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A METHOD COMPARISON FOR ESTIMATION OF SERUM CALCIUM AND PHOSPHORUS LEVELS AND THEIR ALTERATIONS IN CHRONIC KIDNEY DISEASE PATIENTS

LORE WILLAERT[1], DR.VASUDHA K.C[2*], DR.KUSUMA K.S[2], DR. FILIP DUMONT[1], DR. ELS VAN MECHELEN[1] AND MISS RADHIKA K[3]

[1]Biomedische Laboratorium technologie, HoGent, Faculteit Mens en Welzijn, Campus Vesalius
[2]Dept of Biochemistry, M S Ramaiah Medical College& Hospitals, Bengaluru
[3]Dept of Community Medicine, M S Ramaiah Medical College& Hospitals, Bengaluru

ABSTRACT

A method comparison study for estimation of serum Calcium and Phosphorus levels and their alterations in CKD (Chronic Kidney Disease) patients was done. Serum Calcium was estimated by O-cresolphthalein complexone spectrophotometric method and NM - BAPTA autoanalyzer method. Serum Phosphorus was estimated by the Gomorri's spectrophotometric method and Phosphomolybdate UV autoanalyzer method. The need for the study was to find out which of the two methods was more sensitive, specific, cost-effective and had minimal interferences by other metal ions or if one method was as effective as the other one. The study showed that the NM-BAPTA method was an excellent test to measure calcium while both the methods used for phosphorus measurement were fairly good tests. Out of 151 samples, 78 were of CKD and we found that nearly 30.76% of the CKD patients exhibited hypocalcemia and 33.33% of them exhibited hyperphosphatemia by the autoanalyzer methods.

KEYWORDS: Method comparison, calcium, phosphorus, chronic kidney disease.

*Corresponding author

DR.VASUDHA K.C
Dept of Biochemistry, M S Ramaiah Medical College& Hospitals, Bengaluru

INTRODUCTION

A normal adult human body has about 1 to 2 kg of calcium and approximately 600 g of Phosphorus, of which 98% of calcium is in the bones and the teeth and about 85% of phosphorus is in the skeleton[1]. Calcium and Phosphorus are the two major macrominerals which help in the mineralization of bones. Chronic kidney disease is defined as either kidney damage or GFR < 60 ml/min/1.73m^2 for ≥ 3 months. Kidney damage is defined as the structural or functional abnormality of the kidney, including abnormalities in the composition of the blood or urine or in imaging studies[2]. The calcium and phosphorus levels are well maintained in the human body unless there is a disease which affects the regulation of these minerals in the body. Several studies conducted earlier show that in chronic kidney disease there is an alteration of the metabolism of both calcium and phosphorus. There are no studies conducted so far to compare calcium or phosphorus by two different methods. In this study our aim was to measure the serum calcium and phosphorus by two different methods. The need for the study was to find out which of the two methods was more sensitive, specific, cost-effective and had minimal interferences by other metal ions or if one method was as effective as the other one.

MATERIALS AND METHODS

A cross sectional study was conducted in the M.S.Ramaiah Group of Hospitals, Bangalore from March 2014 to May 2014. About 151 patient samples were collected during this period from the Biochemistry section, Dept of Lab Medicine, to estimate serum calcium and phosphorus by two different methods. The ethical clearance for this study was obtained from the Ethical Review Board of M.S.Ramaiah Group of Hospitals.

Sample type:Serum

Sample container: Gel vacutainer.

