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Modified chromogenic method to assess anti-Xa activity in jaundiced patients

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ABSTRACT

Objective: To propose a modification of the chromogenic conventional method (Rorachrom Heparin Stago's Method) to be used in patients with jaundice.

Material and method: An analytic epidemiological study was made. We compared the values of anti-Xa activity in 40 patients with jaundice and without jaundice using a modified chromogenic conventional method. The Pearson coefficient correlation was calculated.

Results: The absorbance of each reading was concurrent with the concentration of low molecular weight heparin added in each tube is not modified by the value of bilirubin with the pNA. The adaptation mentioned could be used in jaundice newborn achieving a more exact measurement of blood level of heparin.

Conclusions: The modified chromogenic conventional method is an excellent tool to have into consideration to measure anti-Xa in patients with jaundice.

Key words: anti-Xa activity, jaundice, modified chromogenic method.

Método cromogénico modificado para medir la actividad anti-Xa en pacientes ictéricos

RESUMEN

Objetivo: proponer una modificación del método convencional cromogénico para usarse en pacientes ictéricos.

Material and método: estudio epidemiológico analítico en el que comparamos los valores de la actividad anti-Xa en 40 pacientes con y sin ictericia usando un método cromogénico modificado. Se calculó el coeficiente de Pearson.

Resultados: la absorbancia de cada lectura fue concurrente con la concentración de heparina de bajo peso molecular agregada en cada tubo y no se modificó por la concentración de bilirrubina con el pNA.

Conclusiones: el método cromogénico modificado es una excelente herramienta a tener en cuenta para medir la actividad anti-Xa en pacientes con ictericia.

Palabras clave: actividad anti-Xa, ictericia, método cromogénico modificado.

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BACKGROUND

The indication of pharmacological thromboprophylaxis fixed-dose with low molecular weight heparin (LMWH) is a standard treatment of numerous clinical conditions and/or surgical procedures with the objective of reducing the venous thromboembolic disease (VTD). LMWH typically does not require laboratory monitoring except in newborn, children, high-risk pregnant, renal failure and obese patients.[1-4]

The recommended method to check the anticoagulant response of LMWH is the measurement of the activity anti-Xa activity whose peak occurs 4 hour after administration by subcutaneous via. The measurement of the activity anti-Xa using the chromogenic conventional method is often undervalued by the interference with elevated bilirubin levels in newborns with jaundice, for example.

The objective of our work is to propose a modification of the chromogenic conventional method (Rotachrom Heparin Stago's Method)[5] to be used in patients with jaundice.

MATERIAL AND METHOD

An analytic epidemiologic study was done. We analyzed the Pearson correlation coefficient (r) which measures the intensity of the linear relationship between the two quantitative variables.

Firstly, we determined and compared the values of anti-Xa activity in anicteric (without jaundice) patients using conventional and modified chromogenic method.

Secondly, we determined the values of anti-Xa activity using only modified chromogenic method in patients with jaundice.

Finally, we compared the values of anti-Xa activity using modified chromogenic method in both kinds of patients: without (anicteric) and with jaundice (icteric).

Setting/participants

This study was made with sample blood obtained from 40 anicteric and icteric newborns (total bilirubin 80mg/L at 200mg/L) patients from CEDEAC Laboratory and 25 de Mayo Clinic at Mar del Plata, City of Buenos Aires Province, Argentina.

The blood was collected in plastic tubes with sodium citrate at 3.2% (relationship between anticoagulant/blood 1/9, respectively) to determine and compare the anti-Xa activity in both groups using conventional and modified chromogenic method. Most of them were newborn with jaundice and thrombosis. In such cases the blood collection was made 4 hours after the enoxaparin injection.

