A Simple Method for the Determination of Serum Albumin with an Anionic Dye 4′-hydroxyazobenzene-carboxylic Acid (2)

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(Received February 19, 1960)

Fractionation of serum protein into albumin and globulin is among the services of clinical chemistry which are the most frequently demanded in the laboratories of ordinary hospitals. A variety of methods different in principle have been invented for that purpose, but salting-out biuret colorimetry[1–3] and paper electrophoresis[4–6] will be the procedures which are the most favored by clinical chemists, because they are relatively simple and rapid. Recently there arose a possibility that a more convenient method for the determination of serum albumin might be developed through the studies on the combination of several anionic dyes, i. e. methyl orange,[7] congo red[8] and so forth,[9] with albumin.[10] The dyes bound to albumin were different in color from the free dyes, and the amount or concentration of combined dyes was measured spectrophotometrically to determine the albumin concentration in a fluid. The attempts on this line had been fruitless because of the absence of linear relationship between the concentration of albumin and the intensity of coloration of the bound dyes until 1954, when Rutstein and his associates[11] discovered another anionic dye 2 (4'-hydroxybenzeneazo) benzoic acid (HABABA) with which they were allegedly successful in the development of a dye method for the determination of serum albumin. The new method was introduced into this country by SAITO (1955)[12], and it was checked in our laboratory with a reagent made of 4′-hydroxyazobenzene-carboxylic acid (2) (HABCA) produced by Daiichi Kagaku Co. (Tokyo) which was identical with HABABA in chemical formula. The result of our experiments was at first somewhat discouraging because it did not compare well with the conventional salting-out (sodium sulfate) biuret colorimetry.

The binding of HABCA to human serum albumin was accordingly studied anew in comparison with paper electrophoresis,[6] and with salting-out of albumin with sodium sulfate[1,3]. The experiments disclosed that the combining capacity of the dye was exclusively displayed with the albumin fraction separated by paper electrophoresis and with the filtrate of the solution of serum protein containing sodium sulfate in a concentration of 26g/dl, which was identical with the albumin of MILNE.[1] It became accordingly apparent that the HABCA-protein combination bare no relation to serum globulin. The HABCA-albumin compound had invariably its peak of absorption of light at 480 mμ with maximum magnitude of absorbance at pH6.2, where LAMBERT-BEER's law was also observed between the dye

compound and albumin over a wide range of variation of the concentration of albumin.[11, 13, 14]

The combination of HABCA with human albumin was remarkably inhibited by sodium chloride. The inhibition was, however, fortunately constant in degree when sodium chloride was added to the HABCA-albumin solution in a concentration in excess of 1.8g/dl.[13] There was, on the other hand, an increasing production of dye-albumin compound with the rise in HABCA concentration of the medium and the fall in ambient temperature (from 37°C to 10°C). The amount of dye-albumin compound varied reversibly with the shift of ambient temperature.[15,16]

Purified bovine serum albumin assumed a somewhat different attitude toward HABCA, although it was quite similar to human serum albumin at various points[17]. It produced a larger amount of dye-albumin compound when the medium was adjusted so as to have a dye concencentration below 4×10^{-4} M and an albumin concentration above 0.1g/dl, while it yielded a smaller amount of the compound when the ambient temperature was maintained below 25°C.[15, 16] It was therefore thpught that bovine albumin should be employed as standard solution for the construction of calibration line, taking its distinct character of this sort into special consideration.

After a contemplation of the results of the afore-mentioned experiments a new procedure for the determination of serum albumin by means of HABCA which was considerably different in principle from that devised by RUTSTEIN and his associates[11] was developed. About 400 determinations performed routinely in our laboratory for the past year and a half proved the new procedure to be among the simplest methods which gave reliable albumin determination. It dispensed with the tedious filtration or centrifugation which was involved in the coventional salting-out biuret method and it contributed much to the reduction of the labor paid by the technicians for the determination of serum albumin.

