Technical methods

Method for the determination of small amounts of bilirubin in liquor amnii and other body fluids

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During an investigation into the bilirubin content of liquor amnii in haemolytic disease, it became obvious that the methods of assessment currently employed were of limited value. These methods consisted of an assessment of the peak of absorption at 450 m μ (Bevis, 1953; Walker, 1957; Liley, 1961; Alvey, 1964), or a direct measurement using either a diazo reaction (Lathe and Ruthven 1958,) or a modification of it (Stewart and Taylor, 1964). The former is not very precise and may be affected by the extent of background absorption and the presence of other substances absorbing at neighbouring wavelengths. In addition the results are usually expressed in terms not easily understood by the clinician. When a direct colorimetric reaction is employed, the concentration of bilirubin in liquor is very close to the limits of sensitivity of the methods routinely employed.

In an effort to find a method which would reflect an accurate measurement of bilirubin in liquor, advantage was taken of its extremely high solubility in chloroform.

METHOD

A 10 ml. sample of liquor was placed into a dark bottle and refrigerated until analysis could be carried out. The sample of liquor was shaken for approximately 10 minutes with 40 ml. chloroform. The solvent was removed by aspiration, placed into a round-bottomed 50 ml. flask and evaporated to dryness under vacuum at a temperature not exceeding 40°C. The residue was taken up in 2.5 ml. chloroform (a four-fold concentration of the bilirubin), and the extract was measured spectrophotometrically at 450 m μ against the solvent. Two further readings were taken at 420 m μ and 480 m μ , and an Allen correction applied. The absorbance of known concentrations of bilirubin in chloroform varying between 0.5 and 1.5 mg./100 ml. was measured and a linear regression curve constructed (Fig. 1). By reference to this curve the corrected absorbance at 450 m μ can be read directly in terms of bilirubin concentration (mg./100 ml.).

ACCURACY OF THE PROCEDURE

The addition of known amounts of bilirubin to liquor

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FIG. 1. Linear regression curve.

has indicated that 90-95% of the added compound can be re-extracted by the method described. Control sera stated to contain either 0.4, 5, or 20 mg. of bilirubin per 100 ml. plasma were reconstituted and used. Four estimations were performed at each concentration by both the diazo method routinely used in this laboratory

TABLE
COMPARISON OF RESULTS USING DIFFERENT METHODS FOR THE ESTIMATION OF BILIRUBIN

Stated Concentration of Bilirubin (mg./100 ml.)	Concentration Using Diazo Method	Mean % Recovery	Estimation Using Chloroform Extraction Method	Mean % Recovery
20	19.6		16.8	
20	20.4		17.6	
20	19.4		17.8	
20	19.6	98.6	17.0	87
5	4.6		4.0	
5	4.8		4.2	
5	4.2		4.0	
5	5.2	94	3.9	80
0.4	0.32		0.34	
0.4	0.35		0.30	
0.4	0.30		0.36	
0.4	0.40	85	0.31	82
Mean % recovery	92%		83%

FIG. 2. Correlation of bilirubin concentrations when estimated by the routine diazo method and the chloroform extraction method.

(Lathe and Ruthven, 1958) and the chloroform extraction method (Table I). If it is assumed that the stated concentration of bilirubin in the reconstituted plasma is correct, it will be observed that at the lowest concentration, that is, at the end of the scale where the diazo method is, in theory, liable to the greatest fluctuation, there is a marked similarity in the results obtained. As the concentration is increased the diazo method reads about 10% higher than the chloroform extraction procedure. This divergence becomes more obvious if the linear regression curves are plotted.

In Fig. 2 the results obtained by the diazo method are plotted against the results obtained by the chloroform extraction method. The regression line of the slope has been drawn together with the theoretical line of 100% correlation $y = x$. From these results it may be calculated that the chloroform extraction readings are 14% lower

than the routine diazo readings. Similar results are obtained when bilirubin is added to specimens of liquor.

COMMENT

The method described makes it possible to determine the extremely small amounts of bilirubin usually found in liquor amnii from cases of haemolytic disease. By suitable dilution procedures it has also been found useful in the determination of bilirubin in plasma.

In order to show that it is bilirubin which alone is being estimated, a specimen was measured and then subjected to ultra-violet light for a period of two hours. Under these conditions bilirubin is destroyed. At the end of this period no peak of absorbance appeared at 450 mμ. when the extract was re-examined spectrophotometrically. Due to the volatile nature of chloroform, it is apparent that it might well be advisable to take the dry residue up in some other solvent. It is suggested that ethanol might be more suitable.

Work is proceeding to improve the recovery and measurement of bilirubin extracted by this method. It will be appreciated that this method measures the indirect reading chloroform-soluble bilirubin, but investigations are currently proceeding to determine if the direct (non-chloroform-soluble) bilirubin can be determined by the use of enzymatic hydrolysis followed by chloroform extraction.

SUMMARY

A simple method is described for the determination of small amounts of bilirubin.

A direct comparison has been made between the concentrations found when both the routine diazo and the chloroform extraction methods are used.

Within the concentration range examined, a good correlation between these two methods has been found, the chloroform extraction method reading about 14% lower than the routine diazo reaction.

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