The samples that came to the laboratory with a request for serum calcium and serum phosphorus were processed in the analyzer, the Cobas® c501 systems. The remaining samples were then transferred from the vacutainer to an Eppendorf tube and stored at 2 - 8˚C till they were subjected to the manual methods of estimation, within 7 days from the date of collection. Serum Calcium was estimated by O-cresolphthalein complexone (O-CPC) spectrophotometric method[3] and NM - BAPTA autoanalyzer method[4]. CPC reacts with calcium to form a purple color in alkaline solution, which is measured colorimetrically at 570 nm. The principle of the NM-BAPTA method is based on the binding of Ca^{2+} ions in the human serum sample with the 5-nitro-5'-methyl-BAPTA (NM-BAPTA) indicator. Every NM-BAPTA molecule binds exactly to one calcium molecule. The calcium-NM-BAPTA complex reacts in the second step with EDTA. EDTA snatches the calcium ion from the calcium-NM-BAPTA complex due to the higher binding affinity. This leads to a calcium EDTA complex and NM-BAPTA is released that is measured spectrophotometrically at 340 nm. The change in absorbance is directly proportional to the calcium concentration present in the sample. Serum Phosphorus was estimated by the Gomorri's spectrophotometric method[5] and Phosphomolybdate UV autoanalyzer method[6]. The principle of Gomori's method for the estimation is based on separating inorganic phosphate from protein by dialysis and allowing it to react with ammonium molybdate at pH 4. The colorless phosphomolybdic acid that is formed is reduced by metol (p-methylaminophenolsulphate) to a blue complex that is measured colorimetrically at 670 nm and is proportional to the inorganic phosphate concentration. The determination of inorganic phosphate on the auto analyzer is based on the reaction of phosphate with ammonium molybdate to form phosphomolybdate complex which is measured at 340 nm (UV spectrum). The unit of measurement for both Calcium and Phosphorus were expressed in mg/dl. The reference range for serum Calcium was taken as 8.4 – 10.2 mg/dl[1] for both the methods. The reference range for Serum Phosphorus was

taken for both children and adults as follows: Adults: 2.5 – 4.5 mg/dl[1] and Children: 3 – 6 mg/dl[1].

STATISTICAL ANALYSIS

A cross sectional study of 151 serum samples selected by simple random sampling was undertaken to compare calcium and phosphorus levels in serum by using two different methods for each parameter.

Rationale for sample size (Calcium)

The reference range for serum calcium from 2years to an adult by the above mentioned methods is 8.4 - 10.2 mg/dl. Based on the assumption of equivalence of the two methods and assuming it to be 0.3 mg/dl, keeping the power of study as 80% with an α-error of 5%, it is estimated that 135 samples need to be studied by each method.

Rationale for sample size (Phosphorus)

The reference range for phosphorus spans from 2.5 – 6mg/dl when both children and adults are considered. Based on the assumption of equivalence of the two methods and assuming it to be 0.3mg/dl, keeping the power of study ~~of~~ as 80% with an α-error of 5%, it is estimated that 137 samples need to be studied by each method.All the continuous variables were summarized as mean, standard deviation, median and categorical variables as proportion. Comparison of the manual and analyzer method for both calcium and phosphorus was done to determine the equivalence of the methods by estimating the sensitivity and specificity and by plotting a ROC (Receiver operating characteristics curve). Patients diagnosed with CKD were studied for the altered metabolism of calcium and phosphorus. The correlation between creatinine, calcium and phosphorus was measured using the Spearman's rho test. Data was entered in MS Excel and was analyzed using SPSS version 20.0 and Med Calc version 13.2.0.0.

RESULTS AND DISCUSSION

The present study involves the comparison between two methods for calcium and phosphorus. Calcium and phosphorus are two important minerals required for mineralization of the bone in the human body. Calcium and phosphorus levels are regulated by vitamin D, PTH and calcitonin[1]. The calcium and phosphorus levels are well maintained in the human body unless there is a disease which affects the regulation of the minerals in the body. Several studies indicate that there is an alteration of the metabolism of both calcium and phosphorus in CKD.

Table 1
Descriptive statistics of all the parameters (total 151 cases and 78 CKD cases) *A=Analyzer, M=Manual

		Age (yrs)	Creatinine (mg/dl)	Calcium A* (mg/dl)	Calcium M* (mg/dl)	Phosphorus A (mg/dl)	Phosphorus M (mg/dl)
Mean	Total	40.79	_	8.90	9.65	4.10	3.78
	CKD	52.33	6.46	8.68	9.52	4.3	4.03
SD	Total	24.46	_	0.90	0.62	1.48	1.40
	CKD	16.91	3.59	0.76	0.44	1.47	1.35
Median	Total	50	_	8.91	9.65	4.10	3.78
	CKD	56	5.89	8.71	9.55	4.07	3.89

Table 1 represents the descriptive statistics of 151 samples, which includes 78 CKD cases. The median age of total 151 samples was 50 years with age range of 0- 85 years and that of 78 CKD cases was 56 years with age range 3- 85 years. Among the 151 samples, there were 57 (37.7%) women and 94 (62.3%) men. Of those 57 women, 18 (23.08%) were diagnosed with CKD and of the 94 men, 60 (76.92%) were diagnosed with CKD. A study conducted earlier in South India indicates that out of 5588 participants, nearly 55.1% of males and 44.9% of females had CKD. Our study also depicts similar findings of more men developing CKD than women[9].

