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Experimental Research

Biochemistry Section

Automation of Ischemia Modified Albumin on Beckman Analyser

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ABSTRACT

Introduction: Early markers for the diagnosis of acute myocardial ischemia are required before necrosis sets in. Ischemia-Modified Albumin (IMA) is one marker which has been shown to rise early before cell necrosis and has been found to be useful in the diagnosis of acute myocardial ischemia.

Aim: The present study was taken up to evaluate the assay of IMA levels using albumin cobalt binding method and to automate the method on Beckman Synchron DxC600 analyser.

Materials and Methods: Seventy patient samples received in the clinical laboratory for biochemical analysis were used after analysis of the required parameters. Cobalt chloride (CoCl2) and Dithiothrietol (DTT) solutions in various concentrations were tested keeping one parameter constant at a time. Spectrum scan was performed, λmax was noted and absorbance measured at 5 min, 10 min, 15 min, 20 min, 25 min. After the method was established, within run, day to day precision, recovery and interference experiments were performed to test the method performance

Results: Experiments showed that CoCl2, corresponding to a concentration of 0.641 mmol/l and DTT corresponding to a concentration of 1.6207 mmol/l in the final reaction mixture gave the maximum absorbance at 470nm. The method was found to be linear upto an albumin concentration of 5.5g/dl. Intra-assay coefficient of variation (CV) and Inter-assay CV were 1% and 2.5% respectively. IMA test showed no interference for triglyceride concentration upto 443mg/dl, bilirubin concentrations upto 7.9mg/dl and haemoglobin upto 3g/dl. IMA analysis in paired serum and plasma showed values to be higher in serum compared to heparinised plasma.

Conclusion: IMA assay can be performed on Beckman DxC 600 analyser. The sample analysis time is 10 minutes and the turn around time is short and thus acceptable. It can therefore be useful in patients with acute chest pain.

Keywords: Automation, Evaluation, Myocardial infarction, Myocardial ischemia

INTRODUCTION

Acute Myocardial Infarction (AMI) is associated with significant morbidity and mortality. Biochemical markers play an important role in the diagnosis and management of these patients. AMI is routinely diagnosed by analysing various non-specific and specific myocardial proteins in the serum which are released in varying amounts from the damaged myocytes during ischemia. Analysis of these markers is useful in predicting the time-course of occurrence of myocardial ischemia progressing to myocardial infarction.

Among the markers currently used, troponin I (TnI) is specific for the cardiac muscle and is an ideal marker of myocardial injury [1] being released 6-8 hours after myocardial injury and peaks at 12-24 hours. However, it is slowly cleared and remains elevated for 7-10 days [2]. Creatine Kinase MB, the isoform (CK-MB) of CK present in the cardiac muscles, rises in the circulation 4-9 hours after the onset of chest pain, peaks at 24 hours and returns to baseline levels by 48-72 hours. Its early clearance helps in diagnosing re-infarction. Hence, serum TnI levels along with CK-MB are routinely used for diagnosing AMI [3,4]. Myoglobin is another useful marker which is released extremely early into the serum i.e., 1 hours after the onset of myocardial injury, peaks at 4-12 hours and returns to baseline values immediately but lacks specificity. However, it can be used to rule out myocardial infarction and can be used in conjunction with troponins and CK-MB [4,5]. All these markers routinely used for detection of myocardial infarction are not suitable for assessing early myocardial ischemia since these are intracellular markers released into the circulation only after complete damage to the myocardium [6,7].

Other markers which have been found to be useful include, Heart-type Fatty Acid Binding Protein (hFABP) [8], Growth Differentiation Factor 15 [9], Pregnancy associated Plasma Protein A [10] and IMA [11,12] Among these, IMA helps in the diagnosis of acute ischemia prior to the onset of myocardial necrosis [11]. It increases immediately after the onset of ischemia and returns to baseline values within 6-12 hours [12].

