Original Article

Modified Jendrassik-Grof Method for Measurement of Direct Bilirubin: An Improvement of In-House Method@

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Modified Jendrassik-Grof Method for Measurement of Direct Bilirubin: An Improvement of In-House Method.

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บทคัดย่อ:
บทนำ: การตรวจวิเคราะห์ direct bilirubin ของหน่วยเคมีคลินิกเป็นการทดสอบประเภทงานประจำด้วยเครื่องวิเคราะห์อัตโนมัติ ซึ่งเดิมใช้น้ำยาตรวจวัดที่เตรียมขึ้นเองในห้องปฏิบัติการ แต่เมื่อทำการควบคุมคุณภาพทั้งชนิดการควบคุมภายใน Internal Quality Control (IQC) และการควบคุมคุณภาพภายนอก External

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Quality Control (EQC) พบว่าค่าที่ได้จะอยู่ในเกณฑ์ค่อนมาทางต่ำ และน้ำยาของบริษัทที่มีขายในท้องตลาดมีบรรจุภัณฑ์ของน้ำยาขนาดเล็ก อายุการใช้งานสั้น ไม่เหมาะกับปริมาณการทดสอบสำหรับห้องปฏิบัติการ
วัตถุประสงค์: เพื่อปรับปรุงและประยุกต์สูตรน้ำยาที่ใช้อยู่เดิมให้มีความถูกต้องและแม่นยำเหมาะสมที่จะนำมาใช้ในห้องปฏิบัติการ
วิธีการ: ปรับปรุงและประยุกต์สูตรน้ำยา ทดสอบความแม่นยำ และทดสอบหาค่า direct bilirubin โดยทำการเปรียบเทียบกับน้ำยาสำเร็จรูปที่ซื้อจากบริษัท ใน serum จำนวน 105 ราย ด้วยเครื่องวิเคราะห์อัตโนมัติ Modular P800 ทดสอบความแตกต่างโดยใช้ paired-t test และหาความสัมพันธ์โดยใช้โปรแกรมทางสถิติ Statistical Package for Social Science (SPSS)
ผลการศึกษา: น้ำยาที่ประยุกต์มีความแม่นยำดี ทั้งชนิด within run precision ได้ค่า % Coefficient of Variation (CV) เท่ากับ 0.77 และ 0.55 และชนิด between run precision ได้ค่า %CV เท่ากับ 2.41 และ 2.56 ในช่วงค่าต่ำและค่าสูง ตามลำดับ ผลการเปรียบเทียบการตรวจ direct bilirubin ระหว่างน้ำยาบริษัทและน้ำยาที่ประยุกต์ได้ค่า mean±SD เท่ากับ 2.22±2.59 และ 2.25±2.50 มก./ดล. ตามลำดับ วิธีทั้งสองมีความสัมพันธ์กันดี มีค่า r=0.998, สมการถดถอยเชิงเส้นตรง y=0.9578+0.1146 และไม่แตกต่างกันอย่างมีนัยสำคัญทางสถิติ (p>0.05)
สรุป: น้ำยาสำหรับการทดสอบ direct bilirubin ที่ประยุกต์ขึ้นใช้เอง มีความถูกต้อง แม่นยำ เทียบเท่ากับน้ำยาสำเร็จรูปของบริษัท

คำสำคัญ: เครื่องตรวจวิเคราะห์อัตโนมัติ, direct bilirubin, น้ำยาสำเร็จรูป, น้ำยาเตรียมเอง

Abstract:

Background: In our lab, the assay for direct bilirubin using the diazotized colorimetric method is routinely performed using automated analyzer. Previously we used reagents that were prepared in-house. However, results of the quality controls tend to be in the low ends. Therefore, we considered commercially available reagents for the bilirubin assay, however, the available commercial reagents were provided only in small volume package and had a short shelf life, and were thus unsuitable for our laboratory. We then decided to improve our in-house reagents for the test.
Objective: The aim of this work was to modify and improve the existing reagent formulas for measurement of direct bilirubin which are suitable for routine clinical laboratory testing as well to produce accurate and reproducible results.
Method: The modified in-house reagents were compared to the commercially available reagents by measuring direct bilirubin in 105 serum samples using the Modular P800 Analyzer. The differences between the results from both methods were assessed using paired-t test. The correlation was determined using linear regression analysis.
Results: The modified in-house reagents showed good precision with within-run precision (%CV = 0.77 and 0.55, N=20) and between-run precision (% CV = 2.41 and 2.56) at low and high level respectively. The comparison of the results measured by commercial and modified in-house reagents were 2.22±2.59 and 2.25±2.50 mg/dL respectively. There was no significant difference

between the results from two methods ($p>0.05$), mean difference -0.0285 and high correlation was obtained ($r=0.998$; $y=0.9578x+0.1146$).

