JOURNAL OF NATURAL REMEDIES
DOI: 10.18311/jnr/2022/31332
RESEARCH ARTICLE

Development and Validation of Analytical Method for Estimation of Calcium in Herbomineral Formulation by Atomic Absorption Spectrophotometry

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Abstract

Calcium determination is normally done by conventional titrimetric methods (complexometric titration). In this study, a simple, precise, accurate, rapid and sensitive method has been developed for calcium (Ca) determination in Maxcal-C Tablet (herbo-mineral formulation) and its raw materials by Atomic Absorption Spectrophotometer (AAS). For complete digestion of the sample, closed vessel wet digestion technique was used with 69% HNO_3 and 30% H_2O_2 (7:1) as digestive solvents. Analysis was performed on AAS (AA-6300) at λmax 422.7 nm. Calcium nitrate $Ca(NO_3)_2$ was used as a standard for Calcium and the linearity of the standard was excellent over a concentration range of 0.5 to 20 μgmL^{-1}. The regression coefficient (r^2) was 0.999. For intraday and interday precision % RSD was obtained between 0.48%-1.95% and 3.48%-5.01%, respectively which complied with the standard range for AAS. Recovery was achieved at 93.24%, 99.73%, 101.75% for 3 levels of spikes (80%, 100%, 120%) respectively, which complied with the standard range 75- 125% for AAS. However, the AAS method produced accurate and reproducible results and also overcame the colour and other ions interference, which are common problems with the titrimetric method. The results of both techniques (the AAS method and the titrimetric method) were compared, which showed good estimation by AAS.

Keywords: AAS, *Bhasma,* Complexometric Titration, Maxcal-C, Wet Digestion

1. Introduction

Active components in herbal drugs, known as marker compounds or reference compounds, represent the quality and efficacy of the herbal drugs. In the case of herbal drugs, there are always hundreds of components, and many of them are in lower concentrations. Chromatography has been the most powerful separation technique for the separation of complex systems into many relatively simple subsystems. Furthermore, hyphenated chromatographic and spectroscopic approaches HPLC-DAD, GC-MS, HPLC-MS show greatly improved performances in separation and elimination of instrumental interferences. Generally, herbal formulations are derived from natural sources, so there is a presence of micronutrients and a chance of heavy metals in the final product. So, to confirm their presence and quality of the product (absence of heavy metals), a unique spectrophotometric technique, Atomic Absorption Spectrophotometry was used. The ease of operation and the speed of analysis, which can yield very accurate results, have made atomic absorption one of the most popular methods for the determination of metals in various samples. Every element has a specific number of electrons associated with its nucleus. The normal and most stable orbital configuration of an atom is known as the "ground state." If energy is applied to an atom, the energy

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Article Received on: 03.09.2021 Revised on: 19.10.2021 Accepted on: 02.02.2022

will be absorbed and an outer electron will be promoted to a less stable configuration known as the "excited state." Since this state is unstable, the atom will immediately return to the "ground state", releasing light energy.

Sufficient amounts of calcium are required for bone strength. The body also uses calcium for the heart, blood, muscles and nerves maintenance. Without the proper amount of calcium intake, the body will strip calcium from the bones where it is stored, causing the bones to get weaker. It is important to note that since the human body cannot produce its own calcium, adequate calcium intake is vital. Calcium supplementation is beneficial during childhood, pregnancy and lactation and is also useful in the treatment of osteoporosis and fracture healing. Maxcal-C, a herbo-mineral dosage form ensures optimum calcium availability during pregnancy, lactation and growing stages of children.

