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ANALYTICAL PROBLEMS OF BILIRUBIN DETERMINATION BY DIAZOREACTION METHOD IN ICTERIC NEONATES IN ALGERIA

El kadi Fatima Zohra, Bouanani Fatima Zohra, Kanoun Khedoudja, Bouarfa Amine, Maaz Asma, Sofiane Bouazza, Meziani Samira

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INTRODUCTION

Neonatal icterus is a yellowish coloration of the teguments and mucous membranes that appears clinically when there is an excess of bilirubin in the blood. The particularities of the bilirubin metabolism in the newborn explain the high frequency with which icterus occurs at this age of life, usually, it is a simple icterus: physiological icterus without severity or consequences (Alkhotani et al., 2014), More occasionally, neonatal icterus can be serious when it is with unconjugated bilirubin and will induce a kernicterus known by chronic or permanent lesions of the brain (Cheng et al., 2012), This bilirubin is dangerous because of its great capacity of cellular diffusion, because it is liposoluble, it is neurotoxic and causes severe encephalopathies (Tsao et al., 2020). The bilirubinemia measurement allows on the one hand to confirm the clinical icterus which can be more or less intense, and on the other hand by distinguishing the conjugated forms called direct and non-conjugated forms called indirect (Rawal et al., 2020). Several methods of bilirubin determination have been described; either by HPLC, electrophoresis or spectrophotometry (Puppalwar et al.,2012); The most used method is the diazoreaction technique; a bilirubin molecule generates two azobilirubins, the most used diazotization agent is sulfanilic acid diazotized by sodium nitrite , different concentrations have been proposed, it has been reported over the time several modifications of the initial technique (Cherian et al., 1981) concerning the diazotization agent, the activator nature and the diazoreaction pH.

Different activators are used to activate the diazotization reaction rate of unconjugated bilirubin such as caffeine, diphyllin , methylsulfoxide ...; they act as surfactants by increasing the bilirubin solubility in the reaction medium (Dhungana et al., 2017).

The use of activator had made it possible very early on to differentiate the non-conjugated form from the conjugated form. The latter form, which reacts directly with the diazo-reagent, can be measured in the absence of activator. Among the disadvantages of this technique, the interference of lipemia, para proteins, carotenes, opalescence or lactescence of the sample and especially the interference of hemolysis which is very abundant in newborns (Lo et al., 2004); to eliminate these interferences a reading at two different wavelengths is practiced. Thus, the method of Hertz-Dybkaer (Hertz et al., 1974), recommends the simultaneous measurement of absorbance at 469 and 521 nm. The icteric index should therefore be reserved for newborns samples. The prevention of kernicterus in newborns requires monitoring of bilirubinemia, levels; whose the assay is often made difficult by some pre analytical and analytical difficulties.

Due to the fact that the pediatricians in the neonatology department were often not satisfied with the results issued by the laboratory, it has been seen necessary to optimize an assay technique that opts for a reliable result that is consistent with the symptomatology of the newborn.

MATERIALS AND METHODS

Biological material

Fresh serum collected on heparin tubes from icteric newborns (100 cases) at term and premature babies in the neonatology department was sent to the medical analysis laboratory of the maternity hospital.

Equipment

1. Chemistry analyzer: ERBA XL 300 mono- and bichromatic measuring machine with multi-wavelength diffraction grating (340-376-415-450-480-505-546-570-600-660-700 and 750 nm).

2. Spectrophotometer: Robert Riele with 06 standard interference filters.

Methods

The bilirubin determination by diazoreaction was performed by two methods:

-Method with the Cetrimide activator of the ERBA Mannheim reagent which is read automatically (ERBA XL300) and manually (Robert Riele spectrophotometer).

- Method with the DMSO activator of the Biomaghreb reagent which is read manually (Robert Riele spectrophotometer).

Determination Principle

The assay technique is the diazoreaction method (Kwo et al., 2017) which was established by Malloy -Evelyn modified by (Walters et al.,1970); bilirubin is converted to azobilirubin using diazotized sulfanilic acid; the two fractions present in serum: bilirubin -glucoronide or conjugated bilirubin / direct bilirubin (DB) and free bilirubin associated with albumin or indirect bilirubin; only the first one reacts in aqueous medium, the second one reacts only by solubilization with a booster which is either cetrimonium bromide in the case of ERBA reagent or dimethyl sulfoxide (DMSO) found in Biomaghreb reagent.

