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VALIDATION OF METHOD FOR CARBON DI OXIDE ESTIMATION

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Abstract-

Background: Carbon di oxide (Bicarbonate) is generally estimated either by using Arterial Blood Gas Analyser or diagnostic kit. The author found out the method described in the kit not producing satisfactory results. Hence, the parameter programming was modified and validated as per the guideline of College of American Pathologist and standard textbooks.

Method: Every step of the parameter programme was assessed, necessary modification was done and checked by repeat testing. Statistical analysis were done to assess the performance status.

Place of Study: Laboratory Services, JMD Diagnostics Pvt. Ltd., Kolkata, WB, India.

Results: Results on every step of method validation were found out to be consistent. Deviations at every step were well within the acceptance limit. Precision checks showed excellent sigma results specially considering the fact that Bicarbonate is a labile parameter.

Conclusion: The modified parameter programme has successfully been validated.

Keywords- CO2, PEP, ABG, IQC, EQAS

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Introduction

Carbon Dioxide (CO_2) is an important marker of acid base balance [1]. It is one of the critical markers. In most of the hospitals CO_2 is being estimated in Arterial Blood Gas (ABG) system. In handful laboratories it is being measured using diagnostic kits. The basic principle of estimation is, phosphoenolpyruvate (PEP) reacts with bicarbonate in the serum/plasma and the enzyme phosphoenolpyruvate carboxylase forms oxaloacetate which in turn is reduced to malate in presence of malate dehydrogenase (Kreb's cycle). During the process of conversion of oxaloacetate to malate, ($NADH+H^+$) reduces oxaloacetate to malate and itself gets dehydrogenated to NAD^+. The reduction in absorbance at 340 nm caused by dehydrogenation of ($NADH+H^+$) is directly proportional to the CO_2 concentration in the sample [2]. During processing of test samples the author found out inconsistency in the sample as well as internal quality control (IQC) results. It has been observed if ten different samples are being processed the results are almost similar. To check the performance both levels of IQC sample were tested at a time. Both results are similar to the calibrator provided in the kit.

The author had no option except to evaluate and validate the method. After evaluation and Validation both IQC and EQAS reports were satisfactory. The replicate assay, bias check, lowest and highest detection limits were estimated, linearity has also been tested.

Materials and Methods

Study Subjects

Patient samples were chosen at random for replicate assay. For validation study the deviations study of the results were determining factors. Clinical history has not been taken. Replicate assay has been done from 5 patient's samples, 10 times replication of each [2,3].

Method of Estimation

The kit was obtained from Randox Laboratories. The samples were collected in tightly capped vacutainer and test performed within 1 hr. of collection. The reagent was reconstituted at least 8 hrs. earlier and kept in sealed container to prevent exposure to air. The procedure was as follows:

1. Twenty samples were tested using method provided in manufacturer's insert and modified method by the author. As per insert the testing range is 0-50 mmol/L. The author obtained direct value up to 37 mmol/L. Moving averages were plotted [4,5].
2. Linearity check has been done using deionized water instead of sample to detect lowest detection limit. The value of calibrator provided in the kit is 25.5 mmol/L. Instead of 5 µl, 10 µl was added in 1000 µl reagent to check linearity of modified method.

3. Precision checking was done using two level IQC samples. Both within run and run to run precision were checked [3].
4. Carryover has been checked by performing the test and blank alternatively [3,4].
5. Replicate testing of 5 patient's sample, 10 replicate for each have been performed.
6. Z-score of the EQAS reports were compared [3,4].

Statistical Analysis

Regression analysis of patients samples could not be done as results following the method provided in the insert were not reproducible. Instead of regression analysis moving average has been plotted. Six sigma precision check for IQC and CV% of replicate samples were performed.

Results

Twenty samples were tested in existing as well as modified method [Fig-1].

Six sigma calculation were performed using BIORAD Level 1 & 2, lot number 14210. Both within run and run to run performance were assessed [6].

