IOSR Journal Of Pharmacy And Biological Sciences (IOSR-JPBS)
e-ISSN:2278-3008, p-ISSN:2319-7676. Volume 19, Issue 3 Ser. 2 (May. – June. 2024), PP 16-21
www.Iosrjournals.Org

Validation Of Calcium Analysis Method In High Calcium Milk Powder By Uv-Visible Spectrophotometry

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Abstract
Background*: Milk is a source of calcium and phosphorus which are very important for bone formation. In general, powdered milk high in calcium often adds macro minerals such as calcium to prevent osteoporosis. To overcome the weakness of titration method, the development of an analytical method for determining calcium levels in high-calcium powdered milk is very necessary. The method that can be used is the UV-Vis spectrophotometric method.*
Materials and Methods *Samples of high-calcium powdered milk were subjected to dry digestion using 6 N of nitric acid. The digestion filtrate was neutralized with 0.1N NaOH. Next, it is reacted with murexide to form a calcium-murexide complex compound which is reddish purple. This complex compound produces maximum absorption at a wavelength of 532 nm.*
Results *Validation of the analytical method of calcium ion in high calcium milk powder has met the validation criteria that linearity with r = 0.996 value, limit of detection (LoD) 0.0048µg/mL, limit of quantitative (LoQ) 0.0163µg/mL, intraday precision value < 2% and accuracy value of 90-120%. Calcium content in the AL sample was 0.189%, the PR sample was 0.146% and the HL sample was 0.200%*
Conclusion: *The method for determining calcium levels using the UV-visible spectrophotometric method is a valid and reliable method that can be used to determine calcium levels in high-calcium powdered milk.*
Keywords: *calcium, UV-visible spectrophotometry, validation*

Date of Submission: 09-06-2024 Date of Acceptance: 19-06-2024

I. Introduction

Calcium is the mineral most needed in the body, namely 1.5-2% of an adult's body weight or approximately 1 kg. Bone density varies with age, increasing in the first part of life and decreasing gradually after adulthood. Due to lack of calcium during growth, it can cause growth disorders, bones are less strong, bend easily and become brittle. Everyone, especially after the age of 30, loses calcium from their bones[1].

The best source of calcium is milk and processed products such as yoghurt, ice cream, cheese; fish eaten with bones such as anchovies, sardines and shellfish; nuts and processed products such as tofu and tempeh; fruit and vegetables such as broccoli, kale, mustard greens, spinach, cassava and seaweed. Most experts believe the body needs about 1 gram (1000 mg) of calcium every day. Half a liter of milk (± 2 glasses) can fulfill three-quarters of this amount[2].

Determination of calcium levels in the Indonesian Pharmacopoeia Edition IV is carried out using the complexometric method. Complexometric titration is a titration method based on the formation of complexes between cations and complex-forming substances. Determination of calcium levels in goat's milk and cow's milk using the complexometric titration method was carried out by Mirna (2009)[3] .This titration method is less effective because it takes a long time, is less sensitive, and is less specific in its implementation [3, 4, 5].

Previous research has examined calcium levels in powdered milk using the Atomic Absorption Spectrophotometry (AAS) method. Based on this, it is necessary to develop and select alternative methods that are better and more appropriate. Validation of analytical methods is also carried out, because method validation is usually intended for analytical methods that are newly created and developed[6]. Therefore, research was carried out using the UV-Visible Spectrophotometry method to analyze calcium from high-calcium powdered milk with murexid reagent which will form a reddish purple complex. Validation tests on standards and samples are also carried out to determine the detection limit, level of accuracy and precision for metals.

II. Material And Methods

Chemicals, reagents and samples

The ingredients used are hydrochloric acid, sulfuric acid, 65% nitric acid, calcium standard $CaCl_2.2H_2O$, 96% ethanol, murexide, 0.1N NaOH and distilled water (all grade p.a).

