A NEW METHOD FOR QUANTITATING URINARY PROTEIN USING GEL CHROMATOGRAPHY AND ULTRAVIOLET ABSORPTION AT 210 nm.

by

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Introduction

Harper (1) reviewed the basic causes of proteinuria. The primary cause of an abnormal amount of protein in the urine is an increase of the permeability of the glomerular filter. This renal type of proteinuria is due to a breakdown of the kidney itself, allowing a greater amount of normal serum proteins to be excreted. Heavy metal poisoning may cause this type of proteinuria. The protein may be from an excess of production elsewhere within the body which is classified as pre-renal proteinuria. Bence Jones proteinuria is of this type. Post-renal production of urinary protein is attributed to lower urinary tract infections or lesions.

Miller (2) stated that proteins of lower molecular weight pass the renal barrier more easily than do the larger proteins. The approximate molecular weight cut off is 70,000. Thus albumin (M.W. 69,000) and hemoglobin (M.W. 64,000) tend to appear in the urine more often than the larger proteins such as the globulins (M.W. 150,000 to 2,000,000).

Thysell (3) compared several of the more common methods for the detection of urinary protein. He used a biuret method as a control. The various "stix" methods do not give consistent results. They depend upon the "protein error" when the protein is in contact with the Brom-phenol blue indicator in the strip. This "protein error" causes a change in the color of the indicator without the need for any pH change. Albustix and Hema-combistix agreed in only 17 % of the urines tested. The variation appeared to be due to the differ-

ence in pH resulting in the indicator strip during the test procedure.

Other methods given by Miller (2) and Hepler (4) include the heat and acetic acid test and Robert's ring test with magnesium sulfate and nitric acid. A semi-quantitative test, the Kingsbury test, is also mentioned. The ring tests (2,4) are dependent upon the formation of a coagulum or cloudy "ring" when urine containing abnormal amounts of protein is layered over the reagent in a tube. Interferences may occur with other common urinary components such as urates. The Exton's and Kingsbury tests are read by noting the cloudiness or precipitate formed when the reagent is mixed with a urine containing protein. Peters and Van Slyke (5) recorded Tsuchiya's modification of the Esbach test for quantitative urinary protein. The precipitate formed in this test is measured in a MacKay tube after centrifugation.

Goodwin and Choi (6) described a quantitative method for amino groups using trinitrobenzenesulfonic acid (TNBS). The method compares well with other standard methods such as U.V. and biuret. However, spinal fluid protein must be precipitated prior to analysis to remove amino acids and presumably the same would have to be done with urinary protein although reference to urine was not made in the paper. The sensitivity of the method was about 0.08 O.D. for 20 mg. of protein per 100 ml. of spinal fluid.

Savory, Pu and Sunderman (7) have given an improved biuret method for quantitating urinary protein with a sensitivity of 0.5 mg. per 100 ml. The procedure is linear up to approximately 27 mg. per 100 ml.

Henry (8), in his chapter on proteins, listed procedures for several standard protein tests along with a good review of the methodology. His urinary protein method uses the turbidity of a mixture of trichloroacetic acid and urine. He gives the method of Daughaday, Lowry, Rosebrough and Fields (9) using the Folin-Ciocalteu reagent for spinal fluid protein. The method is not very linear at 740 nm. and for accurate work a standard curve should be used.

Patrick and Thiers (10) and Plum, Hermansen and Petersen (11) have described drawbacks to the routine methods given. Methods involving precipitation are subject to error from changing albumin to globulin ratios, incomplete precipitation and precipitation or trapping of non-protein interfering substances. In general, albumin gives a larger amount of precipitate per given weight than does globulin. The errors due to the changing albumin to globulin ratios can be as high as 20 %. Methods not using a precipitate or some other method of purifing the protein are subject to large interferences by non-protein nitrogenous substances such as amino acids, urates and uric acid.

Ressler and Goodwin (12) determined the O.D. of cerebrospinal fluid at 210 nm. before and after precipitation of the protein with heat and ethyl alcohol. The difference in O.D. was related to the amount of protein present in the cerebrospinal fluid with which they worked. They corrected for the addition of the ethyl alcohol by using a reagent blank. The method compares better with a TCA turbidimetric method than with a sulfosalicylic acid method.

Jorgensen (13) used gel filtration (Sephadex G-50-coarse) to isolate urinary protein. He quantitated the protein with the Folin-Lowry reagent.

Werner (14) attempted to estimate the protein in cerebrospinal fluid by dilution and reading the sample at 210 nm. before and after ultrafiltration. The procedure was intended to also provide a specimen for electrophoresis.

Tombs, Souter and MacLagan (15) described the absorption of proteins as rising sharply from around 230 nm. to a peak around 190 nm. This absorption is due mainly to the peptide bonds. The peak at 280 nm. measures the amino acids tyrosine and tryptophan.

Patric and Thiers (10) have developed a U.V. method for protein determination in cerebrospinal fluids. The protein is separated from the interfering substances by means of a Sephadex (G-50-medium) column. The resulting protein solution was quantitated at 220 nm. using a standardization curve which showed linearity up to 0.6 O. D. which represented 80 mg./100 ml. of protein.

No matter what method has been used in the past for the determination of urinary protein, there appear to be inaccuracies due to precipitation problems, differences in the chemical behavior of the various proteins, or interferences by non-protein substances. It usually takes a fairly long and laborious procedure to determine urinary protein by the methods currently in use.

It appeared desirable to try to develop a method that would be specific, accurate and simple to set up and run in a clinical

laboratory. The gel filtration method for the removal of interferences seemed to be the best method available. The quantitation by absorption at 210 nm. seemed to be the most sensitive and specific method for a purified protein solution which was available in a clinical laboratory.

Therefore, this investigation has been addressed to the uniting of the two above procedures into a method for urinary protein which would be simple enough to encourage its use as a standard clinical laboratory procedure and yet be accurate enough for use as a reference method if desired.

Materials

Sephadex G-25-80, salt-free egg albumin, bovine albumin and lysozyme were obtained from Sigma Chemical Co.

Folin-Ciocalteu reagent was obtained from Harleco, Hartman-Leddon Co.

Distilled water was used throughout the experiment as elution agent, diluent, solvent for the gel bed and proteins, and as the spectrophotometric blank.

A Beckman DU with Gilford 222 Photometer and power supply was used for the quantitation. The lamp was deuterium and the 1.0 cm. cuvettes were made of fuzed quartz.

A Fractomat automatic fraction collector with drop counter made by Buchler Instruments, Fort Lee, N. J. was used to collect 5 or 10 drop samples from the columns.

The AutoAnalyzer diacetyl monoxime method for urinary urea was used to determine the presence of urea in various samples. The specimens were run along with the routine daily procedure in the clinical laboratory.

A Gilford Model 300 N Spectrophotometer was used for some of the colorimetric determinations not requiring U.V.

The columns were the type used by Curtis Nuclear Corp. for one of their thyroid test kits. (24.4 cm. by 8 mm. I.D.)

The average gel bed prepared was 15 cm. in length with a bed volume of 7.5 ml.

Methods

The Sephadex gel was prepared by swelling in distilled water for three or more hours. The fines were removed several times by aspiration of the supernatant after mixing and partial settling. The columns were poured with a thick slurry of gel particles. Additions of gel to the columns or removal of excess gel was done with a disposable pipette. A piece of filter paper was placed on the top of each column to prevent disturbance of the gel. The fine G-25-80 gel was particularly susceptible to disturbance.

The void volume of the columns was calculated from data given by Determann (16) to be 3.0 ml. for a 7.5 ml. bed volume. This data was checked by use of the fraction collector. A protein sample was applied to the top of the column. Water was added to the top of the column and 5 drop fractions of the effluent were collected. Each fraction was diluted with enough water to enable it to be read in the U.V. spectrophotometer and the resulting solutions checked for absorbance at 210 nm. to determine where the protein first appeared.

In all quantitation procedures requiring a weighed protein sample, the protein was first dessicated overnight or longer under a vacuum.

In all experiments utilizing U. V. measurements, the quartz cuvettes were checked to determine their individual absorbances while filled with water. The water was replaced several times and the readings checked after each time until the absorbances became stable. The cuvette having the lowest absorbance with

water was chosen as the blank for that run of determinations. The absorbances for the other cuvettes were recorded and subsequently subtracted from the test readings to give corrected values which were used in the calculations.