IMA has the clearance of the United States Food and Drug Administration (FDA), of America for use in ruling out Acute Coronary Syndrome (ACS) in low-risk patients. IMA levels

have been shown to be significantly elevated immediately within minutes of the rupture of atheromatous plaque and 2 hours after percutaneous coronary intervention [13]. It has been found to have high sensitivity (85-95%) and a high Negative Predictive Value (NPV) (75-95%) [13].

Laboratory assessment of IMA is usually performed by Albumin Cobalt Binding assay (ACB test). The amino terminal end (N-terminal) of the albumin molecule and especially the aspartyl-alanyl-histidyl-lysine sequence, appears to be the primary binding site for transitional metals, such as cobalt (Co^{2+}), copper and nickel [14,15]. Under conditions of ischemia, as in patients with myocardial ischemia, the N-terminus of the albumin is altered and thus, decreases its binding capacity for metals and thus formation of IMA [15]. Serum albumin of myocardial ischemic patients exhibit reduced binding to Co^{2+} when compared with serum albumin of non ischemic patients [16].

Review of literature suggests that IMA is an important marker for AMI. With this background, the present study was taken up to evaluate the assay of IMA levels using albumin cobalt binding method and to automate the method for its possible introduction for patient care.

MATERIALS AND METHODS

This method evaluation study was done in the Department of Biochemistry, Sri Venkateswara Institute of Medical Sciences, Tirupati, India from January 2014 to June 2014. The study was approved by the Institutional Ethics Committee (IEC no 354/10/02/2014). Seventy patient samples received in the clinical laboratory for biochemical analysis were used after analysis of the required parameters. The serum samples were pooled and pipetted into aliquots and stored at -80 °C until further analysis. These aliquots were used to perform various experiments like within run, day to day precision, recovery and interference study.

Cobalt chloride ($CoCl_2$) (Qualigens), Dithiothrietol (DTT) (Sisco Research Laboratories) and sodium chloride from SD Fine-Chem were used. All the chemicals were of analytical grade and double distilled water was used for preparation of reagents.

The principle underlying this assay is the same as ACB assay which measures the binding of Co to albumin in serum. Concentration of IMA in the assay is determined by the addition of a known amount of exogenous Co($CoCl_2$) to a serum specimen and measurement of unbound cobalt using the chromogen DTT which binds unbound Co. The difference in our method is that it measures native albumin concentration binding to cobalt in the sample. IMA levels in a given sample is the difference in serum albumin level for a given sample and the levels obtained by this assay which represent the native albumin concentration.

A stock solution containing 2g/dl of $CoCl_2$ corresponding to a concentration of 15.403 mmol/l was prepared. Stock solution of $CoCl_2$ was diluted further to test the concentration which yields maximum absorbance. Stock solution containing 3g/dl of DTT corresponding to a concentration of 19.44 mmol/l was prepared. Stock solution of DTT was diluted further to test the concentration which yields maximum absorbance.

Cobalt chloride and DTT solutions in various concentrations were tested keeping one parameter constant at a time. Spectrum scan was performed and λmax was noted. Absorbance was measured at 5 min, 10 min, 15 min, 20 min, 25 min. Experiments showed that $CoCl_2$ corresponding to a concentration of 0.641 mmol/l and DTT corresponding to a concentration of 1.6207 mmol/l in the final reaction mixture gave the maximum absorbance at 470 nm. The program used on Beckman DXC 600 is shown in [Table/Fig-1].

Method Validation

The linearity of the method was tested using human serum albumin from 2-6 g/l. Precision study was done using BioRad controls at two levels (B1 and B2). Ten replicates were analysed for within-run precision and the same were run on 5 consecutive days to calculate day to day precision. IMA test was evaluated for interference from triglycerides upto 7mmol/l, bilirubin upto 135µmol/l and haemoglobin upto 7g/dl. Recovery was tested by adding known concentration of albumin to the samples.

STATISTICAL ANALYSIS

Continuous variables were expressed as mean ± SD. The coefficient of variation for intra assay and inter assay precision studies were calculated as percentages. Analysis of the data was done using Microsoft Excel 2007 for Windows (Microsoft Corporation, Redmond, WA, USA) spread sheets.

