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Icteric human samples: Icterus index and method of estimating an interference-free value for 16 biochemical analyses

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Background: Hemolysis, Icterus, and Lipemia constituting the HIL index, are the most common causes of interference with accurate measurement in biochemistry. This study focuses on bilirubin interference, aiming to identify the analyses impacted and proposing a way to predict nominal interference-free analyte concentrations, based on both analyte level and Icterus Index (I_{ict}).

Methods: Sixteen common analytes were studied: alanine aminotransferase (ALT), albumin (ALB), alkaline phosphatase (ALP), amylase (AMY), aspartate aminotransferase (AST), total cholesterol (CHOLT), creatinine (CREA, enzymatic method), fructosamine (FRUC), gamma-glutamyl transferase (GGT), HDL cholesterol (HDLc), total iron (Iron), lipase (LIP), inorganic phosphorus (Phos), total protein (PROT), triglycerides (TG), and uric acid (UA). Both the traditional 10% change in concentrations from baseline and the Total Change Level (TCL) were taken as acceptance limits. Nineteen pools of sera covering a wide range of values were tested on the Cobas® 6000 (Roche Diagnostics). I_{ict} ranged from 0 to 60.

Results: Eight analytes increased (FRUC and Phos) or decreased (CHOLT, CREA, HDLc, PROT, TG, and UA) significantly when I_{ict} increased. FRUC, HDLc, PROT, and UA showed a linear relationship when I_{ict} increased. A non-linear relationship was found for TG, CREA, and for CHOLT; this also depended on analyte levels. Others were not impacted, even at high I_{ict}.

Conclusions: A method of estimating an interference-free value for FRUC, HDLc, PROT, Phos, UA, TG, and CREA, and for CHOLT in cases of cholestasis, is proposed. I_{ict} levels are identified based on analytical performance goals, and equations to recalculate interference-free values are also proposed.

KEYWORDS
bilirubin, biochemistry, correction, icterus index, interference

1 | INTRODUCTION

Biochemical analysis of body fluids provides clinically useful information. Hemolysis, icterus, and lipemia (HIL) are the most common causes of blood sample inadequacy and interfere with the accurate measurement of various analytes.[1-5]

Icterus interferes via bilirubin (BIL), in two ways: physical interference (light absorption) and chemical interference (with reagent components as H_2O_2).[6] For every assay method, a cut-off value for Icterus (Icterus Index: I_{ict}) is established by the manufacturer and indicated on the insert sheet,[7] or recognized through laboratory experience. Therefore, a sample may be rejected if the I_{ict} value exceeds this threshold. In the case of hemolysis (except if in vivo hemolysis), a new sample – without hemolysis – is required for accurate assay measurements. However, in some pathological circumstances, such as newborn jaundice or hepatic diseases like viral hepatitis or cirrhosis, a new

TABLE 1 Icterus interference cut-off from Roche[7] observed values and TCL (see abbreviation in text). For each analyte (column A), the tested range (column C) with the corresponding units (column B) is done. Roche's icterus interference cut-off at 10% (column D) is compared to the value observed when there is 10% variation (column E). At least, I_{ict} observed at TCL level (column F) is done. The values differ when TCL is not close to 10% (column G). Therefore, the interpretation is adapted to the analytical performance goals of the laboratory

Column A	Column B	Column C	Column D	Column E	Column F	Column G
Tests	Units	Range tested (min-max)	I_{ict} 10% proposal from Roche	I_{ict} observed at 10% variation	TCL (%)	I_{ict} observed at TCL (column F)
ALT	U/L	12-359	60	>60	11.3	>60
ALB	g/L	22.8-37.9	60	>60	5.3	45
ALP	U/L	48-381	60	>60	7.1	58
AMY	U/L	30-158	60	>60	6.3	>60
AST	U/L	14-734	60	>60	9	>60
CHOLT	mmol/L	2.06-6.72	14	14	4.7	7
CREA	µmol/L	55-912	15	13	6.0	6
FRUC	mmol/L	139-228	5	5	9.5	5
GGT	U/L	26-179	20	>60	8.9	>60
HDLc	mmol/L	0.5-1.74	30	20	6.9	12
Iron	µmol/L	5.14-27	60	>60	14.3	>60
LIP	U/L	21-410	50	>60	13.1	>60
Phos	mmol/L	0.73-1.95	60	>60	5.2	34
PROT	g/L	45.2-67.5	20	16	5.2	6
TG	mmol/L	0.81-3.64	10	20	11	21
UA	µmol/L	204-670	40	43	5.3	20

sample without interference is not available. Yet these are precisely the cases where every chemical analysis counts for the physician, and rejection of assays above the cut-off value could hinder treatment.

