized each pooled serum (20 replicates) using trichloroacetic acid, 0.36 mol/l (sample-to-reagent ratio of 1:1), and 0.55 mol/l (sample-to-reagent ratio of 2:1). We measured creatinine in the serum samples and in the supernatants on the CX5, the AXON and the Selectra. The standard deviations

TABLE 1. Description of creatinine methods in various chemical analyzers

Instrument	Vitros DTSC	Beckman CX5		Technicon Axon		Vitalab Selectra
method	CRSC (16)	CREA (17)	CR-T (18)	CREAT (19)	CRE (19)	CREA (20)
Reaction type	Enzymatic	Jaffe reaction	Jaffe reaction	Jaffe reaction	Jaffe reaction	Jaffe reaction
	Rate	Rate	Rate	Twopoint	Rate	Twopoint
Wavelength (nm)	680	520/560	520/560	505/575	505/575	505
Sample volume (µl)	10	20	20	18	18	30
Reagent volume (µl)	Slide	219	250	350[c]	350[c]	220
		(175 + 44)[a]	(200 + 50)[b]			
Volume fraction of serum		0.083	0.074	0.049	0.049	0.120
Sample blank[d]	No	No	Yes	No	No	No
Start (sec)			96			
End (sec)			144			
Reaction read[e]						
Start (sec)	240	16	16	52.5/67.5	45	32
End (sec)	360	64	64	127.5/142.5	120	168
Reagent concentration						
Picric acid (mmol/l)		8.1	8.1	9.5	9.5	38
NaOH (mmol/l)		f	f	193.5	193.5	137.7
Linearity (µmol/l)	1459	2210	2210	1768	1768	1591

[a]Separated bottle of NaOH and picric acid. Mix 175 µl of NaOH witih 44 µl of picric acid before sample addition.
[b]Separated bottle of NaOH and picric acid. Preincubate serum with 200 µl NaOH before addition of 50 µl picric acid.
[c]Prepare reagent as working alkaline picrate solution.
[d]Times after the addition of sample and alkaline solution.
[e]Times after the addition of sample and all reagent components.
[f]Commercial liquid reagent provided only the value of Ph >13.3.

and the coefficient of variations (CV, %) of creatinine obtained from serum and from supernatant were calculated and compared.

RESULTS

Table 2 compares the within-run precisions of creatinine obtained from serum (Nppt) and from supernatant (ppt1 and ppt2) measured by various kinetic methods. The Selectra CREA gave the best precision (CV, % of 1.0–2.1), while the CX5 CREA (CV, % of 1.3–25.0) and the CR-T (CV, % 1.8–19.2) gave the least precision, especially at low creatinine levels. The precision of creatinine determination obtained from serum (CV, % of 0.4–12.6) is better than those obtained from the supernatant (CV, % of 0.7–25). Deproteinzation with TCA, 0.55 mol/l at the ratio of 2:1 gave better precision (CV, % of 1.0–19) than those obtained from TCA, 0.36 mol/l at the ratio of 1:1 (CV, % of 0.7–25).

Figure 1 shows the nature of bilirubin interference on patient serum creatinine obtained from all methods. We found significant negative bilirubin interference on creatinine in the direct kinetic methods (Fig. 1B, 1C, 1D, and 1E). The CX5 CR-T method with serum blank rate gave significant positive bilirubin interference (Fig. 1A). The process of deproteinization prior to analysis could completely correct bilirubin interference in the kinetic Jaffe reactions (Fig. 1F, 1G, 1H, 1I, and 1J).

Table 3 shows the means as well as the range differences of serum creatinine obtained between each kinetic Jaffe and Vitros dry enzymatic method. The Selectra CREA Nppt gave the greatest negative bilirubin interference, followed by the Axon CREAT Nppt, Axon CRE Nppt, and CX5 CREA Nppt, respectively. When deproteinization was introduced before the reaction (ppt2), we found insignificant negative interference from bilirubin on creatinine in all methods. For the CX5 CRT, the deprotenization process before the reaction eliminated the positive bilirubin bias from +14 to –1 μmol/l (+0.16 to –0.01 mg/dl) of serum creatinine. Figure 1 shows the serum creatinine difference between each direct and deproteinized serum creatinine by the kinetic Jaffe reaction and the Vitros enzymatic slide method plotted against bilirubin concentration. The slopes of –0.165, –0.179, –0.160, and –0.267 suggested the negative interference of bilirubin on the direct kinetic serum creatinine (Fig. 1B, 1C, 1D, and 1E). An increase in bilirubin concentration increased the negative bilirubin interference on serum creatinine (Fig. 1B, 1C, 1D, and 1E). The negative interference correlated with bilirubin concentration (r = –0.8370, –0.8498, –0.8422, and –0.7550, respectively). The direct CX5 CR-T was unaffected by negative interference from bilirubin (Fig. 1A); however, we found significant positive bias as shown by the positive slope of 0.021 (Fig. 1A). This positive bias did not correlate to bilirubin concentration (r = 0.2823). For the methods incorporated with deproteinization, we found patient serum creatinine results obtained from all methods accumulated near the zero lines (Fig. 1F, 1G, 1H, 1I, and 1J).