Herbo-mineral dosage form contains herbs and minerals, making it difficult to isolate or extract a particular component (calcium). For extracting the total calcium from the dosage form, a suitable analytical method is required for the estimation of calcium. A literature review reveals that till now so many works have been done for the determination of trace metals from different samples like composts, serum, insulin, urine, soil, water, etc., with the help of Atomic Absorption Spectrometry (AAS)[1-17]. But there is no reported method for estimation of calcium from herbo-mineral dosage form. Generally, calcium content is estimated by titrimetric assay (complexometric) method as per the Indian Pharmacopoeia[18]. It is estimated in the form of $CaCO_3$ and on the basis of that calcium content, it is theoretically determined. Calcium present other than in carbonate form cannot be estimated from the dosage form. While in the AAS method, the proper digestion method converts the metal to free form in solvent media by pre-treatment of the sample and in the AAS Air–Acetylene flame convert the metal into free atomic form and detects the total amount of the metal.

2. Materials and Methods

Maxcal-C was procured from Vasu Research Centre, A division of Vasu Healthcare Pvt. Ltd., Vadodara, India.

2.1 Selection of Measurement Condition for Calcium by AAS

Before the analysis on AAS, parameters were selected to analyze the calcium content in the formulation (Figure 1). The measurement conditions are as shown in Table 1.

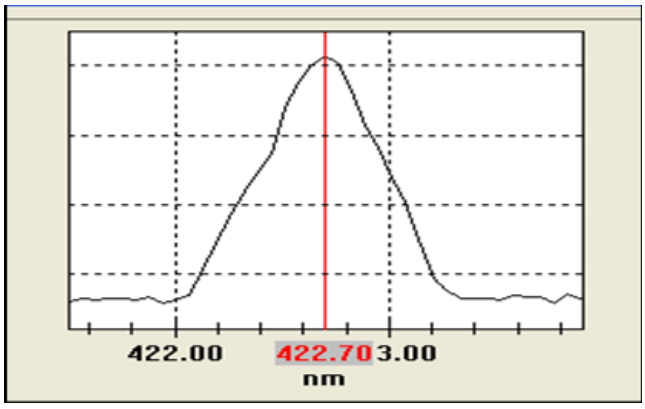

Figure 1. Calcium λ_{max} at 422.7nm.

Table 1. Measurement condition for Calcium

Element	Calcium
Wavelength	422.7 nm
Slit width	0.7 nm
Flame type	Air-Acetylene
Flow rate	2.0 mL/min
Burner height	7 mm

2.2 Preparation of Standard Solution

$Ca(NO_3)_2$ solution of 1000 μgmL^{-1} concentration was used as stock solution. An aliquot of the 1 mL stock solution (1000 μgmL^{-1}) was transferred to a 10 mL volumetric flask. Made up to the mark with distilled water to prepare a 100 μgmL^{-1} solution. This was used as a standard working solution. Aliquots of 0.5, 1.0, 1.5 and 2.0 mL of the working standard (100 μgmL^{-1}) were transferred into each 10 mL volumetric flask, respectively. Volume was made up to the mark with distilled water to form a solution containing 5, 10, 15, 20 μgmL^{-1}. Each dilution was aspirated sequentially into an AAS nebulizer, and absorbance was noted down and a linear curve was plotted (Figure 2).

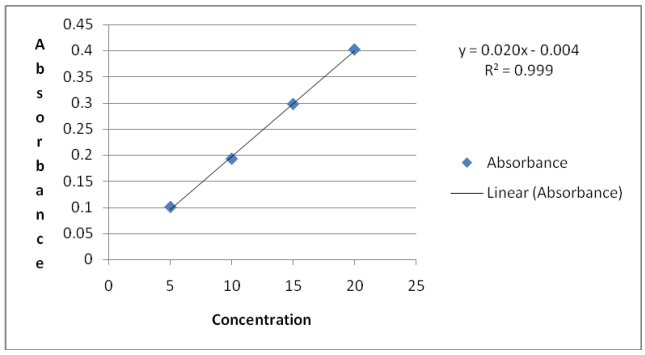

Figure 2. Calibration curve of the calcium standard.

