Analysis of Effect of Composition of Direct Bilirubin Reagent on Observed Values of Direct Bilirubin in Human Serum

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Abstracts: Introduction: While total bilirubin methods generally perform satisfactory across laboratories, there is perceived dissatisfaction over direct bilirubin results, particularly negative results in normal ranges, wide variation across labs and direct bilirubin results exceeding total bilirubin. Variation in reagent composition may be responsible for problems observed in direct bilirubin measurement performance. Method: This study was performed to see effect of various direct bilirubin reagents on observed values. 50 mmol/L, 150 mmol/L and 250 mmol/L HCL, water and saline are compared in the study. The study was done by modified Jendrassik diazo method using various composition of reagent R1 for direct bilirubin measurement. The study stratified results in various ranges. Saline and Water gives negative, constant, systemic bias as compared to 50mmol/L reagent for measurement of direct bilirubin. **Result:** On increasing HCL concentration in the reagent there is increasing negative, proportionate, systemic bias as compared to 50 mmol/L reagent for measurement of direct bilirubin. Discussion: Results of the study indicate need for better uniformity in reagents used for measurement of direct bilirubin to facilitate inter-laboratory comparison of patient result.[Dipti K NJIRM 2017; 8(2):31-34]

Key Words: Modified Jendrassik, Direct bilirubin, Measurement, Bilirubin, Humans, Indicators and Reagents

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Introduction: Direct Bilirubin measurement in serum is one of the most frequently used laboratory examination in clinical chemistry laboratory. It is used in diagnosis, treatment monitoring and judging prognosis of Jaundice and liver disorders and various blood disorders, in order to be useful to the clinicians[1]. The Direct Bilirubin reporting should have performance acceptable to clinicians. Performance of a laboratory test, obviously, highly dependent on equipment and reagents used in the process. There is an observed variation across laboratories in direct bilirubin result. The similar variation is also evident in proficiency testing programs. It is observed that different publications, text books and kitManufacturer uses/recommends different reagents constitutions for measurement of direct bilirubin by modified Jendrassik diazo method[4]. Such difference in reagent constituents may be responsible for lab-to-lab and PT variation observed for direct bilirubin.

Our laboratory uses 50mmol/L HCL as reagent medium for measurement of direct bilirubin. But, different laboratories use different concentrations of HCL and even use water or saline instead of HCL. The purpose of this study is to find up to what extent 50 mmol/L HCL, 150 mmol/L HCL, 250 mmol/L HCL, 0.9% NaCl and water as direct bilirubin measuring medium correlate among each other.

It is observed that different kit manufacturer using different concentration of HCL for measurement of direct bilirubin as shown in below table. This variation is observed in the lab to lab variation in direct bilirubin results.

Table-1: HCL concentration used by kit manufacturer.

No.	Kit manufacturer	HCL mmol /LR1	HCL mmol /LR2	HCLmmol /LFinal
1	Spin react	150		150
2	Agappe	165		165
3	Beckman coulter	50		50
4	Pishtaz teb diagnostic	67		67
5	Teco diagnostics	165		165
6	Bio Lab Diagnostics	145		145
7	Biolabo	130		130
8	Biotechnica	180	60	240
9	Pointe Scientific	164		164
10	Elitech	67		67
11	Erba Mannheim	117.6	117.6	235
12	CliniChem	165		165
13	Spectrum	200		200
14	Stan Bio	50	50	50
15	Tulip	115		115

Methods: The study is performed at New Civil Hospital, Surat Laboratory Services (NCHSLS). NCHSLS is NABL accredited laboratory. Study is performed after approval from institute of research council and ethics committee. Leftover serums of samples received by the laboratory, devoid of patient identification,

wereused to make pool of serum. Everyday starting from 1 st July 2016, leftover laboratory samples were observed for left-over serum volume. Serum with left-over volume of >400 µl were observed for their direct bilirubin results obtained during routine testing. Samples were selected for analysis till following numbers were completed for each groups.

Table-2: Criteria for sample selection

Group	Direct Bilirubin Range (mg/dl)	No.of Samples
A	0.0-0.5	50
B	0.5-2.0	100
C	2.0-5.0	50
D	>5.0	50

We used ERBA XL 640 Multiple wavelength, diffraction spectrophotometer and glass cuvette for absorbance measurements. Photometric accuracy and equipment condition were regularly checked by daily internal quality assessment.

Reagent Preparation: Use analytical grade chemicals, whenever available, and deionized water with conductivity of 0.0 to 0.5 micro-Siemens per meter.

Sulfanilic acid (R2A): 5 gm/L. Dissolve sulfanilic acid in de-ionized water. Add 15 ml of concentrated HCL and dilute it up to 1 L. Stored at 4 °C in refrigerator.

Sodium Nitrite (R2B): 5 gm/ L. prepare freshly every monthly and stored at 4°C in refrigerator.

