Original article

Haematological and micronutrient values in volunteer subjects in Addis Abeba

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Abstract: This haematological and micronutrient study was based on 675 residents of Addis Abeba. Twenty three volunteers (3.4%) were excluded from the main study for the following reasons: BP>140/90mm of mercury, inflammatory conditions, infections or systemic illnesses. Out of the remaining 652 volunteers, stool examination led to exclusion of 207 individuals (31.7%) that were harbouring one or more types of parasite. According to 'reference value' suggested in this paper 50% of parasite infected volunteers were anaemic. Haematological data from 386 clinically 'normal' subjects and micronutrient data from 50 samples selected by stratified sampling from 386 samples were used to calculate 'reference values'. Comparison of our results with that of Peters (15) indicates that both haematological results are similar (p-value = 0.000). Since haematological data based on 50 samples are similar to those of a larger set of samples (n=386), values of folic acid and vitamin B_{12} obtained for the former may apply to the latter. In conclusion, results shown in Table 1 can be used as Addis Abeba 'reference value'. [*Ethiop. J. Health Dev.* 1998;12(3):217-223]

Introduction

Micronutrients, i.e. vitamins and trace elements, are important in the maintenance of health. Vitamins play two major roles in the body. Firstly, they are precursors of some hormones: for instance cholecalciferol is synthesized from vitamin D. Secondly chemically modified, or in a few cases unmodified, vitamins are cofactors of enzymes. The latter role of vitamins is shared by trace elements. One or several processes that require micronutrients is erythropoiesis. This pathway requires vitamin C(1), vitamin E, folic acid, vitamin B_{12}, copper, iron and protein(2). Vitamin C is important for absorption of iron that mainly occurs at ferrous state. Vitamin E is required for the integrity of the membrane's unsaturated fatty acids, thus insuring a normal life span of erythrocytes (3). Copper is an essential ion of a ferroxidase that
converts absorbed ferrous to the ferric form which is the blood transport form of iron. N^5, N^{10}methylene tetrahydrofolic acid is required for conversion of dUMP to dTMP. However, in the absence of methyl cobalamin (methylated vitamin B_{12}), all folic acid is trapped as N^5-methyl tetrahydrofolic thus preventing the synthesis of N^5, N^{10} methylene tetrahydrofolic (4). It is clear from the above that folic acid and vitamin B_{12} are important in DNA synthesis and subsequent cell proliferation, including that of red blood cells. Iron is an essential element of haem.
Numerous global studies were conducted to obtain haematological and serum micronutrient reference values. WHO recommended haemoglobin reference values for six month old infants, six months to 14 year olds, adult males and females are 11,12,13 and 12 gm%, respectively (5). International reference values suggested for folate are 11-48 nmol/L for 2-16 year olds and 7-45 nmol/L for adults. The value for vitamin B_{12} is 148-616 pmol/L whereas the corresponding values of iron for children, adult males and adult females are 400-1200, 650-1700 and 500-1700μg/L, respectively (6). Failure to maintain the normal values stated above would lead to an insufficient number of blood cells to sustain oxygen supply to the body i.e. anaemia (7). In addition to anaemia lack of vitamin B_{12} precipitates methylmalonic aciduria, homocystinuria and neurological

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complications (8,9). Folate deficiency would lead to degeneration of neurons (10). Iron deficiency anaemia is known to have a long term impact on the intelligence of an individual even if the anaemia is corrected (11).