Measurement

Immediately after the blood collection, the blood sample was centrifuged at 2000 G for 10 minutes to avoid the release of platelet factor 4 (PF4), which is a potent inhibitor of heparin. It was established as activity range of anti-Xa prophylactic between 0.2 and 0.6 U/mL, and as therapeutic activity range between 0.6 and 1.0 U/mL.[4]

If we have into account that the presentation of 1mg of is equivalent to 113 IU anti-Xa; we can say a vial of enoxaparin 20mg contains 0.2 mL, which is equivalent enoxaparin to 2260 UI anti-Xa and a vial of enoxaparin 40 mg contains 0.4 mL, which is equivalent to 4250 UI anti-Xa. The dosages were performed in citrated plasmas and added different concentrations of enoxaparin used by us in our centers. To obtain the curves, we diluted 10 µL of enoxaparin 20 mg with 14 mL of saline solution. The final concentration of this dilution was 8.0 UI of anti-Xa. Then, we took 0.1 µL of the before dilution of the enoxaparin 20

(8.0 UI anti-Xa) and added to 0.9 mL of plasma to be analyzed. When we worked with values of the curve greater than 0.8 UI anti-Xa, we made lower dilutions.

Variables

We performed correlation analysis that studies the relationship between two quantitative variables: anti-Xa activity in anicteric and icteric patients.

Chromogenic method

We used the conventional chromogenic method of Stago: Rotachrom heparin and modified the conventional method to compare the results of anti-Xa in icteric and anicteric serum.

Conventional chromogenic method

Samples + excess of factor Xa + specific chromogen (MAPA-Gly-Arg-pNA). The liberation of pNA is inversely proportional to the concentration of anti-Xa present in patient's plasma. The acid medium stops the reaction. We read at 405 nm. The curves are made with known data in IU of anti-Xa with LMWH used for the patients and Stago controls with different concentrations of anti-Xa.

Colouring property is contingent on the presence of molecules of chromophore groups (colour carriers) attached to the azo group (-N = N-).

Modified chromogenic method

Diazotation is carried in acidified pNA liberated in Chromogenic Rotachrom method and subsequent coupling. Giving a pink colour allowing its reading at 540nm, without overlapping with the bilirubin. The pNA is released in the reaction is inversely proportional to the heparin activity.

After the diazotation and coupling the diazoic red colorant is obtained. In the azoic colorants the azo group is the principal chromophore. According to the chromophore we obtain differences in colour and intensity and in acid environment are precipited.

To measure concentration through the light absorption, they were dissolved by HOK alkalization.

The diazotation reaction takes place between an aromatic primary amine in the presence of $NaNO_2$ and in strong acid medium to form diazonium salt intermediates for the formation of azo pigments.

The amount of $NaNO_2$ is stechiometric; acid medium should be in excess to prevent partial diazotation and condensation.

If the diazotation occurs successfully, the amine must be in aqueous acidic solution.

The diazonium salt is not isolated and should be used quickly as diazotation reactions are exothermic and the diazonium salts decompose if the system is not cooled and does not react on time. To obtain diazotation it is necessary to maintain the temperature between 0-5ºC.

Coupling reaction: diazonium salts react with compound couplers to form AZO derivatives, such as phenol and naphthol ethers.

The coupling reaction is carried out at room temperature or 10-20ºC.

Phenols are dissolved in weak alkaline environment and become phenoxides, since phenols are not reactive enough to attack the diazonium salts. Neither amines nor phenols react moderately in an alkaline medium because the diazonium ion becomes diazohidroxide Ar-N-N-OH.

Reaction scheme

1) Diazotation reaction (Figure 1),[6] *2)* Coupling reaction (Figure 2).[7]

The red dye is a red precipitate in acid medium. The pH has to be changed to alkaline medium to be a real solution and coloured.

Experimental procedure

The reagents are prepared and the reaction is made with stechiometric concentrations to continue with the reactions made with the kit of Rotachrom Heparin chromogenic method.

Protocol work

a) Diazotation cooling the solution of amine in an acidic medium obtained in the kit, in an ice water bath. The solution is kept at 0ºC (≤ 10ºC) slowly adding with the micropipette and stirring a solution of sodium nitrite to complete, add two or three minutes to finish the reaction.

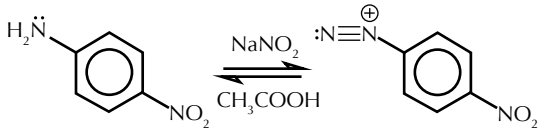

Figure 1. Diazotation reaction.