Diazo reagent (mix): Just before use, mix 600 microliter of Sodium Nitrite with 20 ml of Sulfanilic Acid reagent2.

Direct Bilirubin (R1):

1. 50 mmol/L HCL: 4.3 ml of concentrated HCL added to de-ionized water and dilute it up to 1 litre. Stored at 4 °C in refrigerator.
2. 150 mmol/L HCL: 13.10 ml of concentrated HCL added to de-ionized water and dilute it up to 1 litre. Stored at 4 °C in refrigerator.
3. 250 mmol/L HCL: 21.83 ml of concentrated HCL added to de-ionized water and dilute it up to 1 litre. Stored at 4 °C in refrigerator.
4. Normal Saline
5. De-ionized Water

All reagents were prepared in 1000 ml volume. There were two Direct Bilirubin reagents used for measurement of DirectBilirubin – Direct Bilirubin R1 and Direct Bilirubin R2.

Proportion of Sample, R1 and R2 was 20: 160: 40.Concentrations of all constituents of R2 reagents were same while concentration of reagent R1 was changed according to table.

Table-3: Scheme for preparation of various reagents

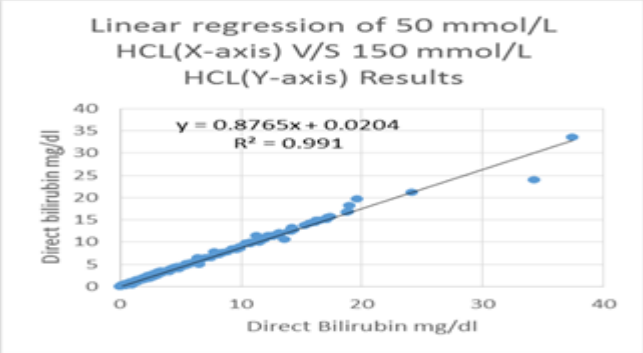
Scheme for preparation of various reagents					
Reagent	**Reagent 1**	**Reagent 2**			**Final HCL mmol/L**
	HCL mmol/L	**HCL mmol/L**	**Sulfanilic Acid gm/L**	**Sodium Nitrate gm/L**	
50 mmol/L	50	187	10	1.25	77.4
150 mmol/L	150	187	10	1.25	157.4
250 mmol/L	250	187	10	1.25	237.4
Water	Water	187	10	1.25	37.4
NaCl	NaCl	187	10	1.25	37.4

Study is performed by modified Jendrassik Groff Method. Method uses caffeine benzoate[1] as an accelerator which splits the unconjugated bilirubin protein complex releasing the bilirubin so that it can react with diazotised sulfanilic acid. A plasma blank was carried out. The reagent blank was zero. In acidic mixture bilirubin react with diazotised sulfanilic acid converts the red colour azopigments which is read at filter 540.Reaction of conjugated bilirubin and

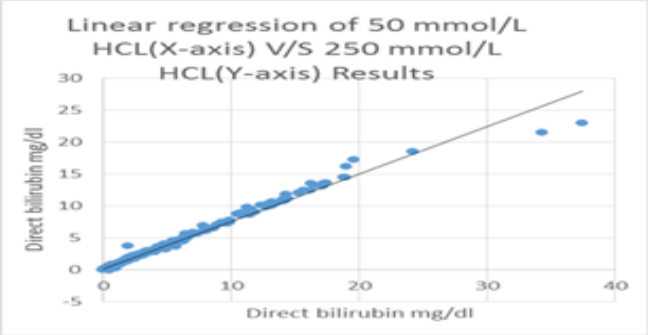
Unconjugated bilirubin with diazo reagent is very much dependent on pH of medium, and incubation period of reagent mixture before adding of diazo reagent. Without Accelerator Only Direct bilirubin reacts with diazo complex and giving colour which is measure at 540nm.In acidic medium indirect bilirubin does not react as direct bilirubin giving lower result compared to water[3].

Result:

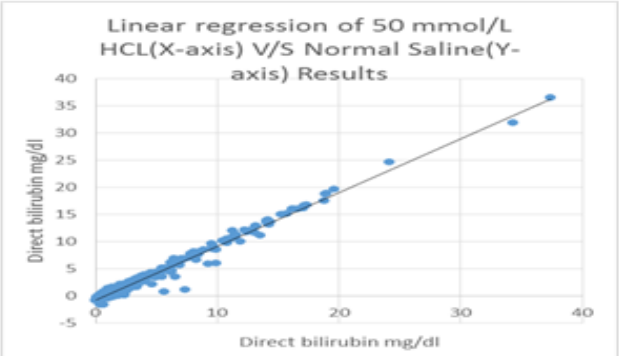
Graph-1: Linear regression of 50 mmol/L HCL vs 150 mmol/L HCL



