USEFULNESS OF FERROXIDASE ACTIVITY OF CERULOPLASMIN IN THE DIAGNOSIS OF WILSON'S DISEASE

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ABSTRACT

Serum ceruloplasmin is one of the most commonly used screening tests for Wilson's disease. However immunological assays for ceruloplasmin are not recommended for diagnosis and management of Wilson's disease through calculation of free copper index. Enzymatic methods using non-physiological substrates have toxicity and stability problems, making them difficult to automate. Ferroxidase assays may be a satisfactory alternative for measuring serum ceruloplasmin. The o-dianisidine hydrochloride manual method for estimation of serum ceruloplasmin enzyme activity was compared with an automated method using the ferroxidase activity of ceruloplasmin in measurement in a double blind study in 91 consecutive patients screened for Wilson's disease. The o-dianisidine and ferroxidase methods both successfully identified 7 patients with Wilson's disease. Values for these 7 patients in the o-dianisidine and ferroxidase methods were median 5.0 (range 0-16.0 U/L) and median 45.0 (range 4-166 U/L) respectively. There were 7 other positive values (<62 U/L) with the o-diansidine method and 2 (<200 U/L) with the ferroxidase method, where WD was not confirmed. ROC curves for both methods showed area under the curve of 0.998 for o-dianisidine and 0.997 for ferroxidase. Using literature cut off values of 62 U/L and 200 U/L respectively both methods had 100% sensitivity and specificity was 91.7% (o-dianisidine) and 97.6% (ferroxidase). For the o-dianisidine assay, specificity was improved to 98.8% using a cut off of 22.5 U/L. In the 84 persons (46 adults and 38 children) in whom the diagnosis of Wilson's disease was not established, the mean value for ceruloplasmin activity by the o-dianisidine and ferroxidase methods was 124.7 ± 48.7 U/L and 571.4 ± 168.1 U/L respectively. There were no significant differences between sex or age of patients ($p > 0.29$). In a subsequent evaluation with 372 specimens, the Pearson correlation coefficient between the assays was 0.908, $p < 0.01$, slope 4.06, intercept 265.8, with the manual assay as the x-axis. The ferroxidase assay is a suitable replacement for the o-dianisidine assay in detecting patients with Wilson's disease.

KEY WORDS

Ceruloplasmin, Wilson's Disease, o-dianisidine, Ferroxidase.

INTRODUCTION

Wilson's disease (WD) is an autosomal recessive disorder of copper overload with impaired biliary excretion of copper leading to hepatic or neuropsychiatric disease in children and young adults. Deficiency leads to accumulation of copper in the liver and eventually in other tissues. It is one of the few

fatal diseases of the liver or central nervous system for which a specific and effective therapy is available and is a diagnosis that should not be missed. However there is no gold standard for the diagnosis. It is usually based on clinical and laboratory findings. Low serum ceruloplasmin, serum copper and increased urinary copper excretion are characteristic biochemical findings of the disorder (1). Diagnosis is straightforward if two of the following symptoms are present Kayser -Fleischer (KF) rings, typical neurological symptoms and decreased serum ceruloplasmin. Diagnosis is far more complex in patients presenting with liver disease, with KF rings, being seen in up to 50% of these patients (2).

Serum ceruloplasmin level can be assessed

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immunochemically or functionally (enzymatic activity). Walshe states that values obtained immunochemically for ceruloplasmin should not be used for the diagnosis and management of patients with WD (1). These methods also measure apoceruloplasmin (which does not contain copper) and so may be misleading in WD. He further states that the most important investigation for diagnostic purposes is the non-ceruloplasmin bound copper (NCC) or "free copper" or copper index. This is a derived parameter using the ceruloplasmin concentration in mg/L. NCC is normally around 1.6 $\mu\text{mol/L}$ (10 $\mu\text{g/dL}$). In symptomatic untreated WD it is much higher, but falls with adequate treatment. Immunological methods give falsely low or even negative values for "free" copper, so they should not be used for diagnosis and management of WD. Walshe (1) states that serum ceruloplasmin in the diagnosis of WD should be measured by its oxidase activity ie as an Fe(II): oxygen oxidoreductase (EC 1.16.3.1). Oxidase methods also have lower detection limits than immunological ones.