METHOD

Reagents

1. Stock solution of HABCA (M/1000). An amount of 0.240g of 4′-hydroxy-azobenzene-carboxylic acid (2) (HABCA, produced by Diichi-Kagaku, Tokyo) is introduced into a 1000ml volumetric flask, to which 800ml of N/100 aqeous NaOH solution. The flask is subjected to a shaking machine for about twenty minutes until HABCA is completely dissolved. The solution is made to the volume (1000 ml) with N/100 aqueous NaOH solution, mixed by inversion, and transferred into a dark colored (brown) bottle. The solution keeps for about 3 weeks when stored in a refrigreator.

2. Buffer solution containing sodium chloride and hydrochloric acid. To the mixture of one volume of SÖRENSEN'S M/15 phosphate buffer (pH 6.2) and 2.4 volumes of 8.0/dl aqueous sodium chloride solution is poured 0.6 volume of N/10

aqueous HCl solution drop by drop under constant agitation with a glass rod. The M/15 phosphate buffer solution (pH 6.2) is prepared by mixing one volume of M/15 aqueous Na_2HPO_4 solution with 4 volumes of M/15 aqueous KH_2PO_4 solution.

3. HABCA working solution. Shortly before use add two volumes of the buffer solution described in (2) to 3 volumes of the stock solution of HABCA (1), and mix.

4. Aqueous solution of sodium chloride (0.9g/dl).

5. Standard solution of albumin. One g of the purified bovine serum albumin is dissolved in 10 ml of 0.9g/dl aqueous sodium chloride solution. The solution, which is to be limpid, is determined for its total nitrogen concentration. The exact concentration (g/dl) of albumin in this solution is obtained by multiplying the total nitrogen concentration (g/dl) by 6.25. This albumin solution is a stock solution which is useful for a month if it is preserved in a refrigerator. On determination of serum albumin, the working standard solutions 1.0, 3.0, 5.0 and 6.0 g/dl in albumin concentration are made from the stock solutin by diluting with 0.9 g/dl aqueous sodium chloride solution. The working standard solutions keep for a week in a refrigerator.

Procedure

Into 3 test tubes (10 × 100mm) A, B and C introduce the serum and reagents as described below.

	Serum	0.9g/dl NaCl solution	HABCA working solution
A	0.1		3.0
B	0.1	3.0	
C		0.1	3.0

(ml)

Mix the contents of the test tubes by inversion and incubate at the temperature of 25 to 30°C for 30 minutes. Measure without delay (to prevent cooling) the absorbance of A, denoted by a, in a photoelectric colorimeter at 480 mμ (Filter S47) against C as a blank solution. The absordance of B, denoted by b, is also measured against distilled water as a blank at the same wave length of light. The concentration of serum albumin (g/dl) is directly read from the calibration curve by applying the difference of absorbances (a–b) to it.

Calibration curve.

Aliquots of 0.1 ml of working standard solutions of bovine albumin (1.0, 3.0, 5.0 and 6.0 g/dl in concentration) are treated in the same way as described in the procedure. Their differences of absosbances corresponding to (a–b) mentioned in the procecure are collated to the relevant concentrations of the albumin to construct a

calibration curve which makes a recti-linear line passing through the origin of the graph.

Correction of the serum albumin concentration for serum bilirubin.

In case of jaundiced serum of which total bilirubin concentration[18] is over 5mg/dl, 0.05 × total bilirubin concentration (expressed in mg/dl) in terms of g/dl is to be added to the serum albumin determination (g/dl) which has been read from the calibration curve, because there is a signiflicant decrease in the production of dye-albumin compound in the presence of a larger amount of bilirubin in the medium.

The aged serum gives an albumin determination identical with that of the fresh serum for at least a week after withdrawal of the blood from the vein, so long as the serum is kept in a refrigerator.