More recently, sophisticated chemical analyzers have automatically detected HIL status and have reported HIL index values. The HIL alert index (also known as the threshold level) is defined as the lowest concentration of HIL that interferes with chemical analyses, yielding a bias >10%.[6]

The aim of this study is to determine which analyses are impacted when I_{ict} increases and to propose a method of predicting, when possible, nominal interference-free analyte concentrations as a function of measured I_{ict}.

2 | MATERIALS AND METHODS

This study was considered a quality assessment project and was therefore deemed exempt from ethics committee approval. It was conducted at La Conception Hospital, Biochemistry Laboratory, Marseille, France. To evaluate the effect of bilirubin on routine analytes, we analyzed the most common analytes reported by manufacturers to be affected by this interferent. We chose the most common, manufacturer-reported interferents and tested different levels of bilirubin. We provide a comprehensive report on the effect of this interference on the analytes listed in Table 1.

We studied the effect of icterus interference on alanine aminotransferase (ALT, IFCC with pyridoxal phosphate), albumin (ALB,

bromocresol green), alkaline phosphatase (ALP, IFCC), amylase (AMY, IFCC), aspartate aminotransferase (AST, IFCC with pyridoxal phosphate), total cholesterol (CHOLT, enzymatic), creatinine (CREA, enzymatic), fructosamine (FRUC, nitroblue tetrazolium), gamma-glutamyl transferase (GGT, IFCC/Szasz), HDL cholesterol (HDLc, direct method), total iron (Iron, ferrozine), lipase (LIP, diglyceride substrat), inorganic phosphorus (Phos, molybdate, UV), total protein (PROT, Biuret), triglycerides (TG, enzymatic), uric acid (UA, uricase). Samples were tested on the Cobas® 6000 (Roche Diagnostics, Meilan, France). All assays were performed according to manufacturer's instructions. Nineteen pools were realized covering a wide range of values. As a preliminary, to be pooled, each serum was analyzed for triglycerides and tested to ensure that it was without detectable concentrations of free hemoglobin and bilirubin (HIL≤1).

A 10 mmol/L bilirubin stock solution was prepared with physiological sera from of commercially available human conjugated bilirubin (ref B850 from Frontier Scientific, Logan, UT, USA) and stored away from light at −20°C. Each pool was aliquoted (about 1 mL) and spiked with 100 µL of an extemporaneous dilution of bilirubin stock solution (with physiological sera) in order to obtain I_{ict} ranging from 0 to 60 in 16 samples and immediately measured for the analytes listed above, as well as for total bilirubin (TBIL) and I_{ict}.

Given the lack of studies on icterus interference using Roche Diagnostics analyzers and the conflicting published results, our three objectives were: (1) to verify I_{ict} quality specifications on Roche Diagnostics analyzers for chemistry testing, considering both
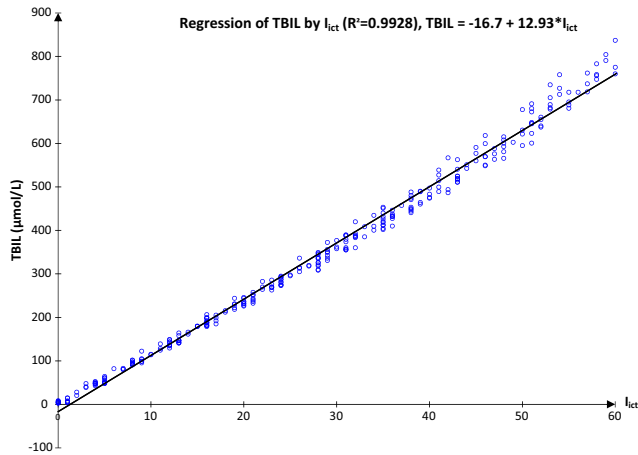
FIGURE 1 Relation between I_{ict} and TBIL (μmol/L; see abbreviation in text). A linear relation is observed between I_{ict} and TBIL. As I_{ict} is always available (and not TBIL), a relation between I_{ict} and the interference on the analytes should be studied

the traditional 10% change in concentration from baseline and the Total Change Level (TCL) as acceptance limits,[8,9] (2) to determine the proportion of tests impacted by icterus beyond these allowable limits and (3) to propose a method of predicting, when possible, nominal interference-free analyte concentrations as a function of measured I_{ict}.