Conclusion: The modified in-house reagents for direct bilirubin assay provide accurate, precise and reproducible results comparable with those derived from the commercial reagents.

Key words: automated analyzer, commercial reagent, direct bilirubin, in-house reagent

Introduction

Bilirubin is an orange-yellow pigment derived from the break down of red blood cells in the reticuloendothelial system.[1] There are two forms of bilirubin in the body: unconjugated or indirect bilirubin and conjugated or direct bilirubin. The unconjugated form is protein bound and insoluble in water while the conjugated form circulates freely in the blood and was transformed into water-soluble bilirubin in the liver by conjugated conjugating with glucuronide and is then excreted into the bile.[2,3] The assay for bilirubin provides diagnostic help for liver diseases, the detection of hemolytic anemia, and assessment of the degree of severity of jaundice.[1,2,4] An increase in direct bilirubin is highly specific for diseases of the liver or bile ducts and may also occurs when there is impaired energy-dependent bilirubin excretion in sepsis, during total parenteral nutrition, and after surgery.[5]

Various methods for bilirubin assay have been described including direct spectrophotometry, colorimetric method using diazotization reaction by Jendrassik and Grof, and Molloy-Evelyn, an enzymatic assay, and High Performance Liquid Chromatography (HPLC). In our lab, the assay for direct bilirubin with diazotized colorimetric method by Jendrassik and Grof was routinely performed using automated analyzers. About 30,000 tests were requested annually. With the use of previously prepared in-house reagents, results of the internal quality controls (IQC) for low level appeared to be lower than expected value. Furthermore, the external quality control (EQC) revealed that the results from our lab tend to be at the low end. Therefore, we considered to use commercially available reagents for the test. However, they were provided only in a small volume package and had short life and thus were unsuitable for the load of this test in our laboratory. Moreover, the cost was quite high. We, therefore, decided to improve our in-house reagents for the test.

The aim of this work was to modify and improve the existing reagent formulas to produce accurate and reproducible results and suitable for voluminous usage in routine laboratory.

Materials and methods

1. The reagent formulas were adjusted and modified.

Original formula:

Reagent 1: 154 mmol/l Sodium chloride (NaCl) 36 ml: 29 mmol/l Sulfanilic

acid; 180 mmol/l Hydrochloric acid (HCl) 10 ml; 150 mmol/l Ethylenediaminetetraacetic acid dipotassium salt (EDTA.K2) 0.4 ml.

Reagent 2: 154 mmol/l NaCl 17.3 ml; 72.5 mmol/l Sodium nitrite 0.7 ml.

The formula was adjusted and modified according to the commercial formulas as follows:

Modified formula:

Reagent 1: 154 mmol/l NaCl 99 ml: 150 mmol/l EDTA 1 ml.

Reagent 2: 29 mmol/l Sulfanilic acid; 180 mmol/l HCl 10 ml: 72.5 mmol/l Sodium nitrite 0.35 ml.

Commercial reagents (Roche Diagnostics, Thailand) are as follows:

Reagent 1: EDTA:150 mmol/l; NaCl: 152.5 mmol/l,

Reagent 2: Sulfanilic acid: 29 mmol/l; HCl: 170 mmol/l,

Reagent 2a: Sodium nitrite 25 mmol/l, mix 6 ml of Reagent 2 with 0.6 ml of Reagent 2a before use. This working reagent is stable for 7 days.

The test is based on the Jendrassik and Grof diazotization method[6] whereby direct bilirubin in the sample reacts with diazotized sulfanilic acid derived from the reaction of acidified sodium nitrite and sulfanilic acid. The intensity of the purple-red color of the derived azo dye is directly proportional to the concentration of direct bilirubin and can be photometrically measured.

Bilirubin + diazonium ion $\rightarrow$ azobilirubin

2. The accuracy and precision of the test measures using modified reagents were evaluated using 2 level quality control samples. The stability of these reagents was also assessed. These in-house reagents were also compared to the commercially available reagents by measuring direct bilirubin in 105 serum samples from patients at Songklanagarind Hospital using the Hitachi Modular P800 Chemistry Analyzer. The differences between the obtained results were assessed using paired-t test. The correlation between the 2 reagent sets was determined using linear regression analysis.

