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A Spectrometric Version of the Total Bilirubin Determination with the Du Pont ACA with Respect to Neonatal Sera

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Summary: A spectrometric modification of the determination of total bilirubin with the Du Pont ACA is described. Accuracy, precision and standardization problems are discussed in comparison with the earlier published diazo modification, as well as other techniques well known in the field of neonatal chemistry.

Spektrometrische Version der Bestimmung von Gesamt-Bilirubin mit dem Du Pont ACA unter Berücksichtigung von Seren Neugeborener

Zusammenfassung: Eine spektrometrische Modifikation der Bestimmung von Gesamt-Bilirubin mit dem Du Pont ACA wird beschrieben. Richtigkeit, Präzision und Standardisierungsprobleme werden im Vergleich mit der früher veröffentlichten Modifikation der Diazo-Methode wie auch anderen für Bestimmungen bei Neugeborenen bekannten Methoden diskutiert.

Introduction

In a previous study we described how the Du Pont Automatic Clinical Analyzer (ACA), TBIL method could be modified in order to make the method suitable for determining total bilirubin in neonatal serum (1). We reported that this modification, called BBIL, worked satisfactorily in the opinion of the clinicians working in our hospital. However, from a clinical chemical point of view we had certain reservations. As we have already mentioned the problem of accuracy was not completely solved (hemoglobin interference). Furthermore, we had the impression that the precision with the ACA could be improved, and finally the correlation study sometimes showed differences that could not be explained very easily. Therefore we decided to study the "state of the art" for the determination of total bilirubin in sera of neonates. In general, there are two ways by which total bilirubin can be determined:

1. spectrometric by measuring the absorbance of the bilirubin colour at about the peak wavelength, and

2. by means of the diazo reaction.

The advantage of the spectrometric method is its speed and ease. However, the advantage of the diazo reaction is a higher sensitivity and specificity.

For the majority of samples coming from neonates one is concerned with elevated unconjugated bilirubin levels. In these samples turbidity and interfering coloured components are usually absent. These reasons and the simplicity of the technique have led to the popularity of the spectrometric method for measuring the bilirubin content of neonatal serum. Because of this fact, it seemed worthwhile to study the possibilities of the ACA with respect to a spectrometric modification, in addition to the ACA diazo modification already described.

Since empty packs (ABS packs) are used in the ACA as a functional check of the system (e. g. sample dilution and absorbance reading), it should be possible to use these packs for a spectrometric method for determining bilirubin concentrations.

Furthermore, the first ACA method for estimating total bilirubin had been comparable to our proposed procedure. We divided the study into four parts i. e.

1. the development of the ACA spectrometric method,

2. the interference of hemoglobin with the techniques used,

3. the estimation of the precision of these techniques,

4. the standardization of the method.

Materials and Methods

Equipment

Du Pont Automatic Clinical Analyzer (ACA). By means of the Computer II the spectrometric method (NBIL) was laced in

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channel 62. For a further description the reader is referred to our earlier publication (1) or to the manufacturer.

Beckman DU-2 spectrophotometer. This well known instrument was checked regularly according to *Rand* (2) with respect to wavelength (didymium) and absorbance (cobalt sulphate).

Materials

ABS packs. The pack header code was changed by wiping off one black bar. As a result of this a new method was "developed".

Solid bilirubin was purchased as a rule from E. Merck Co, catalogue number 24519.

Furthermore we used the standard reference material, SRM 916 from the National Bureau of Standards.

Human albumin solution (200 g/l, salt-poor) was obtained from Institut Mérieux S. A. (France).

The materials used for the methods of *Hertz* (3), *Richterich* (4) and *Michaëlsson* (5) were from E. Merck Co or J. T. Baker Chemical Co.

Commercial sera. The reader is referred to table 3.

Methods

The preparation of bilirubin standards and hemoglobin solutions was described earlier (1).

The methods of *Hertz* (3), *Richterich* (4) and *Michaëlsson* (5) were used as described in the original publications.

