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Research article

A multi-component spectrophotometric method for simultaneous determination of conjugated bilirbuin, unconjugated bilirubin, oxyhemoglobin and methemalbumin in adult human serum

Atef M. M. Attia*, Abd El-Baset M. Sayed, Wael M. Aboulthana, Fatma A. A. Ibrahim, Noha A. Abd El-Latif, Samir W. Aziz, Azhar M. Elwan, Mohamed D. E. Abdelmaksoud, Sherif A. A. Moussa

Biochemistry Department, National Research Centre, 33 Bohouth Street, Dokki, Giza, Egypt (Affiliation ID: 60014618).

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***Corresponding Author: Atef M. M. Attia,** Biochemistry Department, National Research Centre, 33 Bohouth Street, Dokki, Giza, Egypt (Affiliation ID: 60014618).
Email id: yousatef@yahoo.com

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Key words: Total bilirubin, unconjugated bilirubin, conjugated bilirubin, oxyhemoglobin (HbO_2), methemalbumin (Mha), serum, adults.

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Abstract

A new multi-component spectrophotometric method was developed to determine the accurate concentrations of the conjugated bilirubin (CB), unconjugated bilirubin (UB), oxyhemoglobin (HbO_2) and methemalbumin (Mha) in adult human serum of a healthy subject. The method of preparation of serum solutions has been developed, like the use of distilled water as a solvent and centrifugation of serum solutions to clear the sample turbidity. The results of bilirubins were compared to the diazo assay, as a reference method. The formulas used for the calculation of the major components (UB, CB, HbO_2 and Mha) in human serum have been derived based on the theory of multi-component spectrophotometric analysis and the mathematical Gaussian elimination method for matrix calculation. The method of multi-component spectrophotometry (at λ=450, 490, 576, 600 and 700 nm), suggested in this study for determination of total bilirubin (TB), UB and CB showed small % error of values 0.201, 2.772 and 7.81%, respectively, indicating the high accuracy of the method. The method showed small coefficients of variation (CV=3.84-10.5%), indicating the high precision of the method. The method is inexpensive, precise, accurate, and reproducible and has the advantages of small sample volume, simplicity speed and can be computerized.

Introduction

Bilirubin is a degradation product of hemoglobin heme. Bilirubin formed in peripheral tissues is transported to the liver by binding noncovalently to plasma albumin. In the liver, by the enzyme glucuronyltransferase bilirubin is conjugated with glucuronic acid, making it soluble in water. Much of it goes into the bile and thus out into the small intestine. The unconjugated bilirubin is not conjugated with glucuronic acid and also called indirect bilirubin. The majority of unconjugated bilirubin in the circulation is bound to albumin [1]. The sum of the conjugated and unconjugated bilirubins is termed the total bilirubin.

Determination of total and conjugated bilirubin is important for the diagnosis of many diseases associated with hyperbilirubinemia. Hyperbilirubinemia results from the increase of bilirubin concentrations in blood plasma. Clinically hyperbilirubinemia appears as jaundice or icterus. Jaundice can usually be detected when serum bilirubin level exceeds 2.0 to 2.5 mg/dl. When the level of bilirubin is between 1 to 2 mg/dl, it is known as latent jaundice [1]. Causes of hyperbilirubinemia: Total bilirubin: Increased hemolysis, genetic errors, neonatal

jaundice, ineffective erythropoiesis, and hepatocellular disease (viral or drug-induced hepatitis, cirrhosis). Conjugated bilirubin increases in cases of hepatic cholestasis, genetic errors, and hepatocellular disease (viral or drug-induced hepatitis, cirrhosis) [1, 2].

A new multi-component spectrophotometric method has been developed experimentally and theoretically to determine the accurate serum concentrations of the total bilirubin (TB), oxyhemoglobin (HbO_2) and methemalbumin (Mha) in human sera [3]. In this method, absorption at wavelengths 469.8, 576, and 600 nm, which represent the near-maximum absorptions of bilirubins, HbO_2 and Mha, respectively and these wavelengths are the isobestic points of both unconjugated and conjugated bilirubins in adult serum [4, 5]. At these isobestic points the conjugated and unconjugated bilirubins have the same molar absorptivity and therefore both components can be represented as a single one (i.e. total bilirubin), which can be calculated by absorbance measurement at three wavelengths instead of four, taking in account the absorption contribution of the other two major components (HbO_2 and Mha). Multi-component spectrophotometric methods for simultaneous determination of conjugated bilirubin, unconjugated bilirubin, HbO_2, and methemalbumin in adult human serum have not yet been published.