2.3 Preparation of Sample Solution

A sample accurately weighing 0.1g of was transferred into the digestion vessel. Up to 8 mL of the required quantity of acids were added along with a blank. The material was digested in the digester as per the specified conditions (Table 2). After digestion, each vessel's content including blank, was transferred into a volumetric flask of 50 mL respectively and made up to the mark with distilled water. All samples prepared were at a concentration of 2000 μgmL^{-1}. Sample solutions were further diluted by adding 0.25 mL of solution to a 50 mL volumetric flask and making up to the mark using distilled water to form 10 μgmL^{-1} of each sample, including the blank.

2.4 Optimization of Acid Media

Calcium is present in complex form in *bhasma*. For complete extraction of the calcium from its complex form, different acids (HCl, HNO_3, HF, H_2O_2) were used in different ratios and combinations. Each acid has its own chemistry to react with the sample analyzed. Digested formulations with different acids were analyzed in AAS to get the proper recovery of calcium. The acid media in which calcium is completely extracted and stable in that acid media was finalized for further process.

Calcium carbonate ($CaCO_3$) was used as a reference material with a known amount of 40% calcium in each trial along with the formulation to confirm the stability of extracted calcium in digestive media.

3. Results and Discussion

HNO_3 digested the sample by oxidizing the material, and H_2O_2 was added as a supportive oxidizing agent. Moreover, it reoxidises the nitrous oxide and prevents the formation of yellow precipitates by yielding a colourless solution after digestion. Synergist the effects of HNO_3 and digest the material properly. The percentage of calcium in the sample and $CaCO_3$ are shown in Table 3.

Table 2. Digestion condition

Watt	%	Temperature	Ramp	Hold time
800 W	80	200°C	15 min.	10 min.
Acid used: 69% HNO_3 (7 mL) + 30% H_2O_2 (1 mL)				

Table 3. Percentage of calcium in sample and $CaCO_3$

Sample	Absorbance	Concentration μgmL^{-1}	Final Concentration μgmL^{-1}	% Calcium
$CaCO_3$	0.0710	2.9875	373443.4	37.34
Tablet	0.0544	2.5081	312889.1	31.28

3.1 Optimization of Acid Ratio

Once the proper acid media was selected, the proper ratio of combined acid media was set to get good recovery and stability of calcium with a minimum of interference (Table 4).

Table 4. Optimization of Acid ratio

Sample	Absorbance	Concentration μgmL^{-1}	Final Concentration μgmL^{-1}	% Calcium
$CaCO_3$	0.0761	3.4625	420210.8	42.02
Tablet	0.0506	2.3505	293816.2	29.38

3.2 Optimization of Digestion Condition

After optimization of the acid media and acid ratio, the digestion condition was optimized. The well-advanced wet acid, closed vessel method was used here for homogenous and complete digestion of the formulation. The effect of different digestion conditions on the complete solubilization and digestion of the material was noted. Different digestion conditions were tried by changing the temperature and power conditions. To confirm the digestion condition, a sample with $CaCO_3$ was spiked (0.1g of the sample was weighed in 3 digestion vessels. In 1st vessel, only a sample was added. In 2nd vessel, 0.1 mL of 1 μgmL^{-1} $CaCO_3$ solution was added and in 3rd vessel, 0.8 mL of 8 μgmL^{-1} $CaCO_3$ solution was added along with the sample) and digested (Table 5).

Table 5. Optimization of Digestion condition

Vessel	Absorbance	Concentration µgmL^{-1}	Final Concentration µgmL^{-1}	% Calcium	% Recovery
1	0.0557	3.0482	304519.1	30.45	0
2	0.0630	3.4477	344773.6	34.47	99.87
3	0.1208	6.6109	661089.8	66.10	111.32

3.3 Minimization of Ionization Interference

Ionization interference was removed by using an easily ionized moiety like potassium (K) or sodium salt (Na). Normally KCl, KOH or NaCl used but they are easily ionized compared to the interested elements that are to be detected and hence the metal was maintained in a unionized form to reduce the interference.