Lowest detection limit was tested using deionized water instead of sample. The values were less than zero. The sample was diluted 2/4 times using deionized water and tested immediately. As 4 times dilution has not showed consistent results, further dilution was considered to be unnecessary. Similarly highest detection limit has been checked and linearity seems to be upto 37mmol/L. Only double dilution is recommended.Results as supplemented in [Table-2].

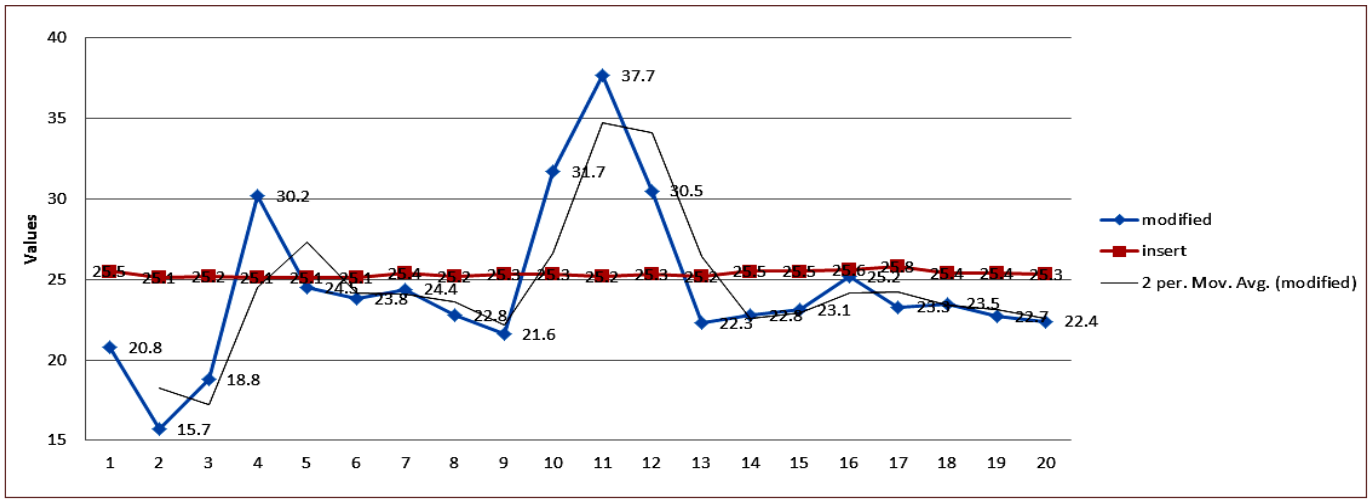

Fig. 1- Comparison of patient report in two methods

Table 1- Precision Check

	Within run		Run to run	
	L1	L2	L1	L2
Mean	30.55	17.18	31.65	18.05
SD	0.45	0.13	0.56	0.17
CV%	1.47	0.75	1.77	0.94
Avg.bias	-0.56	-0.25	0.75	0.45
TEA	9.2	9.4	9.2	9.4
Sigma	5.9	12.2	4.77	9.5

L1(mmol/L)- 26-40; L2(mmol/L)- 13-21; BIORAD,LOT-14410

Table 2- Linearity Check

	L1	L2	Test
LOD	-0.69	-0.66	-0.55
5µl	32.16±0.33	16.98±0.15	23.64±0.31
DD	15.17± 0.17	9.18±0.11	12.18±0.11
4D	9.35±0.21	8.98±0.16	9.58±0.11
7.5µl	37.85±0.55	25.35±0.33	33.68±0.28
10µl	38.15±0.19	33.67±0.29	37.99±0.17

Replicate testing of 5 patients each 20 times were done. The precision check for every patient from ten replicates were done.

Z-score of 6 month EQAS report has been observed. Ten replication of 5 patients were done. None of the samples has exceeded the Zscore limit. Z-scores showed equal distribution in the histogram.