Operating mode

The operating mode of the used techniques is illustrated in Table 1 .

Biomaghreb reagent: R1: sulfanilic acid (30 mmol /L) +Hcl (150 mmol /L); R2: sodium nitrite (20 mmol /L); R3: sulfanilic acid +HCL +DMSO (7 mol /L)

ERBA reagent: R1: sulfanilic acid (28.87 mmol /L) +HCL(23 mmol /L); R2: sodium nitrite (2.9 mmol /L); R3: sulfanilic acid + HCL + cetrimonium bromide (68.6 mmol).

Two spectrophotometer readings were carried out at time T_1= 05 min and T_2=10 min for DB and T_1 =10 min, and t T_2= 15 min for TB, in order to evaluate the influence of the incubation time on the reliability of the assays.

Statistical analysis

The results are presented in M± SD, the statistical analysis is carried out with the software: IBM SPSS; the graphical representations are illustrated with Microsoft Excel 2016, after verification of the normality, the comparison between the concentrations is carried out with the ANOVA test.

RESULTS AND DISCUSSIONS

Bilirubin determination by diazoreaction is the most commonly used method for clinical purposes. It is a simple method, easy for adapting to automatic analyzers and spectrophotometers .However, there are still limitations especially for the determination of serums from newborns with hyperbilirubinemia **(Dhungana et al., 2017).**Such as interference of hemolysis **(Devgun and Richardson , 2016),** the choice of the appropriate activator, the required incubation time, and the sample volume **(Skurup , 2008).**

Influence of the activator nature and reagent

Our results confirm that the serum level of TB is influenced by the different techniques used, with values (112.59±36.7 vs 134.54±52.52 vs 108.74±44.52 mg/L) for: automatic technique ERBA , manual technique ERBA and manual technique Biomaghreb , respectively . Similarly, the serum DB level is unstable with values ranging from 6.13±1.9 to 11.64±6.44 mg/L. This may be due to the use of 4 fold more ERBA reagent in the manual technique compared to the automated technique (excess may confuse the results) (Fig.1.) (Boutwell, 1964).

Table 1. Procedure of the different used techniques for the direct and indirect bilirubin determination

	Automatic method ERBA	Manual method			
		Biomaghreb		ERBA	
		Control	Sample	Control	Sample
DB	200 µL R_1+50 µL R_2 +50 µL serum	1000 µL R_1 + 50 µL serum	1000 µL R_1+ 50 µL R_2+ 50 µL serum	800 µL R_1 + 50 µL serum	800 µL R_1+ 200 µL R_2 + 50 µL serum
TB	200 µL R_3 + 50 µL R_2 +50 µL serum	1000 µL R_3+ 50 µL serum	1000 µL R_3 + 50 µL R_2 + 50 µL serum	800 µL R_3 + 50 µL serum	800 µL R_3 + 200 µL R_2 + 50 µL serum
IT	08 min and 30 secs	5min for DB and 10 min for TB		5 min for DB and 10 min for TB	

BD : Direct Bilirubin ; TB : Total Bilirubin , IT : Incubation Time

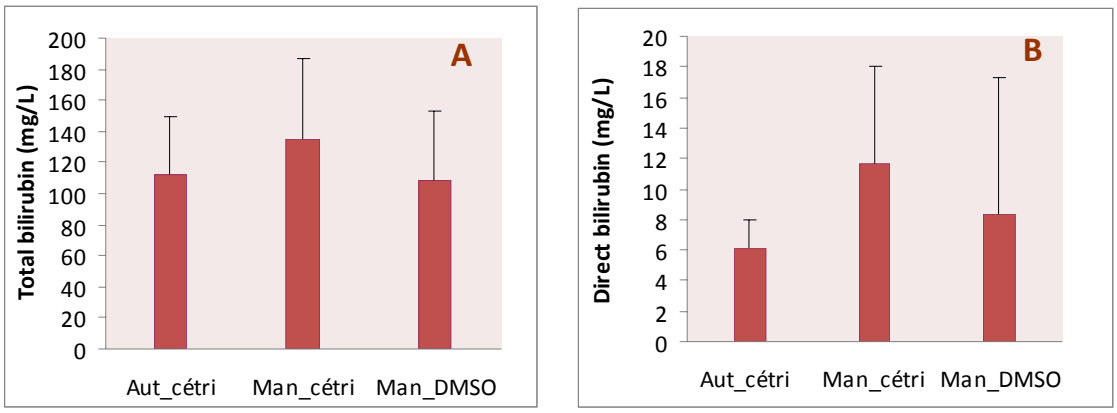

Fig .1 Serum content of total bilirubin (A) and direct bilirubin (B) as a function of the activator accelerant nature, technique and reagent

