Study Protocol

A Study Protocol for Evaluation of Microcytic Hypochromic Anemia by High Performance Liquid Chromatography (HPLC)

ABSTRACT : -

Background- One of the commonest assessment done in the clinical practice is for the state of anemia. The anemias are classified by different levels on the basis of numerical value of hemoglobin, alteration in the morphology and chromia, underlying etiologies, red cell volumetric parameters and functionally depending on patho-physiologic processes of anemia. Microcytic hypochromic anemia (MCHC) have different underlying causes which include iron deficiency anemia, beta thalassemia trait, hemoglobinopathies. Before planning the treatment, assessment of the hemoglobin for its variants and the detection of abnormal hemoglobin are mandatory. High performance liquid chromatography (HPLC) has proved to be significant for evaluation of MCHC because of its superior separation and quantification analytical powers. The present study has been undertaken as the anemia is the most prevailing and commonly treated clinical state that is managed by numerous nutritional supplement without much being done to know the underlying etiology or without evaluation of hemoglobin for its abnormalities.

Methods: This will be an observational (Prospective and Retrospective) study. Blood samples of 100 patients will be evaluated by HPLC Bio-Rad variant and various patterns of hemoglobin, associated hemoglobinopathies will be evaluated.

Results – The observations will be made according to the objectives and will be tabulated which will be subjected to statistical tests for its significance and conclusions.

Conclusion – This study would contribute in understanding and knowing the etiology of MCHC in the population that is served by our tertiary care hospitals.

Keywords- High Performance Liquid Chromatography, Anemia, Microcytic Hypochromic, Bio-Rad, Hemoglobin

Introduction:

One of the commonest assessment done in the clinical practice is for the state of anemia. The anemias are classified by different levels on the basis of numerical value of hemoglobin, alteration in the morphology and chromia, underlying etiologies, red cell volumetric parameters and functionally depending on patho-physiologic processes of anemia. Whatever the scheme of classification maybe, the usual approach to classify anemia is morphological.[1]

The morphological manifestation within the erythrocytes for its size and chromia has long been evaluated for underlying etiology. The appreciation for this morphological alteration is always manual by microscopy of a stained blood film and also by the electronic cell counters in modern hematology laboratories.[2]

The clinicians commonly come across a morphological class of anemia that of microcytic hypochromic anemia (MCHC) alternately called as low cell volume anemia which have plentiful underlying etiologies. The MCHC for its etiologies include the underlying causes of iron deficiency state, beta thalassemia trait, hemoglobinopathies because of HB E, HB D, HB C and many others. Such situation of MCHC before being embarked for the treatment requires assessment of the hemoglobin for its variants and detection of abnormal hemoglobin.[3]

The traditional approach at evaluation of MCHC was to differentiate the underlying etiologies of iron deficiency anemia or beta thalassemia trait, by knowing the red blood cell indices such as Mentzer index, Green and Kings index and many others. However these indices can only be used for screening the limited situation and etiologies. MCHC is having far wide underlying etiologies. For this hemoglobin studies are needed. [4]

Hemoglobin mutation is one of the single most common "single gene disorder" that manifests uniquely as microcytic erythrocytosis (MCHC).[5]

The treatment of MCHC is the iron replacement therapy if the iron deficiency is suspected as its cause. However, the therapeutic iron can produce deleterious effect if the underlying pathology for MCHC is otherwise like, beta thalassemia trait, sickle cell disease or the other hemoglobinopathies.[1-5]

Therefore, a MCHC popularly called in pathology as microcytic erythrocytosis must undergo the evaluation of the hemoglobin for its variant and abnormality before the iron therapy is prescribed.[6-11]

There are a few studies that has evaluated Microcytic erythrocytosis (MCHC) for the result of hemoglobin variant and hemoglobinopathies. [1-11]

The recent years interest has been generated to analyze the hemoglobin variant and abnormal hemoglobinopathies in the situations of microcytic erythrocytosis enabled by advent in technology. There is a shift of evaluation of hemoglobinopathies from simple solubility test to high definition electrophoresis to high performance liquid chromatography (HPLC) with its superior separation and quantification analytical powers. [5,11]

The present study has been undertaken as the anemia is the most prevailing and commonly treated clinical state that is managed by numerous nutritional supplement without much being done to know the underlying etiology or without evaluation of hemoglobin for its abnormalities.