RESULTS AND DISCUSSION

Sera of 45 healthy persons and 418 patients with various diseases comprising 47 cases of jaundice and 9 cases of nephrotic syndrome were determined for albumin concentration by the present HABCA method and simultaneously by the conven-

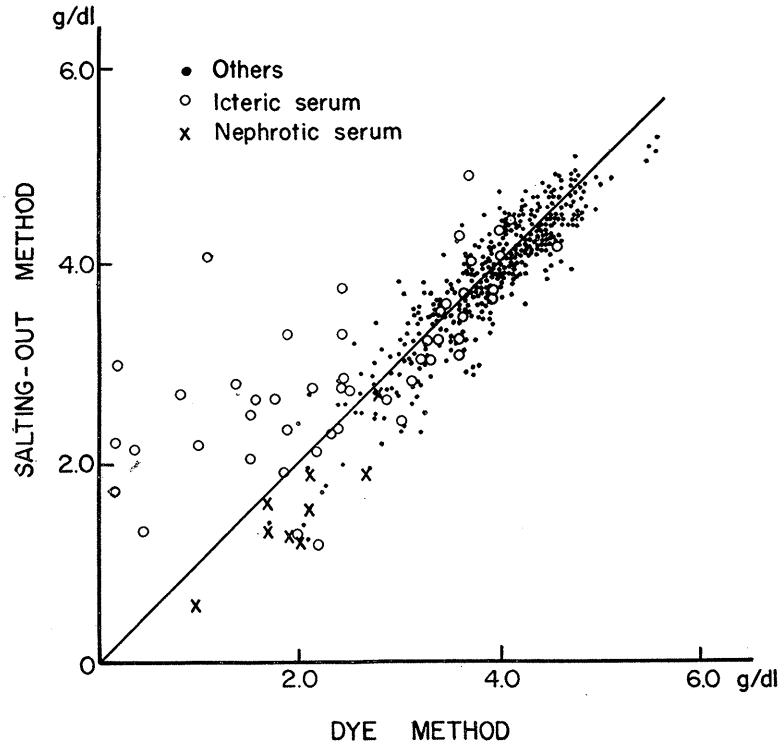

Fig. 1. Correlationship between the dye method and salting-out method.

tional salting-out 26.0 g/dl ($Na_2 SO_4$) biuret colorimetry.[3] The result of the comparative study, which is presented in Figure 1, revealed a close correlationship between the two methods (coefficient of correlation $r = +0.913$), in which 88,4 per cent of the total determinations showed a good agreement with an insignificant discrepancy as small as 0.4 g/dl or less. The average disagreement of the present method with the salting-out biuret method was only +0.013 g/dl. This can be regarded as essentially equal to 0.0 g/dl, if allowance is made to the technical error inherent in the methods. RUTSTEIN and his associates[11] stated that the HABABA method gave lower albumin determinations than the salting-out method, while WRENN[19] and KIOTZ[7] propounded that the reverse would be true. The diverse results in the comparative study of dye method and salting-out method will be accounted for by the difference in the composition of dye solutions specified in their procedures. In our procedure the dye solution (working HABCA solution) seems to have been successfully prescribed and adjusted so that the albumin determinations may compare well with those obtained by the conventional salting-out method.

However, a close scrutiny of Figure 1 reveals that the agreement may be deceptive, because it is apparent that the conformity of the two determinations is not very satisfactory with the sera of nephrotic syndrome (slightly lower determinations in dye-method), and there is also a considerable disagreement in jaundiced sera with frequent deviation of albumin determination to the lower side of concentration. A study of the saltingout biuret methods in comparison with paper electrophoresis of serum protein[6] was therefore made with 24 jaundiced and 125 non-jaundiced sera as meterials to know whether or not the cause of the disagreement in jaundiced sera pointed out above is to be attributed to the defect in the salting-out biuret method.[3] Its result disclosed no difference in the degree of conformation of salting-out biuret method with paper electrophoresis between jaunced and non-jaundiced sera, being entirely negative to the assumption of the defect in the salting-out biuret method. It is acordingly thought that the combination of HABCA with albumin in jaundiced sera might be abnormal. According to KUSUNOKI[20] and HIRAYAMA[21] an albumin possessing diminished dye-combining capacity is produced in severe hepatic diseases (portal cirrhosis). It appears to be compatible with the result of our study. If the discrepancy between the dye-method and salting-out biuret method with jaundiced sera is truly due to the diminution of dye-combining capacity of serum albumin, the determinations by dye-method may well be accepted without correction because they indicate the abnormality in the properties of albumin, which is valuable for the clinical diagnosis, in addition to its mere concentration. It will be pertinent to expect that jaundiced sera associated with low albumin concentration are related to advanced hapatic diseases, and in these jaudiced sera the dye-combining capacity of albumin is decreased remarkably on account of its abnormal defective production in the pathological liver. If this hypothesis is justified, the deviation of dye-method from the salting-out biuret method will progres-