The Boehringer technique (Test-Combination Bilirubin, DPD-method, cat. no. 123943) was applied according to the instructions of the manufacturer.

Results

As we mentioned in the Introduction it should be possible to develop a spectrometric method in the ACA system. Simply by diluting a sample with phosphate buffer 0.15 mol/l, pH = 7.8 (already present in the system) in an empty pack, it is possible to measure an absorbance (filter 452 nm), which can be related to the bilirubin content of the sample.

Correction for hemoglobin can be done by measuring the absorbance either at 540 or 577 nm, because at these wavelengths the absorbances of hemoglobin are comparable to the absorbance at 452 nm.

We did our experiments with both combinations 452–540 nm and 452–577 nm. Because of the better precision, we chose the combination 452–540 nm. Figure 1 shows the calibration graph of the method, which we called NBIL. Each point represents the mean of six samples.

The study of the hemoglobin interference problem was performed as described earlier (1) with one addition: we also included blood additions in which a certain part was assumed to be "fetal" hemoglobin.

These samples were obtained by lysing blood clots from neonatal specimens. No difference was found to exist between the "adult" and the "fetal" additions. The results obtained for spiking with "adult" hemoglobin are shown in figure 2.

The same technique was applied in the measurement of the interference of hemoglobin with two other techniques. These results are also shown in figure 2.

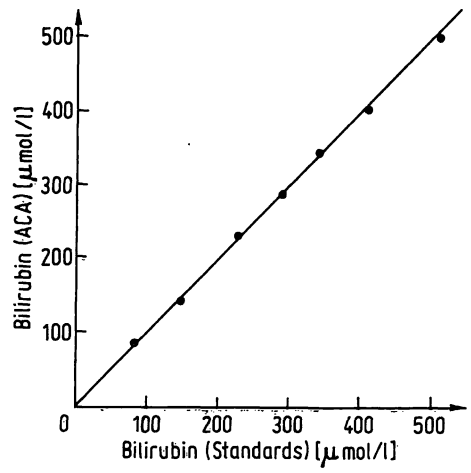

Fig. 1. Calibration graph of the ACA-NBIL method. Each point is the average of six measurements.

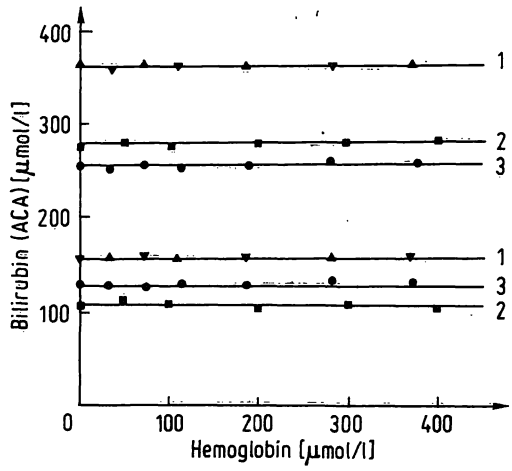

Fig. 2. The interference of hemoglobin with the ACA-NBIL method (1), the *Michaëlsson* technique (2) and the *Richterich* technique (3).

A comparison of the precision for a number of methods was then made. We estimated the "within-run" and the "day-to-day" precision at two levels.

In table 1 all results are tabulated.

At this point we found the spectrometric modification to be comparable in performance with the diazo modification. Therefore we focussed our attention more on this method.

We determined the recovery of bilirubin standards added to patient samples. Because of the small residual volumes of the samples (neonatal sera!) we decided to calculate the recovery data on the basis of the weights of the standard and patient sera. As an average value for the density of serum we used 1.026 kg/l (6). We found with 29 patient samples an average value of 101% for the recovery (standard deviation 6%).

The correlation study for peripheral blood was done with two other techniques: a spectrometric technique described by *Richterich* (4), and the well-established *Michaëlsson* diazo method (5). Figure 3 shows the comparison with the *Richterich* method.