Therefore, this practical study aimed to develop a new direct multi-component spectrophotometric method for accurate and simultaneous determination of conjugated bilirubin, unconjugated bilirubin, HbO_2, and methemalbumin in adult human serum.

Methodology

Subjects

A healthy adult volunteer was investigated in this study. The volunteer had to fulfill the following criteria: not suffering from any hemolytic anemia, hepatitis or liver failure. The study was approved by the Research Ethics Committee of National Research Centre. The ethical approval number was 02920, 5 Mar, 2020. The committee established its regulatory rules for human and animal research ethics based on the Declaration of Helsinki 2000-2008 and WHO regulations 2000-2011.

Blood collection

Blood samples were withdrawn from humans by venipuncture and collected into plastic tubes without anticoagulant. Then, blood samples were allowed to coagulate partially for only two minutes at room temperature. This is important because the long period of coagulation was found to be accompanied with high hemolysis and turbidity. The blood samples were centrifuged at 4000 rpm for 15 minutes to separate the serum. Immediately after separation, serum samples were centrifuged at 6000 rpm for 10 min to eliminate any erythrocytes or lipids that present. Supernatant was removed carefully and centrifugation has been continued for second time to eliminate any remaining erythrocytes. Clear sera were analyzed immediately after blood collection.

Determination of conjugated bilirubin, unconjugated bilirubin, oxyhemoglobin and methemalbumin

The levels of the total bilirubin
, UB, CB, oxyhemoglobin and methemalbumin in human serum were determined by the following multi-component spectrophotometric method.

Materials and sample preparation

For absorbance measurements, 100 µl of the human serum is added to 1 ml of ice-cold distilled water (pH 9.3). After mixing and incubation in a refrigerator at 4 ^{0}C for 10 min, the serum solution was clarified further from any turbidity using centrifugation at 10,000 rpm, 4 ^{0}C for 10 minutes. The clear serum solution was separated for experimental purposes for absorbance measurements, by pouring and pipetting the clear supernatant into another clean tube.

Measurements and calculations

The absorbance measurements for the purified human serum solution were made at five wavelengths (λ = 450, 490, 576, 600 and 700 nm), using a Cary UV/VIS double-beam spectrophotometer (model 100 UV-VIS), manufactured by Agilent Technologies, Australia. The spectrophotometer was adjusted at a spectral band width of 2.0 nm and a quartz cuvette of 1.0 cm light path was used for absorbance measurement. The absorbances A_{450}, A_{490}, A_{576}, A_{600} and A_{700} of the serum pigments in solution were calculated by subtracting the absorbances of the blank from the absorbances of the solution measured at the same wavelengths, against air as a reference.

The four absorbances A_{450}, A_{490}, A_{576} and A_{600} should be corrected for the very low residual turbidity remaining after centrifugation of serum solution, by subtracting from the absorbance at 700 nm (A_{700}), where the serum pigments have negligible absorption, the absorption at this wavelength is mainly due to serum turbidity. The four absorbances should be corrected also for transferrin and carotenoids (in the form of β-carotene) present in human serum, by using the usual reference concentrations and molar absorptivity of these components in human serum [4], as the following:

$$A'_{450} = A_{450} - A_{700} - 0.0180569 \quad [1]$$

$$A'_{490} = A_{490} - A_{700} - 0.0159914 \quad [2]$$

$$A'_{576} = A_{576} - A_{700} - 0.00286364 \quad [3]$$

$$A'_{600} = A_{600} - A_{700} - 0.00109091 \quad [4]$$

Where, A'_{450}, A'_{490}, A'_{576} and A'_{600} are the corrected absorbances of serum solution, and 0.0180569, 0.0159914, 0.00286364 and 0.00109091 are the correction factors for transferrin and carotenoids at 450, 490, 576 and 600 nm, respectively. Since in practice hypercarotinemia is rare [6], we used the usual reference value of β-carotene in adult serum (0.57 μmol/L) [4] to correct for these absorbances.

