SHORT REPORTS

J. Sci. Soc. Thailand, 15 (1989) 121-132

MODIFIED DYE BINDING METHOD FOR MEASURING TOTAL PROTEIN CONCENTRATION OF CEREBROSPINAL FLUID

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(Received 26 August 1988)

ABSTRACT

The present method measures the absorbancy of supernatant after precipitation of protein in cerebrospinal fluid by TCA-Ponceau S dye. The optimum concentration of Ponceau S dye, amount of specimen and method of analysis which give good linearity, sensitivity, accuracy and precision have been determined. This modified method uses 2.94% trichloroacetic acid as in the Pesce-Strande method, 0.006% Ponceau S dye and 50 µl of specimen. The measured absorbancy of supernatant shows linearity up to 250 mg/dl of protein. Optimum condition variance (OCV) are 3.22, 4.65 for high and normal values, while routine condition variance are 5.58, 8.46 for high and normal values. This modified method gives good correlation with the Pesce-Strande dye-binding method. The present method is appropriate for routine use in the laboratory because it is simple and has good accuracy and precision, wide range linearity, and needs only a small amount of sample. It is also suitable for small laboratories because it requires only simple equipments.

INTRODUCTION

The clinical chemist is often faced with the problem of low protein content in a limited sample volume for an accurate and rapid determination of cerebrospinal fluid protein. Several methods have been used for the determination of protein in cerebrospinal fluid (CSF) such as the Biuret reaction,[1] Folin-phenol reagent[2] and turbidimetric method.[3, 4] These methods have problems in precision, sensitivity, linearity and specificity.[5-8] The sulfosalicylic acid or biuret technique is also somewhat impractical and requires a large sample volume, although it shows good precision and linear range.[9]

The Coomassie brilliant blue technique seems to be the most practical method, but relatively low linear range and poor precision are its disadvantages.[9]

The sulfosalicylic acid or sodium sulfate turbidimetry technique is simple to perform but has poor precision[9] and requires a large sample size.

The trichloroacetic turbidimetry technique is a generally good practical method but again requires a large sample and also has a narrow linear range.[9]

The Ponceau S technique has good practicability, requires only a small amount of sample, has good precision and little apparent bias. Human or bovine albumin, serum, or urine, appear to be satisfactory for calibration or quality control.[9] The Pesce-Strande micromethod[10] employs Ponceau S and trichloroacetic acid (TCA) as reagents for determination of protein in CSF, with good performance characteristics and practicability,[9] but it is difficult to remove the supernatant quantitatively without any loss of minimal precipitate.[11]

The objective of this research is to develop a method which has good linearity, sensitivity, accuracy and precision in the determination of small amounts of protein.

The present method is modified from the Pesce-Strande method by keeping the amount of trichloroacetic acid in the working reagent fixed as described in the Pesce-Strande method, but changing the concentration of Ponceau S dye and the procedure to obtain the best linearity and sensitivity.

In the determination of total protein in cerebrospinal fluid in the Pesce-Strande micromethod, the cerebrospinal fluid is mixed with trichloroacetic acid, Ponceau S dye solution, and the red protein precipitate which develops is dissolved in dilute sodium hydroxide solution, to give a violet-colored solution. The color is then measured spectrophotometrically at 560 mm. However, the red precipitate can be lost with removal of the supernatant, either by decanting or pipetting with the pasteur pipet after centrifugation. In the present method, after the cerebrospinal fluid is mixed with the trichloroacetic acid-Ponceau S dye solution, only 0.5 millilitre of supernatant is removed without any contamination of red precipitate. The difference in optical density between the blank tube and the unknown tube is directly proportional to the amount of protein present in the unknown cerebrospinal fluid.

MATERIALS AND METHODS

Chemicals :

All chemicals were analytical reagent grade. Trichloroacetic acid (TCA), Ponceau S and Bovine albumin were obtained from E. Merck AG, Darmstadt, Germany; George T. Gurr Ltd., London, England; and Sigma U.S.A. respectively.

Reagents :

1. Stock trichloroacetic acid, 12% w/v
2. Stock Ponceau S dye, 4% w/v

3. TCA-Ponceau S working reagent : 0.001 to 0.010 g/dl of Ponceau S dye in 2.94 g/dl of TCA.

4. Stock protein standard solution : 10% bovine serum albumin in 0.9% sodium chloride.

5. Working protein standard solution : Reagent 4 was diluted to 25, 50, 100, 150, 200, 250, 300, 400 and 500 mg/dl in 0.9% sodium chloride solution.