A sample accurately weighing 0.1g was transferred into the digestion vessel. Up to 8 mL of the required quantity of acids were added along with a blank. The vessels were tightly closed with cork. The material was digested as per optimized conditions. After digestion, each vessel's content including blank was transferred into a volumetric flask of 100 mL respectively and made up to the mark with distilled water. The Samples' solution was further diluted by adding 0.5 mL solution with 0.5 mL 1000 µgmL^{-1} KCl to a 50 mL volumetric flask and made up to the mark using distilled water (Table 5).

Table 5. Minimization of ionization interference

Sample	Absorbance	Concentration µgmL^{-1}	Final Concentration µgmL^{-1}	% Calcium
Tablet	0.0497	3.4221	341866.0	34.18
Tablet(KCl)	0.0528	3.6355	363189.7	36.31

Again, no significant change in calcium recovery was observed, indicating the absence of ionization interference.

3.4 Minimization of Chemical Interference

Phosphorous is present in most of the cases along with calcium, forms calcium phosphate, which is thermally stable and does not breakdown to prevent the formation of calcium in atom form[44]. Therefore, results were lower than expected. The problem can be solved in two ways. One can use a higher temperature flame, the N_2O-Air flame, which can break down thermo-stable compound, or use releasing agent like Lanthanum or EDTA moiety which form compound with calcium and release the calcium atom, and increase the opportunity for detecting the actual amount of calcium.

Accurately weighed 0.1g of sample and transferred it into a digestion vessel. Up to 8 mL of the required quantity of acids were added along with a blank. The vessels are tightly closed with a cork. The material was digested as per optimized conditions. After digestion, each vessel's content including blank, transferred into a volumetric flask of 100 mL respectively, and made up to the mark with distilled water. Samples solution was further diluted by taking 0.5 mL solution with 0.5 mL 1000 µgmL^{-1} $La(NO_3)_2$ to 50 mL volumetric flask and made up to the mark using distilled water (Table 6 and 7).

Table 6. Minimization of chemical interference (without Lanthanum)

Sample	Absorbance	Concentration µgmL^{-1}	Final Concentration µgmL^{-1}	% Calcium
Kapardika	0.0871	5.0742	505900.3	50.59
Muktasukti	0.0879	5.1208	509530.9	50.95
Kukkundatwak	0.0984	5.7325	574397.2	57.43
Godanti	0.0375	2.1846	218463.5	21.84
Shankh	0.0861	5.0159	501592.3	50.15
Tablet	0.0557	3.0482	304519.1	30.45

Table 7. Minimization of chemical interference (with Lanthanum)

Sample	Absorbance	Concentration µgmL^{-1}	Final Concentration µgmL^{-1}	% Calcium
Kapardika	0.1063	5.3424	532644.5	53.25
Muktasukti	0.0940	4.7243	470074.7	47.00
Kukkundatwak	0.1055	5.3022	531284.3	53.12
Godanti	0.0894	4.4931	449306.4	44.93
Shankh	0.0918	4.6137	461368.3	46.13
Tablet	0.0697	3.5030	349250.4	34.99

3.5 Method Validation

3.5.1 System Suitability

System suitability was performed to confirm the reliability and reproducibility of the instrument. For that standard calcium concentration, $Ca(NO_3)_2$ of 10 µgmL^{-1}, was prepared. The same concentration of solution was aspirated 5 times in the AAS, and absorbance was noted. % RSD was calculated from the results (Table 8).

Table 8. System suitability at 422.7 nm

Sr. no.	Concentration (µgmL^{-1})	Absorbance
1.	10	0.2445
2.	10	0.2429
3.	10	0.2450
4.	10	0.2507
5.	10	0.2504
Mean		0.2457
SD		0.0036
% RSD		1.4533

3.5.2 Linearity

$Ca(NO_3)_2$ solution of 1000 µgmL^{-1} concentration was used as stock solution. Aliquot of the 1mL stock solution (1000 µgmL^{-1}) was transferred to 10mL volumetric flask. Another 1 mL of 1000 µgmL^{-1} $La(NO_3)_2$ solution was added and the volume was made up to 10mL mark with distilled water to prepare 100 µgmL^{-1} solution. Aliquots of 0.05, 0.1, 0.2, 0.5, 1.0, 1.5, 2.0 mL of the working standard (100 µgmL^{-1}) were transferred into each 10mL volumetric flask, respectively. The volume was adjusted to the mark with double distilled water to give solutions containing 0.5-20 µgmL^{-1} (n=7). All standard solution absorbances were recorded using the above set condition for calcium in AAS. Absorbances were recorded at 422.7nm. Calibration curves were constructed by plotting absorbance versus concentrations. Straight line equations were obtained from this calibration curve (Figure 3 and 4, Table 9).