The Folin-Ciocalteu method (9) for spinal fluid protein was used to check the results of the separation procedures. It was also used in the correlation check against the U.V. method for urinary protein. A standard curve was used in the quantitation and was fairly linear at the low concentrations measured. Two-tenths of a ml. of sample solution was used as in the procedure given by Daughaday et al., but the volumes of the other reagents were reduced to one-fifth that of their method.

Experiments were run to determine the reproducibility, recovery, linearity and accuracy of the U.V. method.

The step by step procedure which was followed for the determination of protein in the urines during this research is given as follows:

1. Be sure the column (7.5 ml. gel bed volume) has been well washed (at least 15 ml. of water) since the last test.

2. Check the approximate concentration of protein with Albustix or sulfosalicylic acid. If the approximate concentration is less than 100 mg./100 ml., use 0.2 ml. of sample. If greater, use 0.1 ml. of sample.

3. Add the urine sample directly to the top of the column. Allow the sample to completely enter the column.

4. Wash twice with 0.1 ml. of water allowing each portion to completely enter the column before adding the next one.

5. Add enough water to the column to make up a total of one void volume including the volumes of the sample and washes. It will usually require 2.6 ml. or 2.7 ml. to make up the 3.0 ml. void volume. Allow the water to completely enter the gel bed. Discard the effluent.

6. Place collecting tubes under the columns.

7. Add 2.0 ml. of water to each column used. Collect the effluent. (Data from Tables 1-9 and Figures 2-4 used 3.0 ml.)

8. Add 1.0 ml. of water to each effluent sample to give a volume large enough to read in the spectrophotometer.

9. Read the absorbances of the samples from (8) in the spectrophotometer at 210 nm. Dilute if necessary.

10. The concentration of protein in the effluents in mg./100 ml. is calculated as follows:

$$\text{Protein in effluent in mg./100 ml.} = \frac{\text{O. D.}}{E^{0}_{mg}} \times \text{dilution factor (if any)}$$

$$= \frac{\text{O. D.}}{0.205} \times \text{dilution factor (if any)}$$

11. The concentration of protein in the urine is calculated as follows:

$$\text{Protein in urine in mg./100 ml.} = \text{Protein in effluent in mg./100 ml.} \times \text{Dilution Factor}$$

This Dilution Factor is equal to the volume quantitated (3.0 ml.)

from steps (7) and (8) divided by the sample size applied to the colume (0.1 ml. or 0.2 ml.). The dilution factor (if any) used in the calculation of the effluent protein concentration is from any further dilution necessary in step (9).

12. The total grams of protein per specimen can be calculated by the following formula:

$$\text{Grams/spec.} = \text{Protein in urine in mg./100 ml.} \times \frac{\text{Total volume in ml.}}{100 \times 1000}$$

13. Wash the columns with approximately 15 ml. of water prior to the next run. A siphon arrangement can serve to wash the columns without periodic attention.

Note: The G-25-80 Sephadex gel holds water well enough so that it is not necessary to add one volume of water or sample immediately after the previous one has entered the column. Thus it is not necessary to constantly attend the procedure. Several minutes or even an hour or more may elapse with no readily detectable dehydration of the column.

Results

Figure (1) shows the results of three experiments carried out to determine the separation between the large and small molecules in urine when the urine is run through the Sephadex column. Two-tenths of a ml. of a urine containing approximately 300 mg. of protein per 100 ml. was applied to the column and an excess of water was allowed to enter the column. Five drop fractions of the effluent were collected and diluted with 2.8 ml. of water to give a volume large enough to be read in the cuvettes. Each fraction was read at 210 nm. and the O.D. readings plotted against the fraction number. The solid line on the graph connects the plotted points. The plateau between the peaks is characteristic of the separation (14) and may be involved with the results of the correlation experiment with normal urines to be discussed later.

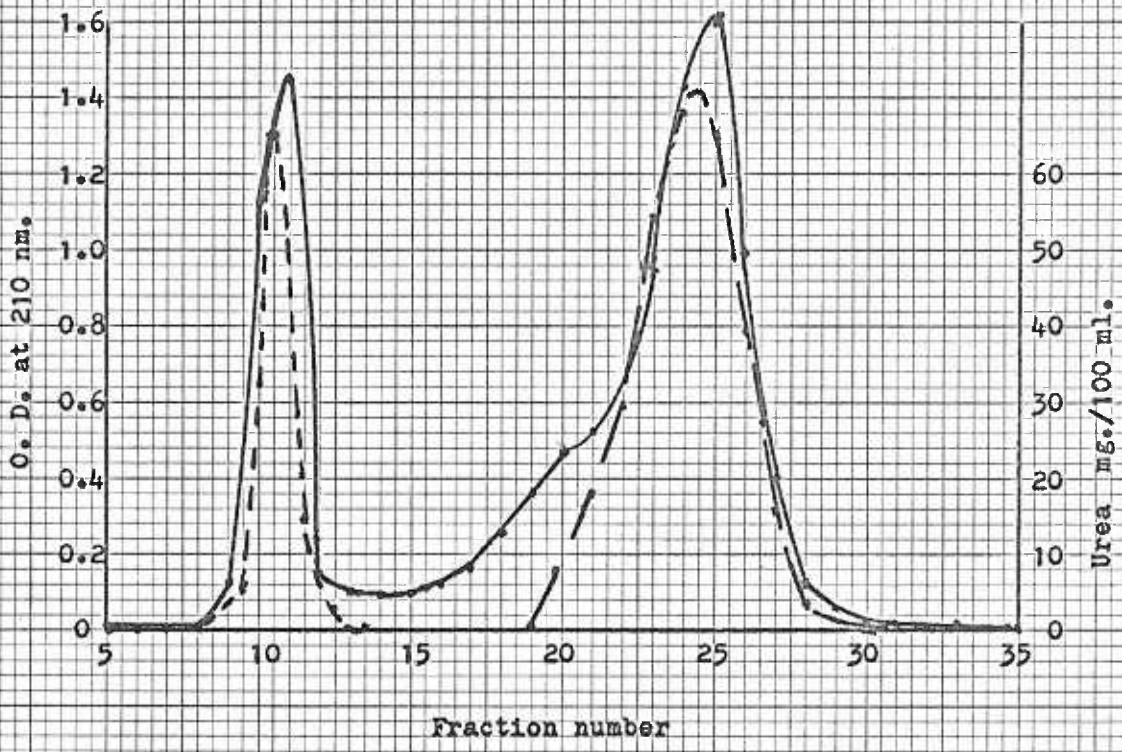
The dotted line in Figure (1) represents 0.2 ml. of a solution of egg albumin applied to the column with the fractions collected and read at 210 nm. in the same manner as the urine above. The protein peak fits nicely within the first peak from the urine with the tail of the peak dropping rapidly to the background levels.

The dashed line in Figure (1) represents the presence of urea in the fractions collected from a urine sample run on the column. Two consecutive fractions were pooled. This gave a large enough sample for the AutoAnalyzer procedure. No urea was demonstrated in the area of the first peak on the separation, with the area and shape of the urea portions fitting well within the second peak.

Figure 1

Separation of urinary components

on a Sephadex G-25-80 column



Solid line = urine

Dotted line = pure protein solution (egg albumin)

Dashed line = urea

Table (1) gives the results of a recovery experiment in which solutions containing known weighed concentrations of egg albumin were applied to columns. The average recovery was 96.7 %.

Table (2) gives the results of a different type of recovery experiment in which varying amounts of an albumin solution were added to different urines and then run on the columns. Following is an outline of a typical set of three samples for the determination on one urine:

Sample	Number	Urine	Albumin	Water
Urine plus albumin	1	2.0 ml.	1.0 ml.	0
Urine plus water	2	2.0 ml.	0	1.0 ml.
Albumin plus water	3	0	1.0 ml.	2.0 ml.

Five different urines were used in the test. The amount of material absorbing at 210 nm. recovered from the urine plus albumin mixture is compared with the sum of the amounts recovered from the other two mixtures. The dilutions made the readings directly comparable without need for other dilution factors. The average recovery is 94.7 %. All concentrations are given in mg./100 ml. The ratio used in Table (2) represents the ratio of the volumes of urine to albumin solutions used in each sample.