Two approaches were used to establish analytical performance goals:

1. The mean percentage deviation was compared to the Acceptable Change Limit (ACL), according to ISO 5725-6.[10] The ACL for interpreting a measured difference is based on the analytical imprecision (CVa), using the formula ACL = 2.77 CVa.
2. The second approach took into account acceptable imprecision, based on intra-individual biological variation. According to the College of American Pathologists recommendations, the imprecision of a method, for individual single and multipoint testing, should be equal or less than one-half of the average within-subject variation (CVb), and this should be the goal for short-term laboratory imprecision (≤0.5 CVb). The CVb of each analyte was taken from the listing of biological variation for 316 analytes by Ricos et al.[11] This database was most recently updated in 2014, but some analytes are still missing.[12]

To monitor changes in an analyte from the same sample for the same individual, we combined the two approaches (analytical and intra-individual imprecisions) by estimating the square root of the sum of the squared analytical and biological imprecisions, defined as the total change limit (TCL):

The TCL is the square root of the sum of squared analytical reproducibility (CVa) (obtained from Roche Diagnostics QC) and biological imprecision (CVb) (found in "Ricos database"[11,12]):

$$TCL = \sqrt{(2.77 \times CVa)^2 + (0.5 \times CVb)^2}$$

Based on the results observed, a function between I_{ict} and the variation in the analyte (in %) is estimated and optimized, depending on whether a linear or differing relationship to level is found.

We define X' as the interference-free calculated value, X as value of the analyte at I_{ict}, and $X0$ as the original value of the analyte (when $I_{ict} \leq 1$) corresponding to the unspiked pool. The analytes variation (in %) when I_{ict} increase is D for the observed values and Z for the calculated values after correction.

Working from the I_{ict} held in the laboratory, this relationship is used to estimate X' from all the observed values and the I_{ict}.

Based on results, the observed variation (D) in the analytes (in %) is $D = 100\,(X–X0)/X0$. Whereas, D is also a function of I_{ict} and is generally a linear equation, so $D = a + b \times I_{ict}$.

X' is calculated from I_{ict} and X. After changing $X0$ for X', the interference-free value is calculated from the relationship: $X' = X/(D + 1)$. Therefore, knowing X and I_{ict} makes it possible to estimate X'.

The % of difference between X' and $X0$ can be calculated. The goal is reached if less than 5% of Z (where $Z = 100\,(X'–X0)/X0$) gives a value below the TCL.

Data analysis was performed in Excel 2013 (Microsoft Inc., Redmond, WA, USA).

3 | RESULTS

As I_{ict} was determinate by a simple measure of two wavelengths (at 505 and 480 nm) with hemoglobin and lipemia correction, however, it is not a direct determination of TBIL level. So, we observe that there is a linear correlation between I_{ict} and TBIL (Figure 1). The same correlation is found with I_{ict} and direct bilirubin (data not shown). Nevertheless, a better correlation is observed with a second degree equation, where TBIL $= 3.89.10^{-2} \times (I_{ict})^2 + 10.6 \times I_{ict} + 5.21$ ($R^2 = 0.9954$).

Different types of relation between an analyte and I_{ict} are observed (Figure 2) as positive interference a negative interference and non-linear variation and two-level interference.

The range of tested analytes and the 10% icterus interference cut-off from Roche Diagnostics and the cut-off from the observed values and from TCL are included in Table 1. Eight analytes increase or decrease significantly when I_{ict} increases. CHOLT, CREA, HDLc, PROT, TG, and UA decrease, while FRUC and Phos increase. Figure 2 shows also for these eight analytes the equations between I_{ict} and D. The allowed cutoff, TCL, is included and the figure shown the impact of I_{ict} before (D) and after correction (Z).