Methods

The effect of dye concentration on the linearity of the reaction

One ml of reagent 3 was added to 100 μl each of standard and blank, centrifuged for 10 minutes at 2,000 rpm and 500 μl of supernatant liquid was removed from each tube with automatic pipets, taking care to avoid contamination with the red precipitate. 500 μl of distilled water was then added to all supernatant tubes to adjust the concentration to within a suitable range of optical density (less than 0.7 O.D. unit).[12] The absorbancy of blank and standard were read against distilled water at 520 mm. Standard curves of $\triangle$OD (absorbance of blank minus absorbance of standard) against concentration were plotted.

The effect of sample volume on the linearity of the reaction

The amount of sample was varied while maintaining the optimum concentration of dye. The procedure followed, was as described above.

Correlation between modified method and Pesce-Strande method

The protein contents of 91 cerebrospinal fluid specimens were determined by the present method and by the Pesce-Stande method.[10] Correlation between the two methods was plotted.

Linearity of modified method and Pesce-Strande method

Optical density was plotted against concentration (12.5, 25, 50, 100, 150, 200, 250 mg/dl) to obtain the linearity of the modified method. Comparison with the linearity of the Pesce-Strande method is shown above.

Accuracy of modified method

Various concentrations (25, 50, 100 mg/dl) of bovine serum albumin were added to known pooled CSF. Each concentration was run 10 times and the percent recovery of protein was calculated.

Precision of modified method

Within-run precision was measured by performing replicate determinations on pooled CSF. Day-to-day precision was measured for a 20 day period on the same series of CSF.

RESULTS

The present method is modified from the Pesce-Strande method. By using 2.94% TCA, the optimum concentration of Ponceau S dye and the optimum amount of specimen for analysis were determined. The effect of dye concentration on the linearity of the reaction is shown in Fig. 1A and 1B. Dye at concentrations of 0.006% and 0.007% seemed to show equal sensitivity. The optimum concentration of TCA-Ponceau S reagent that showed good sensitivity was 0.006% Ponceau S, but the linearity was about 150 mg/dl for a 100 μl specimen. Because the red-colored supernatant of the blank (contains no protein) has high optical density, 500 μl of distilled water was added to each of the 500 μl of supernatant to dilute to optimum optical density for a more accurate result when measuring OD_{520}. The appropriate amount of specimen was also determined : specimens of 50 μl and 100 μl were also analysed. The 50 μl specimen gave higher linearily than the 100 μl specimen as shown in Fig. 2. (linearity was 250 mg/dl for the 50 μl specimen)

Analytical variables of the present method

Linearity : The standard curve obtained, was linear from 0 to 250 mg/dl, compared with the Pesce-Strande method linearity of 0-150 mg/dl.[10] The result is shown in Fig. 4.

Recovery and precision : Recovery data for albumin, added to CSF or standards, were 96-103% as shown in Table 1. For within-run precision, the coefficient of variation of high and normal values were 3.22% and 4.65% respectively. For day-to-day precision, the coefficient of variation of high and normal values were 5.58% and 8.46% respectively.

Comparison with the other method : Results from the present method compared against those from the Pesce-Strande method yielded a good correlation coefficient of $r = 0.85$ and linear regression equation : $y = 18.8 + 1.003X$.

There was no upper limit in the modified method; specimens with protein concentrations of greater than 250 mg/dl could be diluted with distilled water before analysis and the final concentration obtained by multiplying the result with the dilution factor.

DISCUSSION

In this modified micromethod, a single reagent was added to 50 μl of CSF, and the decrease in absorbancy of the reaction mixture was read after centrifugation. The decreased absorbancy of the reaction mixture after precipitation was linearly proportional to the amount of protein present in the sample (up to 250 mg/dl). The coefficient of variation of within-run precision was 3.22%, 4.65% for high and normal values, and the coefficient of variation of day-to-day precision was 5.69%, 8.46% for high and normal values. The present method correlates well with the Pesce-Strande method. The turbidimetric method using trichloroacetic acid is dependent on temperature.[6] In the sulfosalicylic acid method, albumin yielded more turbidity than globulin per unit weight.[4] The Lowry method is not specific because CSF contains many coloring non-protein substances.[8] The Biuret method is not sensitive for determination of protein in CSF.[8] The disadvantages of these methods

have all been described in "Introduction". The Pesce-Strande micromethod is not accurate due to loss of minimal precipitate in the removal of the supernatant.[11] The present method has been shown to correlate well with the Pesce-Strande method as shown in Table 1 and Fig. 4. Thus it would seem to be the most suitable method for routine determinations of total protein in the CSF.