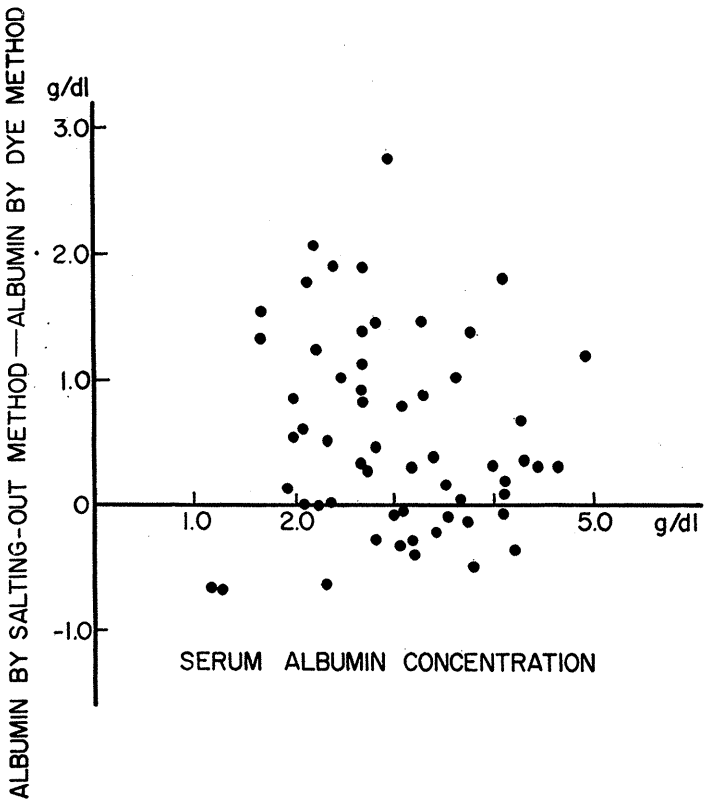

Fig. 2. Correlationship between the deviation of dye method and the serum albumin concentration.

sively grow larger with the decrease in the concentration of albumin in the jaundiced serum. Our study which is illustrated in Figure 2 was however contradictory to the hypothesis, because, as easily seen from the figure, there was no significant inverse correlation of the deviation to the albumin concentration, On the contrary a fairly close correlationship was demonstrated between the deviation of dye method and the serum total bilirubin concentration[18] (Figure 3). The level of serum bilirubin is therefore more important than the change in the properties of albumin as a factor exerting influence upon the determinations of serum albumin by HABCA method. Correction of the albumin determination for serum bilirubin is accordingly required in jaundiced serum.

The deviation of HABCA method from the salting-out biuret method in relation to serum bilirubin is expressed in the following equation.