Table (3) gives the specific absorbances of the proteins available for checking the spectrophotometer. The pure protein was dissolved in water and diluted until the resulting solution could be read in the spectrophotometer. The known concentration and the O.D.

Table 1

Recovery of protein after passage through columns

Weighed value mg./100 ml.	Recovered value mg./100 ml.	Per cent recovery
245	233	95
196	186	95
137	124	94
98	93	95
49	48	98
12.2	13.2	104

Table 2

Recovery of albumin added to urines

Sample number	1	2	3	4	5
Ratio of urine to albumin	2/1	2/1.5	2/0.5	2/1	2/1.5
Albumin plus water mg./100 ml.	5.42	6.90	2.58	5.56	6.95
Urine plus water mg./100 ml.	2.21	1.84	5.98	1.78	2.92
Sum of two above mg./100 ml.	7.63	8.74	8.56	7.34	9.87
Urine plus albumin mg./100 ml.	6.90	8.12	8.16	6.89	9.97
Per cent recovery	90.4	92.9	95.3	94.0	101.0

Table 3

Specific absorbances of various proteins

Protein	Specific absorbance $E^{1\%}_{mg.}$
Egg albumin	0.209
Bovine albumin	0.205
Hemoglobin	0.193
Human albumin*	0.203
Human serum protein*	0.205
Human gamma globulin*	0.213

(*) From Tomba et al. (15)

were used in the following formula modified from Werner (14):

$$E^{o}_{mg} = \frac{O.D.}{mg./100\ ml.}$$

Published values for human albumin, globulin and serum protein are given for comparison. (15)

Table (4) gives the data used to determine the linearity of the U.V. method. Pure protein solutions of egg albumin of known concentrations were added to the columns. The effluents were collected and the absorptions at 210 nm. were determined. The same volume of protein solution (0.2 ml.) was diluted to a total volume of 3.0 ml. with water and read at 210 nm. without passage through the columns. The two effluents containing the larger amounts of protein had to be diluted with equal amounts of water to permit reading in the spectrophotometer. The known concentrations and their theoretical optical densities are also shown. Figure (2) graphs the data from Table (4) to demonstrate the linearity of the method.

Table (5) gives the data from the within day reproducibility study. Samples of one urine were added to 14 different columns and the effluents quantitated. The mean value, standard deviation, range and coefficient of variation are also given.

Table (6) gives the data from the day to day reproducibility study. Four different urines were centrifuged and filtered and ten aliquots of each were frozen. Each day one of the aliquots from each urine was thawed and run on as many columns as were available

Table 4

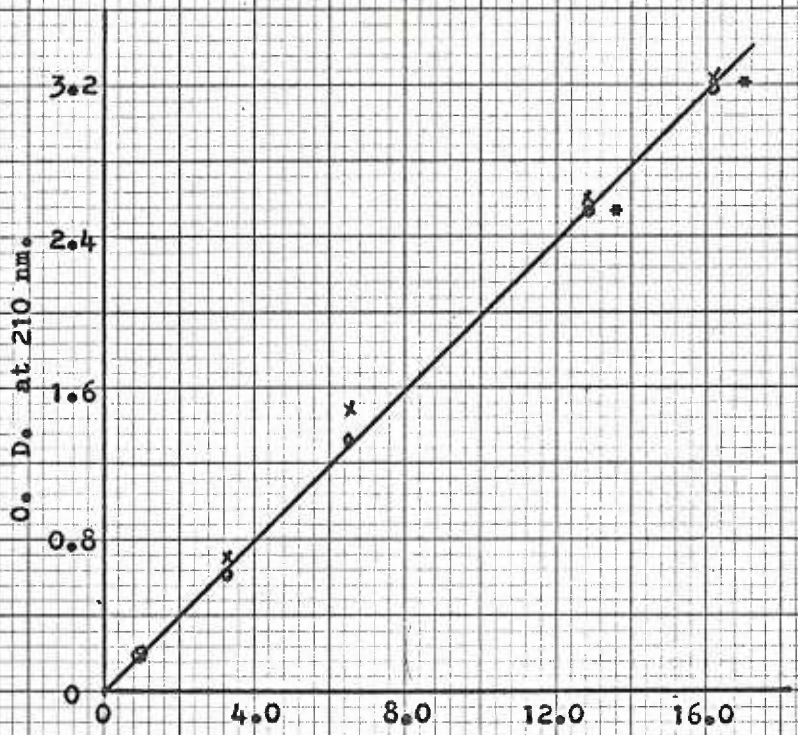
Linearity of the U.V. Spectrophotometer

Concentration mg./100 ml.	O.D. 210 nm. theoretical	O.D. 210 nm. column	O.D. 210 nm. non-column
16.3	3.309	3.203*	3.190*
13.3	2.740	2.576*	2.542*
6.53	1.337	1.345	1.276
3.27	0.664	0.668	0.627
0.82	0.166	0.189	0.176

(*) = the original value in O.D. X 2 (dilution factor)

Figure 2

Linearity of U.V. Spectrophotometer



o = set of standard dilutions

x = same set after running through the Sephadex gel columns

(*) = original O.D. X 2 (dilution factor)

Table 5

Within day reproducibility *

Column number	Protein mg./100 ml.
1	11.04
2	10.95
3	10.66
4	11.23
5	10.51
6	11.26
7	11.54
8	11.01
9	11.68
10	11.38
11	11.06
12	11.43
13	11.38
14	11.47
Mean	11.19
Standard deviation	0.33
Range	1.17
Coefficient of variation	3.0 %

(*) Protein collected in 3.0 ml. effluent volumes

Table 6

Day to day reproducibility *

Urine number	1	2	3	4
Number of determinations for each urine	21	22	22	22
Mean in mg./100 ml.	53.2	43.3	156.3	395.0
Standard deviation in mg./100 ml.	3.47	3.86	5.92	13.9
Range in mg./100 ml.	12.5	18.6	21.1	56.0
Coefficient of variation in per cent	6.53	9.16	3.78	3.54

(*) Protein collected in 3.0 ml. effluent volumes

that day. Sample 1 had a total of 21 determinations over the 10 day period. The other three samples each had 22 determinations. The sample number, number of determinations, means, standard deviations, ranges and coefficients of variation are shown for all four urine samples.

Table (7) gives the data obtained by comparing the U.V. method with Tsuchiya's method for urinary protein. Thirty nine urines containing pathological amounts of protein were quantitated by both methods. The results are shown in mg./100 ml. Figure (3) shows a plot of the data from Table (7) with the line of best fit. The slope of the line is 1.00. The "Y" intercept is - 40. The correlation constant "r" is .986 and "r^2" is .97.

Nine urines from healthy laboratory personnel were quantitated for protein using the U.V. method and the effluents were also quantitated by the Folin-Ciocalteu method. The results are given in Table (8) with the means and standard deviations. Twelve urines collected randomly and screened as negative for protein were also quantitated by both methods. This data is shown in Table (9). The results in mg./100 ml. from both studies are plotted in Figure (4).

An experiment was done to see if the U.V. method and the Folin-Ciocalteu method were measuring different substances at different places during the elution. Two-tenths of a ml. of a urine containing approximately 100 mg. of protein per 100 ml. was applied to a column and six drop fractions were collected as in the separation experiment. The six drops were equal to about 0.42 ml. Two-tenths