DISCUSSION

We select the Vitros CRSC multilayer enzymatic reaction as the reference method according to literature data (11–13). Table 3 confirms the negative bilirubin interference on serum creatinine in four direct kinetic methods. The Merck Selectra CREA method gives the highest degree of the negative interference, followed by the Axon CREAT and CRE, and CX5 CREA methods. Bilirubin interferes with serum creatinine of the twopoint kinetic method (Selectra CREA and Axon CREAT) more than the rate kinetic method (Axon CRE and

TABLE 2. Precision of serum creatinine measured by various methods on the Synchron CX5, Technicon Axon, and Vitalab Selectra

	Apparent creatinine concentration (μmol/l)														
Samples	Synchron CX5						Axon						Selectra		
Within-run	CR-T[a]			CREA[b]			CREAT[b]			CRE[b]			CREA[b]		
(n = 20)	Nppt[c]	ppt1[d]	ppt2[e]	Nppt	ppt1	ppt2	Nppt	ppt1	pp2	Nppt	ppt1	ppt2	Nppt	ppt1	ppt2
P1 Mean	61.0	29.5	36.4	43.6	20.6	27.2	57.3	20.1	29.4	52.7	19.1	24.9	62.7	24.0	31.3
SD	7.7	5.7	5.9	4.7	5.2	5.2	2.7	2.3	1.0	1.5	2.3	1.3	1.3	0.5	0.6
CV (%)	12.6	19.2	16.3	10.8	25.0	19.0	4.7	11.5	3.8	2.8	12.1	5.2	2.1	2.1	2.0
P2 Mean	122.7	62.2	76.3	106.4	52.7	71.9	126.1	55.2	71.0	117.1	52.1	69.5	106.9	56.8	74.6
SD	5.8	6.1	5.9	5.6	5.2	4.9	2.5	1.7	2.2	2.3	2.7	3.7	2.1	0.7	0.8
CV (%)	4.7	9.7	7.8	5.2	9.8	6.8	2.0	3.1	3.1	2.0	5.1	5.3	1.9	1.2	1.0
P3 Mean	531.7	270.5	365.9	536.4	286.7	383.0	524.4	281.7	371.2	529.2	280.8	370.8	508.6	294.9	361.8
SD	9.4	7.4	7.3	7.6	5.9	5.0	2.3	2.0	3.6	4.2	2.8	4.6	4.8	4.9	7.3
CV (%)	1.8	2.8	2.0	1.4	2.1	1.3	0.4	0.7	1.0	0.8	1.0	1.2	1.0	1.7	2.0

[a]Kinetic Jaffee with serum blank rate.
[b]Kinetic Jaffee without serum blank rate.
[c]Nppt, sample without acid precipitation.
[d]ppt1, sample deproteinized with TCA, 0. 36 mol/L (serum:TCA as 1:1 vol/vol).
[e]ppt2, sample deproteinized with TCA, 0.55 mol/L (serum:TCA as 2:1 vol/vol).