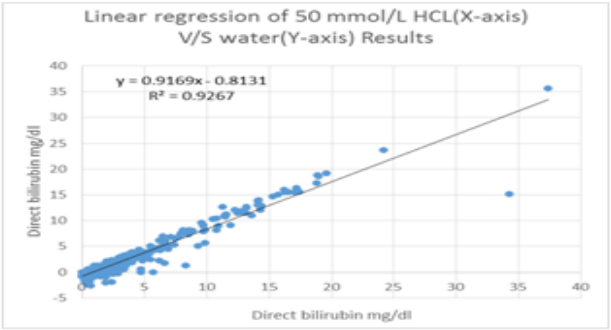
Graph-2: Linear regression of 50 mmol/L HCL vs 250 mmol/L HCL



Graph-3: Linear regression of 50 mmol/L HCLvs Normal Saline



Graph-4: Linear regression of 50 mmol/L HCL vs Deionized Water



Discussion: Graph-1 shows that 150 mmol/L reagent gives approximately 88% of results as compared to 50 mmol/L reagent. The low Y intercept (0.02) denote that 150 mmol/L gives negative proportionate systemic bias as compared to 50 mmol/L reagent.

Graph-2 shows that 250 mmol/L reagent gives approximately 75% of results as compared to 50 mmol/L reagent. The low Y intercept (0.1) denote that 250 mmol/L also gives negative proportionate systemic bias as compared to 50 mmol/L reagent. The negative bias observed is more than that of 150 mmol/L reagent

Graph-3: shows that normal saline reagent gives approximately 0.7 mg/dl results less as compared to 50 mmol/L reagent. The high Y intercept (0.7) together with slop of (0.99) denote that 250 normal saline gives negative constant systemic bias as compared to 50 mmol/L reagent.

Graph 4 shows that normal water reagent gives approximately 0.8 mg/dl results less as compared to 50 mmol/L reagent. The high Y intercept (0.8) together with slop of (0.92) denote that water gives negative constant systemic bias as compared to 50 mmol/L reagent, although possibility of some proportionate bias is indicated by slop of 0.92 (Ideal slop=1).

Saline and Water gives negative, constant, systemic bias as compared to 50 mmol/L reagent for measurement of direct bilirubin. While comparing results of same patient across various laboratories, clinicians needs to be aware of such possibilities. Such differences will be more significant in borderline results (0-1 mg/dl), where two different laboratories using different medium for direct bilirubin measurement can give results crossing the reference range. Considering lack of general consensus about the reagent medium for direct bilirubin measurement, it may be indicated in laboratory reports, water it uses saline, water or 50 mmol/L HCL.

On increasing HCL concentration in the reagent there is increasing negative, proportionate, systemic bias as compared to 50 mmol/Lreagents for measurement of direct bilirubin. While comparing results of same patient across various laboratories, clinicians needs to be aware of such possibilities. Such differences will be more significant across the values, where two different laboratories using different HCL

concentration for direct bilirubin measurement can give results with little mg/dl difference in higher range. Considering lack of general consensus about the reagent medium for direct bilirubin measurement, it may be indicated in laboratory reports, the exact concentration of HCL used.

Considering effect of reagent on direct bilirubin results, it is prudent that clinician use same laboratory for direct bilirubin measurement in same patient for monitoring.

In short, variation in reagent composition greatly affects results in direct bilirubin measurement. John A. Lott et al.[4] also observed that there is considerable variability in the DBILand TBIL methods, and no one uses the preferredmethod for DBIL or the Reference Method for TBIL in routine work. In their view, the most important factorsaffecting accuracy in DBIL assays are calibration, concentration of HCL in the final reaction mixture, reaction times, specimen blanking anddichromatic correction techniques. John A. Lott et al.[4] also noted that the most important requirement for reliable DBIL method is specificity; i.e., the methodmust not measure Bu as DBIL. This is achieved bydiluting the specimen with HC1 (-50 mmol/L) andletting it stand for a few minutes before adding the diazo reagent. Considering variation observed between 50 mmol/L and other reagents in our study and observation of John A. Lott et al.[4], it is advisable that laboratory clearly mention HCL concentration used in their DBIL assay in laboratory report. e.g. instead of writing “modified Jendrassik diazo method” in their lab report , specific methodology “Jendrassik diazo method, 50 mmol/L HCl” or ““Jendrassik diazo method, 0.9% saline” or “Jendrassik diazo method, DI water” should be mentioned in lab report to convey unequivocal information about DBIL methodology.

This laboratory did not have stable direct bilirubin calibrator. The repeat study using stable direct bilirubin calibrator is needed for further confirmation of conclusion of this study.

The study did not use bilirubin reference method for measurement comparison, hence, relative bias observed in the study cannot infer upon which of the reagents used is giving accurate results.

Conclusion: Variation in Direct Bilirubin reagentaffects the patient results. Such variation may affect clinical management, particularly when serial measurement in one patient is done across various laboratories. In order to help clinician understand that different laboratories, using different composition of modified Jendrassik diazo method reagent must mention such differences in their laboratory report to alert clinician about comparison of DBIL results from different laboratories in same patient.

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