Table 7

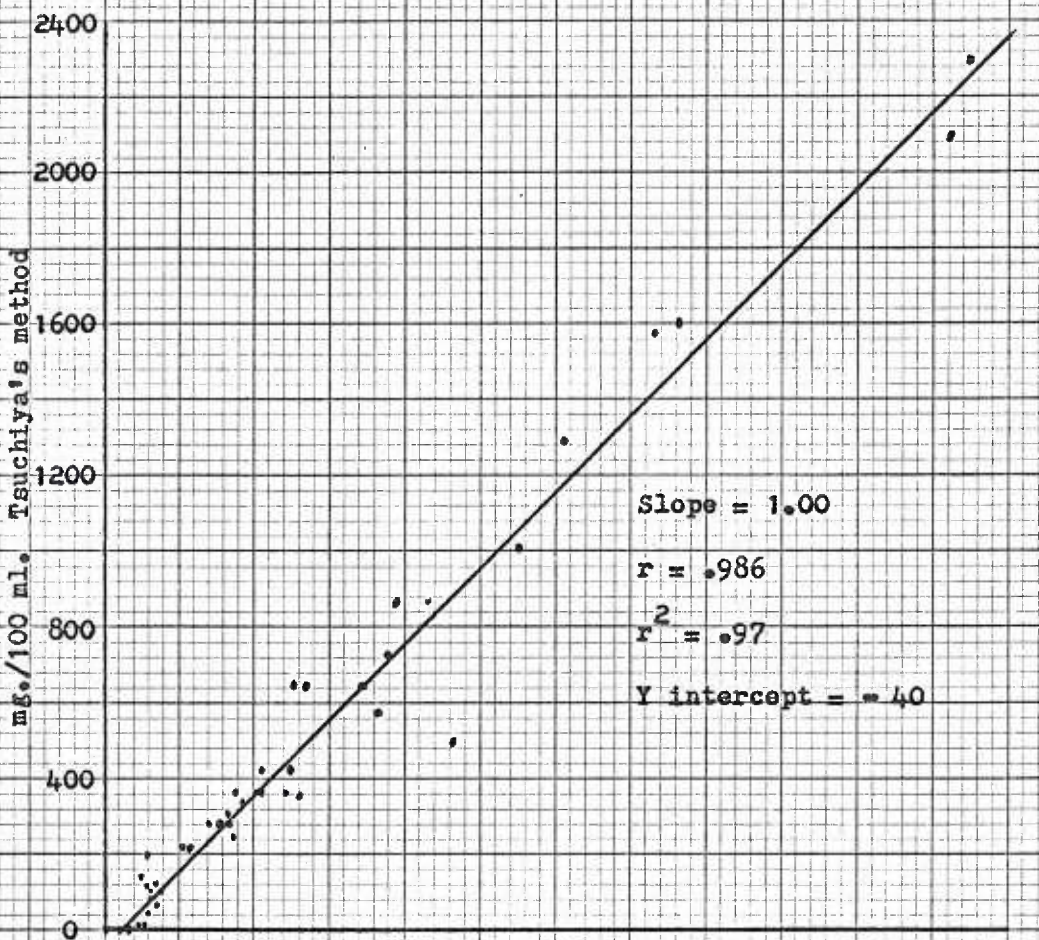
Comparison between the U.V. method and Tsuchiya's method for urinary protein *

U. V. method mg./100 ml.	Tsuchiya's method mg./100 ml.	U. V. method mg./100 ml.	Tsuchiya's method mg./100 ml.
127	86	351	286
151	107	241	216
494	432	308	288
494	361	113	122
2246	2088	1220	1295
1112	1010	346	286
696	650	552	648
1457	1580	520	360
1528	1600	925	518
99	8	753	730
100	143	342	280
128	101	780	865
363	250	158	200
152	109	420	363
415	365	503	648
280	288	730	580
118	47	526	434
76	0	2300	2300
140	72	370	358
206	216		

(*) Protein collected in 3.0 ml. effluent volumes

Figure 3

Statistical correlation between U.V. method and Tsuchiya's method for urinary protein *



(*) Protein collected in 3.0 ml. effluent volumes

Table 8

Comparison of the U.V. method with the Folin-Ciocalteu method on 9 normal 24 hour urine collections *

	U. V. method mg./24 hrs.	Folin-Ciocalteu method mg./24 hrs.
	217	123
	102	33
	97	56
	41	30
	70	26
	78	45
	104	69
	107	27
	56	33
Mean mg./24 hrs.	96.9	49.1
Standard deviation mg./24 hrs.	50.6	31.3
Range mg./24 hrs.	176	97

(*) Protein collected in 3.0 ml. effluent volumes

Table 9

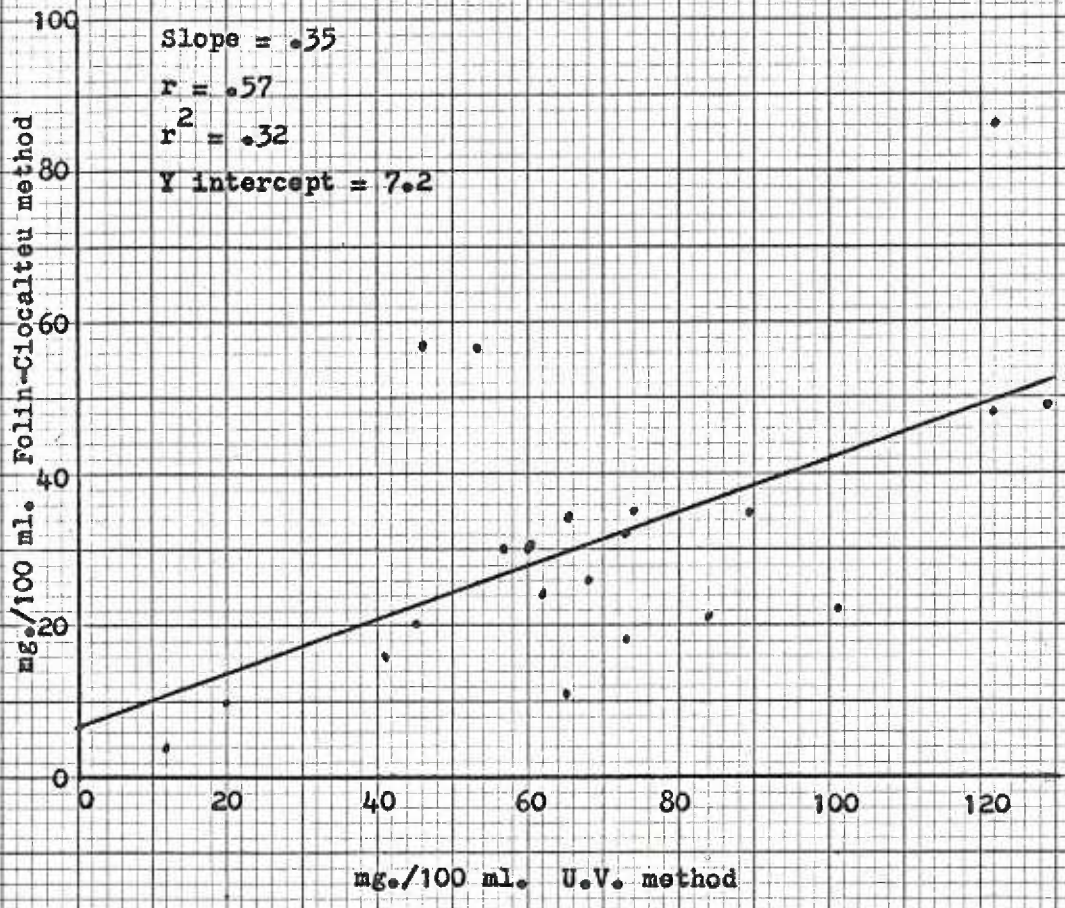
Comparison of the U.V. method with the Folin-Ciocalteu method on 12 random urines *

U. V. method mg./100 ml.	Folin-Ciocalteu method mg./100 ml.
121.5	85.5
72.9	31.5
53.3	57.0
19.9	9.6
41.4	16.1
84.6	20.9
56.7	30.4
45.6	57.0
59.8	30.4
122.0	44.7
65.8	33.6
11.0	4.1

(*) Protein collected in 3.0 ml. effluent volumes

Figure 4

Statistical correlation between U.V. method and the Folin-Ciocalteu method for urinary protein on 21 normal urines *



(*) Protein collected in 3.0 ml. effluent volumes

of a ml. of each effluent fraction were taken for the Folin-Ciocalteu procedure and the remaining 0.22 ml. portions were diluted with 2.8 ml. of water and read in the spectrophotometer at 210 nm. The optical densities from the U.V. method and the Folin-Ciocalteu method are listed for the 20 fractions in Table (10). The ratio of the U.V. to the Folin-Ciocalteu absorbances is also listed for each fraction. The data is plotted in Figure (5).

Examination of the data given in Table (10) and Figure (5) showed that the end of the 3.0 ml. effluent collection included a sizeable portion of the second peak (fraction 14). To determine what effect, if any, this was having on the quantitation of the normal urine proteins, it was decided to reduce the volume to the minimum amount that would allow collection of all the protein peak. Two ml. after the first void volume was chosen for collection and several experiments were done using this volume of effluent.