For human adults, the 16 millimolar absorptivities of conjugated bilirubin (CB), unconjugated bilirubin (UB), HbO_2 and methemalbumin (Mha), determined previously [4], were substituted into four linear equations of the type described by the theory of multi-component spectrophotometric analysis [7], with the four unknown concentrations of serum pigments (C_{UB}, C_{CB}, C_{HbO2} and C_{Mha}).

$$A'_{450} = 47.1C_{UB} + 54.7C_{CB} + 15.4C_{HbO2} + 10.2C_{Mha} \quad [5]$$

$$A'_{490} = 29.7C_{UB} + 24.8C_{CB} + 5.6C_{HbO2} + 8.35C_{Mha} \quad [6]$$

$$A'_{576} = 0.07C_{UB} + 0.07C_{CB} + 14.5C_{HbO2} + 4.95C_{Mha} \quad [7]$$

$$A'_{600} = 0.0C_{UB} + 0.0C_{CB} + 0.8C_{HbO2} + 4.95C_{Mha} \quad [8]$$

The above linear system of equations can be represented in the matrix form as:

$$\begin{bmatrix} 47.1 & 54.7 & 15.4 & 10.2 \\ 29.7 & 24.8 & 5.6 & 8.35 \\ 0.07 & 0.07 & 14.5 & 4.95 \\ 0 & 0 & 0.8 & 4.95 \end{bmatrix} \times \begin{bmatrix} C_{UB} \\ C_{CB} \\ C_{HBO2} \\ C_{Mha} \end{bmatrix} = \begin{bmatrix} A'_{450} \\ A'_{490} \\ A'_{576} \\ A'_{600} \end{bmatrix}$$

This linear system of equations was solved by mathematical manipulation, using the Gaussian elimination method for matrix calculation [8], to yield the following formulas of adults serum pigments:

$$C_{Mha} = \frac{A'_{600} + 0.415057411\times10^{-4}A'_{450} + 6.437625159\times10^{-5}A'_{490} - 0.0552413582A'_{576}}{4.677516177} \quad [9]$$

$$C_{HbO2} = \frac{A'_{576} - 7.513526537\times10^{-4}A'_{450} - 1.1653633\times10^{-3}A'_{490} - 4.932605419C_{Mha}}{14.48190314} \quad [10]$$

$$C_{CB} = \frac{0.630573248A'_{450} - A'_{490} - 4.110828025C_{HbO2} + 1.918152866C_{Mha}}{9.692356688} \quad [11]$$

$$C_{UB} = \frac{A'_{450} - 54.7C_{CB} - 15.4C_{HbO2} - 10.2C_{Mha}}{47.1} \quad [12]$$

$$C_{TB} = C_{CB} + C_{UB} \quad [13]$$

Where A'_{450}, A'_{490}, A'_{576} and A'_{600} are the corrected absorbances measured experimentally at wavelengths 450, 490, 576 and 600 nm, respectively, for purified, diluted, aqueous serum solution and C_{UB}, C_{CB}, C_{HbO2} and C_{Mha} calculated by these equations represent the concentrations (in mmol/L) of various pigments in this diluted serum solution.

The C_{TB}, Mha and HbO_2 concentrations in human serum (in µmol/L) can be determined by using the following equations:

$$C'_{Mha} = 11\times10^3 \times C_{Mha} \quad \mu mol/L \qquad [14]$$

$$C'_{HbO2} = 11\times10^3 \times C_{HbO2} \quad \mu mol/L \qquad [15]$$

$$C'_{TB} = 11\times10^3 \times C_{TB} \quad \mu mol/L \qquad [16]$$

Where 11 is the dilution factor of human serum and 10^3 is the conversion factor for mmol/L to µmol/L.

Determination of total bilirubin by the diazo method

Conjugated (direct) and total bilirubins concentrations were also determined also by the diazo assay, as a reference method, using the bilirubin kit (Diamond Diagnostics Company, Cairo, Egypt), according to the Doumas version of the Jendrassik-Grof method [9].

Data analysis

Data were presented as the mean ± standard deviation (SD) values. Using the statistical program (Statistics Calculator), the *t* distribution was used to calculate the 95% confidence interval of the parameter assessed.

The within run to run reproducibility and precision of the method were assessed by the percentage ratio of SD to the mean-values, coefficient of variation (CV) values and the 95% confidence intervals of the parameter assessed for a serum sample from a single adult subject. The CV values can be used to assess the precision of the individual values while the range of the 95% confidence interval can be used to assess the precision of the mean values of replicates determinations.