Micronutrient deficiency anaemia, particularly of iron, as well as anaemia induced by infections such as malaria are found in Ethiopia (12). In the light of the above, it is necessary to detect and treat anaemia and the underlying disease as early as possible. Hence, the need for reference values. On the other hand one study conducted in the United States (13) demonstrated that mean values of black and white females in America differed. Thus, reference values appear to depend on genetic background. With such awareness a few studies were conducted in Ethiopia to establish normal values; for instance, that of Abdulkadir and Bolodia (14). In a study based on healthy medical students, they obtained a mean haemoglobin value of 16.4 ± 1.5gm%, haematocrit of 47.3±3.5% and MCHC of 34.2 ± 1.9%. A similar investigation by Peters (15) produced the following values: haemoglobin 15.7±1.2gm%, 14.7±1.4gm%, haematocrit of 50.2 ± 2.4%, 45.5±3.1%, and MCHC of 30.8±1.3% and 30.8±1.3% for males and females, respectively. This project reports similar but more extensive work conducted on a sample of healthy Addis Abeba adult residents.

Methods

Materials: Haematological results were obtained using Erma corporation's particle counter Model PC 608, Tokyo, Japan. Reagents needed for this work were also obtained from the same company. Spectrophotometric determinations were conducted on Beckman's Du-64 spectrophotometer. Reagents needed for serum enzyme determination, and creatinine measurements were bought from Boehringer Mannheim GmbH. Test strips for urine analysis were from the same company. Acetic acid, Ascorbic acid, Ethanol, $FeSO_4$, Guanidine thiocyanate, Sodium acetate, Sodium ethylenediaminetetra-acetic acid, Sodium hydroxide, Sodium phosphate and Urea were from Sigma. 2,4,6-Tripyridyl-s-triazine was from Aldrich.

Selection of Subjects: Subjects were recruited in 1994 although vitamin analyses of frozen samples were conducted in Sweden in 1996. To this end, four out of twenty eight weredas (councils) of Addis Abeba were selected by lottery. The same method was used to select five kebeles per wereda. The localities were: wereda 1 Kebeles 4,5,6,7 and 8; Wereda 10 Kebeles 5,8,15,16, and 22. Wereda 17 Kebeles 15,16,18,19, and 21 as well as Wereda 24 Kebeles 11,12,13,15, and 17 (see also Ref. 16 for sampling method). This was followed by a campaign to persuade kebele leaders to assist in convincing individuals about the importance of this study. Furthermore, explanations were given to those willing to subject themselves (volunteers) to this clinical and laboratory study. The aforementioned effort produced 675 individuals: age 18-75 years, 315 (47%) males and 360 (53%) females.

History and Physical Examination: Personal identification, dietary history, and drinking habits of every individual were recorded. Physical examination data included general apperance, symptoms such as headache, dizziness, palpitation, weakness signs, pallor, jaundice, angular stomatitis, glossitis, fine crepitation, gallop, murmur, hepatomegaly, splenomegaly, ascites, and koilonychia of nails were used for excluding clinically 'abnormal' persons from the study. Furthermore any subject that had a blood pressure of 140/90mm of mercury or above, pregnant women, those on drugs and persons suffering from inflammatory conditions, infections or systemic illnesses were excluded.

Stool Examination and Urine Analysis: A stool film was examined by microscopy for ova or cysts of the following parasites and the number per slide was recorded: Ascaris Lumbricoides, E.histolytica, Giardia lamblia, Trichuris trichiura, Hymenolepis nana, and Taenia saginata. However, no other parasite was identified. Glucose and protein content of the urine was analyzed by dip stick technique using Comber -10-test strips.

Haematology: An Erma PC-608 blood analyzer were used. Blood that contained K_2EDTA was used for determination of Hb, HCT, RBC, MCV, MCH, MCHC, WBC, platelets, MPV, and ESR. Haemoglobin was quantified by the cyanomethaemoglobin method at a wavelength of 540 nm (17) on the same equipment. This instrument was calibrated with blood samples whose haemoglobin and hematocrit values were determined manually.

Biochemical: Blood samples devoid of anticoagulants were used to prepare sera. The latter was used to assay enzymes: serum glutamate oxaloacetate transminase, serum glutamate, pyruvate transminase, alkaline phosphatase, iron, urea, creatinine, total protein, serum protein electrophoresis, and vitamins. The sera were stored at -70ºC. Under this condtion (no radiation) both vitamin B12 and folic acid are chemically stable.