A recovery experiment was done to see if the 2.0 ml. effluent volume was enough to recover all the added protein. Table (11) gives the results of this experiment. The average recovery is 99.7 %.

Correlation experiments on normal urines and abnormal urines were done as described previously only using the 2.0 ml. effluent collection. For the normal urines, the U.V. method was correlated with the Folin-Ciocalteu method. The results are given in Table (12) and are shown in Figure (6).

The abnormal urines were quantitated by three methods.

Table 10

Comparison of U.V. and Folin-Ciocalteu

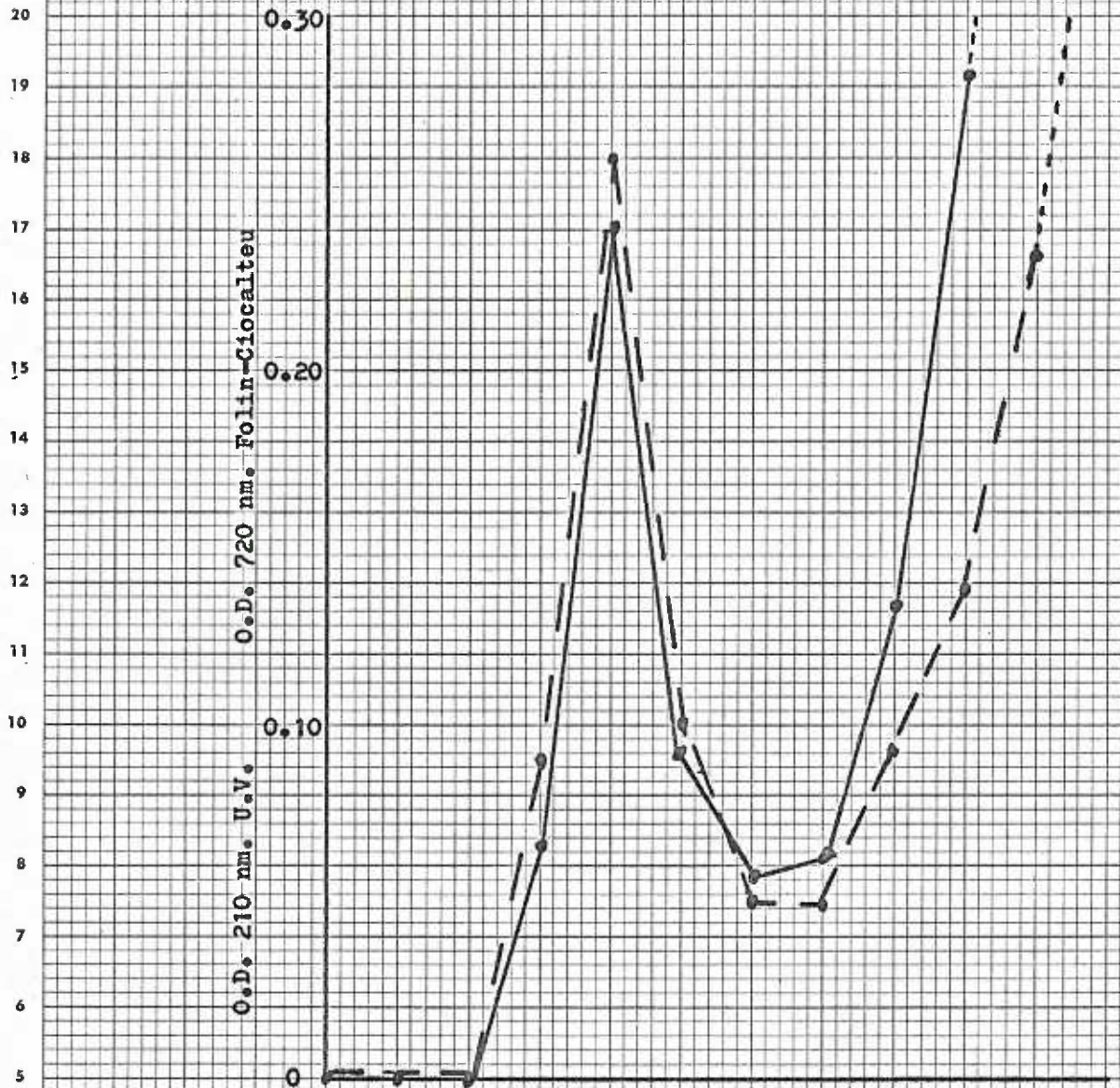
methods on effluent fractions

Fraction number	Optical density U. V.	Optical density Folin-Ciocalteu	Ratio U.V./F.C.
1	0	0	
2	0	0	
3	0	0	
4	0	0	
5	0	0	
6	0	0	
7	0	0	
8	0.066	0.091	0.73
9	0.241	0.260	0.93
10	0.093	0.101	0.92
11	0.057	0.050	1.14
12	0.062	0.049	1.27
13	0.134	0.093	1.44
14	0.283	0.138	2.05
15	0.859	0.233	3.64
16	1.813	0.360	5.03
17	2.709	0.401	6.76
18	4.028*	0.450	8.96
19	4.652*	0.712	6.53
20	3.324*	0.729	4.56

* These readings were made on a one to four dilution. The resulting absorbances were multiplied by four to give the data shown in the table.

Figure 5

Relative values of U.V. and Folin-Ciocalteu methods on effluent fractions



Solid line = U.V. method

Dashed line = Folin-Ciocalteu method

Table 11

Recovery of serum protein added to normal urine in normal amounts. All values are in mg./100 ml. *

Protein added	Protein recovered	Per cent recovered
12.2	13.2	108.2
24.0	24.6	102.4
38.6	35.3	91.5
49.2	47.7	97.0
	average recovery	99.8

(*) Protein collected in 2.0 ml. effluent volumes

Table 12

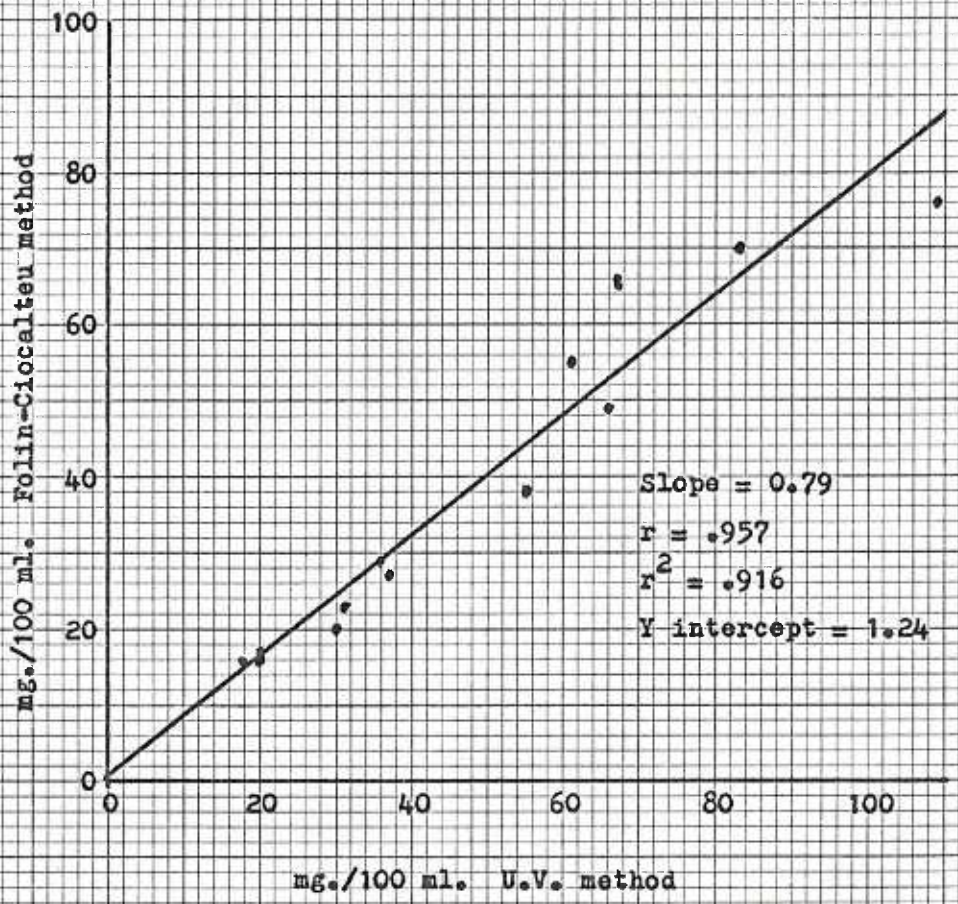
Comparison of the U.V. method with the Folin-Ciocalteu method on 14 random urines *