Influence of incubation time

Two readings were taken at the time: T_1= 5 min and T_2= 10 min for DB; and T_1 =5 min; T_2 = 15 min for TB; the results showed that there was no significant difference for the two manual methods: with the ERBA reagent (p=0.252) and Biomaghreb (p=0.262) for the determination of DB (Fig .2 A) . Similarly, the statistical analysis did not detect any significant difference (p=0.134) between the serum TB values determined by the ERBA manual method, but a highly significant difference in TB levels was detected with the Biomaghreb reagent (p<0.001) (Fig .2 B).

According to our results, the two manual readings with two differents accelerants (DMSO, cetrimide) for the DB determination using the two reagents ERBA and Biomaghreb, showed a reproducibility as a function of the incubation time , This is due to the fact that DB reacts rapidly in aqueous medium 5 min (time indicated in the reagent prospectus), was the time necessary for the coupling of all direct bilirubin with sulfanilic acid. Several studies have recommended an incubation time for DB ranging from 5 min to 10 min (Defreese et al., 1984).

Whereas, serum levels of TB using Biomaghreb reagent (DMSO gas accelerant) revealed non-reproducibility due to the slow reaction of unconjugated bilirubin.

Similarly, the incubation time was insufficient for the reaction to reach its maximum, studies have shown that an incubation time for icteric serums up to 20 to 30 min (Doumas et al., 1985 ; Puppalwar et al.,2012) is necessary to reach the level at which the absorbance remained stable .

The activator nature is also involved, knowing that the dosage of the latter depends on the solvent nature (Westwood, 1991) , for DMSO only unconjugated bilirubin is soluble in this solvent but it is at the same time unstable.

In addition, the results of the TB revealed a reliability at T_1 and T_2 with the activator cetremide. The latter is a quaternary ammonium salt: a mixture of tetradecyltrimethylammonium, dodecyltrimethylammonium and hexadecyltrimethylammonium, which dissociate in aqueous solution forming a large complex of cations is a more powerful surfactant than DMSO although it is used in lesser quantity compared to DMSO (85.07g/L vs. 546.9g/L).

Lolikha and Limpavithayakul, 1977 confirmed that in the presence of low concentrations of combined activator promote the complete bilirubin release ,which leads to the conclusion that the effective gas pedal must be able to interact both ionically and hydrophobically (Novros et al., 1979).

This was also reported in the study by Doumas et al.(1973) ,who determined the advantages of using two solvents at the same time: DMSO and Na_2CO_3. The modifications of the Malloy-Evelyn method made by Meites and Hogg et al.(1959) showed that an increase in the concentration of sodium nitrite, reduces to 10 min the necessary time for the total bilirubin reaction to be complete, which explains the reproducibility of the TB result at time T_1 and T_2 using the ERBA reagent (200µl sodium nitrite) compared to the Biomaghreb reagent (50µl sodium nitrite).

The effectiveness of cetremide was also noted during the observation on the automat of the level reached after 08 min 30 sec ,which shows the release in the cetremide presence of the bilirubin totality contained in the newborns serum which presented a hyperbilirubinemia; one can also note that starting from the linearity limit of the ERBA reagent (23mg /dL) compared to the Biomaghreb reagent (20 mg/L).