RESULTS

Linearity of IMA Assay

Measurement of IMA using different concentrations of human serum albumin from 2-6 g/L was linear [Table/Fig-2]. However, as significant matrix effect was noted calibration was performed using Beckman multi-calibrator serum.

Precision Study

The intra-assay coefficient of variation (CV) for BioRad control material was 1.4 % and 0.6% for B1 (level 1) and B2 (level 2) respectively. Inter-assay CV for control material was 2.73 and 2.28 for B1 and B2 respectively [Table/Fig-3].

Interference Tests

IMA test was evaluated for interference from triglycerides upto 7mmol/l, bilirubin upto 135µmol/l and haemoglobin upto 7g/dl. IMA test showed no interference for triglyceride concentration upto 5mmol/l corresponding to 442.5 mg/dl (recovery: 95.1-100%). However, beyond this a negative interference was noted [Table/Fig-4a]. No interference was noted for bilirubin concentrations upto 135µmol/l corresponding to 7.9mg/dl (recovery:100-104.9%) [Table/

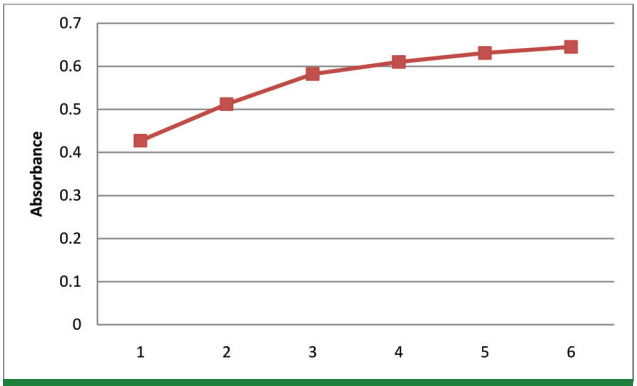
Chemistry Parameters	
Chemistry Name	IMA
Reaction type:	End point 2
Units:	g/ dL
Precision:	x. xx
Reaction Direction:	positive
Math model:	Linear
Primary wavelength:	470 nm
Secondary wavelength:	650 nm
Calculation factor:	1
No. of calibrators:	1
Set points:	Set point of calibrator
Cal Time Limit:	336

Processing Parameters			
	First Inject	**Second Inject**	**Third Inject**
Component:	A	B	C
Dispense Volume (µL):	200	50	50
Inject Time (sec):	-	600	720
Sample Volume (µL):	30	-	-
Read Windows			
Reagent:	**Blank**	**Initial**	**Reaction 1**	**Reaction 2**
Start Read:	-32	721	710	742
End Read:	-16	736	726	758

Error Detection Limits			
	Blank	**Reaction 1**	**Reaction 2**
ABS Low Limit:	-1. 500	-1. 500	-1. 500
ABS High Limit:	2. 200	2. 200	2. 200
Rate Low Limit:	-1. 500	-1. 500	-1. 500
Rate High Limit:	2. 200	2. 200	2. 200
Mean Deviation:	2. 200	2. 200	2. 200
Substrate Depletion			
Initial Rate:	-	99. 999	-
Delta ABS:	-	2. 200	-
Multipoint Spans			
	1-2	0. 000	
Usable Result Range			
Low Limit:	0. 000	-	-
High Limit:	6.000	-	-
The method gives native albumin concentration reacting with DTT to give coloured complex measured spectrophotometrically			
Calculation of IMA concentration: Serum albumin – Serum native albumin (measured using ACB assay above)			

[Table/Fig-1]: Method parameter on Beckman UniCel DxC 600.

Fig-4b]. Haemoglobin upto 3g/dl corresponding to 1.92 mmol/l, did not interfere with the assay (recovery:100-106.3%) [Table/Fig-4c]. Beyond this, positive interference was noted.