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บทคัดย่อ

การหาค่าโปรตีนรวมในน้ำไขสันหลัง โดยการตกตะกอนโปรตีนด้วย TCA และจับกับสีย้อม Ponceau S ตามวิธีที่เสนอนี้เป็นการวัดการเปลี่ยนแปลงของการดูดกลืนแสงหลังจากตกตะกอนโปรตีนด้วย TCA และ Ponceau S โดยใช้ปริมาณ TCA ตามวิธีของ Pesce-Strande ในการทดลองนี้ได้หาปริมาณของ Ponceau S ที่เหมาะสม และหาความเข้มข้นของโปรตีนที่เหมาะสมที่ให้ linearity, sensitivity, accuracy และ precision ที่ดี จากการทดลองพบว่าการใช้ Trichloroacetic acid 2.94%, Ponceau S 0.006% และปริมาณสิ่งส่งตรวจ 50 μl จะให้ linearity สูงถึง 250 mg/dl และมี sensitivity และ precision ดี โดยมีค่า optimal conditions variance = 3.22, 4.65 สำหรับค่าสูงและค่าปกติตามลำดับ วิธีนี้มี accuracy เท่ากับ 96-103% และมีความสัมพันธ์กันดีกับวิธีของ Pesce-Strande ที่นิยมใช้หาปริมาณโปรตีนในน้ำไขสันหลังซึ่งวัดการดูดกลืนแสงของสารละลายตะกอนสีแดง ซึ่งมี linearity เพียง 0-150 มก./ดล. เท่านั้น และมี accuracy ต่ำกว่าวิธีที่เสนอเพราะมีการสูญเสียตะกอนบางส่วนในประหว่างการทดลอง ดังนั้นวิธีที่ได้ดัดแปลงขึ้นนี้เหมาะที่จะนำมาใช้ในห้องปฏิบัติการทั่วไป เนื่องจากเป็นวิธีที่ทำได้ง่าย มีความแม่นยำและความเที่ยงตรงสูงใช้ปริมาณสิ่งส่งตรวจน้อย และใช้เครื่องมือง่าย ๆ ที่มีในห้องปฏิบัติการทั่วไป

TABLE 1 Recovery of albumin added to cerebrospinal fluid (CSF) of present method, compared with Pesce-Strande method

Protein in CSF (mg/dl)	Albumin added (mg/dl)	Found (mg/dl)	Expected (mg/dl)	Recovery %
25	25	49.05	50	96.20
25	50	74.29	75	98.58
25	100	128.60	125	103.60
23	20	43	43	100(10)
23	40	62	63	98(10)
75	19	91	94	84(10)
75	100	178	175	103(10)

TABLE 2 Precision on total protein determination of CSF

Sample	n	Mean ±SD (หน่วย mg/dl)	% Condition Variance
Within run			
Pooled CSF	20	94.13 ± 3.03	3.22
	20	43.05 ± 2.00	4.65
Day-to-day			
Pooled CSF	20	84.20 ± 4.70	5.58
	20	41.38 ± 3.50	8.46