Equipment and apparatus

UV-Visible Spectrophotometer (T70 Spectrophotometer), furnace, hot plate, digital balance, funnel, 10 mL volumetric flask, 25 mL volumetric flask, 50 mL measuring flask, 100 mL measuring flask, 250 mL glass beaker, 500 mL glass beaker, pipette drops, volume pipettes, spatula, watch glass and measuring cup

Preparation of Reagents and Standard Solutions[7].

1. Preparation of Calcium Stock Solution
   0.03675 g of CaCl2.2H2O was weighed and diluted with distilled water to 100 mL, to obtain a concentration of 100 ppm. Then 1 mL of the solution was taken and diluted with distilled water to 100 mL to obtain a calcium standard of 1 ppm.
2. Preparation of 0.1 N NaOH Solution
   A 0.1 N NaOH solution was made by weighing 0.4 grams of NaOH dissolved in distilled water to a volume of 100 mL.
3. Preparation of 0.5% murexide solution
   Weighed 50.0 mg of Murexide, and dissolved it in 10 mL of distilled water, to obtain a Murexid solution with a concentration of 0.5%. Add 25.0 mL of 96% ethanol to the murexid solution.

Sampling

Samples of three brands of high calcium powdered milk were purchased from a shopping centre in the city of Padang.

Sample Preparation Using Dry Digestion

A total of 10 g of powdered milk was put into an evaporator cup, then the sample was heated at 600°C for 2 hours. To obtain white ash, add 10 mL of HNO_3 6N and heat it on a hot plate until the solution is clearer. Filter with Whatman paper No.42. Put the filtrate into a 50 mL measuring flask, and dilute with distilled water to the mark[8].

Qualitative Test of Calcium in Samples[9].

Into 2 test tubes, put 1 mL of the digested sample into each tube, then:

a.In test tube number 1 add a few drops of ammonia, there is no precipitate because the calcium hydroxide dissolves quite a lot. With precipitating agents that have been made for a long time, turbidity may occur due to the formation of calcium carbonate.

b. In test tube number 2, add a few drops of dilute sulfuric acid and a white precipitate of calcium sulfate will form.

Determination of the Maximum Absorption Wavelength of the Complex (Ca 2+(Mu-)2) [(7).]

1 mL of calcium solution with a concentration of 1 ppm was taken and put into a 50 ml measuring flask and 1.0 mL of murexide was added, 2.0 mL of 0.1 N NaOH (check pH 12-13), then distilled water was added until the volume was 50 mL. The solution was shaken until homogeneous, and then the absorbance was read at wavelengths between 400 – 800 nm.

Method Validation [(10,11).]

Linearity test

1.0; 1.5; 2; 2.5; and 3 mL of 1 ppm calcium solution were taken and each was put into a 50 mL measuring flask, 1.0 mL of murexide solution, and 2.0 mL of 0.1 N NaOH were added, increasing the volume with distilled water to the mark. calcium standard solution series with concentrations of 0.02: 0.03: 0.04: 0.05; 0.06 ppm was obtained. The solution was shaken until homogeneous and then the absorbance was read at the maximum absorption wavelength.

Linearity test using a calibration curve, a relationship was made between concentration and absorbance. The correlation coefficient (r) value obtained from the linear regression equation shows its linearity. A good linearity value is $0.99 \leq r \leq 1$[12].

Limit of Detection (LoD) and Limit of Quantitation (LoQ) test

This test is carried out by measuring the lowest standard concentration whose absorbance can be detected.
LoD can be calculated with the formula:

$$LoD = \frac{3\ x\ SD^{X}/_{Y}}{Slope}$$

LoQ can be calculated using the formula:

$$LoQ = \frac{10\ x\ SD^{X}/_{Y}}{Slope}$$

SD = standard deviation
LOD = Limit of Detection
LOQ = Limit of Quantitation

Precision Test

2 mL of 1 ppm calcium standard solution was taken and put into a 50 mL measuring flask, 1 mL of murexide solution and 2 mL of 0.1N NaOH were added (check pH 12-13), then add distilled water to 50 mL, so that the calcium concentration was 0.04 µg/mL was obtained. The solution was shaken until homogeneous and then the absorbance was read. This precision test was carried out 6 times.