Between CHOLT and I_{ict}, the relation is more complex (Figure 2). For CHOLT, for the same decrease, 20% for example, I_{ict} ranges from 20 to 50 depending on CHOLT level. In the same manner, for the same I_{ict}, 30 for example, CHOLT decrease ranges from −12.8% to −24.3%. Therefore, Figure 2g show the complexity of the relation, based on I_{ict} but also on CHOLT level. In this case, Z is done only for CHOLT>4. In this condition, if $I_{ict} \leq 40$, the Z bias is less than 5%, when TCL is used as acceptable limit.
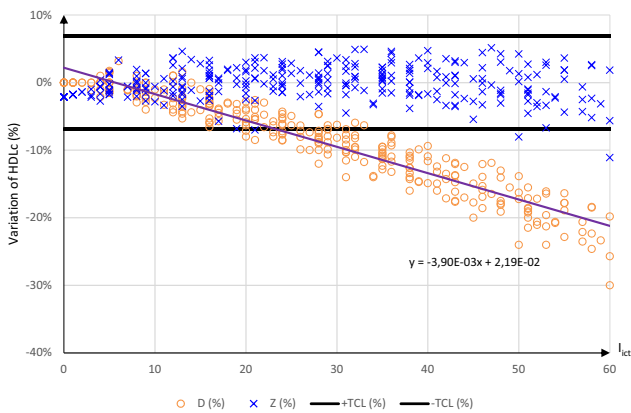
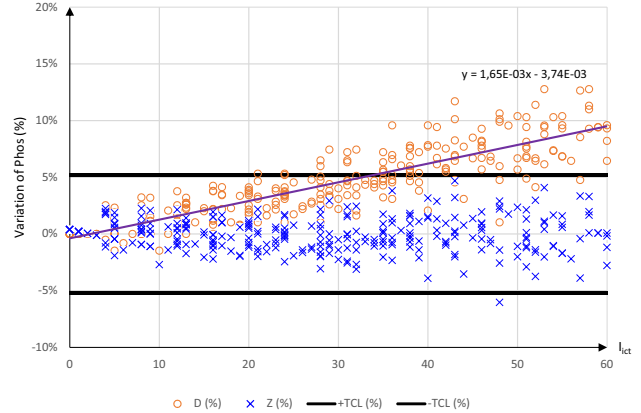**(A)** Variation of PROT by I_{ict} before D(%) and after correction (Z%)

$y = -5,19E-03x + 1,91E-02$
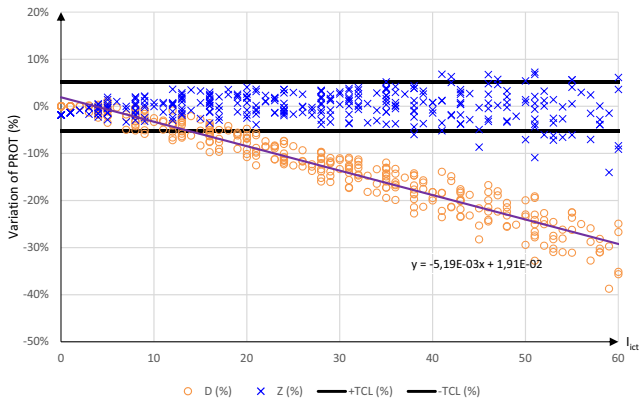
(B) Variation of AU by I_{ict} before D(%) and after correction (Z%)

$y = -2,07E-03x + 1,18E-02$
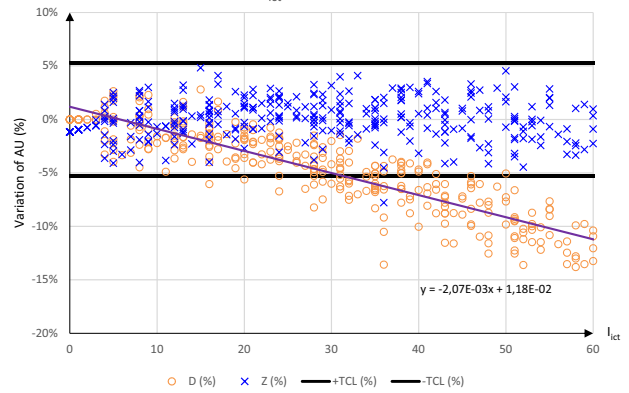
(C) Variation of HDLc by I_{ict} before D(%) and after correction (Z%)

$y = -3,90E-03x + 2,19E-02$

(D) Variation of Phos by I_{ict} before D(%) and after correction (Z%)

$y = 1,65E-03x - 3,74E-03$

(E) Variation of TG by I_{ict} before D(%) and after correction (Z%)