Serum Iron: Total iron was measured by modifying the method of Williams, et al (18). Briefly a protein denaturant was prepared by dissolving equimolar amounts of urea and guanidine thiocyanate. Solution A contained 3.3 moles of urea and 3.3 moles of guanidine thiocyanate in 0.1M sodium acetate buffer at pH 4.8. Solution B was 10gm/L of 2,4,6-tripyridyl-s-trazine (TPTZ) in 10 mM HCl. Solution C was prepared by pouring an aliquot of solution B into solution A such that the final concentration of urea, guanidine thiocyanate and TPTZ were at 3M,3M and 100 mg/L, respectively. In addition to the above, solution C also had ascorbic acid at a concentration of 5gm/L. To avoid oxidation of ascorbic acid, solution C was prepared on a daily basis. Solution D is identical with solution C except lacking TPTZ.

A stock solution of iron was prepared by dissolving iron sulphate such that the concentration of iron added into deionized water was 200mg/L. This solution was used to prepare working solutions needed to draw a standard curve. To this end serial dilutions, leading to the delivery of a desired quantity in a final aquous volume of 0.5ml, were prepared. This was followed by adding 1.5ml of solution C and taking optical density reading

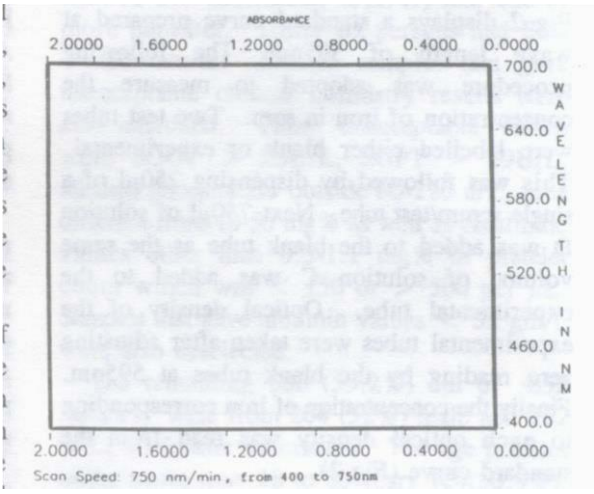

Figure 1a: **Spectrophotometric scan taken using iron reagents in the absence of iron**

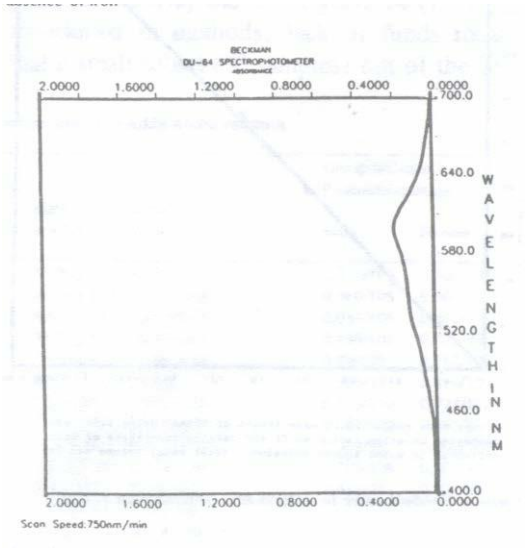

Figure 1b: **Spectrophotometric reading to determine λ max of iron determination**

within half an hour. Unpublished data collected during this work confirmed colour produced by Fe~TPTZ complex is stable and steady for several hours.

When spectrophotometric scan was conducted in the absence of iron, no absorbance could be recorded (fig.1a). On the other hand a scan at various iron concentrations gave λmax of 595nm (Fig.1b). Fig 2 displays a standard curve prepared at wave length of 595nm. The following procedure was adopted to measure the concentration of iron in sera. Two test tubes were labelled either blank or experimental. This was followed by dispensing 250μl of a single serum/test tube. Next 750μl of solution D was added to the blank tube as the same volume of solution C was added to the experimental tube. Optical density of the experimental tubes were taken after adjusting zero reading by the blank tubes at 595nm. Finally the concentration of iron corresponding to each optical density was read from the standard curve (Fig 2).