Accuracy Test

4 mL, 8 mL and 12 mL of 1000 µg/mL $CaCl_2.2H_2O$ standard calcium solution were added to high calcium powdered milk and then dry digestion was carried out. Repeat three times. 1 mL of the filtrate resulting from digestion was taken and put into a 10 mL volumetric flask, neutralized with 0.1N NaOH, add distilled water to the mark. 0.02 mL of the solution was taken and put into a 25 mL volumetric flask and distilled water was added to the mark. The solution was shaken and then the absorbance was measured using a UV-Vis spectrophotometer at a wavelength of 532 nm. This treatment was carried out three times. Accuracy is expressed as the percent recovery of the added analyte.

Determination of calcium levels from samples

1 ml of dry digestion filtrate was taken and then neutralized with 0.1 N NaOH. The volume of the solution was made up to 10 mL with distilled water. 0.02 mL of the solution was taken and put into a 25 mL measuring flask, then 1 mL of murexide solution and 2 mL of 0.1N NaOH were added. The volume of the solution is added with distilled water up to the limit mark. The solution is shaken until homogeneous then the absorbance is read at the maximum absorption wavelength.

III. Result

Table no 1 shows the identification results in samples that were reacted with ammonia did not form a precipitate, with sulfuric acid a white precipitate was formed.

Table no 1: Qualitative Test of Calcium in Samples

No.	Inspection	Condition	Observation
1	The sample was added with ammonia solution .	No precipitate is formed	No precipitate is formed
2	The sample was added with a dilute sulfuric acid solution $Ca^{2+} + SO_4^{2-} \rightarrow CaSO_4\downarrow$	White precipitate of calcium sulfate	White precipitate of calcium sulfate

Figure no 1 shows the result of determining the maximum absorption wavelength of the calcium-murexide complex measured in the wavelength range 400-800 nm was 532 nm with an absorbance of 0.382.

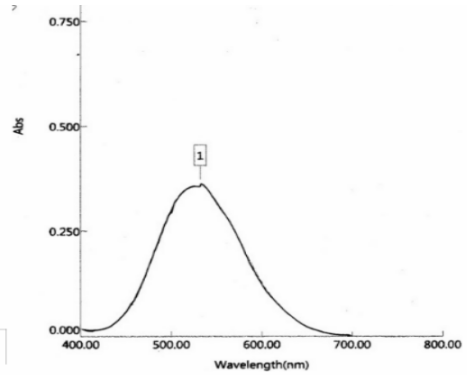

Figure no 1. Absorption spectrum of the complex (Ca 2+(Mu-)2) at a wavelength of 532 nm

Figure no,2 shows the absorbance reading results from the calibration curve at concentrations of 0.02 µg/mL, 0.03 µg/mL, 0.04 µg/mL, 0.05 µg/mL, and 0.06 µg/mL were 0.382, 0.451, 0.545, 0.636 and 0.688. The correlation coefficient (r) is 0.996, regression coefficient b= 7.97, a= 0.2216

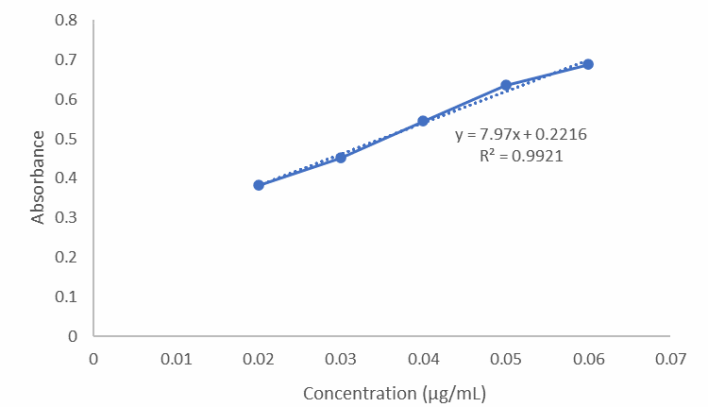

Figure no 2: Calibration Curve of The Calcium-Murexide Complex at λ 532 nm

Table no 2 shows the results of calcium levels in high calcium powder milk brand AL 0.189%; PR 0.146% and HL 0.200% .

