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Biochemistry Section

Analytical Errors in End Point Biochemical Assays – Experimental Analysis and Importance

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ABSTRACT

Introduction: Accurate and precise laboratory test results help immensely in the diagnosis and management of diseases. Though the analytical phase amounts to the least possible error, yet it is imperative to recognize and minimize the possible sources of errors to achieve good quality test results. In this article some points of precautions while running an end point assay have been highlighted.

Material and Methods: The left over blood samples received in our laboratory for routine tests were used for this study. Two most simple and low cost tests i.e. glucose and urea were done.

Result and Discussion: The importance of including a reagent blank and standard in every batch of assay has been demonstrated. In addition some other precautions such as using clear plasma/serum and mixing the thawed serum samples well, before analysis have also been discussed, ignoring which may give erroneous test results.

Conclusion: Since the total analytical error is a cumulative effect of several factors it is better to be cautious at every step of the analysis to have least possible error.

Keywords: Analytical errors, End point assays, Blank, Standard

INTRODUCTION

A correct diagnosis and successful management of a disease depends to a great extent on accurate and precise laboratory test results. The three main phases in laboratory testing are pre-analytical, analytical and post-analytical phase and all these phases have their own importance. Though the analytical phase amounts to the least percentage error [1], yet it is imperative to recognize and minimize the possible sources of analytical errors, what so ever, to achieve good quality test results.

Many biochemical tests are based on end-point assay principle and this requires three types of assay tubes in each batch viz. a blank, a standard and the test samples. In this article the importance of these sets of tubes and some other possible sources of analytical errors are discussed.

MATERIAL AND METHODS

The left over blood samples received in our laboratory for routine tests were used for this study. Two most simple and low cost tests i.e. glucose and urea were done. Plasma glucose test was done by GOD/POD method and serum urea test by urease/Berthelot method using ready made reagent kits. Semi-autoanalyzer was used to read the end point colour.

EXPERIMENTS, RESULTS AND DISCUSSION

Experiment 1 [Importance of reagent blank and standard]:

Every end-point assay includes a blank tube. The blank tube contains the pure solvent or the reagent only and its purpose is to negate the effect of background. The instrument (spectrophotometer/colorimeter/semi-autoanalyzer or fully autoanalyzer) is set at 100% transmission (zero absorbance) with the blank so that the standard and unknown samples (test samples) can be read against the blank of fixed absorbance. Blank tube also checks for contamination / deterioration of the reagent, if any. To demonstrate the importance of the blank tube, the following was done:

Glucose kits (GOD/POD) of three different brands were used for this experiment. Blank and standard(100 mg/dl) was run as per instructions in the procedure insert. Reagent blank was read against distilled water at 5-10 days interval for 30 days and the results are given in [Table/Fig-1]. The results shown in [Table/Fig-1] clearly show that the absorbance (O.D.) of reagent blank gradually increases with passage of time. It was least on day 1 and was maximum on day 30. This change in O.D. was noticed in all the three brands of reagents used. Brand A, B and C showed an increase in

blank O.D. by 23.5%, 14.7% and 23.0% respectively on day 30. Although there was no or negligible change in O.D. up to day 10. This change in O.D. of reagent blank also resulted in change (increase) in O.D. of standard tube against water and so in factor (used to calculate the concentration of analyte in test samples). However, when the standard was read against reagent blank, there was hardly any change in the factor. But when the standard was read against stored reagent blank the factor changed up to 5.9% on day 30.

From this experiment it is very clear that reagent blank should always be included in every batch of assay. The reason is very obvious that the O.D. of reagent blank does change with passage of time and it would be more if the reagent bottle is opened frequently leading to atmospheric exposure of the reagent especially with a large bottle of the reagent. Many times people ignore it and use either the old reading of the blank stored in analyzer or simply read the test samples against distilled water. This may result in gross erroneous results.

Ideally one standard should always be included in every batch of assay [2]. Others have advocated running them twice a week [3]. It checks the quality of the reagent as well as its deterioration, if any, with passage of time. However, if the equipment has the facility to store the standard reading, the stored reading may be used for a week provided the same pipette and the reagent of same lot and batch are used. This is important because relying on pre-calibration, errors can occur as a result of making or buying new reagents, the deterioration or contamination of old reagents, changes in the characteristics of the spectrophotometer itself, etc. [4]. It is pertinent to mention here that the pipettes should also be calibrated time to time.

It is also important to bring the temperature of the reagent and the standard to the room temperature. Many times the temperature of the laboratory varies appreciably in winter and summers especially in the laboratories which are not well air-conditioned. This is important because the temperature affects pipette volume. Air-displacement pipettes over deliver cold liquids and under deliver warm liquids [5]. Under such variable temperature conditions it becomes even more important to run the standard with every batch of assay.

