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ACCELERATED STABILITY STUDY OF ARSENAZO III USED FOR DETECTION OF CALCIUM FROM BIOLOGICAL SYSTEM THROUGH UV-SPECTROPHOTOMETER, BIOCHEMISTRY ANALYZER, PH METER, HPLC AND HPTLC

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Keywords:

In-vitro, linearity, Arsenazo III, Biochemistry analyzer, Shelf life and retention time

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ABSTRACT: Objective: Stability of *In-vitro* Diagnostics (IVDs) reagents was performed to check its quality standards, performance, and linearity. IVDs like Calcium reagent containing Arsenazo III were studied by Accelerated stability studies by considering temperature, pH, and light parameters. **Materials and Methods:** Stability data were obtained by using different instruments like UV spectrophotometer, Biochemistry analyzer, pH meter, HPLC, and HPTLC. This gives information about the degradation that occurred during storage, transportation, *etc*. **Results:** Calcium reagent containing Arsenazo III degrade 90.81% when placed at 42 °C by UV spectrophotometer analysis. The stability conditions' effect on actual serum concentration was measured by taking reagent performance on a biochemistry analyzer. The linearity of reagents decreases at 42 °C and at normal temperature, linearity does not change. HPLC spectra gave degradation of reagent, which was analyzed by its retention time, peak height, and % area. Arsenazo III produces 91.25% remains undecomposed in 3 months when exposed to light. The shelf life of the calcium reagent was found to be 85.36. HPTLC spectra gave degradation of Arsenazo III, which was analyzed by its retention time, peak height. The reagent, during its stability studies, shows a slight change in its pH. **Conclusion:** From HPLC and HPTLC analysis, it is confirmed that the degradation occurred in Arsenazo III after exposed to an accelerated stability study.

INTRODUCTION: In many biological, clinical, environmental, and agricultural systems, the determination of free calcium is very important. There are so many methods been studied, which include ion-selective electrode potentiometry [1] spectrophotometry with indicator dyes, [2-3] equilibrium calculations, filtration [4], solvent extraction [4-5], chromatography, or ion exchange [6].

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The measurement of free calcium in biological cells and tissues is a very important study for the diagnosis of many pathophysiological conditions such as hypo/hypercalcemia, bone diseases, *etc.* [7-8]

Various Azo-dye based on chronotropic acid are widely used as reagents for the photometric determination of various elements like calcium, potassium, *etc*. The reagents containing the arsonous group-AsO, H are much useful and universally applicable, such as “arsenazo”. It was firstly prepared by Kuznetsov in 1941 and named in its abbreviated form. Various improved analogs of arsenazo were synthesized later and tested; these included arsenazo II (a double molecule of arsenazo) andarsenazo III bis-diazo dye based on

chronotropic acid and o-aminophenylarsonic acid The structure of Arsenazo III is given in **Fig. 1** [8-9].

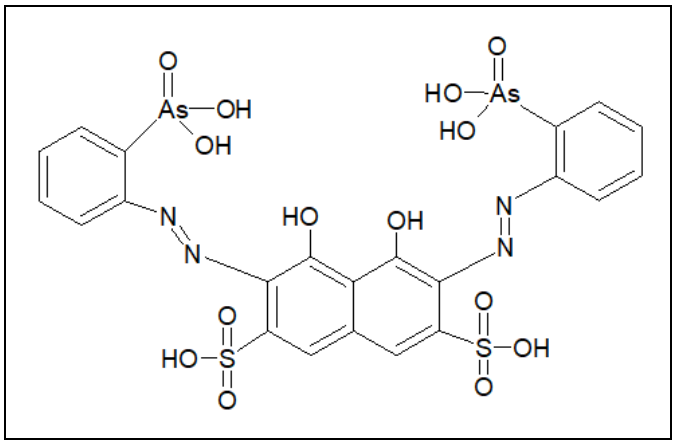

FIG. 1: STRUCTURE OF ARSENAZO III

This dye is a sensitive and selective reagent for calcium and has been studied for the determination of calcium both in solution, in cells, and tissue [10-11]. In the modern era, this reagent is packed with other solution/reagents such as buffer, pH modifiers and called it a diagnostic reagent/pack.

An in vitro diagnostic is a method of performing a diagnostic test outside of a living body in an artificial environment, usually a laboratory. Their purpose is not to have a direct therapeutic effect but rather to provide valuable information on patient's health status. Their values stem from the information they provide. This means that the expertise of the healthcare professional in using the IVDs is crucial to ensure the correct decision-making for patient treatment and care.

This sets IVD as part of what makes them unique amongst healthcare systems/technologies. As these reagents are nothing but chemicals/dyes, the storage condition, time of storage, environmental factors, and incompatibilities may affect the integrity/uniqueness of these reagents. It may lead to false detection/diagnosis. So, there is a need to develop methods for studying the stability of these reagents.