Table no 2: Results of Determining Calcium Levels in High Calcium Milk

Sample	No.	Absorbance	C (µg/mL)	Cs (mg/g)	Cs (%)	Cs (Average w/w) ± SD (%)
AL	1	0.466	0.0304	1.9000	0.1900	0.189 ± 0.01575
	2	0.464	0.0306	1.9125	0.19125	
	3	0.462	0.0301	1.8812	0.18812	
PR	1	0.409	0.0235	1.4684	0.14684	0.146± 0.00704
	2	0.409	0.0235	1.4684	0.14684	
	3	0.408	0.0233	1.4562	0.14562	
HL	1	0.479	0.0322	2.0125	0.20125	0.200 ± 0.00962
	2	0.477	0.0320	2.000	0.20000	
	3	0.476	0.0319	1.9937	0.19937	

Note: C = sample concentration (µg/mL)
Cs = calcium content in the sample (mg/g)
= average calcium level (mg/g)
SD = standard deviation

Table no 3 show the intraday precision test results on a standard solution of 0.04 µg/mL, the SD value is 0.0014 and the CV value is 0.25%.

Table no 3: Determining Intraday Precision

No.	C	Absorbance	Average	SD	CV (%)
1.	0.04 µg/mL	0.545	0.543	0.0014	0.25%
2.		0.545			
3.		0.544			
4.		0.543			
5.		0.542			
6.		0.542			

Table no 4 show the accuracy test results on the addition of 40%, 80% and 120% standard calcium solution obtained % recovery of 117%; 109.37%, and 98.95%

Table no 4: Determination of Accuracy (% Recovery)

Addition of 1000 ppm standard solution	Absorbance	Average	% Recovery
40 %	0.601	0.599	117%
	0.599		

	0.599		
80 %	0.724	0.723	109.37%
	0.723		
	0.723		
120 %	0.809	0.809	98.95%
	0.809		
	0.809		

Table no 5 shows the results of validation of analytical methods

Table no 5: Validation of Analytical Methods

Linearity	
Correlation coefficient	r = 0.996
Intercept	a = 0.2216
Slopes	b = 7.97
Regression equation	y = 0.2216+ 7.97x
Detection limit	0.0048 µg/mL
Quantitation limit	0.0163µg/mL
Precision	0.25%
Accuracy	40% = 117%
	80% = 109.37%
	120% = 98.95%

IV. Discussion

The samples used in this research were high calcium powdered milk from three brands AL, PR and HL. This sample was chosen because calcium is the mineral most needed in the body, and the best source of calcium is milk and its processed products, and milk also has an important role in bone formation. Human bones experience turning over, namely continuous decay and formation. At a young age, bone formation occurs more intensively than resorption. Meanwhile, in old age, resorption occurs faster than formation. To prevent bone loss, regular milk consumption is needed from an early age until old age[13]. Determination of calcium levels in high calcium powdered milk AL, PR and HL was carried out using the UV-Vis spectrophotometer method which began with sample preparation, namely dry digestion. The dry digestion method was chosen because this digestion usually does not require a lot of solvent.

Sample preparation was carried out by weighing 10 g of milk sample. In the dry digestion method, the sample that has been weighed in an evaporator cup is placed in a furnace at a temperature of 600 °C for 2 hours, white ash is obtained, then 10 ml of HNO_3 6N was added. HNO_3 is used as a solvent because HNO_3 is a strong oxidizing agent that can dissolve almost all metals and can prevent the precipitation of elements. At the beginning of adding HNO_3 6N, the solution was cloudy yellow but after being heated for a few minutes the solution turned clear yellow. This indicates that calcium ions have come out of the sample matrix. The reaction equation that occurs can be seen in the following reaction equation.

$$Ca^{2+}_{(aq)} + 3\ HNO_{3(aq)} \longrightarrow Ca^{2+}_{(aq)} + 3\ NO_{3(aq)} + 3\ H^{+}_{(aq)}$$

The filtrate produced in dry digestion is yellow. The filtrate that has been produced in the digestion process is then subjected to a qualitative test to calcium, several drops of the digestion sample are reacted with certain reagents, namely H_2SO_4 and ammonia. In AL milk, PR milk and HL milk with the addition of these two reagents gave positive results namely the formation of a white calcium sulfate precipitate.