Currently we use a manual enzymatic method with o-dianisidine hydrochloride as substrate (3,4). This non-physiological substrate is an aromatic amine whose toxicity is not fully evaluated. Other non-physiological substrates are p-phenylenediamine dihydrochloride (5) and N, N-dimethyl-p-phenylenediamine (DPPD) monohydrochloride (6). These methods have proved difficult to automate and substrates may have to be prepared freshly.

Erel (7) in 1998 automated a method for the Hitachi analyzer using the physiological substrate ferrous ions, which he reports has good stability. Also this method does not require a ceruloplasmin standard for calibration. We carried out this study to compare with our existing enzymatic method in screening patients for Wilson's disease.

MATERIALS AND METHODS

Over the 45 day period of this double blind study, in this large tertiary referral center, 127 patients consecutively presenting, required measurement of serum ceruloplasmin, because of neurological or hepatic complications with inconclusive liver function tests. The breakdown of these 127 patients is shown in Appendix 1.

A retrospective examination was made of all the available clinical records of these patients. Wilson's disease was diagnosed when Kayser-Fleischer (KF) rings were present in the cornea with low serum ceruloplasmin activity, by the o-dianisidine enzymatic method as the gold standard. In the

early stages of the disease when KF rings were absent, low serum copper < 11.0 $\mu\text{mol/L}$ (<70 $\mu\text{g/dL}$) and increased urinary copper excretion >2.4 $\mu\text{mol/24 hr}$ (> 150 $\mu\text{g/24hr}$) were taken as confirmation of copper deficiency. In equivocal cases, liver biopsy with increased dry weight of copper > 3.93 $\mu\text{mol/g}$ (>250 $\mu\text{g/g}$) confirmed the diagnosis.

After this study period, in order to establish the correlation between the two assays of ceruloplasmin activity, a further 372 patients presenting at clinic had specimens estimated by both enzymatic assays. The clinical records of these patients were not systematically examined. Patients were evaluated using a modification of the scoring system developed at the 8th Meeting on Wilson disease, Leipzig 2001, as described by Ferenci (2). The presence of KF rings, serum ceruloplasmin, and urinary copper excretion were used for scoring in our patients. Serum caeruloplasmin by the o-dianisidine method, value < 62 U/L scored 1, < 22.5 U/L scored 2. A total score of 4 or more means that the diagnosis is established, score of 3 means more tests are needed, 2 or less diagnosis is unlikely.

The method used routinely for ceruloplasmin estimation, at the time of the study was the o-dianisidine manual assay (3,4) which has a sensitivity of approximately 6U/L (absorbance change of 0.01) and an upper limit of 440 U/L. Detection wavelength was 540nm, and reaction temperature was 30°C. Ferroxidase activity was measured by the method of Erel (7) on a Hitachi 912 analyser (Roche Diagnostics GmbH). The substrate is ferrous ions and the chromogen is 3-(2-pyridyl)-5,6-bis (2-[5-furyl sulfonic acid])-1,2,4-tri-azine, disodium salt (Ferene). Ferrous ions, which are oxidized to ferric ions by the enzyme and the chromagen forms a highly colored complex with the remaining nonoxidised ferrous ions. Detection limit is 19.0 U/L, upper limit 2400 U/L. The reaction is monitored at 600nm, with 700nm as secondary wavelength, and temperature of 37°C. A two-point inverse calibration was used. Deionized water is used as the first calibrator (zero) and EDTA as the second (2400 U/L.). The zero calibrator gives the absorbance of the total ferrous concentration in the reaction medium and represents initial substrate concentration. The second calibrator all the substrate has been oxidized, equivalent to an enzyme activity of 2400 U/L. The substrate solution was stable for about 1 month at 4°C. Between- day precision for both these assays was based on repeat analysis of patients' samples on the next assay date, according to the formula of Jones and Payne (8). For the manual assay n=36 mean value 147 $\pm$ 48 U/L, % CV = 6.1. For the ferroxidase assay n=16 mean value 721 $\pm$ 398 U/L, % CV = 7.0.