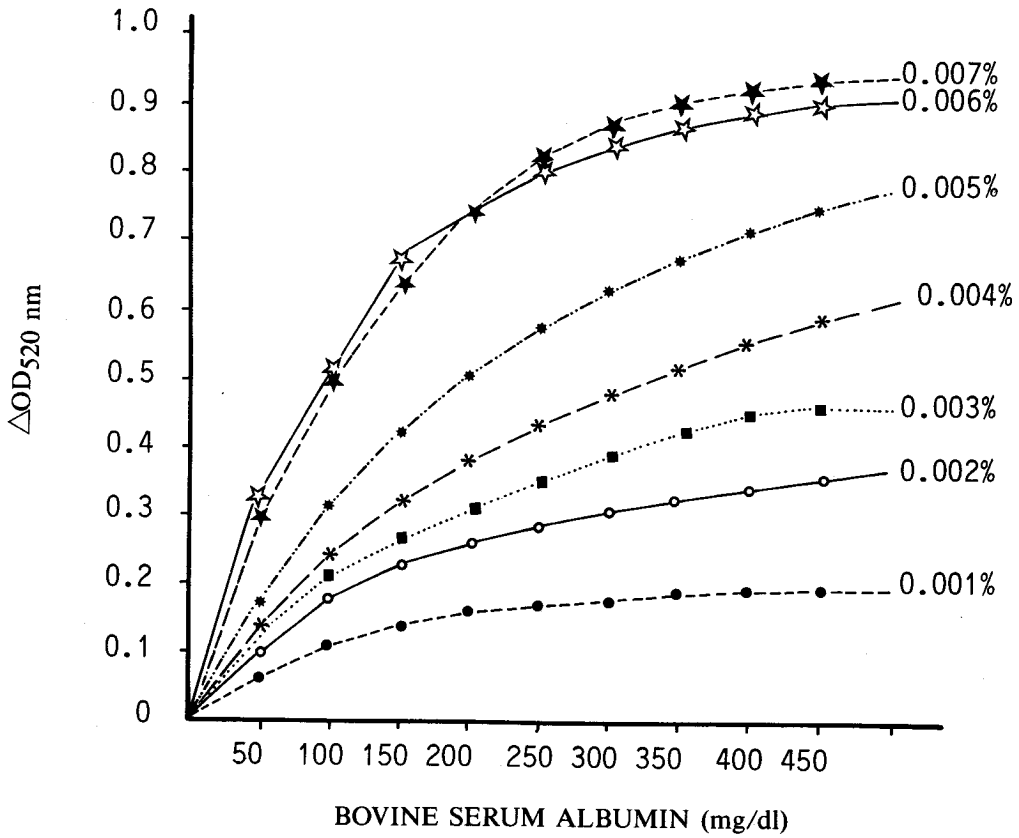

Fig. 1A The effect of dye concentration on the linearity of the reaction. Standard curves with 0.001% to 0.007% Ponceau S concentrations in 2.94% TCA are presented. Sample volume is 100 μl.

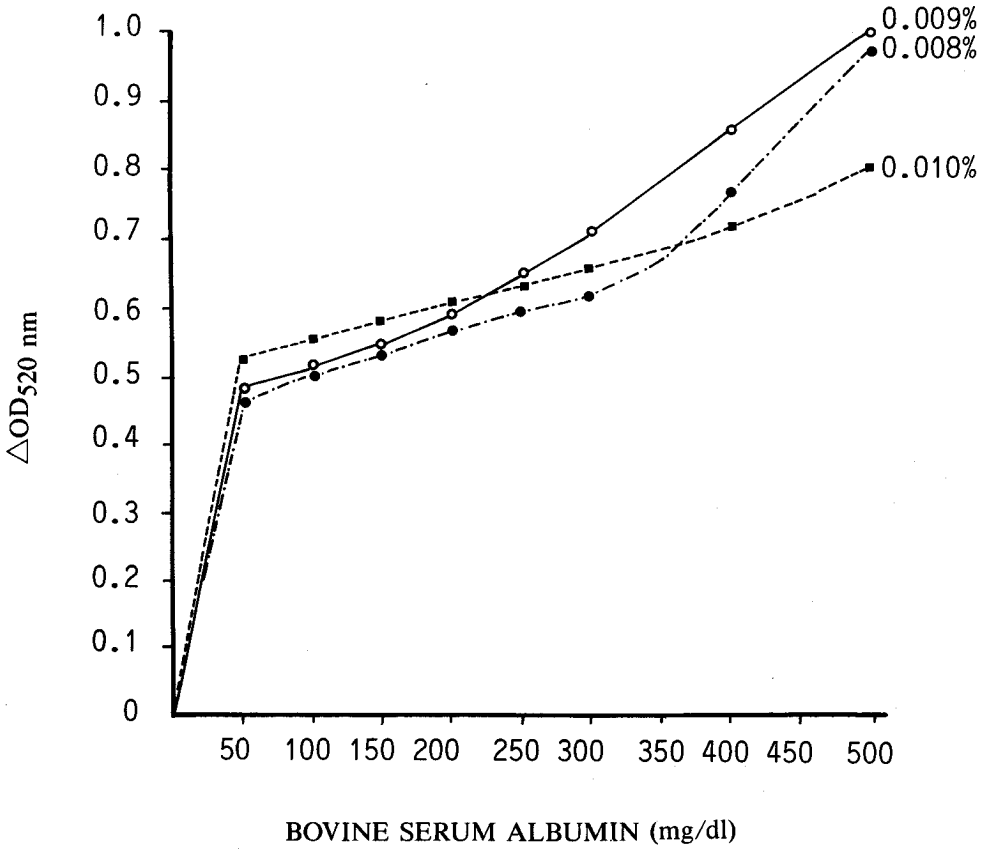

Fig. 1B The effect of dye concentration on the linearity of the reaction. Standard curves with 0.008% to 0.010% Ponceau S concentrations in 2.94% TCA are presented. Sample volume is 100 μl.