[Table/Fig-2]: Linearity of IMA assay.
IMA= ischaemia modified albumin

	Mean ±SD	CV (%)
Within Run Precision		
B1	2.84 ± 0.04	1.4
B2	4.4 ± 0.04	0.6
Day to Day Precision		
B1	2.76± 0.08	2.73
B2	4.22± 0.05	2.28

[Table/Fig-3]: Within-day and between-day precision.
SD= standard deviation; CV= coefficient of variation

	IMA conc.	Recovery %
Baseline IMA	4.1	
TGL conc 1 mmol/L	4.1	100.0
TGL conc 2 mmol/L	4	97.6
TGL conc 3 mmol/L	4	97.6
TGL conc 4 mmol/L	3.9	95.1
TGL conc 5 mmol/L	4.1	100.0
TGL conc 6 mmol/L	3.7	90.2
TGL conc 7 mmol/L	3.6	87.8

[Table/Fig-4a]: Interference study of IMA assay with triglycerides.
(lipemia) (1mmol/L Triglyceride = 88.5mg/dL)

	IMA conc.	Recovery %
Baseline IMA	4.1	
T.bilirubin conc (17.25µmol/L)	4.1	100.0
T.bilirubin conc (33.5 µmol/L)	4.2	102.4
T.bilirubin conc (67.5 µmol/L)	4.2	102.4
T.bilirubin conc (135µmol/L)	4.3	104.9

[Table/Fig-4b]: Interference study of IMA assay with bilirubin (icterus).
(1µmol/L bilirubin = 0.0585 mg/dL)

	IMA conc.	Recovery %
Baseline IMA	4.1	
Hb 1g/dL	4.1	100.0
Hb 2g/dL	4.27	104.1
Hb 3g/dL	4.36	106.3
Hb 4g/dL	5.1	124.4
Hb 5g/dL	5.86	142.9
Hb 6g/dL	6.2	151.2
Hb 7g/dL	6.8	165.9

[Table/Fig-4c]: Interference study of IMA assay with haemoglobin (hemolysis).

IMA Test Results in Plasma v/s Serum

IMA analysis in paired serum and plasma showed values to be higher in serum compared to heparinised plasma [Table/Fig-5]. Values were higher for serum, ranging from 9% to 23.2%.

Heparinized Plasma	Serum	Recovery %
1.92	2.1	109.4
3.1	3.23	104.2
3.26	3.6	110.4
3.42	3.9	114.0
3.63	4.3	118.4
3.82	4.5	117.8
4.14	5.1	123.2

[Table/Fig-5]: Effect of anticoagulant.

Reagent Stability

The reagents were kept on board at a temperature of 2°C to 8°C. DTT was found to be stable for seven days at 2°C to 8°C, while $CoCl_2$ was stable for 15 days at 2°C to 8°C.

DISCUSSION

Several studies have shown IMA to be a useful marker in diagnosing AMI either independently or in combination with standard biomarkers [13,17-19]. The specificity of the test ranges from 31% to more than 95% in different studies. A study by Liyan et al., [13] observed that IMA using the ACB assay may be useful to make an early diagnosis of ACS with a sensitivity of 94.4% and specificity of 82.6% with a cut-off value of 70.0 units/l and area under the curve (AUC) of 0.948. Mehta et al., [17] observed IMA levels to be significantly higher in ischemic chest pain group when compared to non ischemic chest pain group and between ischemic chest pain group and control group. High sensitive-cTnT demonstrated better sensitivity and NPV while IMA showed better specificity and positive predictive value (PPV). Combination of the two markers yielded better AUC (0.884) and a better sensitivity, specificity, PPV, and NPV. Shen et al., [18] showed that IMA may be a useful biomarker for early diagnosis of ACS with a positivity rate of 82.0% for IMA when compared to 40.6% for coronary troponin I (cTnI) within 1 hour of admission. In 72 patients presenting within 3 hours of acute chest pain and negative CTnI, the positivity rate of IMA was 86.1% when compared to 72.2% for electrocardiography (ECG). Thus, IMA showed good utility for early diagnosis of ACS. In another study, Chawla et al., [19] found IMA assay with sensitivity 78.0% and specificity 82.7% when compared to CK-MB assay with a sensitivity of 58% and specificity of 60.0% for detection of myocardial ischemia. Ertekin et al., [20] reported a sensitivity of 83% and specificity of 90% for diagnosing ACS. Anwaruddin et al., [21] reported IMA to be highly sensitive (97%) with a high NPV (92%) only when used in combination with standard biomarkers i.e. myoglobin, CK-MB, TnI in 200 patients with suspected myocardial ischemia admitted to the Emergency Department.