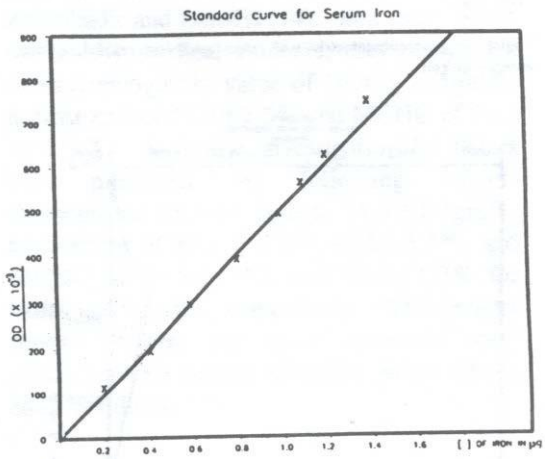

Figure 2: **Standard curve for colorimetric determination of iron**

Total Protein: Total protein was determined by biuret method using assay reagents marketed by Boehringer Mannheim GmbH. In this method cupric ion forms a purple complex with proteins. The intensity of the colour was measured at 550 nm. A standard curve was plotted and the value of the unknown determined.

Enzyme Assays: Serum aspartate transaminase and alanine transaminase were determined by assay reagents marketed by Boehringer Mannheim GmbH, Diagnostic. These assays were adopted on a Coulter Kem-0-Mat II phase II clinical chemistry autoanalyzer (19). Alkaline phosphatase was measured by Boehringer Mannheim GmbH Diagnostic's method (20). In this assay, the rate of production of p-nitrophenol was measured spectrophotometrically at λ405 nm.

Assays of Creatinine and Urea: In alkaline medium creatinine and picric acid form a reddish purple complex whose presence can be measured spectrophotometrically at wavelength of 500 nm. The assay for urea was prepared and processed as described in Boenhringer's manual (21). The same reference (21) was used to measure creatinine.

Serum Electrophoresis: Electrophoresis was conducted by modifying the methods suggested in the references 22 and 23. Strips of 30 cm long four cm wide Whatman no. 3 paper were cut and a sample spot was pencilled in the middle. These pieces were soaked with buffer and excess liquid was blotted out with filter paper. Five strips were mounted on Shandon's electrophoresis apparatus SAE-3225, at any given time. Two hundred ml of 50 mM Sodium phosphate, pH 8.6, was poured in each electrode compartment. Ten μl of serum or standard was carefully applied on a pencil mark avoiding half a centimeter from each edge. Power was switched on immediately thus conducting electrophoresis at 100 volts, 100 mA for 16 hours.

At the end of the electrophoresis, the paper strips were removed and dried in an oven at 80^{o}C for 20 minutes. In order to stain and process the strips the following solutions were prepared: 1% W/v Naphthalene black in glacial acetic acid, 5% acetic acid in three different trays and methanol. A 100% aliquote of each liquid was poured into horizontal solvent trays. The dry filter papers were then submerged in Naphthalene black solution for 20 minutes.

They were dipped in and out for a few minutes. This was followed by passing the strips through three different trays of 5% acetic acid. Finally, they were rinsed with methanol, dried and stored. The strips were sliced such that each piece was associated with one identified band. The pieces were placed in labelled test tubes, where elution was conduced by 5ml of 0.1N NaOH for $\alpha_{1,}\alpha_{2}$ & β and 10 ml for γ-globulin and albumin, for 10 minutes. An equally sized slice with no recognizable band was selected to serve as reagent blank. Optical density reading was taken at 600 nm on a BeckmanModel Du-64 spectro-photometer.