Tab. 1. Precision data.

Method	Sample	Low value		High value	
		Average [μmol/l]	CV [%]	Average [μmol/l]	CV [%]
Within-run precision, n = 20					
ACA-NBIL	20 μl	109	2.2	298	0.7
ACA-NBIL	60 μl	108	1.0	315	0.5
ACA-BBIL	60 μl	87	5.2	263	2.9
Richterich (4)	50 μl	129	2.3	350	1.5
Hertz (3)	50 μl	135	1.9	361	2.3
Michaëlsson (5)	50 μl	76	6.4	214	4.9
Boehringer	40 μl	65	4.4	214	2.2
Ictometer	20 μl	104	1.6	347	0.8
Day-to-day precision, n = 20					
ACA-NBIL	20 μl	66	5.8	242	2.3
ACA-BBIL	60 μl	77	7.2	246	3.7
Hertz (3)	50 μl	81	6.4	238	2.7
Michaëlsson (5)	50 μl	82	5.9	257	3.5
Boehringer	40 μl	67	5.8	233	2.4
Ictometer	20 μl	106	2.9	260	2.6

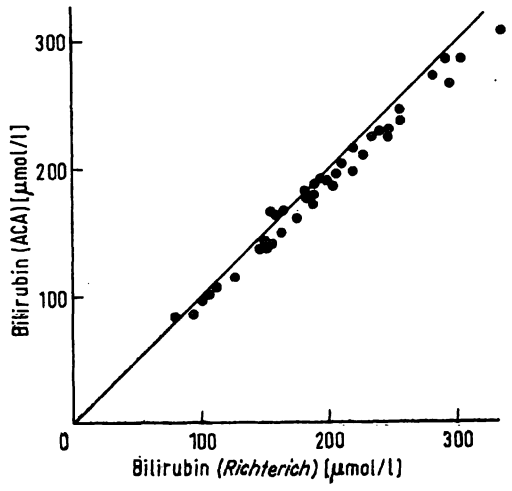

Fig. 3. Split sample comparison between the "NBIL" (y-axis) and the *Richterich* technique (x-axis) (neonatal sera). The straight line represents the "ideal" correlation.

The comparison with the *Michaëlsson* technique was split into two parts. In total we compared 106 samples, 38 sera coming from babies receiving phototherapy, and 68 sera from babies without phototherapy. Figure 4 shows the correlation for all specimens. The regression equations of the two sets of specimens are given in table 2.

We also performed a correlation study for umbilical cord specimens. In figure 5 the results of the comparison between the NBIL and the *Michaëlsson* technique are shown.

In all experiments the results were obtained on the basis of a calibration with bilirubin enriched sera. In practice this is a rather cumbersome method. It is known that the standardization of the bilirubin determination is a difficult problem. The differences in standardization

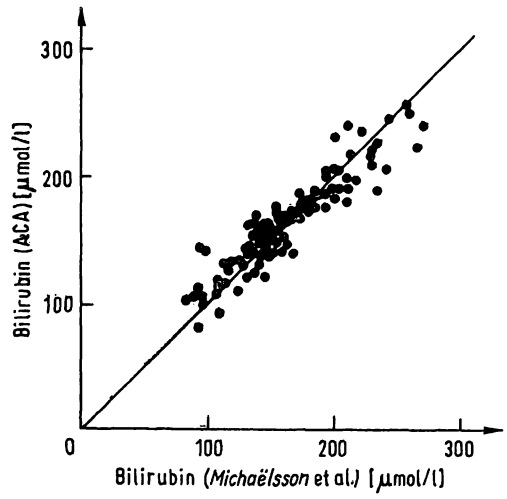

Fig. 4. Split sample comparison between the "NBIL" (y-axis) and the *Michaëlsson* technique (x-axis) for peripheral blood (neonatal sera). The straight line represents the "ideal" correlation.

Tab. 2. Statistical data.