Table 2
Frequency of calcium concentrations by both methods within and outside the normal group ranges, n=151

Group range (mg/dl)	Calcium NM-BAPTA	Calcium O-CPC
5.07 – 6.17	3	2
6.18 – 7.28	8	0
7.29 – 8.39	25	2
8.40 – 8.99	43	11
9.00 – 9.59	52	59
9.60 – 10.20	16	69
10.21 – 12.00	4	8
Total	151	151

Table 3
Frequency of calcium concentrations by both methods, within and outside the normal group ranges in CKD (n=78)

Group range (mg/dl)	Calcium NM-BAPTA	Calcium O-CPC
5.07 - 6.17	0	0
6.18 - 7.28	2	0
7.29 - 8.39	22	1
8.40 - 10.20	52	**73**
10.21 - 12.00	2	4
Total	78	78

Table 2 depicts the total number of 151 samples which were categorized into different group ranges in order to see which group range of calcium concentration had no difference in values. .The study showed that the group range of calcium value between 9.0 – 9.59 mg/dl had almost similar number of samples when measured by both methods (52 & 59). This was the only group range where the two methods seem to have minimum difference in values. It was also observed that the NM-BAPTA method had picked up more samples with calcium concentrations less than 8.40 mg/dl and less number of samples with calcium concentrations more than 10.20 mg/dl when compared to the O-CPC method. It was seen that the NM-BAPTA method had picked up 25 samples with calcium concentrations ranging from 7.29 - 8.39 mg/dl while the O=-CPC method had identified only 2 such samples.This seems to give the impression that the BAPTA analyzer method is more sensitive in identifying hypocalcemic levels (less than 8.40 mg/dl) as compared to the O-CPC method. Table 3 depicts the 78 CKD cases with different group ranges.The NM-BAPTA method has identified 22 samples in the group range of 7.29 – 8.39 mg/dl where as the O-CPC method was able to identify only one sample. The NM-BAPTA method has identified about 52 samples in the group range of 8.4 – 10.2 mg/dl while the O-CPC method was able to identify 73 samples in the same group range. This gives us an impression that NM-BAPTA method is more sensitive in identifying the hypocalcemic levels as compared to the O-CPC method.The group range of > 10.2mg/dl cannot be commented upon because the sample size is insufficient for statistical analysis. This study only compares the 2 methods for both the analytes and is not a method validation study, hence neither of the methods is considered as a gold standard. A pairwise comparison of ROC curves for both methods was done to get the sensitivity, specificity and the diagnostic significance of the area under the curve

Table 4

Pairwise comparison of ROC curves (151 samples) for Calcium analyzer and manual method with a P=0.0060, (P<0.050 is significant) *A=Analyzer, M=Manual

	AUC (Area Under The Curve)	SE (Standard Error)	95% CI (Confidence Interval)
Ca_2BAPTA_**A**	**0.900**	0.0480	0.841 to 0.943
Ca_2OCPC_**M**	**0.768**	0.0513	0.692 to 0.832

Table 4 indicates a pair wise comparison of ROC curves for serum calcium. This gives the information of area under the curve (AUC) for both methods. The area under the curve has a diagnostic significance[10]. The test performed is considered as an excellent test when the area under the curve is between 0.9 – 1.0 and a fair test when the AUC is between 0.7 – 0.8[10].

Figure 1

Comparison of both methods (151 samples) for Calcium by an ROC curve

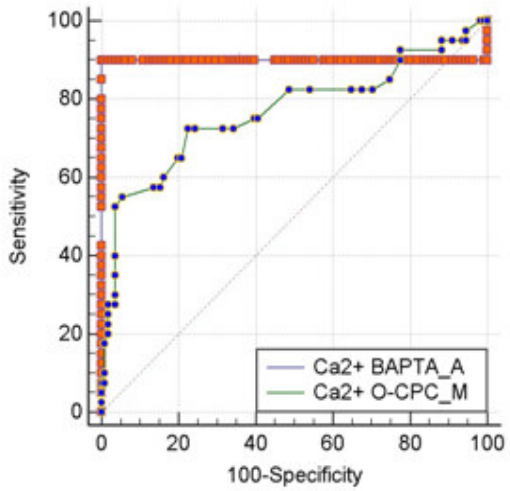

Figure 1 shows the comparison of ROC curves for calcium by both methods for all 151 samples. The NM-BAPTA method has a sensitivity of 90% and specificity of 100% whereas the O-CPC method has a sensitivity of 52% and specificity of 97%.