The guidelines have also been given for studying the stability of these reagents [12-14]. By keeping in view the above all details, we have performed an accelerated stability study of *in-vitro* reagent containing Arsenazo-III and developed analytical methods for the qualitative and quantitative determination of Arsenazo-III.

MATERIALS AND METHODS:

Materials: Pure Arsenazo-III was obtained from Spectrochem Pvt. Ltd., Mumbai, India. A calcium *in-vitro* reagent kit was obtained from Yucca diagnostics, Kolhapur, Maharashtra, India. All other reagents and solvents used in the study were of analytical grade.

Methods:

Equipment: U.V. visible spectrometer was of Agilent Technology Carry 60 and UV-Vis UV-1800 Shimadzu, Japan. The HPLC Shimadzu prominence HPLC system equipped with degasser DGU-20A 5R, low-pressure quaternary pump LC 20 AD, and photodiode array detector SPD- M20 A. Separation was achieved by using reverse-phase C-18 column (Enable 250 × 4.6 mm, 3 µm). HPTLC consisting LINOMAT-V automatic TLC sample applicator CAMAG TLC Scanner 3 equipped with Wincats software (Version 1.4.2), Merk HPTLC plates coated with silica gel 60 F254 (0.2 mm thickness) on aluminum sheets, ILS micro syringe (100 µl) and Biochemistry analyzer of Access 127i.

Selection of Batches for Accelerated Stability Studies: Successive three batches of calcium reagent were selected. They are kept for stability studies at 42 ºC and room temperature. Batch number 1 is kept for in use/open vial stability study. Also, samples from these batches were kept for checking the effect of temperature, light, and pH at 0, 1, 2, and 3 months. The effect of temperature was carried out by keeping samples of different batches at room temperature and 42 ºC for 0, 1, 2 and 3 months. The effect of light was carried out by keeping samples in light for 0, 1, 2, and 3 months. The same procedure was used studying the effect of pH.

Determination of λ_{max} for Arsenazo lll: Double beam UV-Visible spectrophotometer (Shimadzu 1800) was used with matched quartz cells of 1 cm in width. Accurately weighed 10 mg of Arsenazo was transferred to 10 ml volumetric flask containing distilled water, and volume was made up to 10 ml. This solution was treated as a stock solution (1000 µg/ml). 0.2 ml was withdrawn from this stock solution and diluted with water to 10 ml to obtain 20 µg/ml concentrations. The standard solution of Arsenazo lll (20 µg/ml) was scanned in

the range of 500 - 600 nm in 1.0 cm cell against solvent blank, and spectra were recorded. The wavelength of maximum absorbance was considered for further studies.

Standard Calibration Curve for Arsenazo lll: Double beam UV-Visible spectrophotometer (Shimadzu 1800) was used with matched quartz cells of 1 cm in width. Accurately weighed quantity of 10 mg of Arsenazo lll was taken and transferred to 10 ml volumetric flask containing distilled water, and volume was made up to 10 ml. This solution was treated as a stock solution (A) (1000 µg/ml). 2 ml solution was withdrawn from stock solution A and diluted with water up to 100 ml to obtain the solution of concentration 20 µg/ml. This solution was treated as a stock solution (B). 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 ml aliquots were withdrawn from stock solution B and diluted with water up to 10 ml to obtain solutions of concentration 2, 4, 6, 8, 10, 12, 14, 16, 18 and 20 µg/ml. The absorbance of solutions was measured at λ_{max} of 560 nm. A calibration curve was plotted.

Analytical Methods for Determination of Accelerated Stability Study of Arsenazo lll:

UV-Visible Spectroscopic Method:

Effect of Temperature at 42 °C: Samples of 0-month, 1 month, 2 months, and 3 months of Batch 1, 2, 3 were checked using a double beam UV-Visible spectrophotometer (Shimadzu 1800) with matched quartz cells of 1 cm in width. Accurately weighed quantity of 2 ml of Calcium reagent was taken and transferred to 10 ml volumetric flask containing 2 ml water. This solution was treated as a stock solution (A) (1000 µg/ml). 1 ml solution was withdrawn from stock solution A and diluted with water up to 10 ml to obtain the solution of concentration 100 µg/ml. This solution was treated as a stock solution (B). Again 1 ml solution was withdrawn from stock solution B and diluted with water up to 10 ml to obtain a solution of concentration 10 µg/ml. The absorbance of solutions was measured at λ_{max} 560 nm.

Effect of Light: Samples of 0-month, 1 month, 2 months and 3 months of Batch 1, 2, and 3 were checked by the above UV-Visible spectroscopic method. The shelf life was calculated by using the following formula

$$t90 = 0.105 / k$$

Where, t90 = shelf life, k = reaction rate constant

In Use/ Open Vial Stability Study: Samples of 0-month, 1 month, 2 months and 3 months of Batch 1 was checked by the above UV-Visible spectroscopic method.