By using the advanced database (Clipper)-language, with a Clipper compiler and PTLINKER, a computer program was designed, by which we can estimate easily the concentrations of conjugated, unconjugated and total bilirubins, HbO_2 and methemalbumin in human sera, based on the equations (1-16) mathematically derived for human's serum-pigments. The software is characterized by the simplicity, speed, and high accuracy. This software is suggested and available by the author for request.

Results

Precision and accuracy

The results of the reproducibility, precision and accuracy of the method for TB, UB and CB are illustrated in Table 1. The method provided reproducible results of TB, UB and CB at the rates of 3.84%, 7.4 and 10.55%, respectively, indicating the high reproducibility of the method. The small ranges of 95% confidence intervals indicates the high precision of the mean values, while the small coefficients of variation (CV) indicate the high precision of individual values, determined by this method. On the other hand, the small values of % error, 0.201 and 2.772%, for total and unconjugated bilirubins, indicate the high accuracy of the method. The values of total bilirubin assessed by our method are generally close to the reference actual values determined by the diazo reference method.

Table 1. Reproducibility, precision and accuracy of the multi-component spectrophotometric method for the total bilirubin (TB), unconjugated bilirubin (UB) and conjugated bilirubin (CB) in a healthy adult human serum.

Parameter	TB (µmol/L) (Run to run determinations, n=12) (The reference value= 15.39 µmol/L)*	UB (µmol/L) (Run to run determinations, n=12) (The reference value= 11.654 µmol/L) *	CB (µmol/L) (Run to run determinations, n=12) (The reference value= 3.736 µmol/L)*
Mean ± SD	15.359±0.59	11.331±0.839	4.028±0.425
Coefficient of variation (CV)	3.84%	7.4%	10.55%
95% Confidence interval for mean	15.006–15.712	10.828-11.834	3.773–4.283
Range of the 95% confidence interval	0.706	1.006	0.51
% Error	0.201%	2.772%	7.81%

*The values of total bilirubin measured by the diazo reference method.

The % error of the values of bilirubin measured by our method was calculated as the difference between the mean value of 12 determinations measured by this method and the reference actual value measured by the reference diazo method, relative to the reference actual value. n indicates the number of replicates.

Comparison of the method with the reference diazo-method

Figure 1 shows a linear regression plot for serum samples from adult subjects. The results show a good correlation with the reference diazo-method: a slope of 0.981, an intercept of -0.493, a correlation coefficient of 0.998.

Samples from healthy adults and an adult with jaundice were analyzed by both the multi-component spectrophotometric and diazo-reference methods.

Linear-regression analysis was used to determine the best fit line, with the corresponding fit function, with a slope of 0.981, an intercept of -0.493, a correlation coefficient of 0.998.

Reproducibility and precision of HbO2 and Mha concentrations

The results of reproducibility and precision of HbO_2 and Mha concentrations determined by this method are shown in Table 2. Reproducibility (CV) of the method for HbO_2 is within ±8.0% at HbO_2 concentration 4.372 μmol//L (0.070 g/L), which is significantly higher than that of the previous direct spectrophotometric method [10], which showed poor reproducibility within ±25%. Reproducibility (CV) of the method for Mha is within ±48% at methemalbumin concentration 2.668μmol/L. The small values of 95%-confidence interval ranges indicate the high precision of the method.