Table 9. Linearity of Calcium standard at 422.7 nm

Concentration (µgmL^{-1})	Absorbance
0.5	0.0190
1.0	0.0274
2.0	0.0524
5.0	0.1389
10.0	0.2689
15.0	0.3881
20.0	0.5076

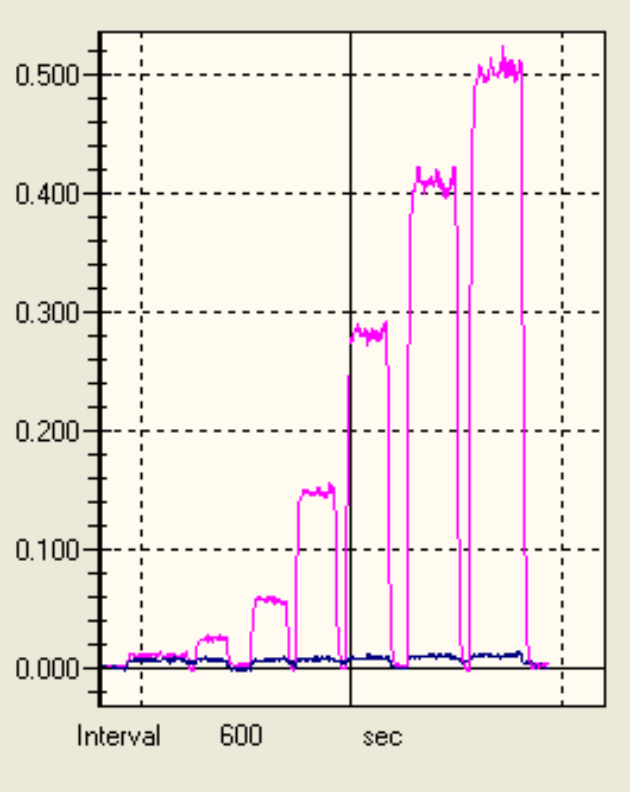

Figure 3. Linearity of Calcium standard.

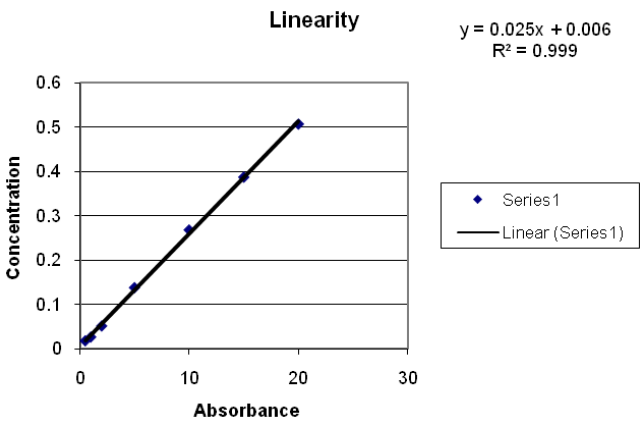

Figure 4. Calibration curve for Calcium Standard.

3.5.3 Specificity

To perform this parameter, the tablet formulation was digested and the total % calcium was calculated. Then each ingredient of the formulation was digested individually following the same procedure as in the formulation and the % calcium was calculated as per they were used in the formulation. Results were compared to confirm that the method was specific for the determination of calcium in formulations (Table 10).