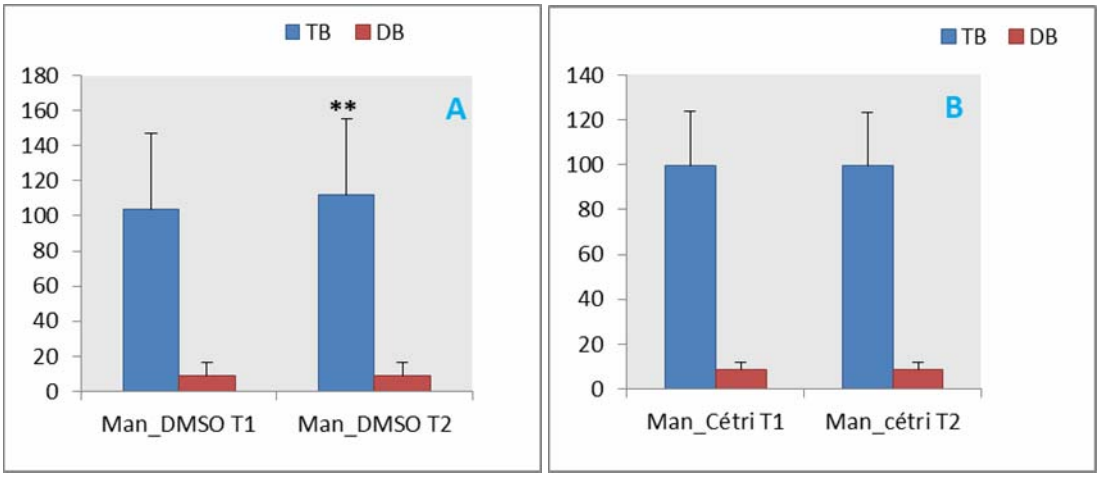

Fig. 2. Serum direct and total bilirubin content (mg/l) as a function of incubation time with DMSO activator (A) cetrimide (B) **p<.001

CONCLUSIONS

In view of the interest of bilirubin determination for the neonatal icterus control, we were requested to ensure the reliability of the results, despite the fact that we were confronted with numerous problems such as the interference of hemolysis, which is very abundant in the newborn samples; the volume of serum was insufficient, whereas 400 ul was needed. The unreliability of the multicalibrants; the lability of the standards; but once this study was done, it demonstrated the importance of respecting the operating mode of the method used, the incubation time as well as the reading time. Also, the choice of the adequate activator which must be adapted with the neonatal hyperbilirubinemia.

On the basis of this comparative study, the automatic method using the ERBA reagent with the cetrimide activator is the method of choice because of the precise reading time, of the effective activator; important linearity limit: 23mg/dL (TB) and 20 mg/dL (DB); the serum volume is very small (12.5 µl); the possibility of repeating the reaction and thus avoiding the need to replicate the newborn; the cells are thermostated at 37°C, mono and bichromatic with multi-wavelength diffraction grating in order to eliminate the hemoglobin interference .

ABSTRACT

The determination of bilirubin remains the key examination to diagnose neonatal icterus and its severity and on which the therapeutic decision is based, The objective of our study is to detect the choice method for the bilirubin determination .To this effect, 100 cases of newborn babies with mucocutaneous icterus at the Neonatology Department of the maternity Hospital of the Sidi Bel Abbés region (Algeria) were subjected to bilirubin determination by diazoreaction manually by spectrophotometry using the ERBA and Biomaghreb reagents and automatically by means of an automaton using the ERBA reagent. Our results showed that the accelerating factor and the incubation time influence the most sensitive fraction of bilirubin which is the total bilirubin, Similarly, the cetrimide accelerant of the ERBA reagent is more efficient than the DMSO of the Biomaghreb reagent. In view of the advantages of the automatic technique and the quality of the reagent used, ERBA and its cetrimide booster, it is chosen as the reference technique for the bilirubin determination in neonates.

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AUTHORS' ADDRESS

FATIMA ZOHRA EL KADI - Process engineering Laboratory, materials and environment, Faculty of Technology, Djillali Liabés University of Sidi-Bel-Abbés, Algeria.

FATIMA ZOHRA BOUANANI , BOUAZZA SOFIANE,SAMIRA MEZIANI - Biotoxicology Laboratory, Biology Department , Faculty of Natural Sciences and Life ,Djillali Liabés University of Sidi-Bel-Abbés, BP N°. 89 Sidi Bel Abbes 22000 Algeria

KHEDOUDJA KANOUN- Molecular Microbiology Health and Proteomics Laboratory, Biology Department , Faculty of Natural Sciences and Life ,Djillali Liabés University of Sidi-Bel-Abbés, Algeria,

e-mail: khedoudja.kanoun@univ-sba.dz and khedi20022002@yahoo.fr

AMINE BOUARFA, ASMA MAAZ -Medical Analysis Central Laboratory of the Mother and Child of Sidi-Bel-Abbés Hospital

Corresponding author:
elkadifatimazohra@yahoo.fr