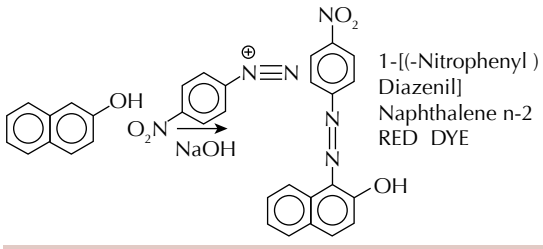

Figure 2. Coupling reaction.

b) *Coupling*: Add slowly the diazonium salt solution to the alkaline solution of 2-Naphtol solution in ice water bath and with constant stirring. A red precipitate occurs during the addition. When the addition is completed, stir for a minute to finish the reaction.

c) After that add 10 µL of solution of 15% KOH to dissolve the precipitate and get a real solution in red colour to be measured in the spectrophotometer to 540nm.

Red colour is inversely proportional to anti-Xa concentration.

The modified method with a diazotization reaction to paranitroaniline (pNA) and coupling to give a color which allows reading at 540 nm and avoiding interference with bilirubin.

Analyses of statistical method

This study was designed to calculate the coefficient of correlation of Pearson (r) between the determination of anti-Xa factor in anicteric and icteric patient using conventional and chromogenic method, respectively.

The Pearson correlation coefficient (r) measures the intensity of the linear relationship between the two quantitative variables. This coefficient (r) varies between -1 and +1. It´s the same to say $-1 \leq r \leq +1$. If $r = \pm 1$; there is a perfect relationship between x and y. We mean, all the points (x and y) they are in a perfect straight line. If we obtain a positive value of r indicates that as you increase the variable makes it the other or to measure that decrease also makes it the other. A negative correlation coefficient indicates that as one variable increases decreases the other and vice versa the opposite. R equal to zero indicates that there is no linear correlation between the variables.

After the determination of the coefficient of correlation of Pearson we determined if such

coefficient is statistically different to zero. For this, we applied the test based on the distribution of the t student.

We calculated the confidence of interval. After that we proceeded to make the inverse calculate to obtain the confidence intervals of correlation coefficient r that were our main finding before the logarithmic transformation (Figure 3).

This test was made to prove if the difference in the measures is zero (anicteric-icteric). The value of r should be different to zero to be sure this job is statistically suitable. Our results reject the null hypothesis which says that the correlation between the two variables is zero. The r was 0.9608273.

Also we presented an estimated value and 95 percent confidence interval for the coefficient of correlation of Pearson. Finally we demonstrated that there is strong evidence that the both samples came from the same population by the Kolmogorov-Smirnov test.

RESULTS

Descriptive data

The curves done with different concentration of LMWH used in plasma samples taken for patients have shown a linear order. The absorbance of each reading was concurrent with the concentration of LMWH added in each tube is not modified by the value of bilirubin with the pNA. The adaptation mentioned could be used in jaundice newborn achieving a more exact measurement of blood level of heparin (Tables 1-3, Figures 4-5).

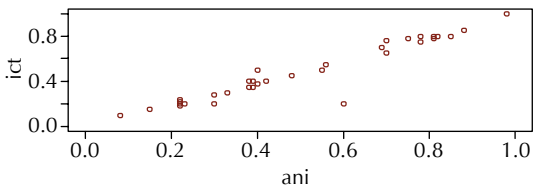

Figure 3. Intervals of correlation coefficent r.

DISCUSSION

Current use of LMWH in neonates as an anticoagulant of choice was the starting point to think about a modification of the conventional chromogenic method, which is used to measure values of anti-Xa. The main problem seen was the interference of bilirubin in icteric newborn using this conventional method. That is why we proposed modify this method.

First of all, we did not realise differences between the values of anti-Xa in anicteric patients using the conventional and modified chromogenic method. Secondly, we did not observe differences between the values of anti-Xa using modified chromogenic method in anicteric and icteric patients.

CONCLUSION

The modified chromogenic method above explained can be used in icteric patients achieving a more accurate measurement of the heparinemia.

Funding

This work was entirely self-funded.