U.V. method mg./100 ml.	Folin-Ciocalteu method mg./100 ml.
82.5	70
66.8	66
67.1	65
60.7	55
30.6	23
17.5	16
36.7	27
65.7	49
35.6	29
55.3	38
109.0	76
19.8	16
29.8	20
20.3	17

(*) Protein collected in 2.0 ml. effluent volumes

Figure 6

Statistical correlation between the U.V. method and the Folin-Ciocalteu method on 14 random urines *



(*) Protein collected in 2.0 ml. effluent volumes

Tsuchiya's method was done in the routine laboratory run during the day. The urine was then quantitated by the U.V. method and an aliquot of these effluents was quantitated by the Folin-Ciocalteu method. The results of these three methods on 11 urines are given in Table (13). Figure (7) shows the correlation between the U.V. method and Tsuchiya's method. Figure (8) shows the correlation between the U.V. method and the Folin-Ciocalteu method. Figure (9) shows the correlation between the Folin-Ciocalteu method and Tsuchiya's method.

A within day reproducibility experiment was done using the 2.0 ml. effluent collection. The results are shown in Table (14).

Table 13

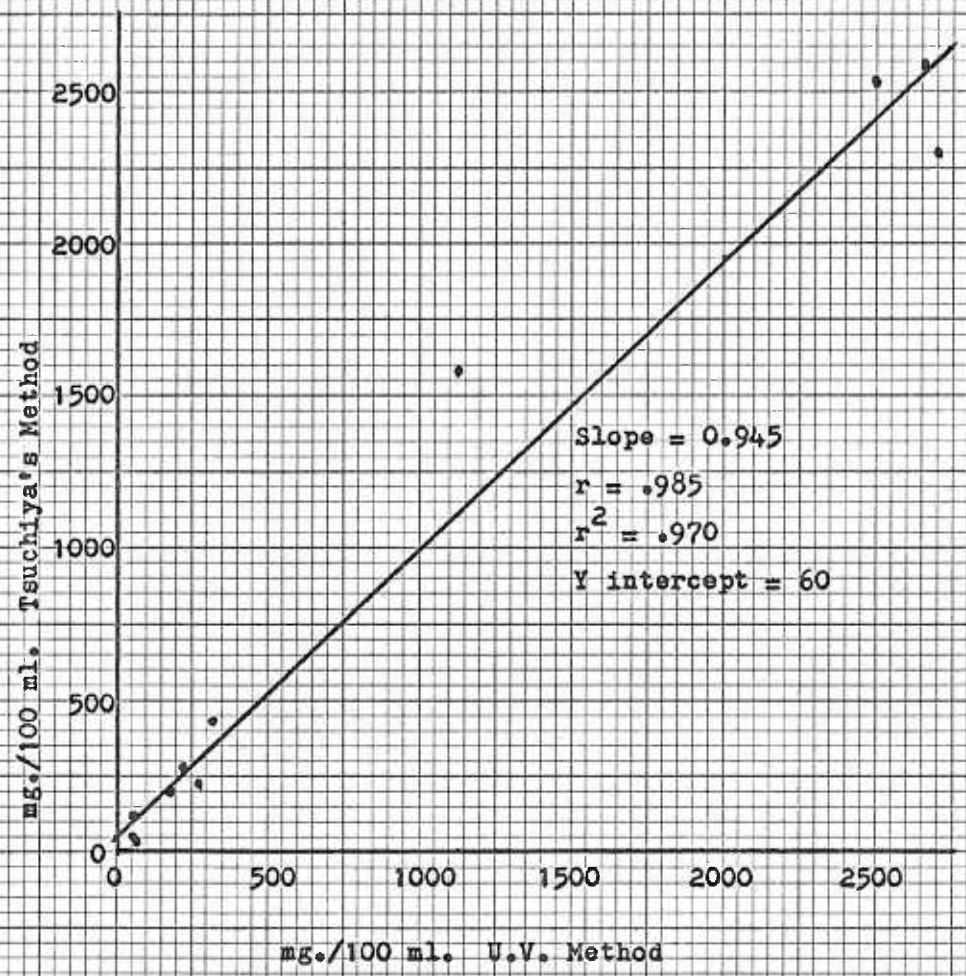
Comparison of the U.V. method, Tsuchiya's method and the Folin-Ciocalteu method on 11 abnormal urines. All values are in mg./100 ml. *

U. V. method	Tsuchiya's method	Folin-Ciocalteu method
1130	1580	990
2700	2300	2000
70	29	70
277	234	240
235	290	180
2500	2540	1870
2660	2590	1910
57	50	52
171	200	90
42	120	20
319	430	290

(*) Protein collected in 2.0 ml. effluent volumes

Figure 7

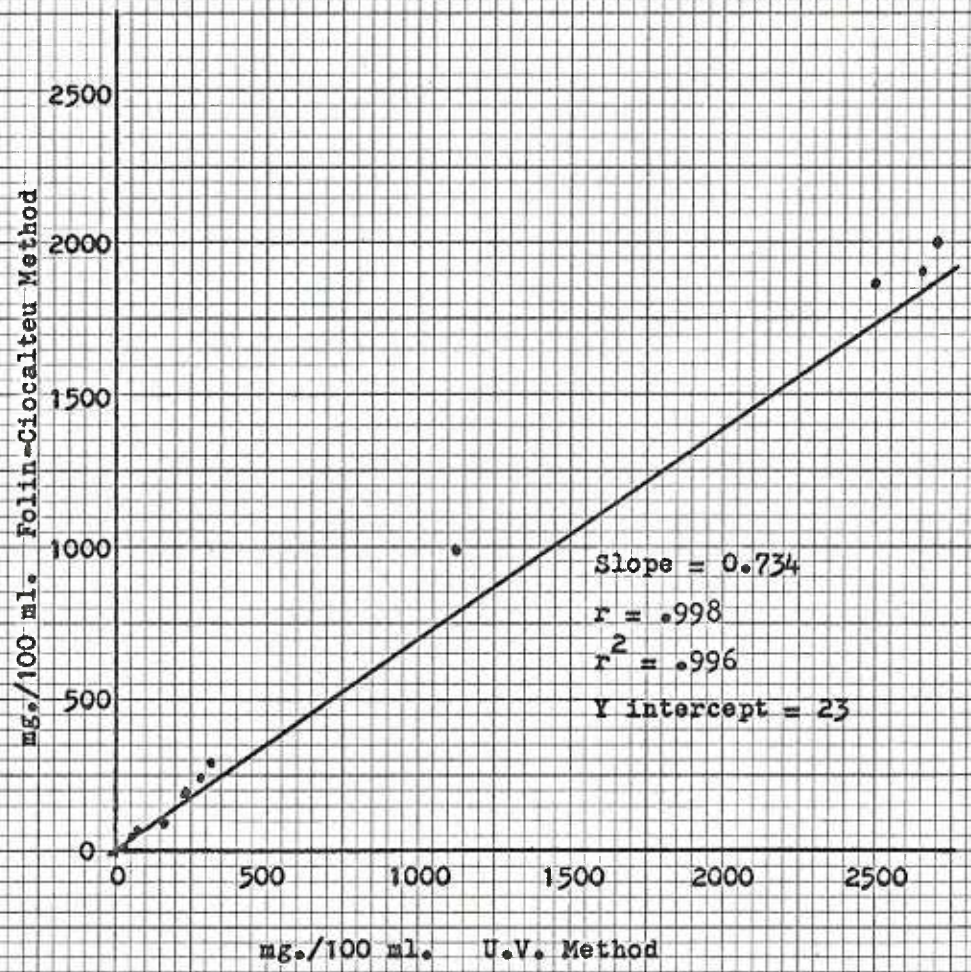
Statistical correlation between the U.V. method and Tsuchiya's method on 11 abnormal urines *