Research gap: The ignorance about the abnormality of hemoglobin results in the wrong treatment of anemia. This is observed always with MCHC which has numerous underlying hemoglobin related abnormalities. The overall treatment by iron is a common danger which can prove not only deteriorating but also fatal if the status of beta thalassemia trait and other hemoglobinopathies are unknown. Such studies that evaluated hemoglobin variants and hemoglobinopathies from India are infrequent. This study would contribute in understanding and knowing the etiology of MCHC in the population that is served by our tertiary care hospitals.

Research questions:

- Do the knowledge of hemoglobin variant and abnormal hemoglobinopathies is required in the evaluation for MCHC on HPLC.
- With this backdrop of the knowledge the reviewed literature, the present study is organized for its aims and objectives below.

Aim and Objectives

Aim:
The present study is aimed at the assessment of MCHC by chromatography (HPLC) for to know associated hemoglobin variants and hemoglobinopathies.

Objectives:
1. To study the MCHC (Microcytic erythrocytosis) for to know its association with hemoglobin variants and abnormal hemoglobins by HPLC.
2. To know the frequencies of the hemoglobin variants and abnormal hemoglobins with MCHC on evaluation by HPLC.
3. The role of HPLC in segregating MCHC of iron deficiency versus beta thalassemia trait. In the cases where hemoglobin electrophoresis was ambiguous for results.

Short Review

The literature search over the topic of assessment of MCHC by HPLC by electronic vial media has shown the multiple studies that has either related with single or multiple parameters pertaining to variants of hemoglobin and hemoglobinopathies. Two such studies is presented as a short review below for its inferential abstract.

Joneja, et al. carried out a study in evaluation of hypothesis that the reasons of hemoglobin variant abnormality (HVA) performed in the patients revealing Microcytic erythrocytosis without accompanying anemia for pluralistic causes. Study comprised of 137 patients in hemoglobin range of 7.20 to 16.1 gm/dl with low or decreased MCV with median value of 64 fl.

The blood samples of those patients were run for high performance liquid chromatography bio-rad variant. The results revealed that 93 of 137 i.e (67.9%) patients could be diagnosed as thalassemia trait and or hemoglobinopathy as a cause of microcytic erythrocytosis.

The common abnormalities those were underlined for MCHC was of beta thalassemia trait, delta/ beta thalassemia trait, hemoglobin E disease, hereditary persistence of fetal hemoglobin (HPFH), possible HPFH, HPFH with beta thalassemia, delta/ beta thalassemia iron deficiency anemia, hemoglobin C trait with beta thalassemia, hemoglobin C trait with possible alpha thalassemia, hemoglobin C with HPFH, unidentified hemoglobinopathy with possible delta/beta thalassemia, hemoglobin S trait combined with possible alpha and possible beta thalassemia trait.

Seventeen patients showed normal hemoglobin pattern on HPLC with pre determined cutoffs. The study concluded that hemoglobin variant analysis provided a very high positive yield in determining the etiology of microcytic erythrocytosis. Therefore the patients diagnosed as MCHC should undergo the analysis for hemoglobin abnormality regularly.

Philip, et al. in their study of MCHC by HPLC have identified the objectives of knowing the underlying hemoglobinopathies. The study was mostly focusing the antenatal cases along with patients of anemia. The study conducted over a span of 4 years was performed and the instrument high performance liquid chromatography BIO RAD variant analyzer. The blood

samples were collected in quantity of 2ml in EDTA vial. The samples went under the complete blood count (CBC), iron stores, high performance liquid chromatography. A part of population statistics, the major abnormality of hemoglobin observed as a cause for MCHC was of beta thalassemia trait (high HB A2). 15.8 cases displayed abnormal hemoglobin pattern of which 20.3 were of the other abnormalities like, beta thalassemia major, beta thalassemia intermedia, sickle cell trait, Hb S/ beta thalassemia, sickle cell disease, HPFH, HbE/ beta thalassemia, homozygous HbE ds, HbD- Punjab trait, homozygous HbD- Punjab HbD/beta thalassemia, HbQ India trait.