Folic acid and Vitamin B_{12}: As described in the results section only 386 volunters out of 675 were clinically 'normal'. Given the expensive nature of vitamin assay, it was not possible to conduct analysis on all the 386 samples. It was therefore decided to select 50 samples (Group II) out of 386 samples (Group I) by stratified sampling technique (16). The selected 50 samples were analyzed by simultaneous determination of folic acid and B12 by radioassay (24), at the Karolinska Institute, Stockholm.

Data Analysis: The data was compiled and analyzed using a World Health Organisation's Epidemological Computer programme called Epi-Info-6.

Results

On the basis of the exclusion criteria specified in section 2.3, 652 volunteers were enlisted. On the basis of urine analysis, seven
individuals with positive reactions for glucose or albumin were dropped. Stool examination showed 207 individuals (31.7%) had one or more parasites. Thirty six persons had ESR > 20 mm/hr. Sixteen samples that gave unacceptable clinical chemistry results were also excluded. Those unacceptable values were SGOT > 25U/L, SGPT > 29U/L, alkaline phosphatase outside 80-220 u/L, urea different

from 10-50 mg% as well as creatinine values other than 0.5-1.1 mg% or platelet count which was < 150 or > 500 per μL. Samples that gave albumin values < 35 gm/L were also deselected.

The remaining 386 (59.2%) out of 652 samples, were from 204 (52%) male and 182 (40.1%) female volunteers. The age profiles of the males were 18 to 30 years 104(50.9%), 31 to 50 years 76(37.3%) and ≥51years 24 (11.8%). Likewise the age profiles of females were 18 to 30 years 85 (46.7%) 31 to 50 years 83 (45.6%) and ≥ 51 years 14 (7.7). As mentioned in methods, lack of funds meant that a small subset (50 samples) out of the 386

Table 1: **Serum Micronutrien and Haematological values determined for a random sample of Addis Ababa residents**

Sample	Group I 386 samples		Group II 50 samples		Group II/Group I P-alue /percentage	
	Male(n=204)	Female(n=182)	Male(n=30)	Female(n=20)	Male	Female
HB (gm/L)	15.23±1.61	13.29±1.45	16.00±1.19	15.03±1.35	0.012/105	0.000/103
HC (%)	45.78±6.33	40.05±4.77	48.24±3.58	44.3±4.65	0.060/105	0.000/101
RBC ($x10^6 \mu L^{-1}$	4.55±0.64	4.05±0.65	4.81±0.38	4.51±0.43	0.031/105	0.000/111
MCV (FL)	98.31±6.39	98.3±4.63	98.56±4.7	98.2±3.23	0.839/100	0.925/99
MCH (pg)	33.21±2.19	32.75≤2.55	33.15≤2.4	33.4≤2.69	0.890/99	0.283/101
MCHC (gm/dL)	33.72≤2.10	33.25≤2.55	33.71≤2.4	34.0≤2.71	0.981/99	0.216/102
WBC x ($10^3 \mu L^{-1}$)	5.71±1.94	5.72±1.83	6.31±2.0	5.87±1.75	0.116/110	0.727/102
PLT (x $10^3 \mu L^{-1}$)	277.43±73.87	294.44±.18	285±7.78	291.83±2	0.603/102	0.891/99
MPL fL	9.58± 6.042	9.34± 0.69	9.21±.76	9.36±0.70	0.004/96	0.902/102
ESR (mm hr^{-1})	3.40±4.18	7.91±7.41	3.61±3.39	4.26±2.26	0.789/106	0.008/53
Iron (μg/L)	806±265	717±252	767±172	722±181	0.435/95	0.931/101
Folic acid (nmol/L)	- -	- -	8.51±2.63	7.87±2.6		
B_{12}(pmol/L)	- -	- -	316±122.2	301.8±102.1		

The values given are Mean ±SD. The result of folic acid and B_{12} were those determined for 50 samples. Percentages are that of mean values of group II divided by mean values of group I.