Results

1. The accuracy of both reagent sets assessed by measuring direct bilirubin in the low level control - Precinorm U lot 1764690 (mean±SD: 0.74±0.12 mg/dl) and the high level control - Precipath U lot 1762870 (mean±SD: 2.25±0.32 mg/dl) showed within range results. The within-run precision was evaluated from measuring many replicates of 2 quality control samples of low and high values in one run whereas the between-run precision was assessed from measuring the low and high value controls once everyday for several days (N=20). Both sets of reagents showed good precision (Table 1).

2. The result of 105 patient serum samples (range 0.00-10.85 mg/dl) measured by the in-house reagents was 2.25±2.50 mg/dl (mean±SD). That measured by the commercial reagents was 2.22±2.59 mg/dl (mean±SD).

3. The correlation between the commercial reagents and the modified in-house reagents for measuring direct bilirubin assessed from regression analysis, showing a highly significant relationship. The regression analysis showed good correlation with r=0.998 and linear regression

equation of y=0.9578x+0.1146 (Figure 1). The comparison of the measured results from the 2 reagent sets using paired t-test showed no statistical significant difference (p>0.05) with the mean difference of -0.0285±0.17 mg/dl (Figure 2).

4. The lower detection limit of 0.03 mg/dL represents the lowest measurable analyte level that can be distinguished from zero by measuring direct bilirubin in 0.9% NaCl. It is calculated as the value lying three standard deviaions above that of the lowest standard. (0.9% NaCl + 3 SD, within-run precision, n=21)

Table 1 Precision data of the commercial reagents and the modified in-house reagents (N=20)

Reagent set	Control sample	Within-run			Between-run		
		Mean (mg/dl)	SD (mg/dl)	%CV	Mean (mg/dl)	SD (mg/dl)	%CV
Commercial reagents	Precinorm U	0.77	0.01	1.08	0.73	0.02	2.91
	Precipath U	2.30	0.01	0.47	2.17	0.07	3.31
Modified in-house reagents	Precinorm U	0.78	0.01	0.77	0.77	0.02	2.41
	Precipath U	2.29	0.01	0.55	2.21	0.06	2.56

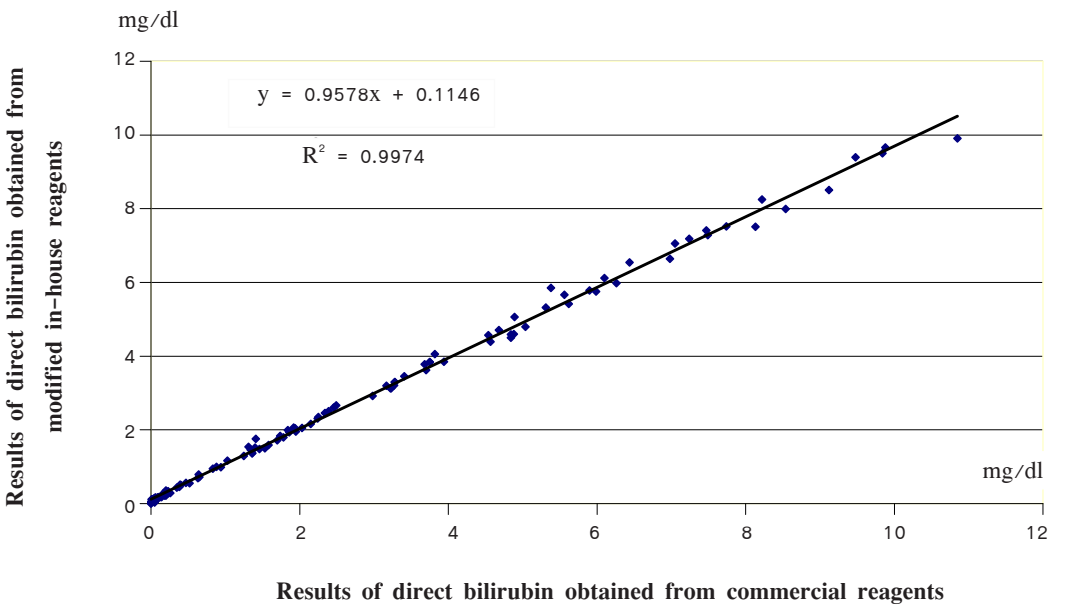

Figure 1 Correlation between the commercial reagents and the modified in-house reagents for measuring direct bilirubin