Biochemistry Analyzer Method:

Principle: Calcium reacts with a dye Arsenazo III at specific pH to form a bluish-purple-colored complex. The intensity of the color formed is directly proportional to the amount of calcium present in sample [15].

Ca^{++} + Arsenazo III ⟶ Ca-Arsenazo III complex (bluish colored)

Procedure: The quantities of reagents and serum/plasma given in **Table 1** were used. Mixed well and incubated at room temperature for exactly 5 min and measured the absorbance of the standard (Abs S) and absorbance of the test sample (Abs. T) against reagent blank (Abs. B), the standard assay parameters were given in **Table 2**.

TABLE1: PROCEDURE FOR CALCIUM REAGENT

Sample	Blank	Standard	Test
Calcium reagent	1000 µl	1000 µl	1000 µl
Calcium std (10 mg/dl)	-------	10 µl	--------
Serum/plasma	--------	-------	10 µl

Assay Parameters:

TABLE 2: ASSAY PARAMETER OF CALCIUM REAGENT

Sr. No.	Parameter	Condition
1.	Mode of reaction	Endpoint
2.	Slope of reaction	Increasing
3.	Wavelength	620 nm
4.	Normal high range	10.5
5.	Normal low range	8.8
6.	Temperature	R.T.
7.	Blanking	Reagent blank
8.	Reagent blank abs.	<0.6
9.	Standard conc.	10 mg/dl
10.	Unit	mg/dl
11.	Reading delay time	5 seconds
12.	Reaction time	5 minutes
13.	Working reagent volume	1000 ul
14.	Sample volume	10 ul
15.	High Linearity	18 mg/dl
16.	Low Linearity	0 mg/dl

Effect of Temperature at 42 °C: Reagents of Batch 1, 2, 3 were checked for its performance on the analyzer by using the above-mentioned method.

Effect of Light: Reagents of Batch 1, 2, 3 were checked for their performance on the analyzer by using the above-mentioned method.

pH Meter: pH of the samples of calcium reagent was measured at 0 months and 3 months for the actual time stability study. The pH meter was calibrated by using a buffer solution. For working of solutions lying between 7-14 pH, 7.00 pH buffer and 9.2 pH buffer used.

The first electrodes were immersed in the buffer for calibration one by one. pH function was selected and knob adjusted to read pH same as that of buffer in which electrode was dipped. Then the electrode is immersed in solution to be analyzed. In this way, pH of all samples were measured. By using above procedure, the following conditions were checked

Effect of Temperature at 42 °C: Effect of temperature for all the batches were checked by using above method on pH meter

Effect of Light: Effect of light for all the batches were checked by using above method on pH meter

RP-HPLC Method:

Preparation of Sample Solution of Arsenazo III: For HPLC analysis, pure chemical Arsenazo III (1 mg) was dissolved in 10 ml of distilled water to form a solution of concentration 100 µg/ml. This solution was filtered and diluted up to 0.2 µl.

Mobile Phase for HPLC Analysis: The mobile phase consisted of Acetonitrile: water (4:1) in isocratic mode with a flow rate of 0.5 ml/min, the detection was at 560 nm, all the data acquisition and post-run analysis were performed by Lab solution software from Shimadzu, Koyoto, Japan. The validation parameters consisted of linearity range, precision, accuracy, and limits of detection and quantification.

The peaks were identified by their retention time, comparing the UV-visible spectra and spiking with standards. Qualitative analysis was done by using a retention time of samples with a comparison of standard Arsanazo III. The linearity range was evaluated by plotting the peak area corresponding to the analyte as a function of concentration introduced. By using above procedure, the effect of temperature at normal and 42 ºC was checked.

HPTLC Method:

Selection of Stationary Phase: Development of standard and isolated CPT was performed on (10 cm × 10 cm, layer thickness 0.2 mm, E- Merk, Darmstadt, Germany) aluminum backed silica gel 60 F254 TLC plates.

Selection of Mobile Phase for Arsenazo III (Calcium reagent): To select a suitable mobile phase, initial experiments were carried out on glass TLC plates in saturated chambers. The solvent system composed of Acetonitrile: Water (4:1 v/v) was used. The above procedure is used for checking the effect of temperature at 42 ºC.

RESULTS AND DISCUSSION:

Batches for Accelerated Stability Studies: All the three batches and samples from respective batches were successfully selected kept at accelerated conditions like temperature, light, and pH. All the batches produce repeatable results with different analytical techniques.

λ_{max} and Calibration Curve for Arsenazo III: The UV-Visible spectrum of pure Arsenazo III is shown in **Fig. 2,** which shows maximum absorbance at 560 nm. So we have selected λ_{max} 560 nm for Arsenazo III.