samples had to be used to assay folic acid and vitamin B_{12}. Thirty of those samples were from male subjects and twenty samples were donated by females. The mean ± SD of the aforementioned results were compiled. Table 1 demonstrates mean values of haemoglobin, haematocrit and RBC for group I and group II are identical (p-value <0.06). On the other hand the correlation using p-value of ESR, blood cell indices, iron, and WBC was poor, although the percentage relationship is acceptable. The poor p-value is due to much higher difference shown by the standard deviation of group I samples, compared to standard deviation of group II samples, but the means are comparable as confirmed by percentage relationships. The mean folic acid value of males to that of females gave p-value of 0.000 and that of vitamin B12 was also similar, hence for the samples under study, there is no significant difference of the level of these two vitamins between the two genders.

Discussion

Haemoglobin and haematocrit values obtained from Addis Abeba volunteers are not much different from those obtained by Abdulkadir and Bolodia (14). Comparison of our data with those authors (Table 2) gave a p-value of 0.000 and 0.090 for haemoglobin and haematocrit respectively. The lower comparablity for hematocrit may be attributed to the fact that our values were based on

random sample of Addis Abeba residents (age 18-72 years) whereas those of reference 14 were based on young medical students aged 21-31 years. The latter are expected to have a better health status than the general population. Similarly, comparison of our results with those of Peters (15) gave a p-value of 0.000. This coincidence can be attributed to the random population base both in our study and that of Peters. However, notwithstanding time and distance, all the three studies revealed more or less the same figures. It is therefore reasonable to use these figures as Ethiopian `reference value'. It may be noted that these values are similar to WHO reference values (5) if variation due to altitude is taken into account.

It is clear from Table 1 that haemoglobin, haematocrit and erythrocyte values for the larger set of samples (n=386) are not much different from those of the smaller set (n=50) drawn from of 386 samples (see Table 1 for P-values). However, coincidence fails for red cell indices and platelet count. This statistical incomparability of the results based on large samples and small samples (Table 1) is mainly due to the difference in standard deviation which heavily influences p-values. Nevertheless, a simple comparison reveals that means of the small samples (n=50) are within 99102 % of the larger set of samples (n=386). On the other hand a similar comparrison for WBC, MPL, ESR and iron gave the following percentages for males and females (110%, 102%), (96%, 102%), 106%, 53%) and (95%, 101%), respectively. The effect of standard deviation is such that the

Table 2: **Comparison of present and recent data by other authors**

G	Paramete	Present study	Abdulkadir & Bolodia (14)	Peters (15)	P-value on Base of d comparison present	Ref. 14
		(n=204)	(n=48)	(n=224)		
	Hb (gm/L)	15.23±1.61	16.4±1.5	15.69±1.23	0.000	0.000
M	CT (%)	45.78±6.33	47.3±3.5	50.2±2.4	0.090	0.000
	MCHC (gm/dL)	33.72±2.1	34.2±1.9	30.8±1.3	0.148	0.000
		(n=182)		(n=80)		
	Hb (gm/L)	13.29±1.45	-	14.1±1.4	-	0.000
F	HCT (%)	40.05±4.77	-	45.5±3.1	-	0.000
	MCHC (gm/dL	33.25±2.55		30.8±1.3	-	0.000

G gender, M male, and F female The values shown are mean ±SD.

p-value of ESR for females is 0.008 although the corresponding percentage is only 53%. Given that a comparison of haemoglobin, haematocrit and erythrocyte values revealed p-values $\leq$ 0.060 and even the means of red cell indices and platelet count are within 99-102 %, results of Group I are comparable with those of Group II. Based on the above, the values of folic acid and B_{12} obtained for Group II samples (n=50) can apply to the larger sample (n=386). Until a larger study is conducted, these figures as well as that of iron can be used as reference values at least for the Addis Abeba population. As mentioned in the results section, 31.7% of our volunteers harboured one or more types of parasite. If one uses our current hematological result, 50% of those individuals were anaemic, thus demonstrating a strong correlation between infection and anaemia.

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