Table 10. Specificity study for formulation at 422.7 nm

Sample	Absorbance	Concentration µgmL^{-1}	% Calcium	Mean ± S.D.	% Calcium calculated per tablet*
Godanti bhasma	0.0897	4.5081	45.08	44.91±0.43	12.62
	0.0884	4.4428	44.42		
	0.0900	4.5232	45.23		
Kapardika bhasma	0.1069	5.3726	53.56	53.24±0.28	8.31
	0.1061	5.3324	53.16		
	0.1058	5.3173	53.01		
Muktashukti bhasma	0.0922	4.6338	46.10	46.97±0.79	5.87
	0.0951	4.7795	47.55		
	0.0947	4.7594	47.37		
Shankh bhasma	0.0915	4.5986	45.98	46.14±0.47	7.20
	0.0911	4.5785	45.78		
	0.0929	4.6690	46.68		
Kukkundatwak bhasma	0.1062	5.3374	53.48	53.12±0.38	1.66
	0.1047	5.2620	52.72		
	0.1056	5.3072	53.17		
Amalaki	0.0	0.0	0.0		
Haritaki	0.0	0.0	0.0		
Excipient	0.0	0.0	0.0		
Total Calcium per Tablet					**35.66**
Maxcal-C Tablet	0.0695	3.4929	34.82	**34.90±0.1**	
	0.0699	3.5130	35.02		
	0.0696	3.4980	34.87		

3.5.4 Accuracy

Accuracy was assessed by the standard addition method at the post-digestive stage. To the aliquots of the digested sample solution, standard was added at three different levels concentrations (80%, 100% and 120% of final sample concentration). A known quantity of standard was added to the pre-analyzed sample and calculated the % recovery by subtracting the sample concentration from spiked sample concentration (Table 11).

Table 11. Accuracy study for formulation at 422.7 nm

Spiking	Sample concentration µgmL^{-1}	Spiked concentration µgmL^{-1}	Final concentration µgmL^{-1}	% Recovery	Mean*
80%	2.6881	6.4723	8.8893	95.81	93.24%
	2.7694	6.6621	8.7809	90.23	
	2.7307	6.6699	8.9552	93.32	
100%	2.6881	8.7731	11.5232	100.71	99.73%
	2.7694	8.8196	11.5262	99.25	
	2.7307	8.9435	11.6045	99.22	
120%	2.6881	11.0777	13.9401	101.57	101.75%
	2.7694	10.9693	13.9324	101.77	
	2.7307	11.0235	13.9634	101.90	

3.6 Detection Limit (LOD) and Quantitation Limit (LOQ)

ICH guideline describes several approaches to determine the detection and quantitation limits. These include visual evaluation, signal-to-noise ratio and the use of the standard deviation of the R^2 value or y-intercept and the mean of the slope of the calibration curve. In the present study, the LOD and LOQ were based on the third approach and calculated according to the 3.3σ/S and 10σ/S criterions respectively, where σ is the standard deviation of the R^2 of regression lines and S is the slope of the calibration curve (Table 12).

Table 12. Detection limit and Quantification limit study at 422.7 nm

Set	R^2	Slope
1	0.9970	0.0243
2	0.9974	0.0254
3	0.9955	0.0258
4	0.9987	0.0256
5	0.9969	0.0248
Mean	–	0.0251
SD	0.0011	–
LOD	0.15 µgmL^{-1}	
LOQ	0.45 µgmL^{-1}	

3.6.1 Precision

About 1 mL of $Ca(NO_3)_2$ in digestion vessel was digestion in similar condition as in case of sample. Then 3 different concentration of 5, 10, 15 µgmL^{-1} $Ca(NO_3)_2$ by sequential dilution was added using distilled water. For intraday precision the solution was aspirated on 3 different times in a day at a 2 hr interval. For interday precision the solution was aspirated on 3 different days at a 24 hr interval (Table 13 and 14).