Copper estimation in serum and urine was carried out using

the AA analyst 100 atomic absorbance spectrophotometer, Perkin Elmer, Shelton, USA, according to the manufacturer's instructions. Daily %CV values for serum of 3.6% and 2.0% were obtained at mean values of 19.5 $\mu\text{mol/L}$ (124 $\mu\text{g/dL}$) and 23.7 $\mu\text{mol/L}$ (151 $\mu\text{g/dL}$) respectively.

Reference values for all assays were as follows: serum ceruloplasmin; o-dianisidine method 62 – 140 U/L, ferroxidase method 200– 1107 U/L, serum copper 11.0 – 26.7 $\mu\text{mol/L}$ (70 – 170 $\mu\text{g/dL}$) and urinary copper excretion < 2.4 $\mu\text{mol/24 hr}$ (< 150 $\mu\text{g/24hr}$)

Correlation data, sensitivity, specificity, positive predictive value, ROC plots, z scores and differences plots were all derived using the SPSS version 11 statistical package SPSS for Windows, version 11 (SPSS Inc., Chicago, IL, USA).

RESULTS

Clinical details were obtained retrospectively and were adequate in only 92 (72%) of the 127 patients. At this time 8 patients were diagnosed as Wilson's disease. However one of these did not have the ferroxidase estimation performed, leaving 91 patients, including 7 Wilson's patients with data complete. The other 84 patients, where the diagnosis of Wilson's disease was not made, consisted of 46 adults (37 male, 9 female) and 38 children (21 male and 17 female). See appendix 1 for details. The results of estimation of both

Table 1: Estimation of serum ceruloplasmin by enzymatic activity with o-dianisidine and ferroxidase substrates.

Patients	No.	o-dianisidine method(U/L) Mean $\pm$ SD	No.	Ferroxidase method (U/L) Mean $\pm$ SD
Adults (non-Wilson's)	46	122.0 $\pm$ 50.3	46	565.5 $\pm$ 128.2
Children (non -Wilson's)	38	128.0 $\pm$ 47.1	38	578.5 $\pm$ 208.1
All non -Wilson's patients	84	124.7 $\pm$ 48.7	84	571.4 $\pm$ 168.1
Wilson's disease (study)	7	mean 4.4 median 5 (range ND-16)	7	mean 53.7 median 45 (range ND-166)
Wilson's disease (all values)	10	6.3 $\pm$ 5.4	10	76.1 $\pm$ 70.1
Ref values		62 – 140		198 – 1107

Values shown are mean $\pm$ SD, except for 7 WD patients where mean, median and range are shown . In the 46 adults and 38 children, in whom the diagnosis of Wilson's disease was were no significant differences between children and adult values, or between sexes, $p > 0.29$.

methods of enzyme activity of serum ceruloplasmin are summarized in Table 1. Six of the 7 confirmed WD patients in the study, had modified Leipzig system scores (2), of 4 or more. Three patients were confirmed neurological WD (patient 2, 6, 7), two were confirmed as Hepatic WD (nos. 3, 4) , and

Fig 1: Ceruloplasmin enzyme activity with two different substrates in patients screened for WD

Number of subjects = 91, Non Wilson's patients = 84, Wilson's patients =7; Query false positive low values in method a) = 7, in method b) = 2

Table 2: Biochemical estimations and Clinical details in Wilsons and non-Wilsons patients