Table 3- Replicate Results

	Patient 1	Patient2	Patient 3	Patient 4	Patient 5
Mean	32.54	20.02	18.804	29.61	27.83
SD	0.41	0.199	0.314	0.56	0.23
CV%	1.26	0.99	1.67	1.89	0.826

Table 4- EQAS Results

Sample number	Results (mmol/L)	Mean (mmol/L)	Z-score
1	18.66	21.6	-1.89
2	21.22	22.7	-0.98
3	18.25	18.2	0.02
4	20.03	19.3	0.57
5	18.09	18.7	-0.35
6	23.82	21.5	1.34

Carry over check has been done by testing sample 1, using saline as sample 2, then running sample 3, repeating saline as sample 4, simultaneously for 5 times.

As every time the results were below zero (-0.69, -0.55, -0.61, -0.57, -0.68) so the method has been considered to be viable.

The incubation time as per insert was 300 seconds. The delta check was done at 60, 120, 180, 240 and 300 seconds. Delta check was done treating EQAS sample 5 times at different time intervals. Better consistency was observed at 60 minutes. Value of EQAS sample is 23.82 mmol/L. Read time is to verify the consistency of

change in absorbance per minute. In the original method as it was stated to be end point method read time was not necessary. But in validated method read time has been given 3 i.e., three change of absorbance per minute would be monitored during testing.

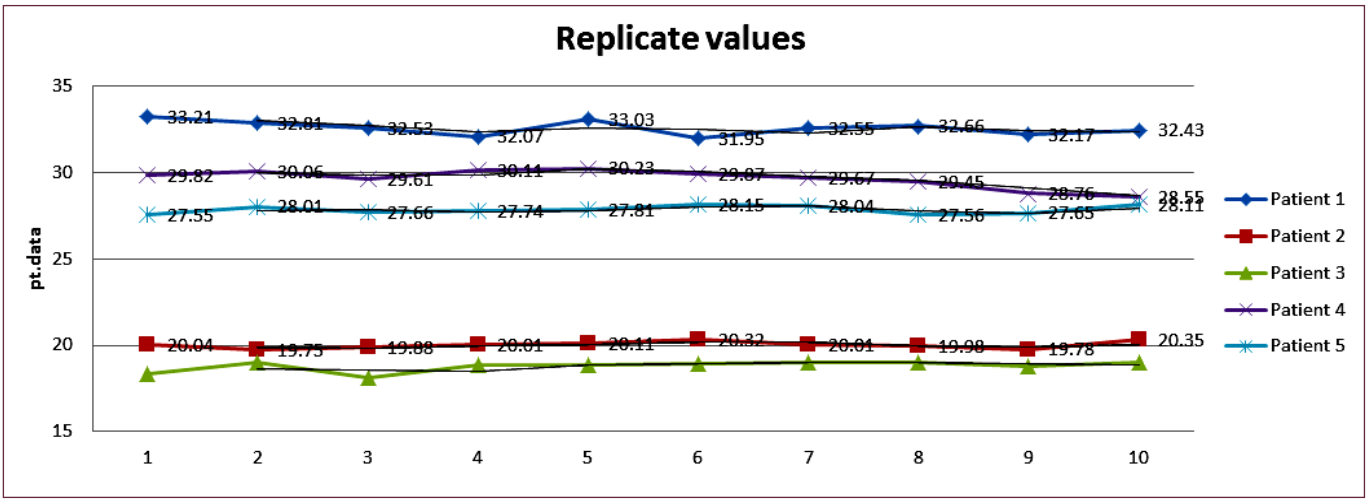

Fig. 2- Moving average of replicate results

Table 5- Comparison of Change in Absorbance/minute

Time (minutes)	60	120	180	240	300
Change in Absorbance/min	-0.469	-0.478	-0.459	-0.488	-0.518
Deviation	0.013	0.048	0.025	0.038	0.03