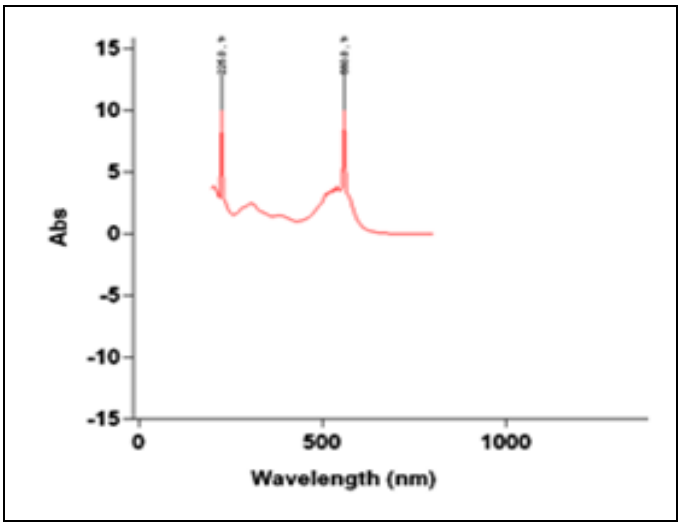

FIG. 2: ABSORBANCE OF ARSENAZO III

The graph of absorbance *vs.* concentration for pure Arsenazo III was found to be linear **Fig. 3** in the concentration range 1-10 µg/ml at 560 nm. The drug obeys Beer-Lamberts law in the range 1-10 µg/ml. Standard calibration curve and value of R^2 of Arsenazo III in water.

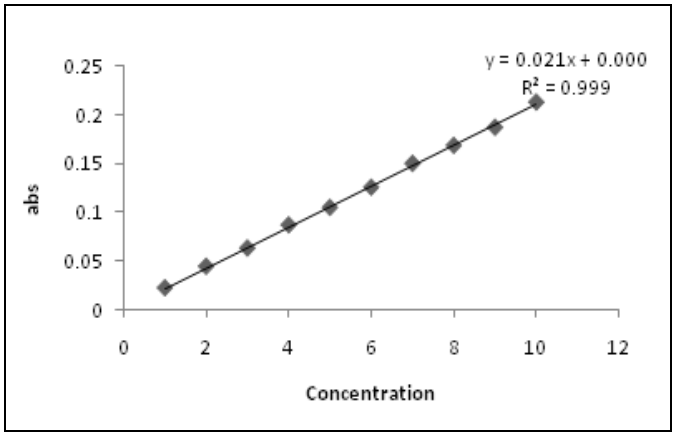

FIG. 3: STANDARD CALIBRATION CURVE FOR ARSENAZO III

UV-Visible Spectroscopic Method:

Effect of Temperature at 42 °C: The Accelerated stability study was performed to check the effect of temperature at 42 °C, which showed that calcium reagent (Arsenazo III) produces 90.81% remains undecomposed for 3 months by UV spectrophotometer.

The results for all three batches are given in **Table 3**, and the comparison in **Fig. 4**, which indicates an increase in % log decomposition for all the batches after 3 months.

TABLE 3: SHOWING % ARSENAZO III REMAIN UNDECOMPOSED AND LOG % ARSENAZO IIIREMAIN UNDECOMPOSED AT 42°C OF BATCH 1, 2 AND 3

Time in Months	Bacth 1			Batch 2			Batch 3		
	Abs.	% Arsenazo remain undecomposed	Log % Arsenazo III remain undecomposed	Abs.	% Arsenazo III remain undecomposed	Log % Arsenazo III remain undecomposed	Abs.	% Arsenazo III remain undecomposed	Log % Arsenazo III remain undecomposed
00	0.013	100	2	0.014	100	2	0.014	100	2
11	0.013	98.46	1.99	0.013	98.64	1.99	0.014	97.97	1.992
22	0.013	94.93	1.977	0.013	94.29	1.97	0.013	95.21	1.978
33	0.012	93.4	1.97	0.012	89.33	1.95	0.013	89.71	1.952