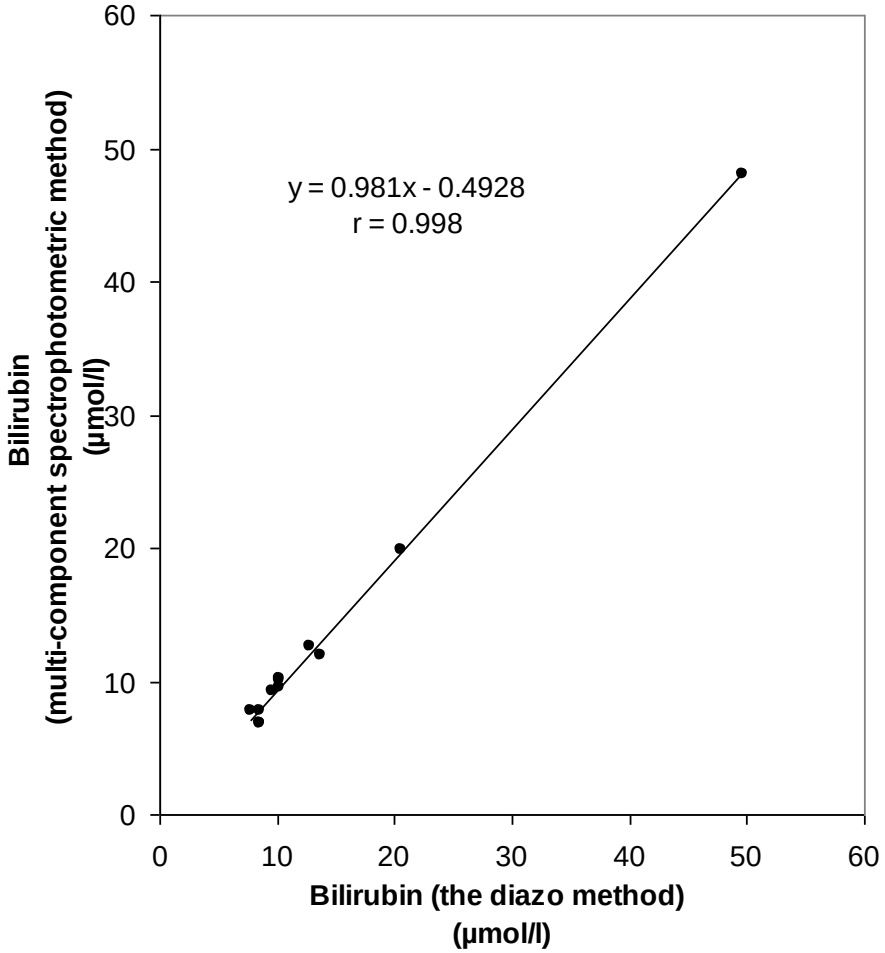


Figure 1. Comparison of the multi-component spectrophotometric method and the diazo-reference method used for determination of total bilirubin in adult human sera.

Table 2. The reproducibility and precision of the multi-component spectrophotometric method for oxyhemoglobin (HbO_2) and methemalbumin (Mha) concentrations in a healthy adult human serum.

Parameter	HbO_2 (µmol/L) (n=12)	Mha (µmol/L) (n=12)
Mean ± SD	4.372±0.35	2.668±1.3
Coefficient of variation (CV)	8%	48.7%
95%-Confidence interval for mean	4.162-4.582	1.889-3.447
Range of 95%-confidence interval	0.42	1.558

-n indicates the number of replicates.

Discussion

Our method allows determination of the major serum pigments, which have high absorption coefficients, like bilirubins, HbO_2 and methemalbumin [4]. Moreover, it allows correction for the minor components, which have lower absorption coefficients, like transferrin, or lower concentrations like carotenoids (in the β-carotene form), which may interfere [4]. Therefore, it yields more accurate values of total bilirubin as compared to previous direct spectrophotometric methods [6, 11, 12, 13].

Furthermore, this method yielded values of total bilirubin with a high accuracy and reproducibility. Moreover, our method is precise as indicated by the small CV (3.84%), in contrast the previous direct spectrophotometric method [13], which showed a high imprecision (CV) of 15% at a bilirubin concentration of 21 µmol/L (1.2 mg/dl).

Moreover, our method allows precise determination of serum HbO_2 concentration, which increases in cases of intravascular hemolysis [14] and therefore our method has the importance of diagnosis of this disease.

Conclusion

In conclusion, the multi-component spectrophotometric method, suggested in this study for determination of TB, UB, CB, HbO_2, and Mha in adult human sera, is highly reproducible and precise. In addition to high reproducibility and precision, the method is characterized also by high speed as well as small sample volume. Furthermore, the method is inexpensive, since it is based on using distilled water as a solvent, and the conventional spectrophotometer. In addition, the method is simple, and can be computerized. Moreover, for the first time, the method can be used, simultaneously, to determine concentrations of the TB, UB, CB, HbO_2 and Mha concentrations in adult human sera.

Declaration of interest

There are no declared conflicts of interest by the authors who are responsible for content and writing of this manuscript.

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