1. ACA-NBIL vs. *Richterich*, peripheral blood
2. ACA-NBIL vs. *Michaëlsson*, without phototherapy
3. ACA-NBIL vs. *Michaëlsson*, with phototherapy
4. ACA-NBIL vs. *Michaëlsson*, peripheral blood (total 2 + 3)
5. ACA-NBIL vs. *Michaëlsson*, umbilical cord blood

	Regression equation (y on x)	Correlation coefficient r	Number of determinations n	Average x [μmol/l]	Average y [μmol/l]
1.	y = 0.92 x + 5	0.99	40	194	184
2.	y = 0.80 x + 34	0.93	66	162	161
3.	y = 0.82 x + 31	0.92	39	169	170
4.	y = 0.85 x + 26	0.94	105	166	164
5.	y = 1.10 x + 1	0.93	43	29	26

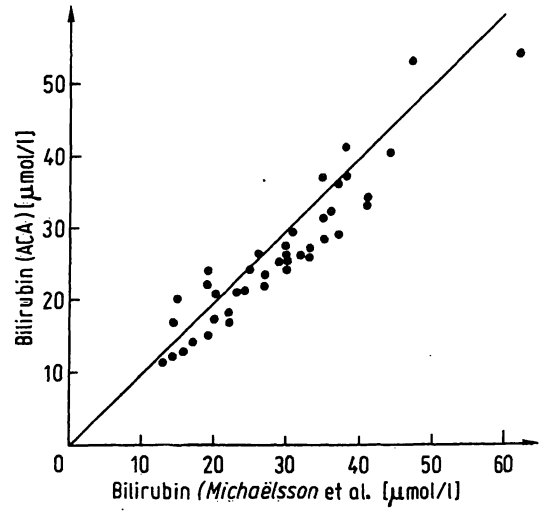

Fig. 5. Split sample comparison between the "NBIL" (y-axis) and the *Michaëlsson* technique (x-axis) for cord blood. The straight line represents the "ideal" correlation.

can be caused by incomplete dissolution of the solid bilirubin and/or the presence of compounds which interfere with the diazo coupling reaction.

Therefore we decided to simplify the preparation of the standard sera. The protein base was changed to human albumin 50 g/l instead of human sera. This solution is more constant and no check on the colour is needed.

Secondly, we studied the possibility of using commercial sera in calibrating the ACA. In fact we use two ways of determining total bilirubin i. e. the "adult" way (TBIL) and the "neonatal" way (NBIL). In practice this means that at regular intervals (to be determined by the laboratory) the ACA must be calibrated, owing to the arrival of new lotnumbers of packs. We studied 11 commercial sera divided into two concentration ranges: a low level between 50 and 200 μmol/l and a high level between 300 and 400 μmol/l.

The details of the commercial sera are presented in table 3. Figure 6 shows the comparison of the ACA values against the assigned bottle values for the commercial sera.

The ACA was calibrated with bilirubin in human albumin (50 g/l). Solid bilirubin was bought from Merck. We compared this product with bilirubin from the National Bureau of Standards (Standard Reference Material, S. R. M. 916) and found as molar absorptivities ($l \cdot mol^{-1} \cdot cm^{-1}$) in chloroform: 61,500 for the Merck product and 62,100 for the N.B.S. product respectively. Furthermore we estimated the molar absorptivity of the azobilirubin by the *Jendrassik-Grof* reaction. The method we used is the one recommended by the Dutch Standardization Committee on Clinical Chemistry and is the same as described by *Doumas* et al (7). The coefficients ($l \cdot mol^{-1} \cdot cm^{-1}$) were 72,200 for the Merck product and 71,700 for the S.R.M. 916. We found these values suitable for the calibration of the TBIL and the NBIL method (12).

Tab. 3. Description of commercial sera used.