Patient No. Age(y)/sex	Ceruloplasmin Activity (U/L) o-dianisidine	Ceruloplasmin Activity (U/L) ferroxidase	Serum copper $\mu\text{mol/L}$ ($\mu\text{g}/100\text{ml}$)	24hr urine copper $\mu\text{mol}/24\text{hr}$ ($\mu\text{g}/24\text{hr}$)	KF Rings	WILSONS SCORE	Clinical Details
Wilsons							
1. 5y F	10	166	5.97 (38)	7.9 (504)	bilat+	5	WD
2. 12y M	<6	56	-	-	Bilat+	6	NeuroWD
3. 9y F	<6	<19	3.77 (24)	4.22 (269)	NR	3	Hepat WD
4. 11y M	8	61	3.93 (25)	4.13 (263)	bilat+	5	Hepat WD
5. 20y M	<6	45	-	14.3 (912)	NR	4	WD
6. 19y F	16	39	5.34 (34)	2.06 (131)	bilat+	4	Neuro WD
7. 20y F	<6	<19	2.83 (18)	17.80 (1134)	bilat+	6	Neuro WD
8. 13y M after study	9	163	7.22 (46)	-	bilat+	4	Neuro WD
9. 33y M after study	11	192	3.45 (22)	-	NR	2	WD
10. 4y M after study	<6	25	3.61(23)	1.53 (98)	neg	2	Hepat WD
Thought not to be WD							
11. 2y F	53	438	-	-	neg	1	Prox RTA
12. 18y M	44	141	-	2.73 (174)	ND	2	psychosis
13. 21y F	29	121	7.53 (48)	1.96 (125)	ND	1	WD CBRO CLD
14. 27y M	50	483	-	3.61(230)	neg	2	DJ
15. 30y M	15	628	15.39 (98)	6.01(383)	neg	3	Acute hep B
16. 41y M	39	598	13.19 (84)	0.72 (46)	neg	1	WDCBRO CLD
17. 27y M	31	459	12.09 (77)	3.43 (219)	neg	2	WD CBRO Chronic hep
18. 11y M after study	46	634	-	5.87 (374)	ND	3	WD CBRO Chronic hep
19. 12y F after study	44	514	5.65 (36)	0.50 (32)	ND	1	NO INFO
20. 6y M after study	15	44	-	-	ND	1	WD CBRO
21. 11y M after study	6	261	14.29 (91)	8.48 (540)	ND	3	CLD
22. 11y M after study	19	532	-	-	neg	1	Dengue
23. 27y F after study	6	533	10.21(65)	-	ND	1	NO INFO
24. 3y M after study	12	190	5.65 (36)	5.02 (320)	ND	3	WDCBRO CLD

Biochemical data for 24 patients with low values in the o-dianisidine assay are shown in the table. Totally there were 10 patients with WD. 7 Wilsons patients in study, 3 WD found after study. Patient 3 had a modified Leipzig score of 3, but had a family history of WD. The presence of KF rings was not recorded (NR) in this patient and in two other patients with WD, no. 5 and 9. In patient 1, 5 and 9 WD classification (Neuro or Hepatic) was not recorded. (Neuro WD) Neurological WD. (Hepat WD) Hepatic WD. Totally 14 patients were thought not to be WD, 7 during the study period and 7 afterwards. In 6 patients WD could not be ruled out (WDCBRO), because not all tests were carried out. In 2 of these patients there was no follow up, so no information was available (NO INFO). In most of these patients assessment of the presence of KF rings was not done (ND). Prox RTA- Proximal renal tubular acidosis; DJ- Dubin Johnson syndrome; Acute Hep B- Acute hepatitis B; CLD- Chronic liver disease.

for two patients the information was not found. There were 3 males (1 adult) and 4 females (2 adults).

Of the 84 non-Wilson's patients 7 had caeruloplasmin results < 62 U/L by the o-dianisidine method (patients 11-17) and 2 of these also had values < 200 U/L by the ferroxidase method (patients 12, 13). A diagnosis of WD could not be ruled out in

3 of these patients (patients 13, 16 and 17).

All the biochemical data for these patients and their diagnosis is shown in Table 2. The enzymatic data for the 91 study patients is shown graphically for both enzymatic assays in Fig. 1a and 1b.

Fig 2: Correlation of O-Dianisidine and Ferroxidase assays

Fig 2a) 91 specimens, Pearson correlation 0.732, slope 2.752, intercept 213.7, (Spearman correlation 0.648).

Fig 2b) 372 specimens, Pearson correlation 0.908, slope 4.065, intercept 265.8, (Spearman correlation 0.924).