These studies show that IMA can be used as a biomarker in patients with AMI. The ACB assay commonly employed for measuring IMA, measures the cobalt binding capacity of albumin in the given serum sample where a known concentration of cobalt is added followed by DTT. This results in binding of any remaining unbound cobalt and the intensity of colour developed is measured spectrophotometrically. Thus, the ACB test measures the capacity of albumin to bind cobalt where IMA has less capacity to bind cobalt than normal albumin. Therefore, the ACB test is an indirect test for IMA [15,16].

In the present study, Beckman multi-calibrator was used to calibrate the method. This method thus measures native albumin concentration binding to cobalt in the sample. Serum albumin levels were measured for the same sample and IMA was calculated as the difference between the patient's albumin and levels obtained by the IMA assay which represent the native albumin concentration. Human serum albumin was not used for calibration as the method showed significant matrix effect on diluting with normal saline. This was further confirmed by analysing human serum albumin and Beckman controls corresponding to the same albumin concentration. Thus, Beckman multi-calibrator which is serum based was used for calibration in order to minimize the matrix effect.

The method was evaluated in terms of linearity, imprecision, recovery along with sample and reagent stability. The linearity study using human serum albumin however showed good linearity upto albumin concentration of 5.5 g/dl. The mean Intra- and inter-assay CVs were found to be 1.0% (0.6 and 1.4) and 2.49% (2.28 and 2.73) respectively, which meets the 1999 National Academy of Clinical Biochemistry Committee recommendations (<5%) for performance of assays for new cardiac markers for coronary artery diseases [22]. IMA test showed no interference for triglyceride concentration upto 5mmol/l corresponding to 442.5mg/dl (recovery: 95.1-100%). However, beyond this a negative interference was noted. No interference was noted for bilirubin concentrations upto 135µmol/l corresponding to 7.9mg/dl (recovery:100-104.9%). Haemoglobin upto 3g/dL did not interfere with

the assay (recovery:100-106.3%). Beyond this, positive interference was noted. Gidenne et al., found no interference with haemoglobin upto 220.4µmol/l and triglycerides upto 7mmol/l [23].

The sample of choice for the assay was selected based on the findings of the assay on serum and heparinised plasma samples drawn from the same individuals. Ethylene Diamine Tetraacetic Acid (EDTA) and citrate were not tested due to the fact that they chelate $CoCl_2$ used for the analysis. Serum samples showed higher values compared to plasma. This agrees with Gidenne et al., [23] and Lefevre et al., [24] who reported higher values for IMA tested in serum compared to plasma. Hence, only serum samples should be used for analysis of IMA by the ACB assay.

The stability of the reagents were tested on the Unicel DxC 600 analyser. DTT was found to be stable for 7 days at 2°C to 8°C, while $CoCl_2$ was stable for 15 days at 2°C to 8°C. The sample stability was not evaluated in the present study. Previous studies recommend that the total duration from blood withdrawal to instrument result must be less than 5 hours [23]. Blood must be centrifuged within 1 hour and specimen tubes should remain closed and kept at 4°C at all times. ACB test values have been shown to be stable upto 6 hours at ambient temperature and 12 hours at 4°C [24].

LIMITATIONS

Application of the method in patients of myocardial infarction could not be performed. This will further throw light on utility of this assay compared to the current markers in patients with MI.

CONCLUSION

IMA assay can be performed on Beckman DxC analyser as seen from the precision, accuracy and interference experiments. The sample analysis time is 10 minutes and the turn around time is short and thus acceptable. It can thus be useful in the management of patients with acute chest pain.

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