$$Y = 0.05\,X$$

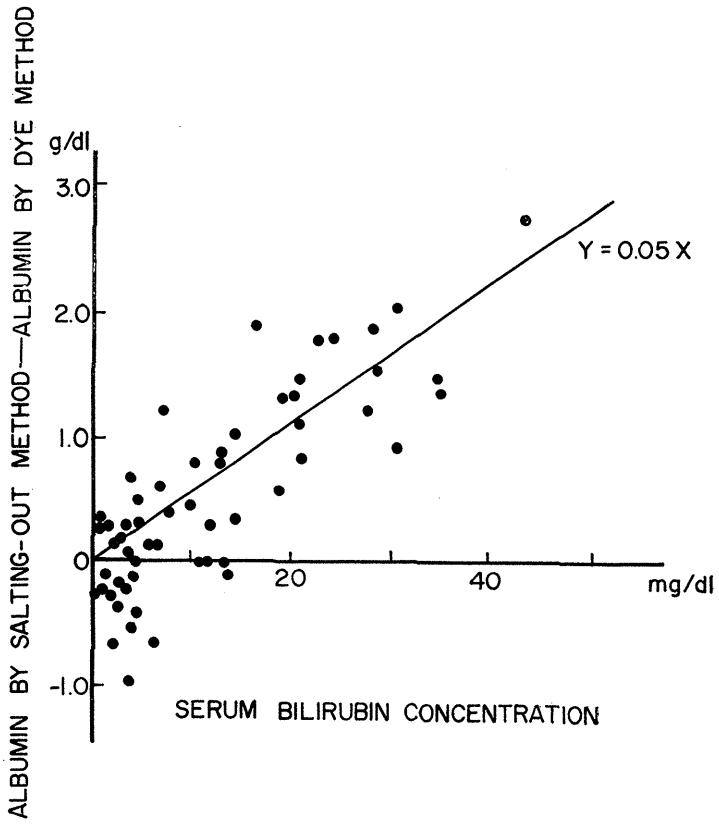

Fig. 3. Correlationship between the daviation of dye method and the serum bilirubin concentration.

x: concentration of total serum bilirubin in mg/dl
y: subtraction of HABCA albumin concentration from salting-out biuret albumin concentration in g/dl

This is the correction of the determination of serum albumin for bilirubin in jaundiced serum.

Albumin concentration of jaundiced serum
Albumin concentration read from the calibration curve (g/dl) + 0.05 × serum total bilirubin in terms of mg/dl (g/dl).

In the jaundiced serum of which bilirubin concentration is below 5 mg/dl, the correction $Y=0.05x$ is so small in size that may be overshadowed by the technical error which is inherent in the albumin determinations. It is therefore justified to omit the correction when serum bilirubin is lower than 5 mg/dl. A useful measure to remove the inhibition of bilirubin upon the combination of HABCA with serum

albumin is desired, but unfortunately no attembt has as yet been successful along this line.

The discrepancy of duplicate determination in the present HABCA method was less than 2 per cent, and the repeated determinations made with the same blood sera kept in a refrigerator over a period of a week proved to give an essentially constant value with a fluctuation not surpassing 0.1 g/dl. The reproducibility was more excellent with this HABCA method than with the conventional salting-out biuret method. The calibration curve remained constant on every determination so far as the same stock HABCA solution was used for the preparation of the working HABCA standard solution. The calibration curve once having been constructed with a batch of stock HABCA solution was repeatedly useful until it was displaced by a new batch, but it is desirable to prepare calibration curves on every determination lest it should be disturbed by some unexpected accident.

Observation on 18 healthy adult males and 27 healthy adult females revealed that the normal range of serum albumin determined by the present HABCA method lay within the range of concentration between 3.7 g/dl and 5.2 g/dl.

SUMMARY

A new simple method for the determination of serum albumin employing a solution of an anionic dye 4-hydroxyazodenzene-cardoxylic acid (2) which compared quite well with the conventional salting-out (sodium sulfate) biuret method was presented. Three volumes of the dye solution (M/1000) were diluted with 2 volumes of the mixture of SÖRENSEN's M/15 phosphate buffer solution (pH 6.2), 8.0 g/dl sodium chloride solution and N/10 HCl (1 : 2.4 : 0.6), and to its 3.0 ml was added 0.1 ml of blood serum. The absorbance of the serum-added dye solution was measured for its absorbance at 480 mμ against a blank consisting of 3.0 ml of 0.9 g/dl sodium chloride solution. The absorbance of the serum (0.1 ml) diluted with 0.9 g/dl sodium chloride solution (3.0 ml) was similarly measured. The concentration of serum albumin was directly read from a calibration curve constructed with standard solutions made of bovine albumin, by applying the difference of the two absorbances. In case of jaundiced serum over 5 mg/dl in bilirubin concentration, the determination of serum albumin was corrected by adding 0.05 × bilirubin concentration (mg/dl) g/dl.

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