The study concluded that there exists a high prevalence of hemoglobinopathies amongst the patients revealing MCHC especially so in patients in antenatal care.

The diagnosis of high performance liquid chromatography helped in preventing unnecessary iron loading as well as unwanted blood transfusions. The study recommended the regular use of high performance liquid chromatography in evaluation of MCHC in avoidance of inappropriate treatment.

Material and Methods:

The following material and methods will be adopted for present study:-

1. **Recording of preliminary data in proforma with following details:-**
   - I. Name
   - II. Age
   - III. Gender
   - IV. Ward
   - V. OPD
   - VI. Unit in charge
   - VII. MRD
   - VIII. Complains
   - IX. Comorbid conditions
2. **Place of study-**
   Department of Pathology JNMC, Sawangi Meghe, Wardha.
3. **Duration of study** – Two years
4. **Study Design** –Observational study design.
5. **Sample size** – Ninety seven cases by formula rounded to 100 cases.
   $n = (Z^{a/2}) 2 \times p \times (1- p)/ d2$
   Where, $Z^{a/2}$ is the level of significance at 5 % i.e. 95% confidence interval.
   p = Prevalence of breast carcinoma
   d = desired error of margin
   n = sample size
6. **Subject characteristics** - Described below for inclusion and exclusion
   Inclusion criteria:
   - i. Patients with microcytic hypochromic anemia (MCHC) as determined by values of cell counter and blood film microscopy.
   - ii. The patients refractory to the conventional treatment of MCHC by iron.

iii. The patients with microcytic hypochromic anemia (MCHC) on two consecutive laboratory evaluations spanned over a period of one month.

Exclusion criteria:

I. The patients with known comorbid causes of MCHC.
II. The patients who have received the blood transfusion as a treatment of MCHC.
III. Patients under the age of one year.

7. Investigations:

I. The complete blood count to be performed on automated electronic cell counters by standard methods.
II. Microscopic evaluation of RBCs on the stained preparation of peripheral smear.
III. The investigation of high performance liquid chromatography (HPLC) to be carried out on whole blood sample

8. Technical method of HPLC in detection of hemoglobin variant and abnormal hemoglobin[12]

1. Switch on the HPLC, and look for the screen on HPLC for Min 0 and Max 380.
   After switching on the computer, double click on the LC solution software on desktop.
2. Then double click on PDA (1) and click OK
   There is a sound of beep, which means instrument is connected to computer (LC solution software) system
3. Completed window with one or two graphs will open (click on instrument parameter if window does not appear)
4. Click advanced button which is below the graph
5. Click on pump. Change the "Total pump A flow" to 3ml/min
6. Then click on PDA just to check that the lamp is on OFF mode.
7. Then click on file – save method file as – click our method name – click on save – yes
8. Press download, then half turn the knob on pump carefully and press purge button. Purging will start automatically which is already set by system for 5 min.
9. During purging "purging line" appear on screen of pump
10. After the completion of purging Min 0- Max 380 pressure limits appear on screen of pump. Return the knob as it is.
11. Click on the pump and change the "Total pump A flow" to 1 ml/min on computer. Press download- Yes
12. Then press "oven" key on oven and "pump" key on pump and look for the increasing pressure on the screen of HPLC
13. Washing of column with distilled water starts. Wash the HPLC column with filtered and sonicated DW for 30 min. at 1 ml/min. flow rate. The 30 min. time is to be maintained by the researcher.
14. After that wash the column with filtered and sonicated ACN:DW (70:30) for valproate assay and MeOH: DW (70:30) for PBT, PHT, CBZ and LTG assay for 30 min. at 1ml/min. by placing the filter above HPLC into the beaker containing desired solution. The 30 min. time is to be maintained by the researcher
15. Prepare the mobile phase as per the assay given in SOP.
16. Filter the mobile phase and sonicate for 15 min.
17. Saturate the column with mobile phase for 10 min. at flow rate 1ml/min. the 30 min. time is to be maintained by the researcher.