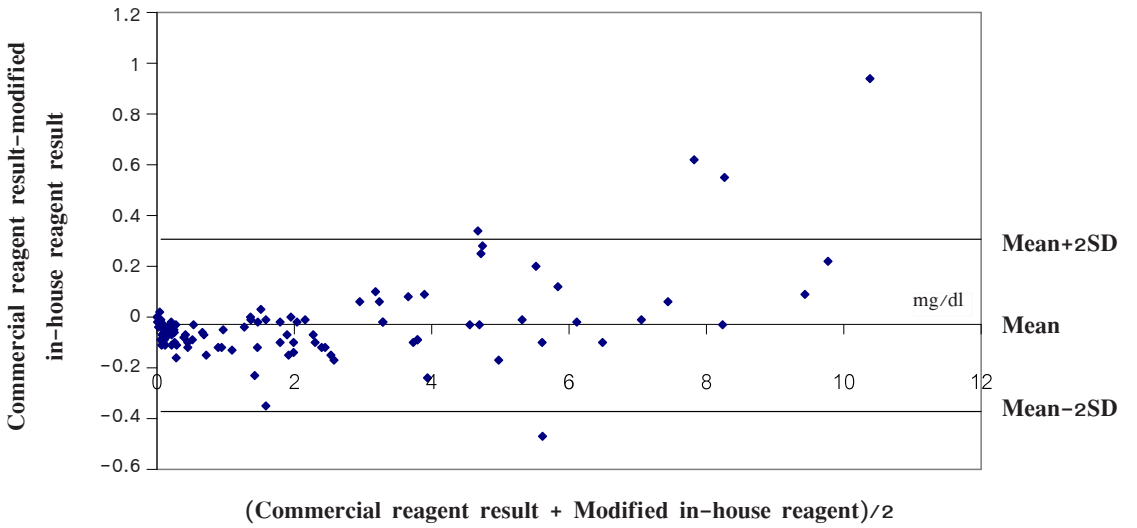

Figure 2 The difference between results of direct bilirubin measured by the commercial reagents and the modified in-house reagents

Discussion

Accurate determination of bilirubin in serum appears to be more difficult than for any other substances.[7] This is due to its sensitivity to many factors such as light, oxygen, hemoglobin concentration, pH, its high-affinity to proteins, the technique used to obtain the blood sample,[8-10] types of automated analyzers and methods employed.[11] Furthermore, a lack of reference materials or direct bilirubin[12,13] due to the unavailability of primary standard bilirubin glucuronides makes the absolute accuracy of any method for measuring direct bilirubin even more difficult.[14] There are several methods for the determination of bilirubin. The method widely used in most clinical laboratories is based on the colorimetric method using diazotization reaction[15,16] because it is cheap, easy and convenient to apply to use with automated analyzers. Our laboratory also decided to use the colorimetric method using diazotization reaction by Jendrassik and Grof. The diazo reagent, however, could not directly determine the indirect bilirubin therefore it is obtained from subtracting direct bilirubin from total bilirubin. This is a limitation of this method compared to the standard method, the high performance liquid chromatography (HPLC), which can separate fractions of bilirubin, but this method is labor-intensive and not practical for routine use.[10,17] It is inappropriate to apply to our laboratory because of the high cost of the equipment, the requirement for skilled and expert personnel, and the method can be performed only on a few cases per day. From these reasons, it is important to determine total bilirubin and direct bilirubin with high accuracy and precision as the levels of direct and indirect bilirubin are used in diagnosis and decision making for the patients.

In our study, the commercial reagents and modified in-house reagents showed good accuracy and good precision for both within-run and between-run as seen in Table 1 and in this study from 105 serum, the means of commercial reagents and modified in-house reagents were 2.22 and 2.25 mg/dl respectively. The result showed good correlation with r=0.998 (y= 0.9578x+0.1146) and no significant difference (p>0.05). Bland-Altmand plot demonstrated good agreement in comparison values between two methods with mean difference of -0.0285 mg/dl.

In this study, the lower detection limit of the modified in-house reagents was 0.03 mg/dl that was similar to the result in the commercial reagents (0.10 mg/dl). Serum with lipidemia or hemolysis interfere the reaction in both reagents.

The original in-house reagent formula from Jendrassik and Grof method was modified and the proportion of the reagents adjusted and then applied for use in an automated analyzer in our laboratory. The stability of reagent 1 was at least 6 months at room temperature and that of reagent 2 at least 1 month at 4 °C. The cost per test of the modified in-house reagent was only 0.02 Baht whereas that of the commercial reagent was 4.50 Baht.

Conclusion

The modified in-house reagents for direct bilirubin assay showed good accuracy and precision with reproducible results comparable to those derived from the commercial reagents. Furthermore, they are economic, easily prepared, have long shelf life, and applicable to automated analyzers.

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