After the study: For the 372 additional patients where both assays were performed, there were 52 low values in the manual assay, 24 of which also had low ferroxidase. There were no patients with a measured low ferroxidase activity where the manual assay value was not also low.

The clinical records of a further 10 patients who had low values in the o-dianisidine assay (< 62 U/L) were examined, after the study (patients 8-10 and 18-24). Three of them had a definite diagnosis of WD, one with neurological WD, one hepatic WD, one data not found. All 3 had low ferroxidase values (patients 8-10). When these 3 patients were included, totally 10 patients with confirmed WD had enzymatic analysis of ceruloplasmin by both methods (Table 1). Totally, three of the 10 WD patients, (nos. 3, 9, 10) had Leipzig scores less than 4. Two of these three had hepatic WD.

Of the query false positive values examined after the study period (patients 18-24), two of them also had low ferroxidase assay values. There were 5 males (1 adult) and 2 females (1 adult). WD could not be ruled out in 3 of these patients (nos. 18, 20, 24). All the biochemical data and clinical diagnosis for these patients is also shown in Table 2. All these patients thought not to be WD had modified Leipzig scores (2) of 3 or less. The regression analysis data between the two assays is shown graphically in Figure 2. Figure 2a) shows the comparison in the initial 91 patients and Figure 2b) the subsequent comparison of 372 patients. Both correlations are significant at the 0.01 level.

The area under the ROC curve for both methods is shown in Figure 3. The area under ROC curve for o-dianisidine activity is 0.998. The Local cut off value 22.5 U/L has 100% sensitivity and 98.8% sensitivity. Using the literature cut off value of 62 U/L gives 100% sensitivity and 91.7% sensitivity. Positive Predictive value with literature cut off is 50.0%, with the local cut off it is 87.5%. The area under ROC curve for Ferroxidase

Fig 3: ROC curve of O-Dianisidine activity and Ferroxidase activity

Source of the Curve

----- Reference Line O-dianisidine method — Ferroxidase method

Fig 4: Difference plot using z scores of O-Dianisidine and ferroxidase assay

Difference plot using z scores of o-dianisidine and ferroxidase assays; Average z score as x-axis, o-dianisidine minus Ferroxidase z score as y-axis (Fig 4a). 91 specimens, intraclass correlation 0.7319, mean difference $\pm$ SD 0 ± 0.73 ;
Fig 4b). 372 specimens, single measure intraclass correlation 0.9075, mean difference $\pm$ SD 0 ± 0.43

activity is 0.997. At the local cut off value of 208 U/L there was 100% sensitivity and 97.6% sensitivity. The literature cut off value of 200 U/L has 100% sensitivity and 97.6% sensitivity. Positive Predictive value with literature cut off and local cut off is 77.8%.

Since the two substrates produce widely different activities, and use different assay temperatures, the absolute difference plot between the o-dianisidine and ferroxidase assay values was calculated using average z score and the difference between z score ie o-dianisidine z score minus ferroxidase z score. This data is shown in Figure 4a) for the initial 91 patients and 4b) for the subsequent comparison of 372 patients.

DISCUSSION

Both enzymatic methods employed in this study detected seven patients with Wilson's disease; however the positive predictive value, using the literature cut offs, was better for the ferroxidase method. The use of this ferroxidase assay, using Ferene as the chromagen was has previously reported in only 1 WD patient (7). We present data from 10 WD patients. Other workers have reported low ferroxidase activity respectively in 4 male subjects (9) and 28 subjects (10). The latter study (10) measured serum ferroxidase activity, using a

different chromagen, ie the absorbance at 377nm of quinolins complexed with ferric ions produced in the reaction. In 28 patients with WD, they found reduced levels in the range 20-282 U/L.

Totally we have described 14 patients, who had low o-dianisidine assay, of whom 4 also had low values in the ferroxidase assay. In 6 of the patients WD could not be ruled out. These were mainly patients with hepatic presentation.