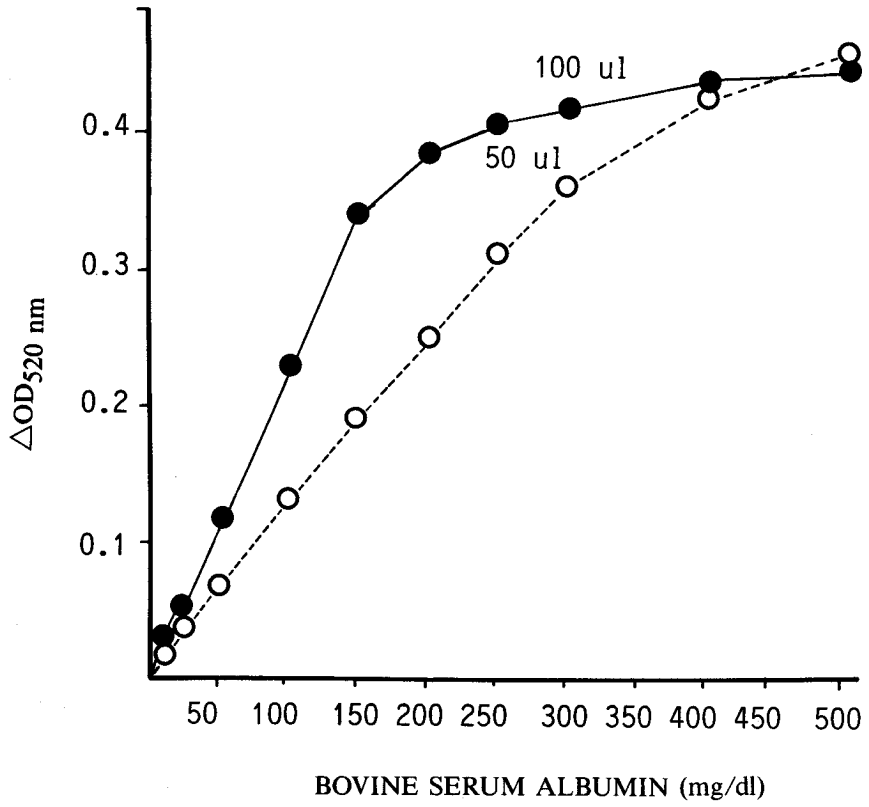

Fig. 2 The effect of sample volume on the linearity of the reaction. Standard curves with 50 μl and 100 μl sample volumes are presented. Concentrations of dye and TCA are 0.006% and 2.94% respectively.