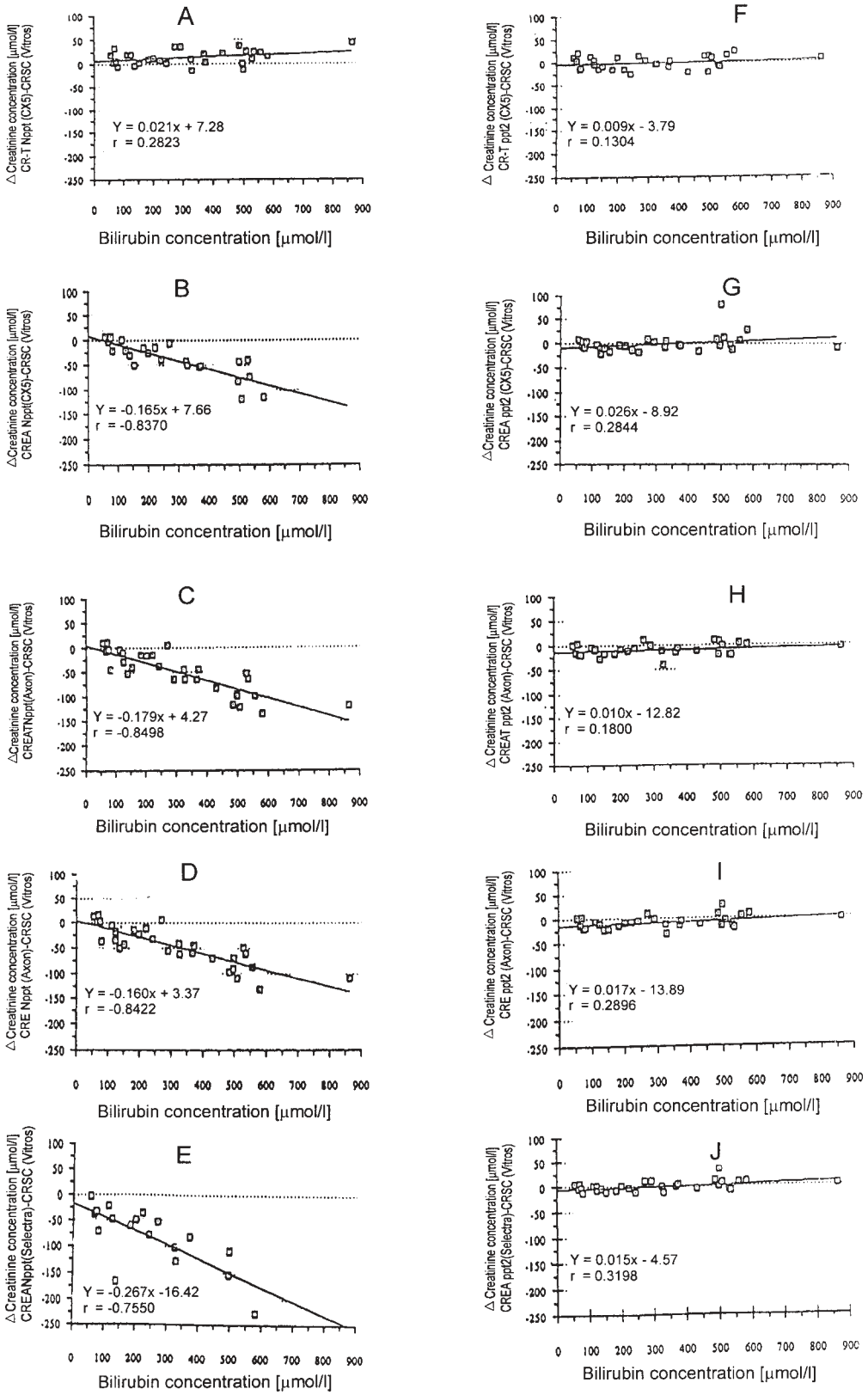

Fig. 1. The difference of patient serum creatinine between the Vitros CRSC enzymatic method and other automated methods treated with (**F–J**) and without (**A–E**) deproteinization process prior to the kinetic Jaffe reaction.

TABLE 3. Comparison of creatinine mean and range differences among 30 icteric patient sera between the Vitros DTSC (CRSC) and the Kinetic Jaffee methods on the Beckman CX5 (CREA, CR-T), Technicon Axon (CREAT, CRE), and Selectra (CREA) with and without deproteinization

	Differences of serum creatinine value			
	Mean difference		Range difference	
Serum creatinine	μmol/l	mg/dl	μmol/l	mg/dl
Without deproteinization				
CR-T Nppt[a] (CX5) vs. CRSC[b] (Vitros)	+14[c]	+0.16	–15 to +42	–0.17 to +0.48
CREA Nppt (CX5) vs. CRSC (Vitros)	–36[d]	–0.41	–120 to +7	–1.36 to +0.08
CREAT Nppt (Axon) vs. CRSC (Vitros)	–51[d]	–0.58	–135 to +1	–1.53 to +0.12
CRE Nppt (Axon) vs. CRSC (Vitros)	–46[d]	–0.52	–132 to +15	–1.49 to +0.17
CREA Nppt (Selectra) vs. CRSC (Vitros)	–79[d]	–0.89	–222 to –3	–2.51 to –0.03
With deproteinization				
CR-T ppt2[e] (CX5) vs. CRSC (Vitros)	–1	–0.01	–27 to +23	–0.31 to +0.26
CREA ppt2 (CX5) vs. CRSC (Vitros)	–1	–0.01	–22 to +81	–0.25 to +0.92
CREAT ppt2 (Axon) vs. CRSC (Vitros)	–10	–0.11	–40 to +10	–0.45 to +0.11
CRE ppt2 (Axon) vs. CRSC (Vitros)	–9	–0.10	–33 to +26	–0.37 to +0.29
CREA ppt2 (Selectra) vs. CRSC (Vitros)	+0	+0	–13 to +33	–0.15 to +0.37

[a]Nppt, sample without acid precipitation.
[b]CRSC, dry chemistry method assayed by Vitros DTSC single-slide creatinine method.
[c]Significant positive bias, no correlation with bilirubin concentration ($P < 0.05$).
[d]Significant negative bias, correlated with bilirubin concentration ($P < 0.05$).
[e]ppt2, sample deproteinized with TCA, 0.55 mol/l (serum:TCA = 2:1 vol/vol).