(*) Protein collected in 2.0 ml. effluent volumes

Figure 8

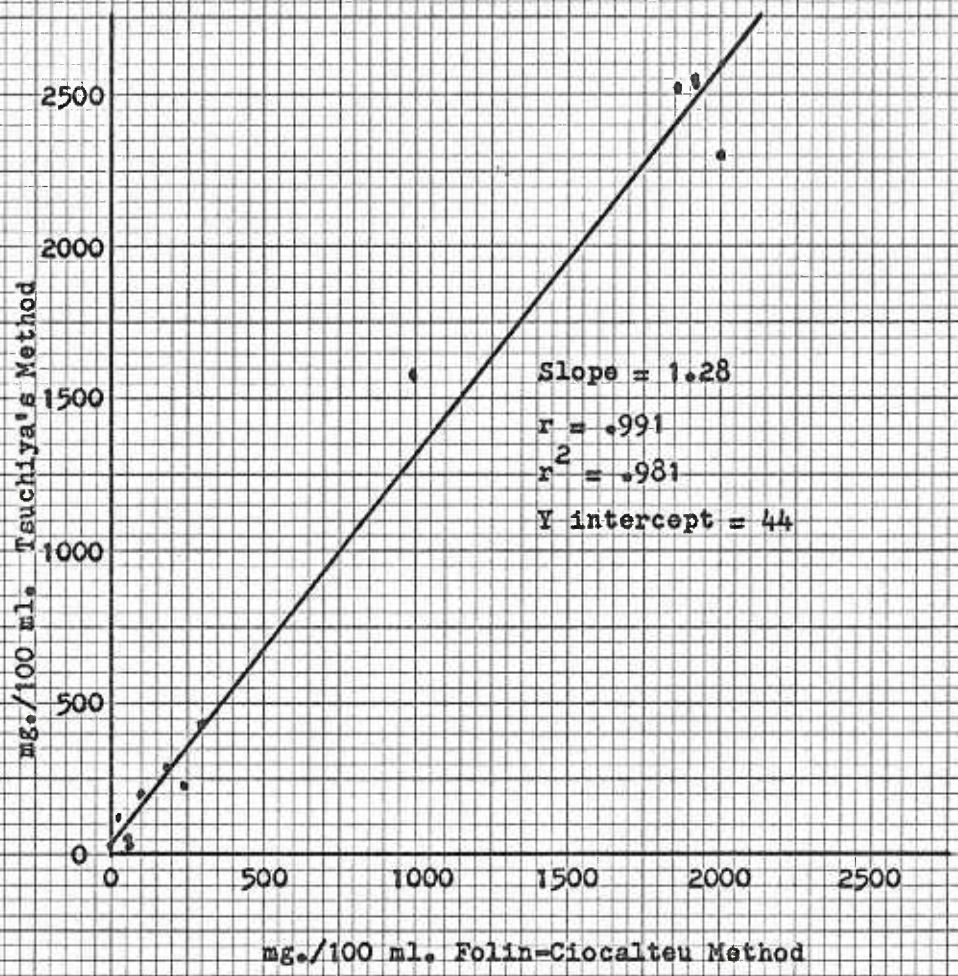
Statistical correlation between the U.V. method and the Folin-Ciocalteu method on 11 abnormal urines *



(*) Protein collected in 2.0 ml. effluent volumes

Figure 9

Statistical correlation between the Folin-Ciocalteu method and Tsuchiya's method on 11 abnormal urines *



(*) Protein collected in 2.0 ml. effluent volumes

Table 14

Within day reproducibility *

Column number	Protein mg./100 ml.
1	155
2	149
3	152
4	158
5	165
6	165
7	162
8	149
9	156
10	144
11	145
Mean	154.5
Standard deviation	7.6
Range	21
Coefficient of variation	4.92 %

(*) Protein collected in 2.0 ml. effluent volumes

Discussion

The good biuret methods which have been used for urinary protein determinations have some drawbacks for use as a standard clinical method. One of these drawbacks is the length of time necessary to perform the test. The method published by Savory et al. (7) in 1968 required ice baths, blank and standard tubes, 60 ml. of cold ethanolic phosphotungstic acid, 60 ml. of absolute ethanol, three centrifugations and a biuret reaction for one sample. Each additional sample would require another 20 ml. each of the acid and alcohol reagents.

Such a procedure is quite lengthy for any number of samples. Reagent volumes are large and there is an appreciable amount of hand work. Because of these conditions, clinical laboratories have come to depend upon unreliable screening tests (3) to eliminate the necessity for performing many of the quantitative urine protein determinations requested.

Experience has shown that the common screening methods, "stix" and sulfosalicylic acid, are subject to errors in interpretation. These errors may be due to the lighting available in the laboratory, color perceptiveness, and dirty or scratched glassware. The "stix" methods are primarily sensitive to albumin. Other proteins may not be detected even when present in relatively large amounts. One urine tested during the present research was completely negative for protein when tested by Combistix. Several technologists and the head of the chemistry section of the clinical laboratory viewed

the test under various lighting conditions. The same urine was negative for protein when first tested by sulfosalicylic acid. Upon standing for a minute or two, the reaction became very strong indicating over 400 mg./100 ml. of protein. The original requisition was turned out as negative for protein and the patient was discharged before follow up work could be done.

This experience may be an extreme example, but it does illustrate the problems facing a laboratory which depends upon screening procedures to reduce the workload for quantitative urinary protein measurement.

There existed a need for a procedure which would be simple enough to eliminate the dependence upon screening tests and yet accurate enough to be used with confidence. It would be preferable if the test could measure a common factor in all protein molecules such as the peptide bond. It should not depend upon the dye binding properties which can vary from protein to protein. It should be free from the errors of precipitation and yet should remove all substances which might interfere with the quantitation method used. Reagent expense should be minimum to keep down fixed costs. Correlation with other methods should be acceptable and the tests for reproducibility and accuracy should be within reasonable limits.

The method proposed as the result of this research measures the absorption of the resonating peptide bond. It is not necessary to make allowance for the different sizes and shapes of the serum protein molecules. Their specific absorbances ($E^{1\%}_{gm.}$) at 210 nm.

vary from 203 for albumin to 213 for gamma globulin (15). The average for serum protein is 205 ± a standard deviation of 1.4. The 205 value was chosen for all the experimental results in this research except where otherwise noted.

At 280 nm., the other wavelength commonly used for protein and amino acid measurement, the total serum protein specific absorbance is about two-thirds again as large as that of albumin. Gamma globulin gives about two and one-half times the absorption per unit of weight as does albumin. The sensitivity at 280 nm. is approximately fifteen times less than at 210 nm. (15)

The peak absorption due to the peptide bond is near 190 nm. (15) However, this wavelength is not generally available in the clinical laboratory and was not available for this work. The lowest practical wavelength available for use was 210 nm. and there is some published data concerning protein absorption at 210 nm. which could be used to compare with our work.

Table (3) shows that egg albumin, bovine albumin and hemoglobin have specific absorbances close to the published data for human serum protein fractions. These proteins which we measured were salt free and soluble in water. Human serum protein fractions and Tamm-Horsfall mucoprotein were not available in the necessary form for our work.

There are a few problems associated with measurement at 210 nm. Stray light and the strong absorption of many biological substances at 210 nm. are most prominent. Stray light would allow more light

to be detected by the photocell and thus give an apparently lower optical density for a given sample. The linearity of the plot of absorbance vs. concentration shown in Figure (2) indicates that stray light isn't a large problem with our spectrophotometer.

Because of the strong absorbance of non-protein substances at 210 nm., distilled water was used for all the portions of the experiment requiring elution, dilution or a blank rather than to use a buffer of some type. Also, this absorption of non-protein substances at 210 nm. emphasizes the need for separation between the protein and non-protein fractions eluted from the column.

As deuterium lamps age, their light output decreases. This decrease can be observed by noting the increasing slit width necessary to blank the instrument from day to day. Small changes will be seen regularly. This was quite noticeable in the present experiment where the lamp had to be placed in the housing each run and removed afterwards to accommodate routine work in the laboratory. However, a marked or sudden shift while using the same lamp with the same cuvettes indicates the need to use a new lamp. (15) If more than one person is to run the procedure or if several days may elapse between runs, it would be a good procedure to record the slit widths with each run.