$y = -1,14E-02x - 1,74E-03$

$y = 1,15E-04x^2 - 1,46E-02x + 2,09E-02$

(F) Variation of CREA by I_{ict} before and after correction (Z%)

$y = -4,92E-03x + 2,60E-02$

$y = -6,34E-03x + 3,18E-02$

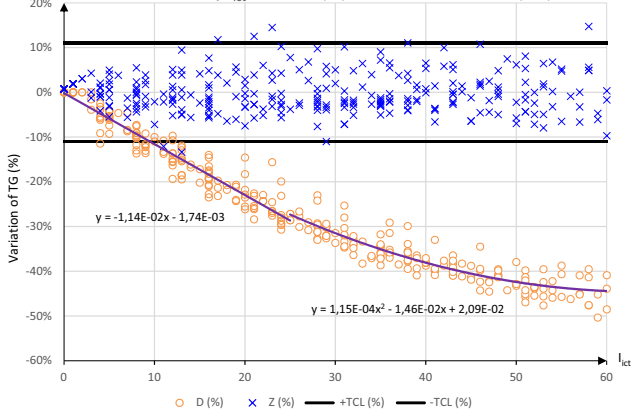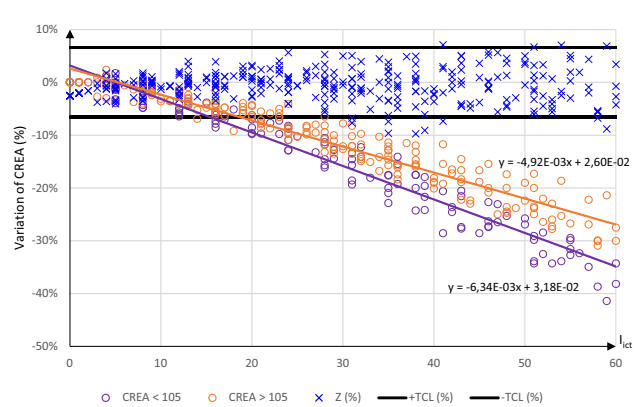**(G)** Variation of CHOLT by I_{ict} before and after correction (Z%)

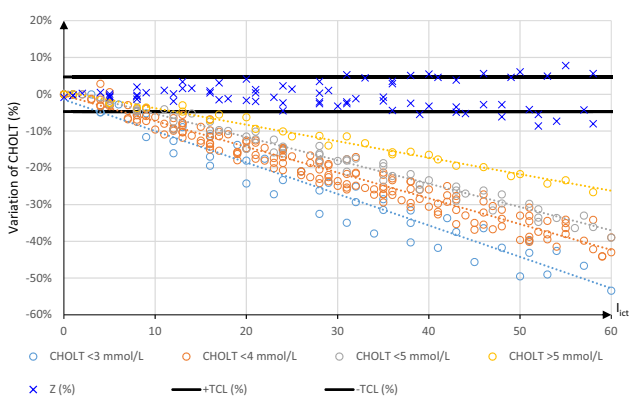**(H)** Variation of FRUC by I_{ict} before D(%) and after correction (Z%)
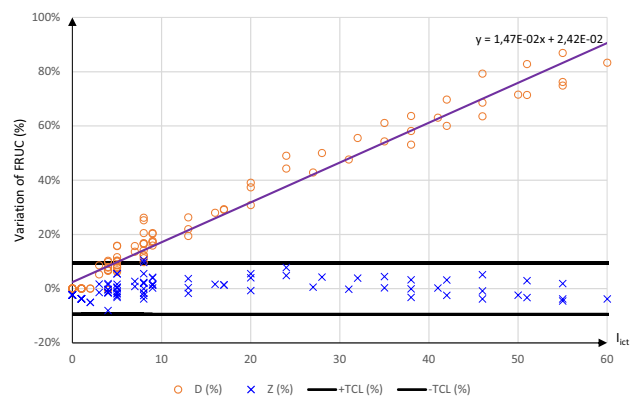
$y = 1,47E-02x + 2,42E-02$