Table 6- Comparison of Parameter Programme

Parameter programme	Manufacturer's insert	Modified method
Wavelength (nm)	340	340
Cuvette	1 cm light path	1 cm light path
Reaction type	End point	Fixed time/rate
Reaction mode	Decreasing	Decreasing
Sample/Standard volume (µl)	5	5
Reagent volume (µl)	1000	1000
Incubation time (seconds)	300	60
Read time	Not mentioned	3
Lowest limit of detection (mmol/L)	Not mentioned	0
Highest limit of detection (mmol/L)	50	37
Aspiration volume (µl)	500	500
Sensitivity	0.001=0.004mmol/L	0.0196= 1 mmol/L
Interference	Not mentioned	Standard interference conditions were chosen. Hb-upto 0.5 g/dl Icterus-55 mg/dl Lipaemic index-550 mg/dl. The container of sample and reagent to be tightly capped.

Discussion

The major discrepancy noted in the method was the reaction type. As the basic principle is measurement of phosphoenolpyruvate carboxylase activity so the reaction type has to be a rate reaction. If a kinetic reaction is being monitored for a certain period of time then the proper reaction mode may only be a fixed time, rate mode. The discrepancy of results were due to wrong choice of reaction type [7,8]. The regression analysis which is customarily done for comparison of two methods in same instrument is not applicable as reaction mode was erroneous which is evident from [Fig-1]. So, ten different patient samples were chosen and moving average of results were shown in [Fig-1]. The different samples showed different results whereas insert method showed replication of same value and approximately close to the calibrator value.

Considering the corrected reaction type is acceptable, for authentication the author needs to check precision and accuracy. The sigma values of two level IQC both within run and run to run [Table-1] show the excellence of the level of precision. Specifically for CO_2 measurement where chance of aerial contamination is the major source of error. The accuracy level is also shown to be satisfactory [Table-4].

The basic principle of carryover check is to run deionized water (Blank sample)as a sample in between sample run [2]. If the deionized water performance produces some data then %carryover is calculated by eliminating Blank 1 from Blank 2. In the present study as every blank value was less than zero, the possibility of carryover has been ruled out.

Linearity check data shows:

- As blank checks during carryover testing were < 0, so lowest detection limit may be considered as zero.
- The double dilution values of both the levels were consistent [Table-2]. The level 1 value is consistent upto 4 times dilution [Table-2]. So, apparently upto 9 mmol/L result of CO_2 the reaction is linear. In practice, the samples are being directly tested. So, linearity upto 4 times dilution seems to be acceptable. Similarly, from [Table-2] it is evident that upper level of linearity is 37 mmol/L. As per manufacturer's insert the upper limit of linearity is upto 50 mmol/L. But the Biological Reference Interval for serum CO_2 is 23-29 mmol/L.

Hence, sample having value exceeding 37 mmol/L is difficult to get. If ever necessary the sample may be tested by double dilution. The lowest detection limit does not seem to be a limitation of the procedure. Hence reportable interval is 0-37 mmol/L.

Regarding incubation time, the kit method suggested incubation period of 300 seconds. But that was end point reaction mode. In fixed time mode, the delta, which is change in absorbance/minute approximately remains constant for a particular concentration, only with variation of incubation period the calibration factor changes. So, within run variation of change in absorbance/minute were assessed. The minimum deviation was seen in 60 seconds incubation

period. So, 60 seconds was decided to be the incubation time. Read time is 3 for any standard fixed time reaction mode [Table-5].

The replicate assay of patient sample [Table-3], [Fig-2] and EQAS reports [Table-4] were the final assessment before computation of the parameter programme [Table-6]. The SD, CV% and graphical presentation show excellent consistency and the deviation from one result is so small there is no chance of affecting clinical decision. Each patient's sample was tested 10 times and precision was excellent. Accuracy test also correlates as per [Table-4].

Conclusion

The modified parameter programme has nicely been validated. If it is accepted and published in the Manufacturer's insert the laboratories using the kit would be benefitted.

Generally, the Arterial Blood Gas analysers are being used in large laboratories. But medium and small laboratories who cannot afford the expensive Arterial Blood Gas analysers are mainly users of this kit and such laboratories also need to provide a good service support.

Acknowledgement

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