Table 5

Mc Nemar test for calcium by both methods in 78 CKD patients. *Values within parenthesis indicate percentages

		Calcium BAPTA method		
		Within the range	Outside the range	Total
Calcium O-CPC method	Within the range	48(65.75)	25(34.24)	73
	Outside the range	4(80.00)	1(20.00)	5
	Total	52	26	78

Figure 2
Mc Nemar test for Calcium in CKD patients

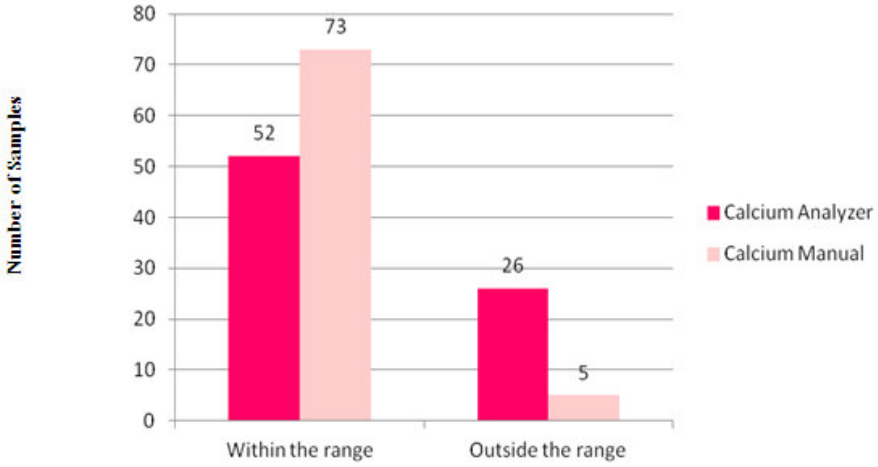

Table 5 and Figure 2 represent the comparison of both the methods used for calcium determination by Mc Nemar's test in CKD patients. Of these 78 patients, 73 cases were found to be within the normal range by O-CPC method, whereas 52 were within the range by BAPTA method. 25 (34.24%) cases are outside the normal range by BAPTA method, whereas 4 (80%) cases fall outside the range by O-CPC. The difference in the proportion of cases detected by both the methods was statistically significant with a P-value < 0.01. The clinical implication of this finding is that the BAPTA method being more sensitive helps to detect even a small decrease in serum calcium aiding in early therapeutic intervention to confer calcium homeostasis.

The evaluation of NM-BAPTA method for plasma total calcium measurement on Cobas® 8000 by Chloe Bourguignon et Al. has shown that the co-efficient of variation (CV) for NM-BAPTA assay showed good analytical performance with a CV less than 1.5%, which is in agreement with the proposed and interim European biologic goals[10]. However, in our study, the variance between the two methods has not been calculated since, the sample was not subjected to multiple runs. The same authors have opined in the international application published under the patient cooperation treaty that the sensitivity of O-CPC method is very dependent on pH (=10.7) and hence it requires more calibrations in order to ensure correct measurement. Also, O-CPC is rather non-selective and binds magnesium and other metals like gadolinium which can interfere with the measurement. The O-CPC method has a limited linearity of maximum 10 mg/dl and a limited on-board stability due to the pH sensitivity of the test reaction. At these alkaline pH values, the reagent still readily absorbs ambient CO_2. The absorption of CO_2 which combines with water to form H_2CO_3, gradually reduces the reagent pH and eventually renders the reagent non-functional for calcium measurement. The BAPTA-type chelator exhibits advantageous properties that give a more appropriate measurement of calcium ions. It has excellent storage stability, is free of the environment pollution by arsenic, does not show interference by magnesium and other metals, gives rapid accurate determination of calcium over a broad range of concentrations, it has high linearity, low reagent blank absorbance, has high sample throughput and the method is stable in the pH range of 8.5-11.5[11].