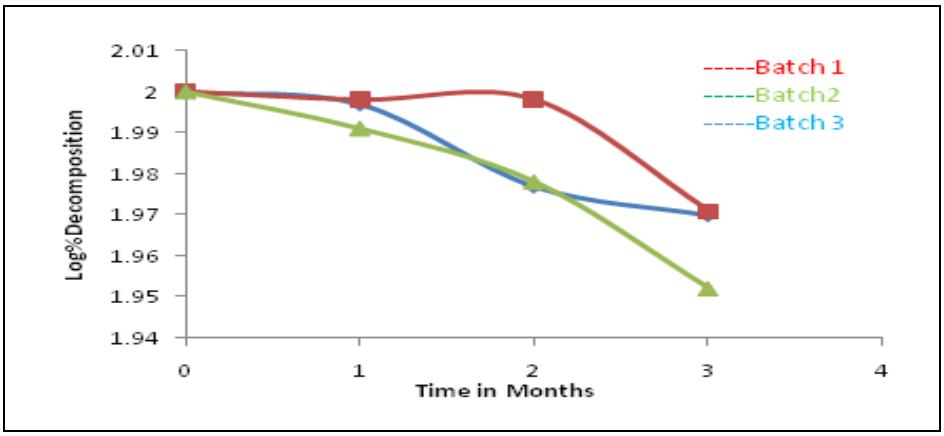

FIG. 4: LOG % DECOMPOSITION *vs.* TIME IN MONTHS OF CALCIUM REAGENT SHOWING EFFECT OF TEMPERATURE BY USING UV SPECTROPHOTOMETER

Effect of Light: In an accelerated stability study of the effect of light on Arsenazo III produces 91.25 % remains undecomposed in 3 months by UV spectrophotometer. The Shelf life of Arsenazo III was found to be 85.36. The results are shown in **Table 4** and **Fig. 5,** which show a gradual decrease in the log of % decomposition from 2 to 1.96 as time moves from 0 to 3 months.

TABLE 4: SHOWING % ARSENAZO III REMAIN UNDECOMPOSED AND LOG % ARSENAZO III REMAIN UNDECOMPOSED OF BATCH 1 (EFFECT OF LIGHT)

Time in month	Measured absorbance	Arsenazo III remain undecomposed	Percentage Arsenazo III remain undecomposed	Log percentage Arsenazo III remain undecomposed
0	0.0137	0.652	100	2
1	0.0135	0.642	98.47	1.993
2	0.0128	0.609	93.4	1.97
3	0.0125	0.595	91.25	1.96

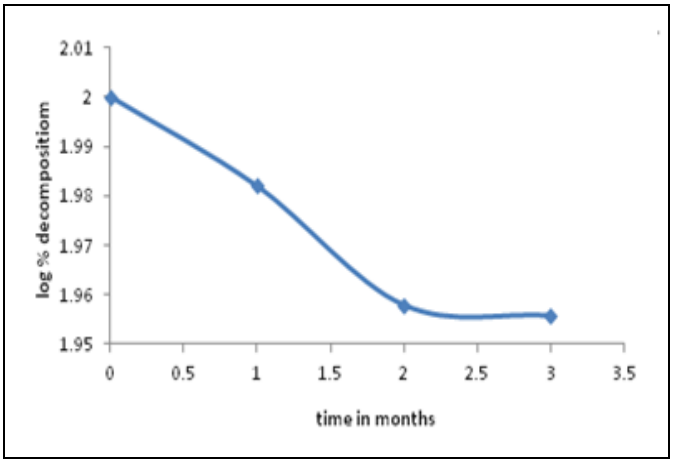

FIG. 5: LOG % DECOMPOSITION *vs.* TIME IN MONTHS OF CALCIUM REAGENT SHOWING EFFECT OF LIGHT BY USING UV SPECTRO-PHOTOMETER

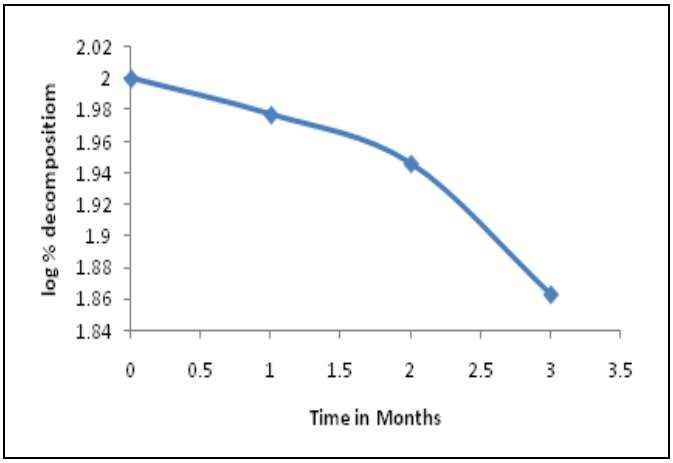

FIG. 6: LOG % DECOMPOSITION *vs.* TIME IN MONTHS OF CALCIUM REAGENT SHOWING OPEN VIAL STABILITY BY USING UV SPECTROPHOTOMETER

In Use/Open Vial Stability Study: Open vial stability study of Arsenazo III produces 73.00% remains undecomposed in 3 months by UV spectrophotometer. The results are shown in **Table 5** and **Fig. 6**.