The spectrophotometer used in this research was linear to around 1.3 O.D. as shown in Figure (2). Because of this and the agreement between the specific absorbances which we measured and those published for human proteins in Table (3) it was unnecessary

to run standard curves for the quantitation. If the spectrophotometer were not linear, it would be possible to make a standard curve from a pure protein solution of known concentration. (10)

The use of gel chromatography to separate various components of urine and spinal fluid has been used by several investigators. Among these are Patrick and Thiers (10), Jorgensen (13), Werner (14), Tombs et al. (15), MacLean and Petrie (17), Burtis, Goldstein and Scott (18), and Davis, Flynn and Platt (19).

The results of the experiments shown in Figure (1) indicate that the protein from the urine is contained in the first peak and that substances which might interfere are contained within the second peak. In order to achieve satisfactory separation as shown in the figure, it may be necessary to change the relative volumes of the gel beds and the samples. A satisfactory relationship for this research was a gel bed of 7.5 ml. and a sample size of 0.1 ml. or 0.2 ml. The G-25 gel gives a molecular weight cut off around 5000. (16) Molecules larger than 5000 in M.W. and globular in shape are excluded from the inner matrices of the gel and therefore get eluted more rapidly than the smaller molecules which can enter the matrices and thus be held back for a while within the column. Other experimenters have demonstrated the same type of separation as is shown in Figure (1). (10,13)

The recovery experiments with data listed in Tables (5) and (6) show over 90 % recovery in all phases with an average recovery around 95 %.

The U.V. method shows good linearity as is demonstrated by Table (4) and Figure (2). The linearity of the set of standard dilutions after having been run through the columns is almost equal to that of the set which was diluted to the same volumes but not run through the columns. Table (4) also shows the theoretical absorbances which should be given by the concentrations listed. The concentration times the coefficient of .203 for albumin gives the theoretical O.D. There is good agreement between the actual and the theoretical absorbances.

A comparison was run checking the U.V. method results on pathological urines against the results of Tsuchiya's method as done in the clinical laboratory. Thirty nine urines were run on both methods. The plot of one method against the other is shown in Figure (3). The slope indicates that the two methods are measuring the same substances in relatively the same amounts and the predictable correlation of "r^2" is .97. Table (7) lists the data for each method.

The above type of experiment was done using several normal urines checking the U.V. method against the Folin-Ciocalteu method. The data is listed in Tables (8) and (9) and the results are shown in Figure (4). The Folin-Ciocalteu method was used because Tsuchiya's method will not detect such low levels of protein. The agreement between these two methods is not good with the U.V. giving more than twice the value of the Folin-Ciocalteu method.

To try to elicit more information concerning the nature of the disagreement, the experiment shown in Table (10) and Figure (5) was

performed. This experiment was done to determine if both the U.V. and Folin-Ciocalteu methods were measuring the same substances at various points during the elution procedure. Figure (5) shows that the lines cross on the descending side of the protein peak and the Ratio column in Table (10) shows a steadily increasing U.V. to Folin-Ciocalteu ratio from fraction 11 through fraction 18. This would indicate that the two methods are not measuring the same substances at all points throughout the elution. Werner (14) suggested that the tailing of the protein peak was due to peptides. The ratio change observed in the above experiment would indicate that at least not all of the tailing is due to peptides or else the two methods should parallel each other throughout the entire elution.

In checking the experiment shown in Figure (5), it became apparent that 3.0 ml. of effluent after the first void volume would include part of the second peak through fraction 14 with the fraction sizes used. On the basis of this knowledge, the effluent size was reduced to 2.0 ml. and several studies were done to see if acceptable results would be obtained by this method.

The recovery as indicated by Table (11) averages 99.7 % in the normal range of urinary protein.

The correlation experiments with normal urines using the U.V. method and the Folin-Ciocalteu method as given in Table (12) and Figure (6) show a marked improvement over the previous study as shown in Tables (8) and (9) and in Figure (4). The predictability has increased from .32 to .916 and the slope has gone from .35 to .79. This slope was repeated in the correlation between the same

two methods on abnormal urines as shown in Table (13) and Figure (8).

The U.V. method produces higher results than the Folin-Ciocalteu method in both normal and pathological urine samples. The ratio of these results is about the same for both high and low concentrations of protein. This indicates a bias in methods and not an interference caused by incomplete separation of proteins from samll molecules. If the discrepancy between methods were caused by incomplete purification of the proteins, it would be expected that the results on low protein urines would be affected considerably more than the high protein urines. However, this is not the case since the ratio of U.V. to Folin-Ciocalteu results is essentially constant for all values of protein. This indicates that the U.V. method results for low protein values are valid.

In correlating with the Folin-Ciocalteu method, egg albumin was used as a standard. Saifer and Gerstenfeld (20) indicated that various proteins give quite different absorbances when quantitated with the Folin-Ciocalteu reagent. If egg albumin gives a higher absorbance per weight than urinary proteins, this might help to explain the bias between the two methods.

A comparison of the three correlations shown in Figures (7), (8) and (9) shows that both the U.V. and Tsuchiya's methods yield higher values than the Folin-Ciocalteu method. The predictability of the three methods compared with each other appears good with all of the "r^2 values greater than .97.

The within day reproducibility using the 2.0 ml. effluent col-

lection as shown in Table (14) is comparable with that given in Table (5).

These experiments indicate that the U.V. method is acceptable for quantitating urinary protein over any concentration range when just the protein peak is included in the determination. Each investigator attempting to use this method would have to determine how wide the protein peak was as eluted from his columns. The present investigation had been almost completely done before discovering that the effluent volume was larger than could be acceptable. Part of the reason was the data from Figure (1) which indicated that a second void volume would cut somewhere between the two peaks around fraction 18. This, then, appeared to be a good and easily determined volume to collect. Such was not the case and much of the experiment had to be repeated using only that portion of the elution which contained the protein peak.

In addition to the demonstrated accuracy and reproducibility with varying concentrations of protein, the U.V. method has great advantages in time, simplicity and cost of reagents. The total time from first addition of the urine to the column until the sample is ready to be read in the spectrophotometer is usually less than 45 minutes. The largest portion of this time is in waiting for the larger portions of water to enter the column completely. The columns do not have to be closely attended. The technologist may add one portion at a time and then return to add the second portion of water at a convenient break in other work. The preparation of the

cuvettes and determination of blank values usually takes less than 15 minutes including time to wash the cuvettes with detergent. If the cuvettes are reserved for this test and are left well rinsed, the blanking part of the procedure may be reduced to only a few minutes.

Each sample may be read in approximately a minute including time to rinse the cuvettes twice with water. All of the calculations are simple multiplications or divisions and should present no difficulties to the technologist that are not present in other methods. Several urine protein determinations may be done completely in less than an hour if adequate flow rates are maintained.

Flow rates may slow after using a column several times. This problem can be at least partially resolved by stirring up the top portion of the column and allowing the gel to settle again. Preservation of the column against growth by fungi can be accomplished by use of 2.5 % (w/v) NaF. (10) This should prevent any growth but it does present a drawback in that the columns would have to be completely washed with about 15 ml. of water before use. This could take over an hour. Keeping the columns cool and away from sunlight could also help to reduce growth. The present research has not used any of the preservation methods. Some of the columns have been in use for more than three weeks without the necessity of removing the gel from the columns for washing, or repouring the columns with new gel.

Gel cost per column is less than 10¢ and many tests can be run

upon the same column. The only other reagent needed is distilled water. The method's simplicity, economy of time, and accuracy recommend this procedure for use on every urine sent to the laboratory for quantitative urinary protein without the necessity for dependence upon a screening procedure with the possibility of overlooking some pathological urine.

Summary

A new method for quantitating urinary protein is described which uses gel filtration to remove interfering substances and which quantitates the protein by means of the absorption of the peptide bonds at 210 nm. Correlation with a precipitation method is good for abnormal urines. Correlation with the Folin-Ciocalteu method on both normal and abnormal urines gives a bias with the U.V. method giving about 25 % higher values than the Folin-Ciocalteu method when egg albumin is used as a standard. The method is simple to run. It requires little technologist time to perform and is accurate and reproducible. Recovery is greater than 90 % and averages about 95 %.

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