Name	Manufacturer	Kind of protein base	Assigned value (only *Jendrassik-Grof*) [μmol/l]
Monitrol II	Dade	Human serum	60, 62 and 64
Calibrate 2	General Diagnostics	Human serum	53
Calibrate 3	General Diagnostics	Human serum	79
Wellcomtrol 2	Wellcome	Horse serum	99
Baker Bilirubin Standard	Baker	Bovine albumin	136
SMAC Control	Technicon	Bovine serum	173
Versatol Pediatric	General Diagnostics	Human serum	296
Q-PAK Bilirubin Control	Hyland	Human serum	352
Precibil	Boehringer	Human serum	354
Bilirubin Control	Dade	Human albumin	357
Wellcomtrol 3	Wellcome	Bovine serum	374

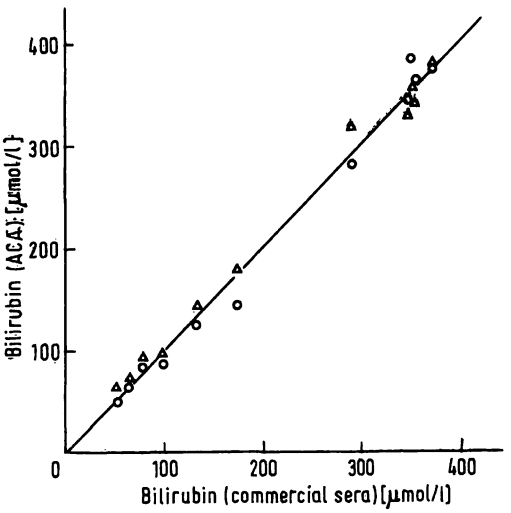

Fig. 6. Graph representing the results obtained with commercial sera
x-axis: data given by the manufacturer
y-axis: ACA-results, ○ = TBIL, △ = NBIL.

Discussion

The literature on the determination of bilirubin is very extensive. A comprehensive survey of the available literature can be found in the publications of *Hertz* and *Dybkaer* (3, 11). In general a preference exists for the determination with the help of the diazo reaction (7). One exception concerns the neonatal field.

Because of the absence of disturbing factors the serum matrix with respect to bilirubin is well defined in neonatal sera; this has resulted in the development of many spectrometric methods. Speed and simplicity are the reasons for the wide popularity of these methods.

We started the study of the determination of total bilirubin on the ACA with neonatal sera by modifying the existing method (TBIL) as we described earlier (BBIL). The method worked well, but because of the reasons mentioned in the Introduction, we wished to know if some kind of a spectrometric modification could be worked out in the ACA; and if so, whether the method would be comparable or better in performance with respect to the BBIL. The present study therefore describes the development of a spectrometric determination of total bilirubin with the ACA. Comparing this modification with our diazo modification we conclude

that the spectrometric method has some practical advantages for several reasons:

1. The main problem in neonatal bilirubin analysis, namely hemolysis, is solved in a better way as can be seen from figure 2.

2. The precision is better as can be seen in table 1, even if the sample volume is 20 µl (also advantageous!).

3. The comparison with two other techniques, *Richterich*'s spectrometric method and the diazo method published by *Michaëlsson* et al. is very favourable (table 2).

4. Additional reagents are not needed.

Another two points are relevant. As one can see in figure 1 the calibration graph is linear up to at least 400 µmol/l which is, clinically speaking, sufficient. Furthermore as can be seen from table 2 we were not able to detect differences between samples coming from babies treated with phototherapy and samples from babies who were not treated with phototherapy. This is in accordance with the work of *Ebbesen* (8). The correlation coefficients for umbilical cord blood and peripheral blood analyses are comparable, when using the *Michaëlsson* technique and the NBIL method. However, there is a difference in the slopes of the regression lines (table 2). We cannot give an explanation for this phenomenon. It is possible that there is a serum matrix effect.

At the moment no further work is planned to determine the reason for the observed differences, because the determination of bilirubin in cord blood is becoming less important. This is due to the general use, since 1969, of anti-D-immunoglobulin in Holland in cases of rhesus incompatability (9).