In the literature non-WD causes of a low ceruloplasmin activity include primary genetic deficiency and secondary deficiency such as severe liver disease, nephrotic syndrome, and protein losing enteropathy, dietary copper deficiency, and Menkes disease (1). We used scores in a modification of the Leipzig system (2) to give scores for our WD and query WD patients. We did not include neurological symptoms in the score. Not all investigations were completed in all patients where WD could not be ruled out. Since completing this study we have switched over to the ferroxidase say for ceruloplasmin and WD patient work up now includes mutation analysis, which by itself can give a score of 4 (2).

A recent report has reiterated the point of view that measuring

ceruloplasmin immunochemically gives a large number of negative values (20.1%) for non-caeruloplasmin bound copper (NCC) in patients (9). This group state that the draw backs of immunological methods for measuring caeruloplasmin. There is no standardized reference method for ceruloplasmin, immunologic methods cross react with apoceruloplasmin, there are method related differences including bias, precision and specificity, which all have a larger effect on calculated (NCC) than previously thought. They recommend extreme caution in using indirectly derived NCC in routine clinical practice, until direct measures of free copper can be introduced.

The alternative way to calculate NCC is recommended by Walshe (1). He was able to convert ceruloplasmin oxidase activity in U/L to terms of mg/L ceruloplasmin by standardizing the ceruloplasmin activity assay against recrystallised human ceruloplasmin. When this was done he found NCC values to be very reliable, with no false negative values. We were not able to estimate ceruloplasmin concentration from our ferroxidase assay since we did not have purified recrystallised human ceruloplasmin. We plan to obtain this material were obtained and determine the ferroxidase activity of the recrystallised human ceruplasmin. From this conversion it would then be possible to calculate free copper index as described by Walshe (1).

The consistently higher values for the ferroxidase method compared to the o-dianisidine method in our study, is explained by the use of different substrates, and by the difference in incubation temperature. The Pearson correlation coefficient shows a significant agreement between the assays. Agreement is better in the larger series of patients. We found that the ferrous substrate has only 1 month stability, not 6 months as listed in the method (7). As the substrate deteriorates, ferroxidase activity is falsely elevated. Absorbance of the blank fell after this time resulting in falsely elevated enzyme activity values. The substrate was prepared freshly whenever the blank absorbance fell by more than 10% from the initial value measured when the substrate was freshly prepared. Erel (7) made a comparison of the ferroxidase method with p-phenylenediamine dihydrochloride (PPD) as the other method, using 200 serum samples and this had a correlation ($r = 0.96$). However it did not appear to include any ferroxidase values below about 380 U/L. It is at the low levels of both assays that we found most discrepant values.

The absolute difference plot showed good agreement between the two assays with no observed bias. The agreement is again better when a larger number of samples were used, with lower

SD values for z score differences.

The major weaknesses of this study are the lack of complete clinical information. WD could not be ruled out in 3 of 7 patients in the study period and 3 of 7 after the study, who had low values in the o-dianisidine assay. This means the performance of especially the o-dianisidine assay (in terms of positive predictive value and specificity) could have been better if these patients were reclassified as WD. Both assays had 100 per cent sensitivity in detecting WD patients. Specificity was 77.8% for the ferroxidase assay. Using a lower cut off value for the o-dianisidine assay, 22.5 U/L instead of 62 U/L, improved specificity from 91.7 to 98.8 percent. If we were persisting with this assay, then this change would be worth introducing. One of the drawbacks of the ferroxidase assay was the requirement for daily calibration, because the substrate deteriorates with time.

Appendix 1 : Specimen breakdown In study

84 persons, 46 adults and 36 children, 81 with a diagnosis of Wilson's disease excluded.

There is no quality control material available in India for measuring ceruloplasmin activity. Quality control material only lists values in concentration unit and did not have a suitable value for enzymatic analysis. For both methods we used SD from duplicate analysis (8). Hence only precision of the assays and not accuracy could be assessed.

Despite these drawbacks we consider the ferroxidase assay to be an acceptable automated method to replace the tedious o-dianisidine manual procedure. Since it is estimating enzyme activity if fulfils the requirement for measuring ceruloplasmin by a non-immunological method (1). We have now replaced the o-dianisidine method by the ferroxidase method for ceruloplasmin in our investigation of patients with WD.

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