Table 6

Frequency of phosphorus concentrations in total 151 samples and in 78 CKD patients within and outside the normal group range

Group Ranges (mg/dl)	Phosphorus Analyzer (Total)	Phosphorus Manual (Total)	Phosphorus Analyzer (CKD)	Phosphorus Manual (CKD)
1.15 - 3.00	20	34	11	19
3.01 - 6.00	117	113	58	54
6.01 - 9.00	12	2	8	4
9.01-12.00	1	1	1	1
> 12.01	1	1	0	0
Total	151	151	78	78

Table 6 depicts the various group ranges for phosphorus by both methods spanning the minimum and maximum values and the number of values(concentrations) that fall within that particular group range for the total 151 samples and 78 CKD cases. It is evident that within a group range of 3.01 - 6.00 mg/dl, the numbers of phosphorus concentrations detected by both methods are nearly the same. The same group range of 3.01 - 6.00 mg/dl for 78 CKD patients also indicates comparable phosphorus concentrations.

Table 7

Pairwise comparison of ROC curves (151 samples) for Phosphorus analyzer and manual method with a P=0.6130, (P > 0.050 is not significant) *A=Analyzer, M=Manual

	AUC (Area Under The Curve)	SE (Standard Error)	95% CI (Confidence Interval)
Phosphomolybdate UV_A	0.702	0.0608	0.622 to 0.773
Gomorri_M	0.692	0.0583	0.612 to 0.764

Figure 3

Comparison of both methods (n- 151) for Phosphorus by an ROC curve

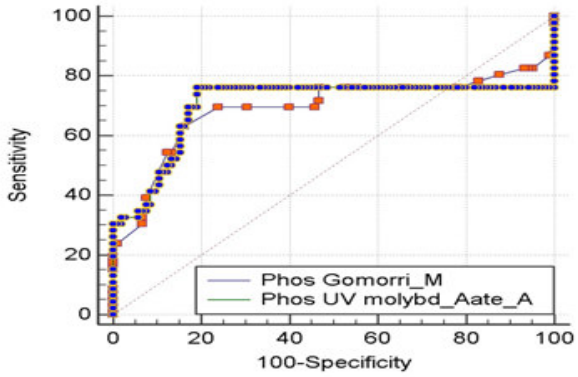

Table 7 and Figure 3 depicts the pair wise comparison of ROC Curves. The AUC are 0.702 and 0.692 for the analyzer and manual methods respectively. This shows that both methods used to measure phosphorus are fairly good tests. The Phosphomolybdate UV analyzer method shows a sensitivity of 75% and specificity of 82% and the Gomorri's method shows a sensitivity of 70% and specificity of 77%. The above results indicate that the analyzer method is slightly more sensitive and specific but a pairwise comparison of the two methods gives a P-value equal to 0.613 which is not statistically significant.

Table 8
Mc Nemar test for phosphorus by both methods in 78 CKD patients.
****Values within parenthesis indicate percentages***

		Phosphomolybdate UV method		
		Within the range	Outside the range	Total
Gomorri's	Within the range	41(87.23)	6(12.76)	47
method	Outside the range	5(16.12)	26(83.87)	31
	Total	46	32	78