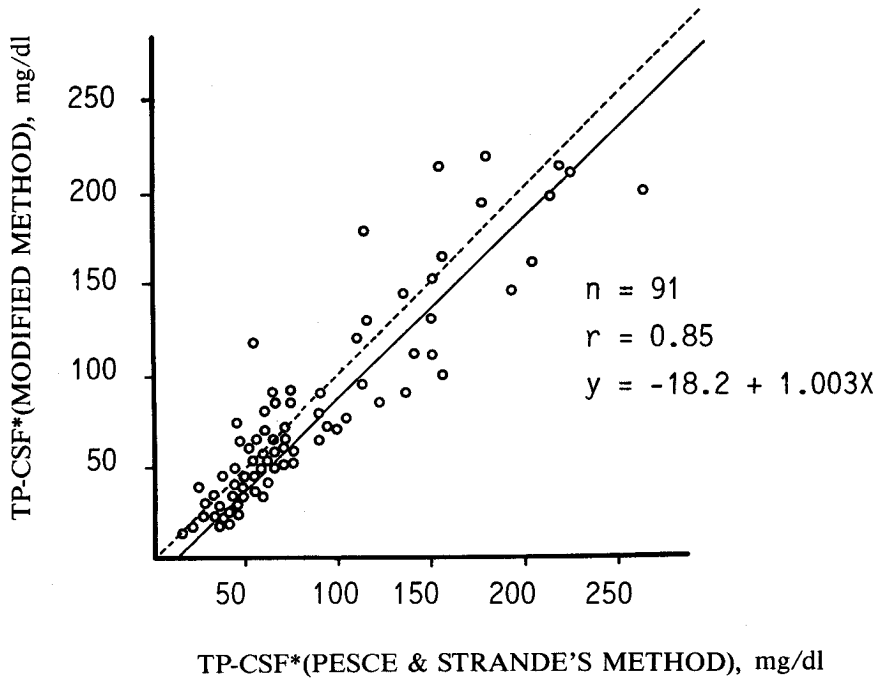

Fig. 3 Correlation between TP-CSF as determined by modified method and Pesce and Strande's method. TP-CSF* = total protein concentration of cerebrospinal fluids.

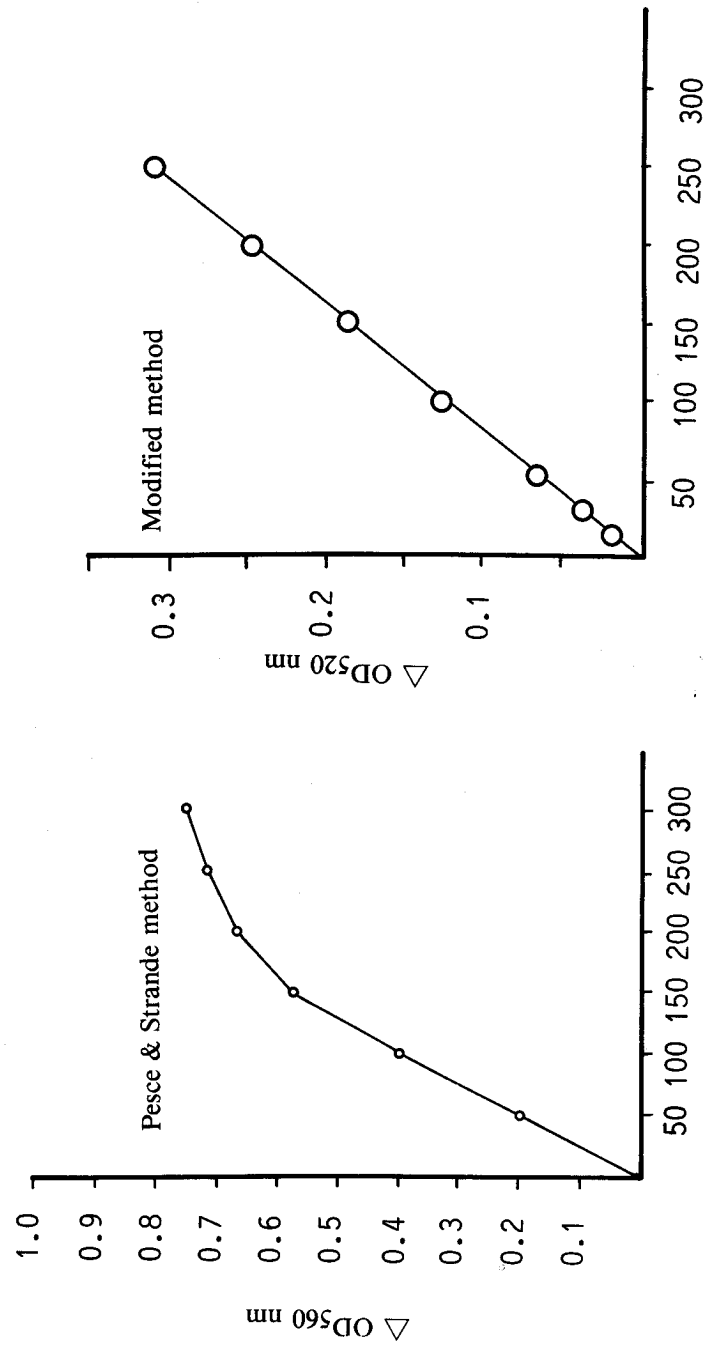

Fig. 4 Linearity of Pesce & Strande method compared with modified method from protein concentration 0-250 mg/dl.
Modified method : Dye concentration 0.006%
TCA concentration 2.94%
Sample volume 50 μl