FIGURE 2 Relation between I_{ict} and analytes. Observed variation before D (%) and after correction Z (%), allowable cut-off as $\pm$TCL (%) and equations between I_{ict} and D (%) are shown for PROT (A), AU (B), Phos (C), HDLc (D),TG (E), CREA (F), CHOLT (G) and FRUC (H). CHOLT is subdivided from <3 mmol/L to >5 mmol/L. The interference with CHOLT depends on level of this analyte. For CHOL (G), Z (%) is valid only when CHOLT >4 mmol/L and $I_{ict}\leq$40. In this last case (G), equation is $8.38 \times 10^{-3} - 5.41 \times 10^{-3x}$ (see abbreviation in text)

4 | DISCUSSION AND CONCLUSION

While previous studies indicate that the chemical properties of bilirubin, whether conjugated (cBIL) or unconjugated (ucBIL), do not contribute to differences in interference,[5] Roche Diagnostics gives differing levels of interference for I_{ict} with cBIL and ucBIL. Bilirubin may be present in some pathological circumstances, and therefore sera analysis could be impacted.

Our conclusion here is that Roche Diagnostics suggested icterus interference cut-off yield a bias $\geq$10%, except for GGT and TG (Table 1). For GGT, Roche Diagnostics indicates an I_{ict} cut-off at 20, as no influence was observed in our study, which is in agreement with.[13] For TG, Roche Diagnostics suggested cut-off is half the value we observed. Roche Diagnostics consistently refers to Glick et al. [14] on HIL interference. In this publication, TG seems to increase when bilirubin is added. However, data show the opposite (Figure 2). In the latest available dataset,[7] Roche Diagnostics indicates that a decrease is observed when I_{ict} is above 10. Moreover, Roche Diagnostics even refers to Glick et al. on HIL interference for FRUC, HDLc and Iron, which are not tested in Glick's publication. For CREA, although there is now an enzymatic method, Roche Diagnostics still refers to the same study (CREA with Jaffé method). Manufacturer's data are not always confirmed in laboratory practice.[1,2,15] The conclusion differs when TCL is used as acceptance limit. Interference cut-off is therefore recalculated and generally emerges as lower than the arbitrary 10% cut-off. However, even with TCL cut-off, we observed that some analytes are not impacted by plasmatic bilirubin, even at high concentrations. Parameters not impacted by plasmatic bilirubin are ALP, ALT, AMY, AST, GGT, Iron, and LIP (Figure 2). These results are in agreement with the Roche Diagnostics dataset [7] and other studies.[3,5,16] One parameter is affected (ALB), but only at high I_{ict}, above 45, which corresponds to about 563 µmol/L of bilirubin (Figure 1).

For analytes that decrease, we found that the degree of icterus interference differs according to analyte. A linear relationship was found for FRUC, HDLc, PROT, and UA. A non-linear relationship (Figure 2) was found for TG, and for two analytes (CREA and CHOLT), where it also depended on analyte concentrations.[17] For five analytes, FRUC, HDLc, PROT, Phos and UA, a simple correction with a linear equation gives the interference-free value (X'). The equations are easy to use and only require the values for the analyte regarding I_{ict} level and I_{ict}. As these two parameters are available, it is possible to estimate the true analyte level. However, Ali et al.[18] show that there are two levels of interference for PROT, tested at 30 and 74 g/L, which are outside our studied range. For TG, due to the nature of the relationship, with I_{ict} and % of interference, two equations are needed. One equation applies to low levels of I_{ict} with a linear relationship, such as FRUC, HDLc, PROT, P, and UA. However, when I_{ict} is above 25, the relationship is not linear, and a second-degree equation is

needed. In both cases, the interference-free value (X') remains easy to estimate. For CREA, the correction is more complicated. CREA from Roche Diagnostics involves an enzymatic method used at the end of reaction, a Trinder reaction. For the same I_{ict}, the lower CREA is, the greater the decrease. Therefore, X' requires an intermediate step to be calculated. Because the decrease in CREA depends on both CREA level and I_{ict}, a cut-off level at 105 µmol/L, the upper limit reference value, must be calculated. If (107.73−0.5167 × I_{ict}<105) then the equation for low CREA could be used, and the second linear equation, for high CREA, otherwise. Therefore, the correction would take both normal and pathological CREA values into account. A corrected value for CREA can be used to estimate the glomerular filtrate rate (GFR) more precisely, including CKD-EPI, with obvious impact on clinical interpretation. For all these parameters (FRUC, HDLc, PROT, Phos, UA, TG, and CREA), the interference-free value (X') is close to the "true" value. The Z bias is always less than 5% when TCL is used as an acceptable variation (Figure 2). For CHOLT no simple relationship has been found.[19] When icterus is due to cBIL (cholestasis), cholesterolemia generally increases and the X' value must be calculated with the equation using high cholesterol (>4 mmol/L) spiked samples. In this condition, if I_{ict} ≤40, the Z bias is less than 5%, when TCL is used as acceptable limit. The proposed correction is not suitable if cholesterol is lower than 4 mmol/L or if I_{ict} >40.