Experiment 2 [To show the importance of centrifuging the blood samples to get clear plasma/serum]:

It is very important to centrifuge the blood specimens for obtaining clear serum / plasma. Sometimes people avoid this important step especially anti-coagulated specimens and take the plasma separated on standing by simple gravitation. To show this, two sets of plasma samples were used: one separated on standing by simple gravitation and the other set used clear plasma obtained by centrifuging the blood samples. Plasma glucose was estimated in 10 such samples and each sample was done in triplicates. The results are shown in [Table/Fig-2]. Perusal of [Table/Fig-2] shows that uncentrifuged plasma gave higher plasma glucose values

Brand	Tube	Day 1		Day 5			Day 10			Day 20			Day 30		
		O.D.	Factor	O.D.	Factor	% Change	O.D.	Factor	% Change	O.D.	Factor	% Change	O.D.	Factor	% Change
A	Reagent Blank (Against DW)	0.085	-	0.086	-	1.1	0.092	-	8.2	0.098	-	15.2	0.105	-	23.5
	Std. (against DW)	0.466	214.5	0.467	214.2	0.14	0.473	211.4	1.4	0.478	209.3	2.47	0.487	205.3	4.3
	Std. - Reagent Blank	0.381	264.4	0.381	264.4	0.0	0.381	264.4	0.0	0.380	263.1	0.49	0.382	261.7	1.0
	Std. - Stored Reagent Blank	0.381	264.4	0.382	261.7	1.0	0.388	257.7	2.5	0.393	254.4	3.8	0.402	248.7	5.9
B	Reagent Blank (Against DW)	0.061	-	0.061	-	0.0	0.063	-	3.2	0.066	-	8.2	0.070	-	14.7
	Std. (against DW)	0.466	224.2	0.446	224.2	0.0	0.448	223.2	0.4	0.451	221.7	1.1	0.456	219.2	2.2
	Std. - Reagent Blank	0.385	259.7	0.385	259.7	0.0	0.385	259.7	0.0	0.385	259.7	0.0	0.386	259.1	0.23
	Std. - Stored Reagent Blank	0.385	259.7	0.385	259.7	0.0	0.387	258.4	0.5	0.390	256.4	1.3	0.394	253.8	2.3
C	Reagent Blank (Against DW)	0.052	-	0.052	-	0.0	0.054	-	3.8	0.059	-	13.4	0.064	-	23.0
	Std. (against DW)	0.427	234.2	0.427	234.2	0.0	0.429	233.1	0.47	0.435	229.8	1.8	0.440	227.2	2.98
	Std. - Reagent Blank	0.375	266.6	0.375	266.6	0.0	0.375	266.6	0.0	0.376	265.9	0.26	0.376	265.9	0.26
	Std. - Stored Reagent Blank	0.375	266.6	0.375	266.6	0.0	0.377	265.2	0.52	0.383	261.1	2.06	0.388	257.1	3.3

[Table/Fig-1]: Blank and Standard absorbance (O.D.) on different days. DW - Distilled water and Std. - Standard

as compared to those of well-centrifuged plasma samples. The uncentrifuged plasma gave 9.9 – 24.1% over estimation of glucose concentration. Moreover, the coefficient of variation in uncentrifuged plasma samples was quite higher than centrifuged plasma (6.8% versus 1.1%) showing poor precision with uncentrifuged plasma. This may be due to presence of blood cells in uncentrifuged plasma.

Experiment 3 [To show the importance of mixing the thawed serum before analysis]:

Many times serum samples are kept in freezer and before running the test the serum samples are thawed to room temperature. At this stage it is very important to mix the thawed samples well. To show this, 10 serum samples in duplicate were kept in freezer. On the day, when the test was to be done, all the frozen samples were thawed. One of the duplicate serum was properly mixed before the test, while the other one was used without mixing. Urea was estimated in these serum samples and the results are shown in [Table/Fig-3]. The results showed that serum urea was lower in unmixed serum samples as compared to those in properly mixed serum. The percentage under estimation varied from 13.3 to 50% and it was more at lower urea concentration. Moreover, the coefficient of variation in unmixed serum varied from 2.4% to 8.2% at different concentration while in the mixed serum it ranged from 0.33 % to 0.38 % showing poor precision in unmixed serum. Others have also reported lowest concentration of different analytes in the upper most layer and the highest in the lower most fraction and this is because thawing of frozen serum results in dilution in the upper part and concentration in the lower part of the serum [6]. [Table/Fig-4] shows the different layers (diffused) in the thawed but unmixed serum sample. This could be the reason for under estimation and poor precision of urea in these serum samples in our study too.

Centrifuged plasma	Uncentrifuged plasma	% over estimation in uncentrifuged plasma
65	72	10.7
72	88	22.2
75	90	11.2
88	100	13.6
94	108	14.9
118	129	9.3
130	150	15.4
171	188	9.9
245	304	24.1
248	278	12.1

[Table/Fig-3]: Glucose in centrifuged clear plasma and uncentrifuged plasma Glucose (mg/dl).
Each value is the mean of triplicates

Thawed & mixed serum	Thawed but unmixed serum	% under-estimation In unmixed serum
32	18	43.7
34	17	50.0
40	25	37.5
47	30	36.0
57	38	33.3
80	65	18.7
91	75	17.6
118	101	14.4
142	123	13.3
165	140	15.1

[Table/Fig-3]: Serum urea in frozen serum samples
Each value is the mean of triplicates

[Table/Fig-4]: Showing diffused layers in two thawed but unmixed serum samples

CONCLUSION

To conclude, there are some minute but important precautions which need attention by the analyst to avoid analytical errors:

1. Both coagulated and uncoagulated blood samples should be properly centrifuged to get clear serum and plasma respectively.
2. The frozen serum / plasma samples should be thawed, brought to room temperature and mixed properly before analysis.
3. The reagents and standards should be at room temperature at the time of analysis.
4. Every batch of assay should include a reagent blank tube and the test samples should be read against the reagent blank, set at 100% transmittance(0.0 O.D.). Reading test samples against stored reagent blank or water blank should be avoided.

5. Every batch should include a standard or at least once a day if several batches are to be run every day.
6. Stored reading of the standard should be avoided or should not be used for more than 1 week.

Since the total analytical error is a cumulative effect of several factors it is better to be cautious at every step of analysis to have least analytical error.

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