The comparison between *Richterich*'s technique and the NBIL method raises the question of accuracy. Although the coefficient of correlation is excellent (0.99) there is a difference in result (fig. 3). Work is in progress to try and solve this problem. One point which may be important is that the calculation of the results in the manual method is based on *Richterich*'s formula with absorbances measured on a Beckman DU-2 spectrophotometer and in the case of the ACA by means of standard specimens. The same question of accuracy arises in considering figure 4. The problem is: "how equal" is a spectrometric determination of bilirubin to a diazo measurement? The results we obtained were certainly comparable to each other which is also the conclusion of *Hertz* (3) and *Blumberger* (10).

This brings us to the method of standardization. When using a diazo reaction, such as the modification described by *Doumas*, or the *Michaëlsson* technique, there is no difference between albumin-based and serum-based bilirubin standards. This was shown by *Doumas* (7), and it is in accordance with our own experience. *Dybkaer*, however, in his spectrometric modification, advocates suitable serum diluent instead of human albumin solution (11).

At the present time, for practical reasons, we have chosen bilirubin standards in albumin, because their preparation is easy and reproducible. In addition, preliminary studies show no appreciable difference between serum-based and albumin-based calibration graphs in the ACA.

However, it is our intention to study this part in more detail, not only because of *Dybkaer*'s statements (though he doesn't mention data), but also because of differences seen in the preparation of the bilirubin standards. Comparing the Merck product with the N.B.S. bilirubin we noted that the Merck bilirubin dissolved much slower than the N.B.S. product. Furthermore the Merck standard is orange coloured while the N.B.S. standard is brownish orange. Both differences may be caused by the existence of isomeric forms and/or the presence of traces of impurities.

However, the calibration with some commercial sera gave results which were not acceptable. In the lower range we measured differences up to 20 µmol/l and in the higher range up to 37 µmol/l.

The turbidity of some samples (Monitrol, Calibrate, Versatel Pediatric and Hyland) is, in our opinion, one important cause of these differences.

Summarizing, we now prefer our NBIL modification to our earlier BBIL solution, with respect to the determination of neonatal total bilirubin, although the standardization needs further investigation.

Acknowledgements

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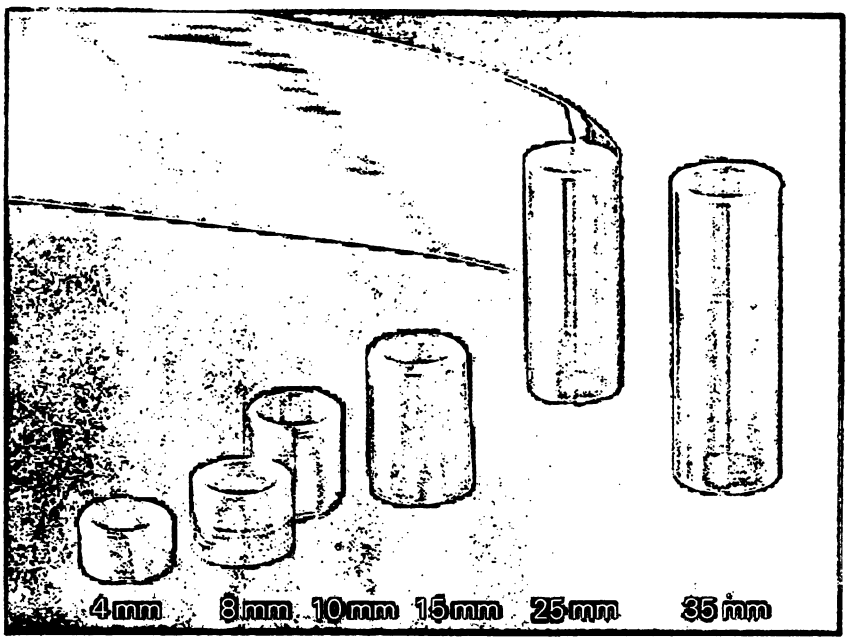

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