The protocol used here is both simple and efficient, requiring only the dilution of cBIL with physiological sera to spike the pool of sera. We chose to spike the sera pool with cBIL because it is totally water-soluble. Previous studies have often used ucBIL.[5,6,20] However, in this case, ucBIL needs to be solubilized at high pH due to NaOH. The resulting pH increase could induce:

1. Spectral change in bilirubin,[21-23] transformation of bilirubin to biliverdin.[22,24]
2. Possible change in analytical conditions for some analytes.

A possible explanation for Bilirubin's interference is the following. CHOLT, CREA, HDLc, TG, and UA involve the same steps for determination in the analyzer. At the last step of the reaction, catalyzed by oxidase or peroxidase, the liberated hydrogen peroxide reacts with compounds to form a quinone imine chromogen (TRINDER reaction). The color intensity of the quinone imine chromogen formed is directly proportional to the analyte concentration in the reaction mixture.

However, Bilirubin interferes in oxidase/peroxidase-based test systems.[6,19] Proportionally to its concentration, bilirubin reacts with H_2O_2 formed in the test system, which in turn generates systematically lower results in enzymatic procedures used for the measurement of CHOLT, CREA, HDLc, TG, and UA.

In a strongly acidic solution, the absorption of conjugated bilirubin shifts to the UV wavelengths (340 nm). Therefore, bilirubin interferes

in the determination of Phos via the phosphomolybdate method through its reducing effect,[25-27] because of the sulfuric medium. In an alkaline solution, the increase in absorption as a result of oxidation of bilirubin to biliverdin, which shows a broad band at about 650 nm,[28,29] is the main cause of bilirubin interference[25] with FRUC and PROT. Furthermore, to some extent, ucBIL in alkaline solution can react with copper to form Bil-Copper complex, which can contribute to PROT decrease.[30-33]

This study presents some limitations. First, the results are based on assays using Roche Diagnostics analyzers for chemistry testing. Further assays realized on other diagnostic analyzers might alter the conclusions. Based on Roche Diagnostics documentation,[7] others analytes in biochemistry that could be impacted by icterus are acid phosphatase, creatinine with Jaffé method, lithium and glucose (GOD PAP). None of these analytes are tested in our laboratory with these methods. Second, our results are validated on the studied range (table 1), and extrapolations outside this range should not be made. Third, results are validated for the TCL used in our laboratory. TCL values are calculated in such a way as to always obtain less than 5% of Z outside TCL after correction. Fourth, no overall solution appears for CHOLT when I_{ict} increases. Finally, a totally enzymatic determination of creatinine plasmatic values would probably not involve the interference shown here.

In conclusion, this study shows that only some biochemical laboratory tests are impacted by icterus. For FRUC, HDLc, PROT, Phos, UA, TG, and CREA, we propose a method of estimating the interference-free value. Only a partial solution was found for CHOLT. Based on I_{ict} and the observed value, a corrected value can be reached, as is done for calcium using protein level. The suggestion to correct laboratory values that have interferences present is generally considered to be ill-advised this concept has been explored previously for hemolysis[34] and, in this case, the consensus is that correction may work for a data series, but would fail for individual patients. However, the methodology used here for Icterus, with significant data (n>300 values), the determination of the interference-free value which was under TCL, may be used for individual patients. The equation can easily be integrated into the MPL Roche Diagnostic analyzer®. I_{ict} levels are based on analytical performance goals, and equations to recalculate interference-free values are also proposed. It appears that the impact of the arbitrary 10% threshold differs according to analyte (sodium vs LIP, for example). This suggests that cut-off levels should be assessed using the most appropriate methods as they evolve (CREA via enzymatic vs Jaffe, for example) and according to analyte. Finally, for the most important parameter, CREA, proper estimation is essential to establish the correct posology regarding GFR. With regard to icterus, CREA is generally underestimated, but our estimating method offers a good interference-free value suitable for clinicians.

ACKNOWLEDGMENTS

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