The filtrate resulting from digestion was neutralized with 0.1N NaOH, the pH of the solution was adjusted to 12-13, because murexid can form a complex with calcium at alkaline pH. Next, the absorption of the calcium-murexide complex was measured using a UV-Vis spectrophotometer. Measurements were carried out in triplicate to get precise results. The sample measurement results obtained are then used to calculate the sample concentration and calcium levels measured from the sample. The calcium content in high calcium powder milk brands AL, PR and HL were 0.189%; 0.146% and 0.200%.

The first step that must be taken in measuring calcium is determining the maximum absorption wavelength. This is very important to know the level of sensitivity of a measurement. Wavelength determination was carried out using a UV-Vis spectrophotometer. The maximum wavelength is indicated by the wavelength

that has the greatest absorbance. The wavelength in this study was determined from the murexide calcium complex. This reaction can be seen in the reaction equation as follows.

$$Ca^{2+}_{(aq)} + 2\ NH_4C_8H_4N_5O_{6(aq)} \longrightarrow [Ca(NH_4C_8H_4N_5O_6)_2]^{2+}_{(aq)}$$

Determination of the maximum absorption wavelength of calcium murexide was carried out in the wavelength range 400–800 nm. The results show that the maximum absorption wavelength of the complex is at a wavelength of 532 nm. The calcium-murexide complex forms a reddish-purple color that absorbs at this wavelength.

The calibration curve was made by measuring the absorbance of the calcium-murexide complex at the maximum wavelength with variations in the concentration of the calcium standard solution of 0.02 µg/mL, 0.03 µg/mL, 0.04 µg/mL, 0.05 µg/mL, and 0.06 µg/mL. Then a calibration curve was created with the x-axis being the calcium concentration (µg/mL) and the y-axis being the absorbance. The calibration curve has a regression equation y = 0.2216 + 7.97x with a correlation coefficient (r) = 0.996. A correlation coefficient of 0.996 states that the test results are linear and there is a close correlation between concentration and absorbance.

Validation of the calcium analysis method in high calcium powdered milk AL, PR and HL was carried out on several parameters including: Linearity is an analytical method that must be tested to determine the existence of a linear relationship between substance levels and detector response[14]. Based on statistical calculations of the linear regression calibration curve, the linear regression equation was obtained: y = 0.2216 + 7.97x with a correlation coefficient (r) = 0.996. The correlation coefficient obtained shows linear results, because it meets the acceptance criteria, namely $0.99 \leq r \leq 1$, so that this method can be used for analysis with good results[15].

The detection limit (LOD) is the smallest level of a compound that can be analyzed which can provide a significant response. Meanwhile, the quantitation limit (LOQ) is the smallest number of compounds that can be analyzed[16]. From the test results, the detection limit was obtained at a concentration of 0.0048 µg/mL and the quantitation limit was at a concentration of 0.0163 µg/mL. Determination of the detection and quantitation limit values is very dependent on the value of b (slope of the line), where an ideal linear relationship is achieved if the value and r = 1 or r = -1 depending on the direction of the line. The analytical method is said to be less sensitive if b is negative so that it gives a larger LOD and LOQ[17]. The results obtained b have a positive value, namely 7.97, indicating that the LOD and LOQ values are significant.

The precision test was carried out by measuring the precision of a standard calcium solution of 0.04 ppm. Precision is carried out intraday, at a concentration of 0.04 ppm the CV value = 0.25%. Precision describes the closeness between the results of one test and another (Harahap, 2010). The precision test was carried out based on the concentration from the calibration curve, namely 0.04 ppm. In this precision test, the absorbance of each concentration is measured intraday. Next, the coefficient of variation (CV) is calculated. Intraday precision meets the precision test criteria with a CV value ≤ 1%, so it can be concluded that the method is quite precise.