Table 1. Determination of anti-Xa in anicteric patients using conventional and modified method

Patients	Conventional method	Modified method	Patients	Conventional method	Modified method
1	0.3	0.3	21	0.4	0.4
2	0.1	0.08	22	0.8	0.85
3	0.3	0.33	23	0.5	0.48
4	1	0.98	24	1.6	1.5
5	0.6	0.55	25	0.8	0.82
6	0.55	0.56	26	0.2	0.22
7	0.7	0.69	27	0.4	0.4
8	0.4	0.38	28	0.8	0.82
9	0.8	0.78	29	0.4	0.42
10	0.66	0.7	30	0.2	0.22
11	0.8	0.78	31	0.4	0.39
12	0.9	0.88	32	0.8	0.81
13	0.3	0.3	33	0.4	0.39
14	0.7	0.7	34	0.2	0.22
15	0.8	0.78	35	0.8	0.75
16	0.2	0.15	36	0.4	0.38
17	0.4	0.42	37	0.2	0.22
18	0.7	0.7	38	0.35	0.39
19	0.2	0.23	39	0.78	0.81
20	0.2	0.22	40	0.35	0.39

Table 2. Values of anti-Xa using modified chromogenic method in patients with jaundice

Patients	Modified chromogenic method	Patients	Modified chromogenic method
1	0.28	21	0.5
2	0.1	22	0.8
3	0.3	23	0.45
4	1	24	0.2
5	0.5	25	0.8
6	0.55	26	0.22
7	0.7	27	0.38
8	0.4	28	0.8
9	0.75	29	0.4
10	0.76	30	0.24
11	0.8	31	0.35
12	0.85	32	0.78
13	0.2	33	0.35
14	0.65	34	0.18
15	0.8	35	0.78
16	0.15	36	0.35
17	0.4	37	0.2
18	0.65	38	0.35
19	0.2	39	0.8
20	0.2	40	0.4

Table 3. Values of anti-Xa using modified chromogenic method in anicteric and icteric patients

Patients	Anicteric	Icteric	Patients	Anicteric	Icteric
1	0.3	0.28	21	0.4	0.5
2	0.08	0.1	22	0.85	0.8
3	0.33	0.3	23	0.48	0.45
4	0.98	1	24	0.6	0.2
5	0.55	0.5	25	0.82	0.8
6	0.56	0.55	26	0.22	0.22
7	0.69	0.7	27	0.4	0.38
8	0.38	0.4	28	0.82	0.8
9	0.78	0.75	29	0.42	0.4
10	0.7	0.76	30	0.22	0.24
11	0.78	0.8	31	0.39	0.35
12	0.88	0.85	32	0.81	0.78
13	0.3	0.2	33	0.39	0.35
14	0.7	0.65	34	0.22	0.18
15	0.78	0.8	35	0.75	0.78
16	0.15	0.15	36	0.38	0.35
17	0.42	0.4	37	0.22	0.2
18	0.7	0.65	38	0.39	0.35
19	0.23	0.2	39	0.81	0.8
20	0.22	0.2	40	0.39	0.4

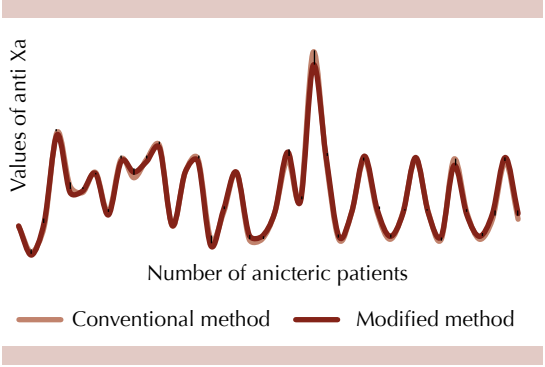

Figure 4. Values of anti-Xa in anicteric patients using conventional and modified chromogenic method.

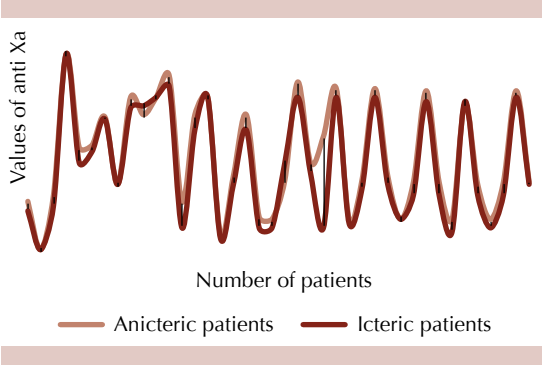

Figure 5. Values of anti-Xa using modified chromogenic method in anicteric and icteric patients.

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