Figure 4
Mc Nemar test for Phosphorus in CKD patients

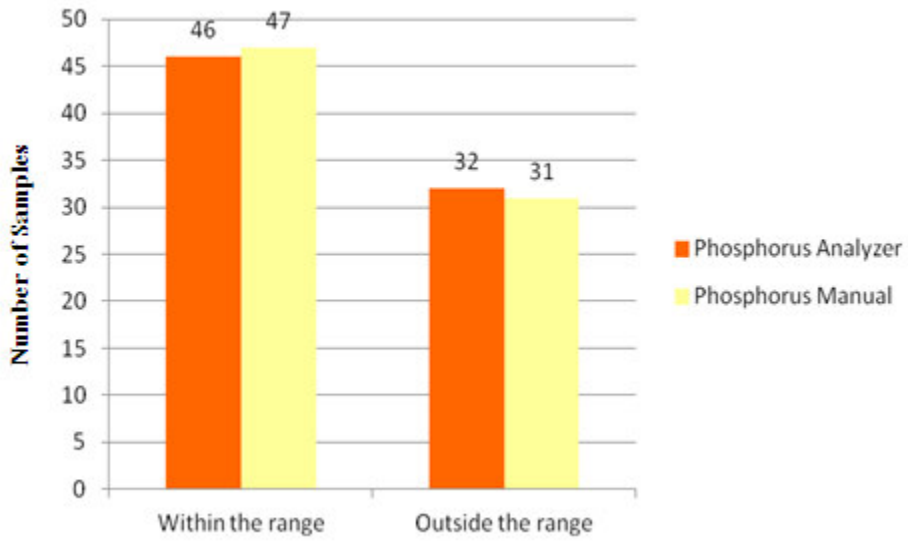

Table 8 and Figure 4 depict the Mc Nemar test for phosphorus in CKD patients. The test represents the comparison of both methods used for phosphorus determination in 78 CKD patients. Of these 78 patients, 47 cases fall within the normal range by the manual method and 31 cases outside the normal range by manual method. 46 cases fall within normal range by the analyzer method and 32 cases fall outside the normal range by analyzer method. The difference in the proportion of cases identified by both the methods was not statistically significant (P-value > 0.05).

Gomorri's method is preferred over other manual methods because it is easy to prepare metol, it is not affected by the concentration of acid used to precipitate proteins and has color stability for a longer time. The principles of both methods vary slightly in that, molybdate is reduced by metol in Gomorri's manual method while in the phosphomolybdate UV method the unreduced phosphomolybdate is measured[5]. The calcium and phosphorus levels are well maintained in the human body unless there is a disease which affects the regulation of these minerals in the body. Several studies conducted earlier show that in chronic kidney disease there is an alteration of the metabolism of both calcium and phosphorus.

Table 9
Correlation of creatinine concentrations with calcium and phosphorus concentrations by both methods used for determination

Relation between	Correlation coefficient (□)	P-value
Creatinine & Calcium Analyzer	-0.003	0.977
Creatinine & Calcium Manual	-0.037	0.745
Creatinine & Phosphorus Analyzer	0.121	0.295
Creatinine & Phosphorus Manual	0.197	0.084

Table 9 shows the Spearman rank correlation coefficient test and P values of creatinine with calcium and phosphorus. The table also shows the negative correlation between creatinine and calcium by both methods, while there is a positive correlation between creatinine and phosphorus by both methods. The above findings are depicted in the graphs below (Fig 5, Fig 6, Fig 7 & Fig 8).

Fig 5 & Fig 6 represents a weak fall in the slopes and it indicates a weak negative correlation between creatinine & calcium.