TABLE 5: SHOWING % ARSENAZO III REMAIN UNDECOMPOSED AND LOG % ARSENAZO III REMAIN UNDECOMPOSED IN OPEN VIAL STABILITY OF BATCH 1

Time in month	Measured absorbance	Arsenazo III remain undecomposed	Percentage Arsenazo III remain undecomposed	Log percentage Arsenazo III remain undecomposed
0	0.0137	0.652	100	2
1	0.0130	0.619	94.93	1.977
2	0.0121	0.576	88.34	1.946
3	0.0100	0.476	73.00	1.863

Biochemistry Analyzer Method:

Effect of Temperature at 42 °C and Light: In accelerated stability studies showing the effect of temperature and light, there was an increase in the linearity of Arsenazo III from 17.4 mg/dl to 18.63 mg/dl and 17.92 to 19.3 respectively. This is because of the increase in reagent blank absorbance, which indicates small degradation in Arsenazo III. The results are shown in **Table 6** and **7**.

TABLE 6: SHOWING SERUM CONTROL READING AT 42 °C OF BATCH NO.3

Period	RB	Factor	Normal mg/dl	Abnormal mg/dl	Linearity mg/dl
0 Month	0.455	25.45	9.15	12.15	17.4
1 Month	0.465	25.6	9.81	12.34	17.91
2 Month	0.481	27.4	10.31	12.56	18.18
3 Month	0.53	28.51	10.4	13.15	18.63

TABLE 7: SHOWING SERUM CONTROL READING BATCH NO. 1 (EFFECT OF LIGHT)

Period	RB	Factor	Normal m/dl	Abnormal mg/dl	Linearity mg/dl
0 Month	0.479	24.31	9.37	12.18	17.92
1 Month	0.49	25.5	9.85	12.43	18.37
2 Month	0.53	26.1	10.1	13.11	18.8
3 Month	0.541	27.23	10.8	13.91	19.3

TABLE 8: SHOWING CHANGE IN pH OF ARSENAZO III 42 °C

Period	Batch 1	Batch 2	Batch 3
0 Month	7.07	7.17	7.06
1 Month	7.17	7.2	7.11
2 Month	7.25	7.31	7.29
3 Month	7.21	7.35	7.32

TABLE 9: SHOWING CHANGE IN pH OF ARSENAZO III

Period	Batch 1 (pH)
0 Month	7.07
1 Month	7.18
2 Month	7.2
3 Month	7.25

pH Meter Analysis:

Effect of temperature at 42 ºC: There was a slight change in pH of the reagent in accelerated stability studies showing temperature and light affects pH. The results are shown in **Tables 8** and **9**.

HPLC:

Effect of Temperature at 42 ºC: The HPLC chromatogram of pure Arsenazo III shows a retention time of 0.869. The peak height (209323) and percent area (100%) are shown in **Fig. 7** and **Table 10**.

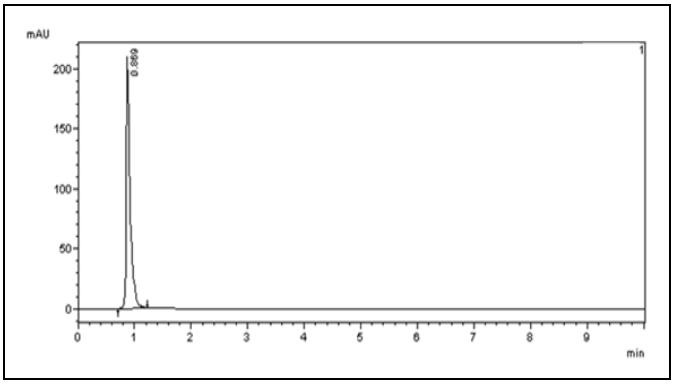

FIG. 7: HPLC SPECTRA OF PURE ARSENAZO III AT NORMAL TEMPERATURE

TABLE 10: HPLC ANALYSIS OF PURE ARSENAZO III AT NORMAL TEMPERATURE

Name	Ret. Time	Conc.	Channel	Height	Peak Start	Peak End	Area %	Area
RT0.869	0.869	100	Ch1 562 nm	209326	0.704	1.227	100	977309

Accelerated stability study of Arsenazo III at 42 °C by HPLC produces a decrease in retention time 0.869 to 0.606, peak height 209323 to 22385, and percent area from 100% to 19.59 % showing **Fig. 8** and **Table 11**. There was degradation reported as compared to pure Arsenazo III by using HPLC. It indicates that at high temperatures, more degradation occurred.