Accuracy is a measure that shows the degree of closeness of the analysis results to the actual analyte levels. Accuracy is expressed as percent accuracy (% recovery value). Here the accuracy is only carried out on HL samples with the addition of 1000 ppm calcium solution of 40%, 80% and 120% to the sample. The treatment was carried out three times in repetition. The results obtained show that the % recovery of calcium with the addition of a 40% standard solution is 117%, at a percentage of 80% it is 109.37% and at a percentage of 120% it is 98.95%. These results meet the accuracy requirements where the requirement for % recovery is 80-120% [18].

V. Conclusion

Validation of the analytical method for determining calcium levels in powdered milk by forming a complex with murexid using the UV-Visible Spectrophotometry method meets the criteria of linearity, detection limit, quantitation limit, precision and accuracy. The calcium levels found in the three milk samples were brand AL = 0.189%, PR = 0.146% and HL = 0.200%.

References

[1] Almatsier S. Basic principles of nutritional science. Jakarta: Gramedia Pustaka Utama; 2001.
[2]. Compston J. Prevention and treatment of osteoporosis: clinical guidelines and new evidence. JR Coll Physicians Lond 2000;34:518-21
[3]. Mirna AS. Determination of calcium and magnesium levels in goat's milk and cow's milk using complexometry, Faculty of Pharmacy, University of North Sumatra, Medan. 2009.
[4]. Miefthawati NP, Gusrina L. Determination of calcium levels in fresh mackerel fish and salted mackerel fish using complexometry, Journal of Clinical Health Science Analysis.2013;I(1):1-9.
[5]. Toledo, M. Basics of Titration, Titration Theory, Schwerzenbach, Swizerland.2009.

[6]. Miller JC, and JN Miller. Statistics for analytical chemistry. 2nd ed., *t*ranslated by Drs. Suroso, M.Sc. Bandung: Institut Teknologi Bandung;1991.
[7]. Rahayu WS, Utami PI, Kurniawati A. Validation of determination of calcium levels in multivitamin tablets using the uv-vis spectrophotometer method. Jurnal Pharmacy.2011; 8(2): 34-41.
[8]. National Standardization Agency. Formula Milk Quality Requirements.S I 01-2970-2006,Jakarta; 2006.
[9]. Vogel AI. Vogel's textbook of macro and semi micro qualitative inorganic analysis London: Longman;1979.
[10]. Chavan SD, and Desai DM. Analytical method validation: A brief review. World Journal of Advanced Research and Reviews, 2022;16(02):389–402.
[11]. U.S. Department of Health and Human Services Food and Drug Administration. Analytical Procedures and Methods Validation for Drugs and Biologics. Silver Spring, MD; FDA:2015.
[12]. Gandjar IG, and Rohman A. Analytical pharmaceutical chemistry. Yogyakarta: Pustaka Pelajar;2007.
[13]. Givens DI. Milk symposium review: The importance of milk and dairy foods in the diets of infants, adolescents, pregnant women, adults, and the elderly, Journal of Dairy Science. 2020; 103 (11): 9681-9699,
[14]. Harahap Y. Sample Preparation. Jakarta:Departemen Farmasi UI; 2010.
[15]. Priyambono B. Industrial Pharmaceutical Management.Yogyakarta:Global Pustaka Utama; 2007.
[16]. European Medicines Agency.ICH Topic Q2(R1) Validation of Analytical Procedures:Text and Methodology. London: EMEA;1995
[17]. Ibrahim S. Use of Statistics in the validation of analytical methods and their application, Proceedings: National Scientific Meeting in the Pharmaceutical Science. Bandung; ITB:1997.
[18]. Carr GP, Wahlich JC. A practical approach to method validation in pharmaceutical analysis. J Pharm Biomed Anal. 1990;8(8-12):613-8.