Figure 5
Spearman rho test creatinine/calcium analyzer

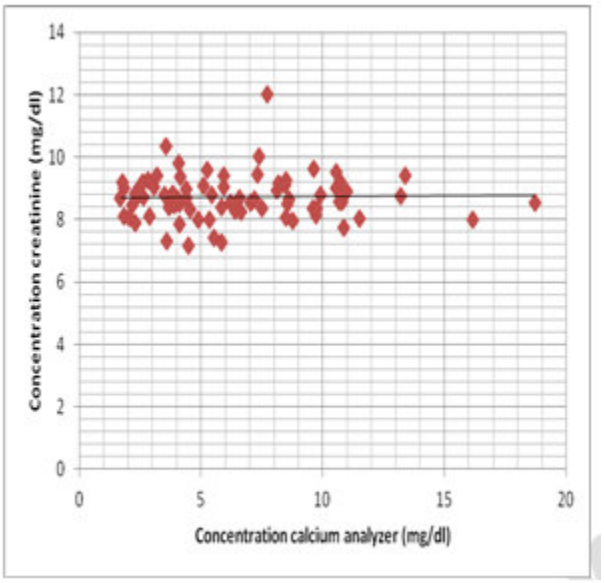

Figure 6
Spearman rho test creatinine/calcium manual

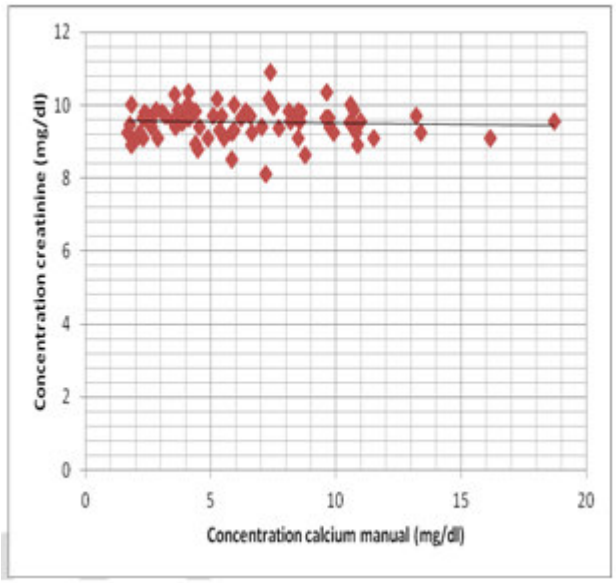

Figure 7
Spearman rho test creatinine/phosphorus analyzer

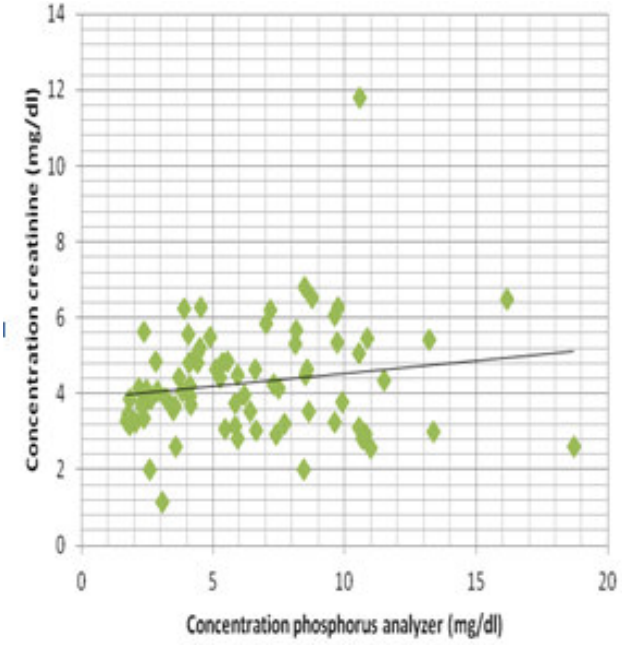

Figure 8
Spearman rho test creatinine/phosphorus manual

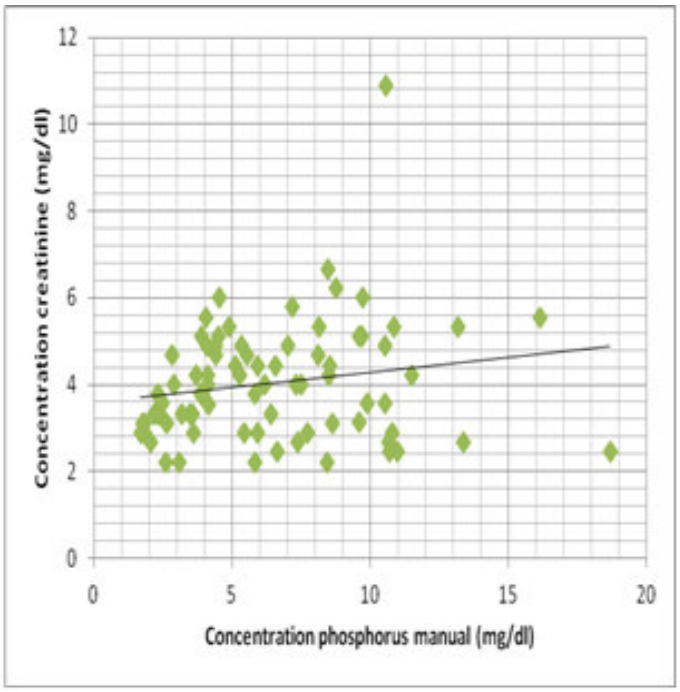

Fig 7 & Fig 8 show a weak positive correlation between creatinine and phosphorus. However these correlations between creatinine and calcium, phosphorus were not found to be statistically significant. The altered metabolism of calcium and phosphorus would have been more statistically significant with a larger CKD population in the study group. It was observed that among the 78 CKD patients, nearly 30.76% were hypocalcemic and around 33.33% were hyperphosphatemic.

CONCLUSION

In this study it can be concluded that NM-BAPTA analyzer method is dependable for determination of serum calcium, while serum phosphorus determined by either of two methods is acceptable. Serum creatinine was increased in all 78 CKD patients. As an altered metabolism, it is expected these CKD patients exhibit hypocalcemia and hyperphosphotemia. In our study it is seen that nearly 30.76 % of CKD cases exhibited hypocalcemia and 33.33 % of the CKD cases exhibited hyperphosphatemia. A further study with larger number of CKD samples can be done to look for a stronger correlation between creatinine and calcium, phosphorus.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this paper."

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