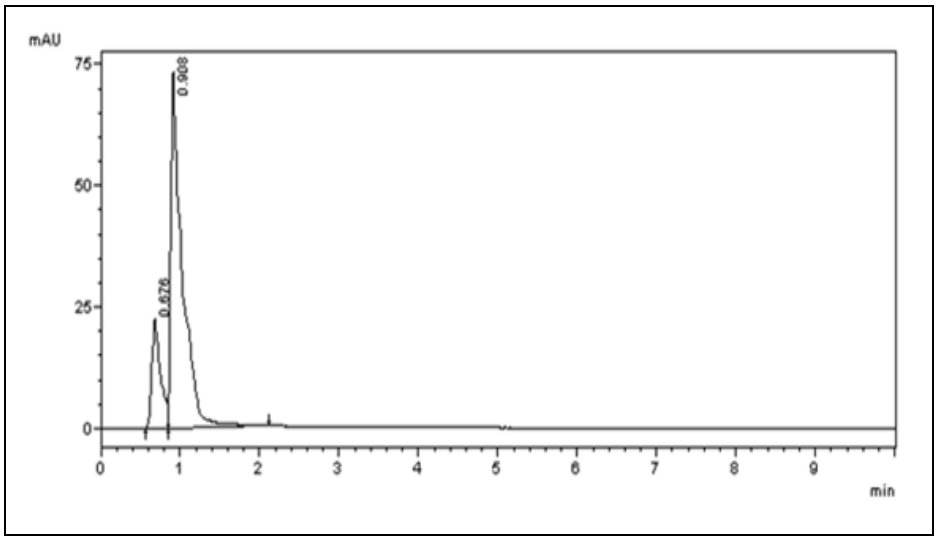

FIG. 8: HPLC SPECTRA OF 3 MONTHS SAMPLE OF CALCIUM REAGENT AT 42 °C

TABLE 11: HPLC ANALYSIS OF 3 MONTHS SAMPLE OF CALCIUM REAGENT AT 42 °C

Name	Ret. Time	Conc.	Channel	Height	Peak Start	Peak End	Area %	Area
RT0.676	0.606	19.5945	Ch1 562nm	22385	0.555	0.853	19.5945	175402
RT0.908	0.908	80.4055	Ch1 562nm	73245	0.853	2.123	80.4055	719757

HPTLC:

Effect of Temperature at 42 ºC: The HPTLC chromatograph of pure Arsenazo III at normal temperature produces peak area 9852 and % decrease in area 100 % **Fig. 9** and **10**. In the accelerated stability of Calcium reagent (Arsenazo III) at 42 °C by HPTLC, a decrease in peak area was observed. Percent peak area was decreased from 100% to 50.76 %.

It indicates degradation occurred more at high temperatures. The results are tabulated in **Table 12**.

The HPTLC chromatogram of Arsenazo III at 42 ºC for 3 months is shown in **Fig. 11**, which indicates a decrease in the area of separation.

The comparative decrease in peak area and height is shown in **Fig. 12**.

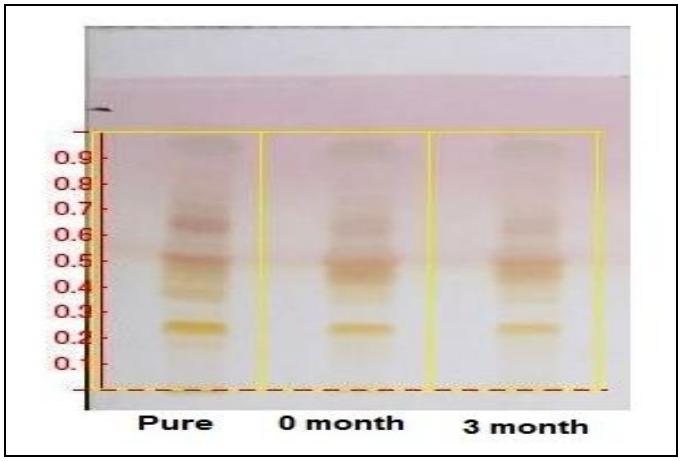

FIG. 9: HPTLC CHROMATOHRAPH OF PURE ARSENAZO III

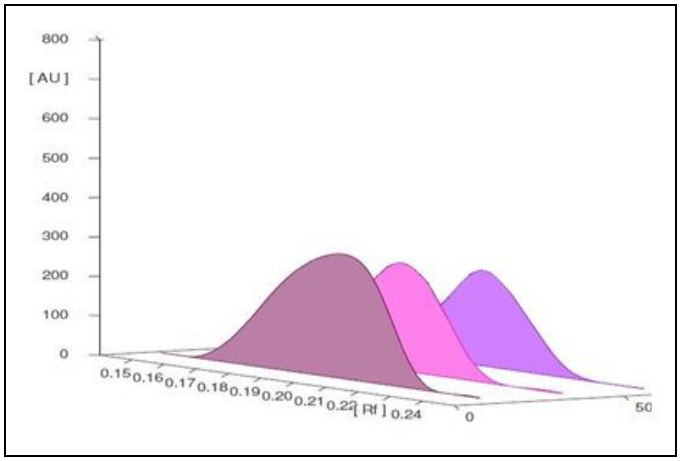

FIG. 10: GRAPHICAL REPRESENTATION OF HPTLC CHROMATOGRAPH OF PURE ARSENAZO III

TABLE 12: AVERAGE VALUES OF THE ARSENAZO III PEAK AREA OBTAINED IN THE ACCELERATED STABILITY STUDY AFTER 3 MONTHS AT 42 ºC