Synchron CREA) does. According to Table 1, reaction time in the twopoint kinetic method (Selectra CREA and Axon CREAT) is longer than the rate kinetic method (Axon CRE and Synchron CREA). The longer the reaction time, the more the bilirubin molecule is oxidized by NaOH to biliverdin (5). Moreover, the Selectra CREA uses large sample volume (30 μl) which increases the bilirubin molecules. Oxidation of bilirubin to biliverdin causes a decrease in absorbance that is opposed to the absorbance increase due to creatinine picrate complex at 520 nm.

Bilirubin does not negatively interfere with creatinine results obtained from the Synchron CR-T incorporated with serum blank rate (Table 3 and Fig. 1A). The CR-T method preincubates serum with NaOH and bilirubin is oxidized to biliverdin before picric acid addition. Biliverdin gives maximum absorbance at 630 nm, while creatinine picrate complex gives maximum absorbance at 520 nm. Therefore, biliverdin does not interfere with serum creatinine determined by the kinetic Jaffe reaction. However, we find significant positive interference from serum bilirubin on creatinine results derived from the Synchron CR-T method. False-positive interference is due to the process of sample blank correction in the Synchron CR-T method.

We propose deproteinization of icteric serum before subjecting it to the kinetic Jaffe reaction. The process can eliminate all forms of bilirubin interference on creatinine in patient serum. Deproteinization can eliminate both the positive and negative bilirubin interferences on creatinine obtained from all kinetic Jaffe methods. Because bilirubin is precipitated with precipitant, it does not interfere with the reaction of creatinine with alkaline picrate. In the Vitros CRSC, bilirubin cannot penetrate the spreading membrane layer to react with enzymatic reagent layer. We found an insignificant difference of creatinine results between all kinetic methods, incorporated with deproteinization to the Vitros CRSC enzymatic method (Table 3).

The Synchron CREA and the Selectra CREA cannot provide negative creatinine results, while the Technicon Axon can. If absorbance detected by the Synchron CREA or the Selectra CREA method is lower than the lower limit set by the manufacturer, results are flagged with a suppress (S) or reject (R) sign. It is our responsibility to find an appropriate solution to report any serum creatinine with a flagged sign. We used the direct acidification method (9,22) to correct the problem on the Labsystems analyzer (Laboratory OY, Research Laboratories, Helsinki, Finland) during 1990–1991. Deproteinization by trichloroacetic acid was used during 1991–1993 then we switched from the Synchron CREA to the Synchron CR-T method during 1993–1995. At present, we reanalyze serum creatinine that has total bilirubin of 171 μmol/l (10 mg/dl) or greater using the method on the Vitros dry chemical analyzer.

Although the time to complete deproteinization is around 20–30 min, the process is simple and is applicable to all sizes of clinical labs. However, we must carefully dilute patient samples with the precipitant using the accurate and precise automatic pipette, such as the SMI pipette (Scientific Manufacturing Industries, Emeryville, CA). Tricholoacetic acid (TCA) is the precipitant of choice; it is a simple reagent available in all labs (7). We use serum to TCA (0.55 mol/l) at the ratio of 2:1, then multiply the result by 1.5. TCA at this concentration used at the ratio of 2:1 can eliminate bilirubin inter-

ference and give better creatinine precision than can using the TCA (0.36 mmol/l) at the ratio of 1:1 (Table 2), as suggested in the previous study (7). Although deproteinization by TCA manually is tedious and cumbersome, it is the most viable solution for labs that do not have the Vitros dry chemical analyzer. In our lab (600–700 patient samples per day), we found that between 1 and 9 samples a day (an average of 3) had a total bilirubin of 171 µmol/l and greater. This infrequent finding does not disrupt the flow of work in the clinical labs.

CONCLUSION

It is advisable to screen creatinine samples for abnormally high bilirubin. If total bilirubin concentration is 171 µmol/l (10 mg/dl) or higher, we must repeat serum creatinine with the appropriate method available in each lab. We suggest the use of the creatinine method on the Vitros dry chemical analyzer as the first choice. If the instrument dose is not available, deproteinization of the icteric serum before subjecting supernatant to the reaction is the next best approach. The process can correct all forms of bilirubin interferences on serum creatinine.

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