18. After 10 min. change the flow rate (total pumpA flow) as per test protocol on computer.
19. Click PDA. Change the lamp to D2. Then right click on chromatogram- display setting- PDA – change the wavelength as per test protocol- OK – Apply- Ok- download- Ok.
20. Check the D2 lamp (on) on PDA instrument (Detector)
21. Wait for 10 min. click on plot (on upper right side corner) to check the baseline.
22. After that click acquisition- single run- sample name – sample ID- OK
23. Then put the sample syringe into injector- turn the injector to load position - inject the sample- turn the injector knob to inject position (downward)
24. Graph will be plotted automatically. Repeat the steps v and w for next sample
25. After the last sample put oven off and pump off- change the flow rate to 3ml/min- put the (PDA) lamp off- download- OK.
26. Repeat the procedure 8,9,10,11,12,13

Statistics:

Statistical Tools: The correlation will be carried out by statistical tests along with values of significance compatible to said objectives. (p-value), Fischer exact test, Univariate comparisons.

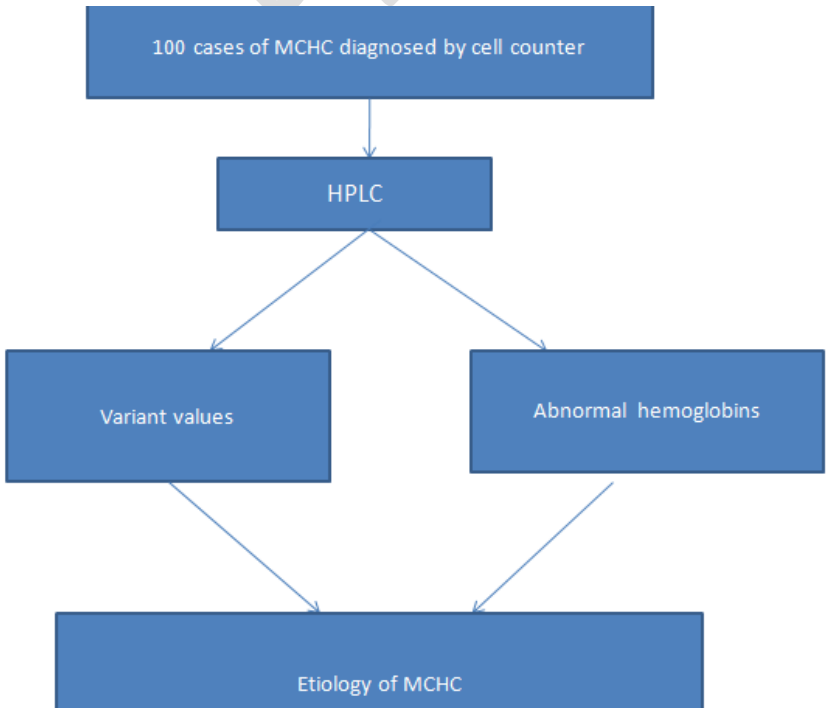
Consent:

The investigations over the blood sample in AVBRH are carried out by informal consent. The investigations specified in this work do not involve infringement and harm to human subjects participating as a patients in present study.

Ethics:

Study doesn't involve major or minor issues offending to human subjects.

Fig. 1. Scheme of Methodology:



Results:

The observations will be made pertaining to the objectives and will be tabulated. These observations will be subjected to statistical tests for its significance and conclusions.

Discussion:

The results of the present study will be compared with the studies published in the literature with similar objectives.

Joneja, et al. carried out a study in evaluation of hypothesis that the reasons of hemoglobin variant abnormality (HVA) performed in the patients revealing Microcytic erythrocytosis without accompanying anemia for pluralistic causes. Study comprised of 137 patients in hemoglobin range of 7.20 to 16.1 gm/dl with low or decreased MCV with median value of 64 fl.

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Sain et. al. reported on discriminant indices for distinguishing beta thalassemia trait from iron deficiency anemia [17]. Few other studies related to anemia were reported [17-20].

The diagnosis of high performance liquid chromatography helped in preventing unnecessary iron loading as well as unwanted blood transfusions. The study recommended the regular use of high performance liquid chromatography in evaluation of MCHC in avoidance of inappropriate treatment.[22-25]

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