Table 13. Intraday Precision study at 422.7 nm

Concentration (µgmL^{-1})	Absorbance	Mean ± SD	% RSD
5	0.1343	0.1374 ± 0.0026	1.95
	0.1389		
	0.1390		
10	0.2675	0.2688 ± 0.0013	0.48
	0.2689		
	0.2701		
15	0.3933	0.3869 ± 0.0069	1.80
	0.3881		
	0.3795		

Table 14. Interday Precision study at 422.7 nm

Concentration (µgmL^{-1})	Absorbance	Mean ± SD	% RSD
5	0.1374	0.1398 ± 0.0070	5.01
	0.1343		
	0.1477		
10	0.2630	0.2705 ± 0.0094	3.48
	0.2675		
	0.2811		
15	0.3762	0.3915 ± 0.014	3.72
	0.3933		
	0.4052		

3.6.2 Robustness

For robustness, the change in acid ratio and the change in temperature conditions during the digestion process in the developed method were estimated (Table 15).

Table 15. Robustness study at 422.7 nm

Parameter	Change	Absorbance	Mean	SD	% RSD
Acid ratio (7 mL 69% HNO_3 + 1 mL 30% H_2O_2)	6.9 mL 69% HNO_3 + 1.1 mL 30% H_2O_2	0.0646	0.0688	0.0703 ± 0.0013	1.84
		0.065			
		0.066			
	7mL 69% HNO_3 + 1mL 30% H_2O_2	0.0858	0.0712		
		0.0880			
		0.0750			
	7.1 mL 69% HNO_3 + 0.9 mL 30% H_2O_2	0.0760	0.0709		
		0.0764			
		0.0802			
Temperature (180°C)	178°C	0.0855	0.0699	0.0716 ± 0.0016	2.23
		0.0854			
		0.0855			
	180°C	0.0896	0.072		
		0.0904			
		0.0895			
	182°C	0.0849	0.0731		
		0.0859			
		0.0835			

3.7 Assay of Calcium by AAS Method

3.7.1 Standard Preparation

$Ca(NO_3)_2$ solution of 1000 µgmL^{-1} concentration was used as stock solution. Aliquot of the 1 mL stock solution (1000 µgmL^{-1}) was transferred to a 10 mL volumetric flask. 1 mL of 1000 µgmL^{-1} $La(NO_3)_2$ solution was added and made up to 10mL mark with distilled water to prepare 100 µgmL^{-1} solution. Aliquots of 0.5, 1.0, 1.5, 2.0 mL of the working standard (100 µgmL^{-1}) were transferred into each 10 mL volumetric flask, respectively. The volume was adjusted to the mark with double distilled water to give solutions containing 5-20 µgmL^{-1} (n=4). All standard solutions absorbance were recorded using the set condition for calcium in AAS. Absorbance was recorded at 422.7 nm. Calibration curves were constructed by plotting absorbance versus concentrations. Straight line equations were obtained from this calibration curve (Figure 5).

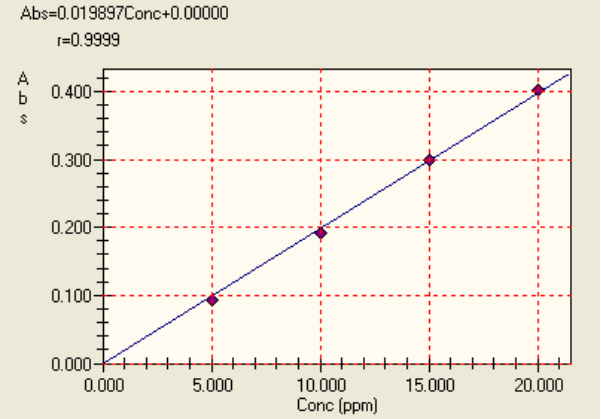

Figure 5. Calibration curve for Assay.