Time	Start Rf	End Rf	Peak Area	% Decrease in Area
Pure Arsenazo III	0.20	0.24	9852	100
1 Months	0.20	0.24	7239	73.47
3 Months	0.20	0.24	5001	50.76

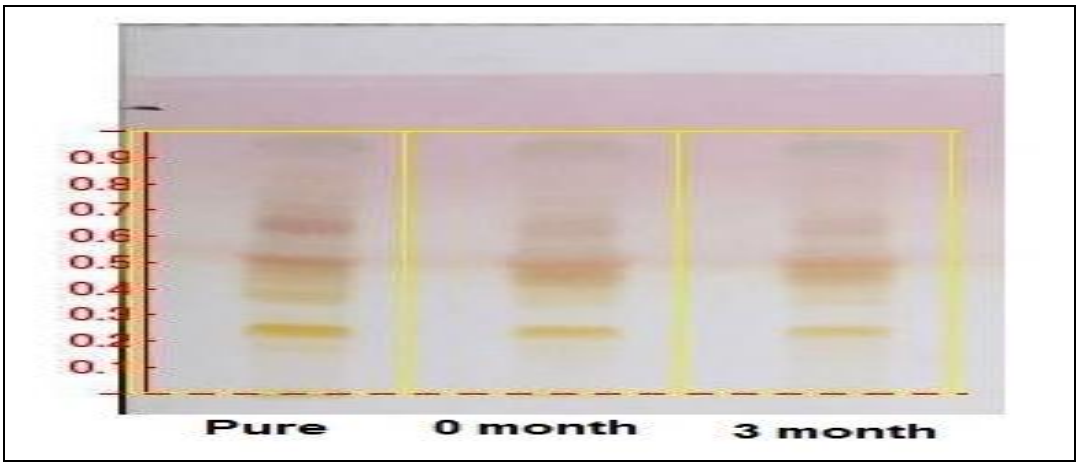

FIG. 11: HPTLCCHROMATOGRAPH OF ARSENAZO III AT 42 ºC FOR 3 MONTH

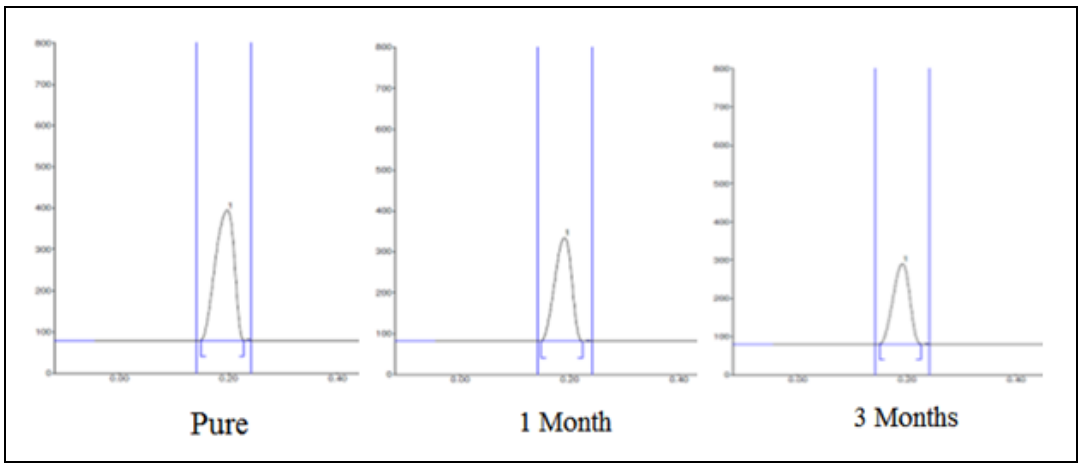

FIG. 12: COMPARISON OF ARSENAZO III PEAKS FOR PURE, 1 MONTHS AND 3 MONTHS AT 42 ºC

CONCLUSION: Accelerated stability study was successfully performed for *in-vitro* diagnostic reagent, Arsenazo III. UV-visible spectroscopy, Biochemistry analyzer, pH, HPLC, and HPTLC

techniques were successfully utilized for checking the degradation in Arsenazo III at different conditions.

The HPLC and HPTLC data showed degradation occurs in Arsenazo III at 42 ºC in three months storage sample. This novel approach of using different analytical techniques can be used to check the stability of in-vitro diagnostic kits and other medical aids.

Ethical Approval is not required for this manuscript

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CONFLICTS OF INTEREST: Not any.

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