3.7.2 Sample Preparation

Twenty tablets were triturated to yield tablet powder. 0.1g tablet powder was weighed and transferred into a digestion vessel. 8 mL of 69% HNO_3 and 1 mL 30% H_2O_2 was

added. After completing digestion, vessels were taken out and the contents were transferred to a 100mL volumetric flask. Volume was made up to the mark with distilled water to form a 1000 μgmL^{-1} concentration solution. The solution was filtered through Whatman filter paper, and the filtrate of 0.1 mL solution was taken and transferred into a 100 mL volumetric flask, to which 1 mL of 1000 μgmL^{-1} $La(NO_3)_2$ was added in the same volumetric flask. Volume was made up to the mark with distilled water to form a 10 μgmL^{-1} concentration sample solution. Same dilution pattern were followed for blank also. Samples was aspirated in AAS nebulizer and absorbance was noted down. Calcium concentration was calculated from the formula obtained from the calibration curve (Table 16).

Table 16. Assay of calcium by AAS method

Sample	Absorbance	Concentration (μgmL^{-1})	% Calcium	Mean ± SD
Maxcal-C Tablet	0.0690	3.4678	34.67%	35.42% ± 0.66
	0.0715	3.5935	35.93%	
	0.0710	3.5680	35.68%	

3.8 Assay of Calcium by Complexometric Titration Method

Accurately about 0.1g of the tablet powder was weighed in conical flask and 3 mL of dilute HCl and 10 mL water was added. It was boiled for 10 minutes, cooled and 50 Ml of water was added. It was titrated against 0.05 M EDTA up to 2mL. 8mL of 20% NaOH solution and 0.1g of calcon mixture was added and continued titration until the colour of the solution changed from pink to blue colour. Burette reading noted down. Blank reading was deducted from the sample reading (Table 17).

1 mL of 0.05 M EDTA = 0.005004g of $CaCO_3$.

Table 17. Assay by complexometric titration method

Sample	Burette reading	% Calcium	Mean
Maxcal-C Tablet	12.2	24.56	25.12 ± 0.49
	12.6	25.30	
	12.7	25.50	

3.8.1 Comparison of the Methods

The results obtained by both methods for each individual ingredient and the tablet formulation assay were compared (Table 18, Figure 6).

Table 18. Comparison of the methods

Sr. no.	Sample	% Calcium (titrimetric method)	% Calcium (AAS)
1.	Godanti bhasma	29.68 ± 0.57	44.91 ± 0.43
2.	Kapardika bhasma	40.76 ± 1.20	53.24 ± 0.28
3.	Muktashukti bhasma	42.43 ± 2.11	46.97 ± 0.79
4.	Shankh bhasma	38.54 ± 0.95	46.14 ± 0.47
5.	Kukkundatwak bhasma	50.37 ± 0.50	53.12 ± 0.38
6.	Maxcal-C Tablet	25.12 ± 0.49	34.90 ± 0.1

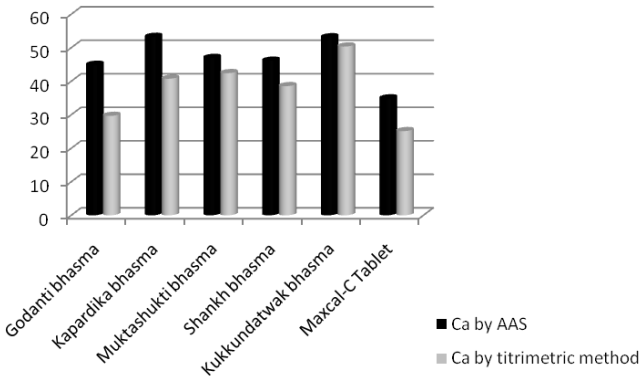

Figure 6. Result comparisons of AAS and titrimetric method.

4. Conclusion

Assay results showed that % calcium in sample found by AAS method was more than titrimetric method. It reveals that proper digestion is necessary for complete extraction of calcium from bhasma and herbo-mineral formulation. Furthermore, AAS detects the calcium without any ion and colour interference which is common problem in case of titrimetric method.

5. Acknowledgement

The authors express their sincere thanks to the Parul Institute of Pharmacy and Research, Vadodara, Gujarat. The authors also thanks Vasu Health Care, G.I, D.C, Makarpura, Vadodara, Gujarat for the laboratory facilities and support.

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